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Participación de los Receptores tipo Toll 2 y 4 en la respuesta de anticuerpos contra antígenos de *Salmonella typhi*

TESIS

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PRESENTA

Q. F. B. LUISA CONSTANZA DEL ROSARIO CERVANTES BARRAGÁN

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RESUMEN

Los receptores tipo toll (TLRs) inician la respuesta inmune innata al reconocer una extensa variedad de componentes microbianos, y son de gran importancia para la inducción y regulación de la respuesta inmune adaptativa subsecuente. Sin embargo, su impacto y mecanismo de acción durante la respuesta de anticuerpos contra antígenos bacteriales no se han estudiado a profundidad. Estudios previos en ratones han demostrado que las porinas de *Salmonella enterica* serovar Typhi *(S. typhi*) inducen una respuesta de anticuerpos específica de larga duración. Por ello se han propuesto a las porinas como un candidato a vacuna. Los estudios clínicos posteriores han demostrado la producción de anticuerpos en voluntarios inmunizados con porinas de *S. typhi*. En el presente trabajo se muestra que la inmunización de ratones deficientes de TLR4, MyD88 o TRIF, con porinas de *S. typhi* induce una respuesta de anticuerpos deficiente. La inmunización de ratones $TLR2^{-/-}$, también induce una producción reducida de IgG anti-porinas, siendo la reducción de IgG3 la mas pronunciada. La transferencia de linfocitos B $TLR2^{-/-}$ o $TLR4^{-/-}$ en ratones deficientes de linfocitos B y su posterior inmunización con porinas, demostraron la contribución directa de la señal de TLR4 durante la respuesta primaria de IgM, mientras que el estímulo vía TLR2 en linfocitos B es importante para la producción de IgG. Adicionalmente los resultados sugieren que las porinas de *S. typhi* son ligandos de TLR2 y que son capaces de activar eficientemente linfocitos B y células dendríticas. Considerando todo lo anterior, las porinas de *S. typhi* no solo representan un antígeno apropiado para la vacunación, sino que muestran funciones estimulantes del sistema inmune innato TLR dependientes, que aumentan y modulan la respuesta de anticuerpos.

ABSTRACT

Toll-like receptors (TLRs) directly induce innate immune responses by sensing a variety of microbial components and are critical for the fine-tuning of subsequent adaptive immune responses. However, their impact and mechanism of action on antibody responses against bacterial antigens is not yet fully understood. *Salmonella enterica* serovar Typhi (*S. typhi*) porins have been characterized as inducers of long-lasting specific antibody responses in mice. They have been also used as a vaccine candidate inducing the production of specific antibodies in humans. In this work is shown, that immunization of TLR4-deficient ($TLR4^{-/-}$), $MyD88^{-/-}$ and $TRIF^{-/-}$ mice with *S. typhi* porins led to significantly reduced B cell responses. $TLR2^{-/-}$ mice, as well, showed reduced IgG titers with a more pronounced impairment in the production of IgG3 anti-porin antibodies. Adoptive transfer of $TLR2^{-/-}$- or $TLR4^{-/-}$- B cells into B cell-deficient mice, followed by immunization with *S. typhi* porins, revealed a direct effect of TLR4 on B cells for the primary IgM response, whereas stimulation of B cells via TLR2 was important for IgG production. Furthermore, the results strongly suggest that *S. typhi* porins are a TLR2 ligand, and able to efficiently elicit maturation of B cells and $CD11c^{+}$ conventional dendritic cells (DCs). Taken together, *S. typhi* porins represent not only a suitable B cell antigen for vaccination, but exhibit potent TLR-dependent stimulatory functions on B cells and DCs which help to further enhance and shape the antibody response.

LISTA DE ABREVIATURAS

7AAD	7-Amino actinomicina D
AID	Citidin-deaminasa inducida por activación
AP-1	Activador proteína 1
APC	Célula presentadora de antígeno
APRIL	Un ligando inductor de proliferación
BAFF	Factor activador de linfocitos B de l familia de TNF
BAFFR	Receptor de BAFF
BCMA	Antígeno de maduración de linfocitos B
BCR	Receptor de linfocito B
BMDC	Células dendríticas derivadas de médula ósea
BSS	Solución salina balanceada
BTK	Tirosina cinasa de Bruton
CARD	Domino de reclutamiento de caspasas
CLP	Célula progenitora común linfoide
CLR	Receptores tipo c-lectinas
CR	Receptor de complemento
CRP	Proteína c reactiva
CSR	Cambio de Isotipo
CTLD	Dominio de lectinas tipo C
DAG	Diacilglicerol
DD	Dominio de Muerte
DNA	Ácido desoxirribonucleico
dsRNA	Ácido ribonucleico de cadena doble
DTH	Hipersensibilidad retardada
ERK	Cinasa regulada por señales extracelulares
E. coli	*Escherichia coli*
FDC	Célula dendrítica folicular
GC	Centro germinal
GM-CSF	Factor estimulante de colonia granulocítica-monocítica
GPI	Proteína anclada a glucosilfosfatidilinositol
HIGM	Síndrome de hiper-IgM ligado a X
IFN	Interferón
IFN-I	Interferón tipo I (α/β)
IFNAR	Receptor de interferón tipo I
Ig	Inmunoglobulina
IκB	Inhibidor del potenciador del gen de la cadena κ en linfocitos B
IKK	Inhibidor de IκB cinasa
IL	Interleucina
IMF	Intensidad media de fluorescencia
iNOS	Oxido nítrico sintasa inducible
IP3	Inositol trifosfato
IRAK	Cinasa asociada al receptor de interleucina 1
IRF	Factor regulador de interferón
ITAM	Motivos ricos en tirosinas asociadas a la activación de inmunoreceptor
JNK	Cinasa N-terminal de c-jun
LBP	Proteína de unión al lipopolisacárido
LPS	Lipopolisacárido
LRR	Repeticiones ricas en leucinas
LSC	Cadena ligera surrogada
MAL	Adaptador similar a MyD88

MAPK	Cinasa activada por mitógenos
MBL	Proteína de unión a manosa
MDA-5	Gen asociado a la diferenciación de melanoma 5
MHC	Complejo principal de histocompatibilidad
μMT	Deficiente del exón transmembranal de la cadena μ
MyD88	Gen de respuesta primaria de diferenciación mieloide 88
MZB	Linfocitos B de zona marginal
NACHT	Dominio central de unión a nucleótidos
NALPs	NACHT, LRR y PYD 1
NBD	Dominio de unión a nucleótidos
NEMO	Modulador esencial de NF-κB
NF-AT	Factor nuclear de linfocitos T activados
NF-κB	Factor nuclear de la cadena κ en linfocitos B
NIK	cinasa inductora de NF-κB
NLR	Receptores tipo NOD
NOD	Dominio de oligomerización de unión a nucleótidos
Omp	Proteína de membrana externa
OVA	Ovoalbúmina
PAMPs	Patrones moleculares asociados a patógenos
PBS	Amortiguador de fosfatos salino
PCR	Reacción en cadena de la polimerasa
PI-3K	Cinasa de 3- fosfatidilinositol
PIP2	Fosfatidilinositol-4,5-difosfato
PIP3	Fosfatidilinositol trifosfato
PIgR	Receptor polimérico de inmunoglobulinas
PKC	Proteína cinasa c
PKR	proteína cinasa activada por dsRNA
PRRs	Receptores de reconocimiento de patrón
PYD	Dominio de pirina
RAG	Gen de activación de la recombinación
RF	Factor reumatoide
RHIM	Motivo de interacción homotípica de proteína de interacción de receptor
RIG-I	Gen I inducible por ácido retinoico
RIP-1	Proteína de interacción al receptor 1
RLR	Receptores tipo RIG-I
rpm	revoluciones por minuto
RT	Transcriptasa reversa
S. typhi	*Salmonella enterica* serovar Typhi
S. typhimurium	*Salmonella enterica* serovar Typhimurium
S1P	Receptor de esfingosina 1 fosfato
SARM	Proteína conteniendo motivo estéril alfa y armadillo
SDS	Dodecil sulfato de sodio
SDS-PAGE	Electroforesis en gel de poliacrilamida y SDS
SHM	Hipermutación somática
SLE	Lupus eritrematoso sistémico
SPF	Libre de patógenos específicos
ssDNA	Acido ribonucléico de cadena sencilla
T1/2	Transicional 1/2
TAB	Proteína de unión a TAK1
TACI	Activador transmembranal modulador de Ca e interactor ligando de ciclofilina
TAK	Cinasa activada por el factor de crecimiento transformante β
T-bet	Factor de trascripción caja T expresado en células T

TBK	Cinasa de unión al miembro de la familia de TRAF activador de NF-κB
TCR	Receptor de linfocito T
TGF-β	Factor de crecimiento transformante β
Th	Linfocito T cooperador
TICAM	Proteína adaptadora conteniendo dominio TIR
TIR	dominio toll/receptor de interleucina 1
TIRAP	Proteína adaptadora con dominio TIR
TLR	Receptor tipo toll
TNF-α	Factor de necrosis tumoral α
TNFR	Receptores de TNF
TNP-Hy	Trinitrofenol hemocianina
TRAF	Factor asociado al receptor de TNF
TRAM	Molécula adaptadora relacionada a TRIF
TRIF	Proteína adaptadora con dominio TIR inductora de IFN-β
ubc13	Enzima conjugadora de ubiquitina 13
uev1A	Enzima conjugadora de ubiquitina E2 variante 1 isoforma A
VLP	Partículas similares a virus

INTRODUCCION

Con base en los sistemas de reconocimiento antigénico, la respuesta inmune se divide en innata (receptores codificados en línea germinal, que no sufren rearreglos y de expresión no clonal) y adaptativa (receptores que sufren rearreglos génicos y de expresión clonal) [1]. Cada una de estas ramas puede ser subdividida en celular o humoral, encontrándose en la primera los receptores de membrana e intracelulares y en la segunda las proteínas extracelulares solubles. Las ramas del sistema inmune innato celular y humoral se subdividen subsecuentemente en aferente y eferente, perteneciendo al primer grupo las moléculas involucradas en el reconocimiento de microorganismos y señales de peligro y al eferente las moléculas con características efectoras [2]. Algunas proteínas pertenecientes a cada grupo se encuentran ejemplificadas en la Figura 1.

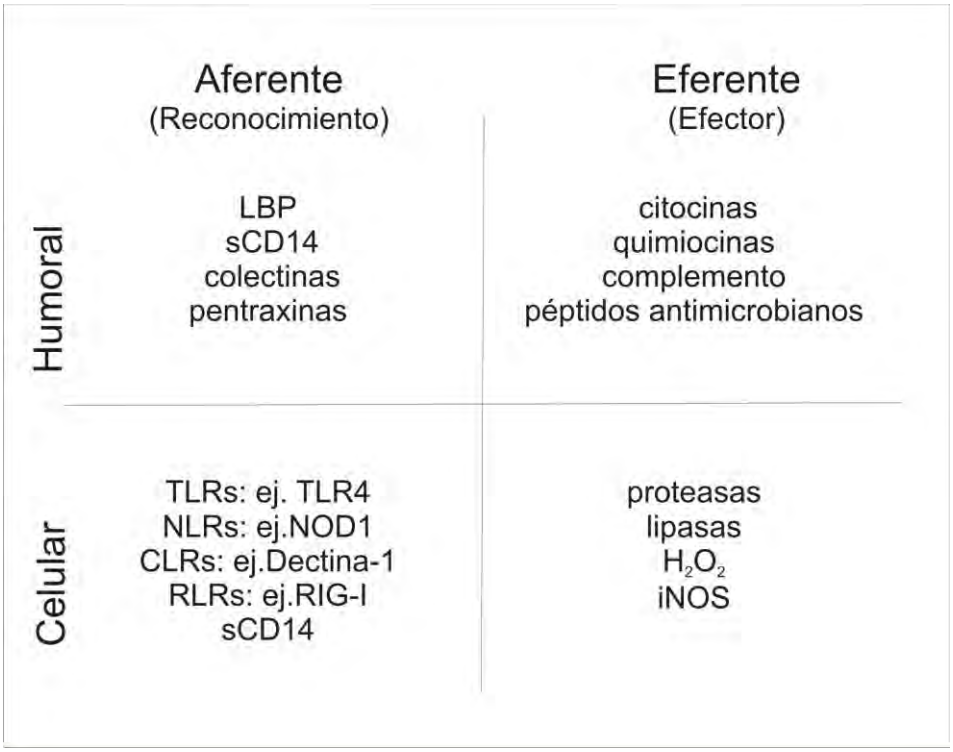


Figura 1. Ramas del sistema inmune innato y algunos ejemplos de moléculas pertenecientes a cada una. LBP: Proteína de unión al LPS, NLRs: Receptores tipo NOD, NOD: Dominio de Oligomerización de unión a nucleótidos 1. CLRs: Receptores tipo c-lectinas. RLRs: Receptores tipo RIG-I. RIG-I: Gen I inducible por ácido retinoico. iNOS: Óxido nítrico sintasa inducible. Adaptado de Beutler et al. [2].

Aún cuando el sistema inmune innato se describió desde hace más de un siglo, el conocimiento de la respuesta inmune innata ha avanzado reconociblemente en las últimas décadas. Actualmente se conocen las funciones de diferentes poblaciones celulares, asi como la expresión, función y vías de señalización de receptores, y las propiedades efectoras de moléculas solubles pertenecientes a este sistema.

Como se ha mencionado anteriormente, los receptores del sistema inmune innato se expresan en las células de manera no clonal y se encuentran codificados en línea germinal. Estos receptores llamados colectivamente receptores de reconocimiento de patrón (PRRs) se encargan del reconocimiento de patrones moleculares asociados a patógenos (PAMPs), que son moléculas altamente conservadas, expresadas en los microorganismos [3]. Aun cuando los PRRs pertenecen al sistema inmune innato, su expresión no se limita a las células de este sistema sino que se encuentran también expresados en las células del sistema inmune adaptativo.

Los receptores del sistema inmune innato aferente se pueden clasificar en cuatro familias principales: Receptores tipo toll (TLR), receptores tipo c-lectinas (CLR), receptores tipo NOD (NLR) y receptores tipo RIG I (RLR), [4-6]. Además existen otros tipos de PRRs como: Integrinas (ej. Receptor de complemento 1, 2), complemento (C3), proteínas inducidas por IFN (ej. Proteína cinasa activada por dsRNA; PKR), proteínas ancladas a glucosilfosfatidilinositol (GPI) (ej. CD14), colectinas (ej. Lectina de unión a manosa; MBL), pentraxinas (ej. Proteína c-reactiva; CRP) y transferasas de lípidos (ej. LBP) [7].

RECEPTORES TIPO TOLL (TLRs).

Los receptores tipo toll reciben este nombre por su similitud con el receptor Toll expresado en *Drosophila melanogaster*. La función de Toll en la organización dorso ventral del embrión de *Drosophila* fueron descritos en 1985 [8]. Mas tarde, en 1996 se descubrió su papel en la respuesta

antimicótica en *Drosophila* adultas [9]. Ese mismo año, se comenzaron a describir posibles homólogos de Toll y el receptor de IL-1 en mamíferos, primero llamados TIL (Toll/IL-1) aunque finalmente se adoptó el nombre de receptores tipo toll [10-12]. Sin embargo, su importancia en el campo de la respuesta inmune innata de mamíferos se conoció hasta que en 1997 Ruslan Medzhitov, en el grupo de Charles Janeway, describió y clonó el primer homólogo de esta proteína en humanos (hToll) y lo relacionó con la producción de citocinas pro-inflamatorias [13]. Posteriormente, se caracterizaron otros homólogos en el genoma humano (TLR1-5), considerándolos entonces como una familia de receptores separados del receptor de IL-1, con el cual comparten su región intracelular pero difieren en la extracelular [14]. Finalmente en 1998, Poltorak y Beutler describieron al TLR4 como el buscado receptor de lipopolisacárido (LPS) y gen afectado en los ratones C3H/HeJ y C57BL/10ScCr [15;16], (ratones con mutaciones espontáneas e hiporrespondedores al estímulo con LPS [17;18]). Iniciado una nueva era en el estudio del sistema inmune innato, ya que aun cuando se conocía el LPS desde 1892 cuando fue descubierto por R. Pfeiffer, fue solo hasta 1998 cuando se encontró su receptor [19].

A la fecha, se conocen 13 TLRs en el ratón y 11 en el humano [19], asi como sus ligandos a excepción del TLR10, 12, y 13 (Tabla. 1), y sus vías de señalización. Algunos TLRs (principalmente los encargados de reconocer productos bacterianos como TLR1, TLR2, TLR4, TLR5 y TLR6) se encuentran expresados en la membrana celular, mientras que otros (como TLR3, TLR7 y TLR9, encargados del reconocimiento de ácidos nucleicos) se encuentran expresados mayoritariamente en las membranas del compartimiento endosómico [20;21]. La importancia de los TLRs radica principalmente en su capacidad de reconocimiento, que abarca una amplia gama de microorganismos, ya que pueden reconocer lípidos, carbohidratos, proteínas y ácidos nucleicos de origen bacterial, viral, micótico, y protozóico; y en su participación en muchos de los procesos de la respuesta inmune, como la maduración de células dendríticas y expresión de moléculas co-estimuladoras, la producción de citocinas pro-inflamatorias como IL-6 e IL-12, la producción de Interferones tipo I (IFN I), la producción de quimiocinas (ej. CCL5-RANTES) y migración de células del sistema inmune innato [21].

TLR	Ligandos	Ref
TLR1/ TLR2	• Lipoarabinomanana **LAM** (*Mycobacterium tuberculosis*) • Triacil-lipopéptidos **Pam_3CSK_4** • Porinas (*Neisseria meningitidis*, *Haemophilus influenzae*) • Fosfolipomanana (*Candida albicans*)	[22;23] [24;25] [26-28] [29]
TLR2	• Lipofosfoglicana **LPG** (*Leishmania major*) • Lipopeptidofosfoglicana **LPPG** (*Entamoeba histolytica*) • Zymosan	[30;31] [32] [29;33]
TLR2/ CD14	• Anclas de glicosilfosfatidilinositol **tGPI** (*Tripanozoma cruzi*) • Lisofosfatidilserina **LisoPS** (*Schistosoma mansoni)* • Peptidoglicana **PGN** • Acido Lipoteicoico **LTA** • Proteínas de Envoltura (Citomegalovirus, CMV) • Lipopolisacáridos **LPS** (Porphyromonas gingivalis) *	[34] [35] [36] [37] [38] [39]
TLR2/ TLR6	• Diacil-lipopéptidos **Pam_2CSK_4** • Lipopéptido activador de macrófagos de 2kD **MALP-2** *(Mycoplasma fermentans)* • Lipoproteinas ej. **OspA** (*Borrelia burgdorferi*)	[25;40] [41] [42;43]
TLR2/ CD36	• Acido lipoteicoico **LTA** • **MALP-2** *(Mycoplasma fermentans)*	[44]
TLR3	• Poli(I:C) • dsRNA	[45]
TLR4/ CD14/ MD2	• Lipopolisacárido **LPS** • Glicoinositol fosfolípidos **GIPL** (*Tripanozoma cruzi*) • Proteínas de fusión (Virus Respiratorio Sincicial RSV)	[15] [46] [47]
TLR5	• Flagelina	[48]
TLR7	• ssRNA • PoliU • Análogos de guanosina: Loxorribina • Imidazoquinolinas: Imiquimod, R848, Gardiquimod	[49;50] [50] [51] [52]
TLR8	• ssRNA • PolyU • Imidazoquinolinas: Imiquimod, R848, Gardiquimod	[50] [50] [51]
TLR9	• dsDNA (CpG) • Hemozoina (*Plasmodium falciparum*)	[53] [54]
TLR11	• Bacterias uropatogénicas • Profilina (*Toxoplasma gondii*)	[55] [56]

Tabla 1. **Principales ligandos de TLRs** reportados.*En controversia.

Estructura de los TLRs.

Los TLRs son glicoproteínas de membrana tipo I. Están formados por una región extracelular encargada del reconocimiento de los ligandos y/o moléculas accesorias y una región citoplásmica capaz de señalizar, formada por un dominio Toll/receptor de IL-1 (TIR) encargado de la unión a las moléculas adaptadoras. La región extracelular esta compuesta por un número variable de dominios de repeticiones ricas en leucinas (LRR), compuestos por 19-25 motivos de LRR en tándem, cada uno de los cuales contiene 24-29 aminoácidos con el motivo LxxLxLxxN [57]. Cada dominio LRR forma una lámina β-plegada, mientras que las regiones variables entre ellas forman alfa hélices conectadas por asas, formando la estructura de una herradura [58] (Figura 2). Según su secuencia y la homogeneidad de sus LRRs, los TLRs se pueden dividir en dos grupos: El primer grupo formado por TLR 1, 2, 4 ,6 y 10 que contienen dos o tres regiones discontinuas de LRR, lo que les confiere una forma un poco diferente a la herradura clásica y que probablemente les permite la unión a lípidos. El segzndo grupo esta formsdo por TLR3, 5, 7, 8 y 9 que tienen una forma de herradura plana típica, similar a otros miembros de la familia de LRR donde la unión al ligando se encuentra en la región N-terminal y C-terminal de la región extracelular. A la fecha se han reportado las estructuras de TLR2/1, TLR3 y TLR4. En la figura 2b, 2c y 2d se puede observar la interacción de estos TLRs en dímero con sus ligandos. [59]

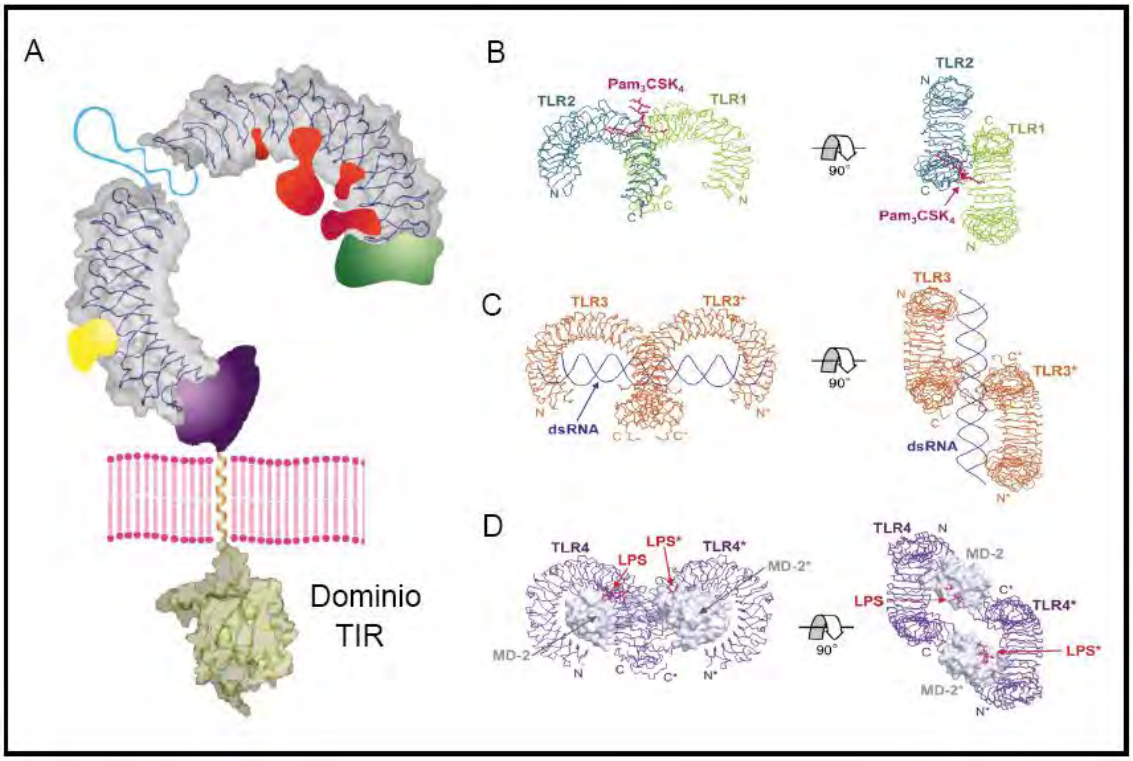


Figura 2. Estructura de los TLRs. (A) Estructura general de los TLRs mostrando el dominio extracelular de LRR y el intracelular TIR. (B) Estructura del heterodímero TLR2/TLR1 y Pam_3CSK_4. (C) Estructura del homodímero TLR3 y dsRNA. (D) Modelo propuesto para el complejo TLR4/MD-2/LPS. Adaptado de Bell JK et al. y Jin MS et al. [58;59].

Moléculas adaptadoras en la vía de señalización de TLRs

Las señales de los TLRs se transmiten a través de 5 moléculas adaptadoras: Gen de respuesta primaria de diferenciación mieloide 88 **(MyD88**) [60], adaptador similar a MyD88 (MAL) también llamada proteína adaptadora don dominio TIR (**TIRAP)** [61;62], proteína adaptadora con dominio TIR inductora de IFN-β (**TRIF,** también llamada proteína adaptadora conteniendo un dominio TIR, TICAM 1) [63-65], molécula adaptadora relacionada a TRIF (**TRAM** también llamada TICAM 2) [66;67] y proteína conteniendo motivo estéril alfa y armadillo (**SARM**) [68]. Estas moléculas contienen un dominio TIR mediante el cual se unen a la región intracelular de los TLRs en interacciones TIR-TIR. La vía de señalización continúa, en el caso de MyD88 por dominio de muerte (DD) y TRIF por un motivo de interacción homotípica de proteína de interacción de receptor (RHIM) que continúan la interacción con las proteína-cinasas río abajo.

MyD88.

Fue descubierta en 1990 por Lord K *et al.* [69]. Primero se describió su función en la vía de señalización del receptor de IL-1 y después en la señalización de TLRs. Todos los TLRs, excepto TLR3, señalizan total o parcialmente (TLR4) a través de esta molécula [70]. Los ratones deficientes de esta proteína son resistentes a los efectos tóxicos del LPS [71] y se encuentran severamente comprometidos en la respuesta a patógenos como: *Staphylococcus aureus* [72], *Toxoplasma gondii* [73], *Listeria monocytogenes* [74], *Leishmania major* [75] o Herpes simplex virus 1 (HSV1) [76].

TIRAP.

Es la segunda molécula adaptadora descubierta y es necesaria para la señalización de TLR2 y TLR4 ya que sirve como puente entre estos TLRs y MyD88 [77]. Tiene un sitio de unión a Fosfatidilinositol-4,5-difosfato (PIP2) lo que permite su reclutamiento a la membrana citoplásmica [78]. Los ratones deficientes de esta molécula no se encuentran inmunocomprometidos como los ratones deficientes de MyD88, sin embargo son mas susceptibles a

algunas infecciones bacterianas como *Klebsiella pneumonieae* [79].

TRIF.

Esta molécula adaptadora fue descubierta después de MAL/TIRAP cuando se demostró que MAL/TIRAP unía la señal de TLR4 con MyD88 pero no controlaba la producción de IFN-β [70]. TRIF controla la señalización MyD88 independiente de TLR4 y también es la molécula adaptadora exclusiva de TLR3 que conlleva a la producción de IFN-β [64]. Los ratones deficientes de TRIF [65] o con una mutación puntual conocida como *lps2* [63], presentan una mayor mortalidad tras la infección con Citomegalovirus (CMV). TRIF también juega un papel importante en el aumento de la expresión de moléculas co-estimuladoras y MHC II inducido por LPS [80].

TRAM.

Es la cuarta molécula adaptadora conocida. Es la más restringida ya que tiene función únicamente en la vía de señalización de TLR4 [66;67]. Funciona como puente entre TLR4 y TRIF cuando TLR4 se encuentra en el compartimiento endosómico [81]. Se postula que su sitio de miristoilación es importante para su localización en la membrana endosómica, similar a la función de PIP2 para TIRAP. Los ratones $TRAM^{-/-}$ presentan una deficiencia en la producción de citocinas inducidas por el estímulo con LPS. También es importante mencionar que probablemente la unión a TLR4 de MAL o TRAM sea mutuamente excluyente modificando así el patrón de respuesta a TLR4 dependiendo de las moléculas adaptadoras que se unan a su región intracelular, que depende finalmente de la localización de TLR4 [81].

SARM.

Es la quinta molécula adaptadora, y a diferencia de las otras cuatro; no induce la activación de IRF3 o NF-κB. En células humanas se postula que tiene una función inhibidora en la vía de señalización de TRIF mediante la interacción directa con esta molécula [68]. Sin embargo, estudios en ratones deficientes de SARM demostraron su expresión casi exclusivamente en neuronas, donde participa en la inducción de apoptosis mediada por estrés

(por privación de oxígeno y glucosa). En estos ratones la respuesta a ligandos de TLR2, TLR3, TLR4 y TLR9 son normales [82] por lo que la participación y función de SARM en las vías de señalización de TLRs requiere confirmarse y podría ser especie dependiente.

Las señales de todos los TLRs son transducidas por solo 2 moléculas adaptadoras, MyD88 para todos los TLRs excepto TLR3 y TRIF para TLR3 y parcialmente para TLR4, lo que se ha comprobado con ratones deficientes de MyD88 y TRIF que carecen de cualquier señal de TLRs [83].

Vía de señalización de TLR4.

El reconocimiento de LPS a través de TLR4 por células de mamíferos sucede tras la interacción de diferentes proteínas relacionadas con TLR4. La proteína de unión al LPS (LBP) es una proteína soluble acarreadora que se une directamente al LPS y facilita su interacción con CD14. CD14 es una proteína anclada por GPI que facilita el transporte de LPS al complejo receptor formado por dímeros de TLR4 y MD-2 [84;85]. Tras el reconocimiento del LPS, el complejo se oligomeriza y recluta las moléculas adaptadoras TIRAP y MyD88 mediante interacciones TIR-TIR. TIRAP funciona como una molécula de selección ya que recluta MyD88 a la membrana celular por su habilidad de interactuar con fosfatidil-inositol 4,5 difosfato (PtdIns(4,5)P2) [78] abundante en la membrana celular. MyD88 se une subsecuentemente, mediante interacciones DD-DD, con 2 cinasas asociadas a IL-1R: IRAK-4 e IRAK-1. Una vez fosforilado IRAK-4 se disocia del complejo e interactúa con TRAF-6. TRAF-6 es una E3-ligasa que forma un complejo con Ubc13 y Uev1A para sintetizar cadenas de poliubiquitina que activan a TAK1, una proteína cinasa activada por mitógenos (MAPKKK). TAK1 en combinación con TAB1, TAB2 y TAB3 activan 2 vías de señalización: el complejo IKK y la vía de las MAP cinasas. El complejo IKK esta compuesto por dos unidades catalíticas: IKKα e IKKβ y una subunidad reguladora IKKγ/NEMO. La activación del complejo y la fosforilación de IKKβ

catalizan la fosforilación de IκB lo que induce su degradación que libera el complejo NF-κB. Esto permite su translocación al núcleo, que controla la expresión de varias citocinas proinflamatorias entre otros genes. En la vía de las MAPcinasas, TAK1 activa a otras cinasas como la cinasa N terminal de c-Jun (JNK), p38 y ERK, produciendo la activación de AP-1 y así la expresión de citocinas [6;21;70;83]. Finalmente la asociación de IRF5 con MyD88 y TRAF6 también participa en la inducción de citocinas proinflamatorias aunque los detalles de la vía de señalización todavía no se conocen [86]. De este modo la vía de señalización MyD88 dependiente de TLR4 induce la producción de citocinas proinflamatorias a través de la activación temprana de los factores nucleares NF-kB, AP-1 e IRF5.

Aproximadamente después de 30 minutos del inicio de la señal por LPS, el complejo proteico del TLR4 + LPS es internalizado en un proceso mediado por dinamina GTPasas, las cuales controlan la invaginación de la membrana citoplasmática creando endosomas tempranos. TLR4 y TRAM viajan independientemente hacia los endosomas tempranos, sin embargo una vez ahí interactúan [81]. Posteriormente, TRAM, que se une a los endosomas por su miristoilación, recluta a TRIF al TLR4. La región C-terminal de TRIF recluta a RIP1 y TRAF6 que a su vez activan a TAK1 cuya señal continúa como se ha descrito previamente, permitiendo la activación tardía de NF-κB y la producción de citocinas proinflamatorias. La región N-terminal de TRIF por su parte, interactúa con TRAF3 que recluta a las moléculas IKK no canónicas TBK1 e IKKι, las cuales fosforilan a IRF3 que forma un dímero y es translocado al núcleo induciendo la producción de IFN-β [6;21;70;83] (Figura 3).

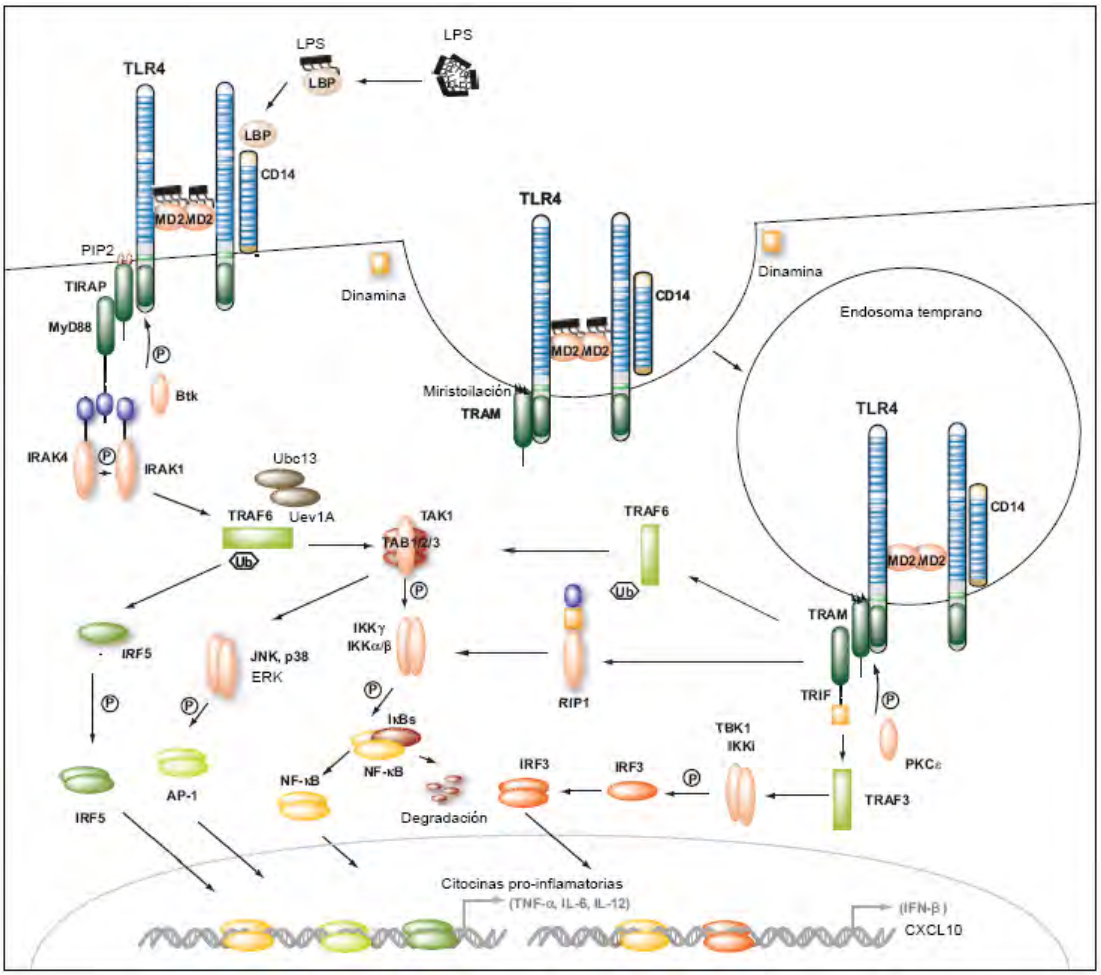


Figura 3. Vía de Señalización de TLR4. LBP: Proteína de unión al LPS, **PIP2**: Fosfatidil inositol 4,5 difosfato (PtdIns(4,5)P2), **TIRAP**: Proteína adaptadora con dominio TIR, **PKCε**: Proteinacinasa C ε, **IRAK**: Cinasa asociada a IL-1R, **TRAF**: Factor asociado al receptor de TNF, **Ubc13**: Enzima conjugadora de ubiquitina 13, **Uev1A**: Enzima conjugadora de ubiquitina E2 variante 1 isoforma A, **TAK**: Cinasa activada por el factor de crecimiento transformante β (TGF-β), **TAB**: Proteína de unión a TAK1, **IKK**: Inhibidor de IκB cinasa, **JNK**: Cinasa N terminal de c-Jun, **ERK**: Cinasa regulada por señales extracelulares, **IRF**: Factor regulador de interferón, **AP-1**: Activador proteína-1, **IκB**: Inhibidor del potenciador del gen de la cadena κ en linfocitos B, **NF-κB**: Factor nuclear de la cadena κ en linfocitos B, **TRIF**: Proteína adaptadora con dominio TIR inductora de IFN-β, **TBK**: Cinasa de unión al miembro de la familia de TRAF activador de NF-κB (TANK), **RIP1**: Proteína de interacción al receptor 1, **TNF-α**: Factor de necrosis tumoral α, **IFN-β**: Interferón β, **P**: Fosforilación, **Ub**: Ubiquitinación. Adaptado de Trinchieri G *et al.*, Kawai T *et al.*, O'Neill LA *et al.*, Kenny EF *et al.* [6;21;70;83].

Vía de Señalización de TLR2

Una de las peculiaridades de TLR2 es su capacidad para formar heterodímeros con TLR1, TLR6 y TLR10 [87], así como interactuar con moléculas como CD14, CD36 o Dectina-1 para aumentar la señal a través de TLR2 [7]. Esto explica la amplia variedad de ligandos que pueden ser reconocidos por esta molécula, sin perder la especificidad característica de lo receptores del sistema inmune innato. Ya que por ejemplo, puede distinguir entre triacilpéptidos reconocidos por el complejo TLR1/TLR2 y diacilpéptidos reconocidos por TLR6/TLR2 [25]. Los heterodímeros de TLR1/TLR2 y TLR2/TLR6 se encuentran pre-ensamblados en la membrana citoplasmática de las células, mientras que CD36 o CD14 se unen al complejo mediante la unión con el ligando, lo que inicia la cascada de señalización [88]. TIRAP es reclutado a la membrana citoplasmática al interactuar con PIP2 [78]. TIRAP a su vez, recluta a MyD88 a la membrana celular. MyD88 se une subsecuentemente a IRAK-4 e IRAK-1. IRAK-4 se disocia del complejo e interactúa con TRAF-6. TRAF-6 se une a Ubc13 y Uev1A que activan TAK1. TAK1 en combinación con TAB1, TAB2 y TAB3 activan 2 vías de señalización: el complejo IKK y la vía de las MAP cinasas. La activación del complejo canónico y la fosforilación de IKKβ catalizan la fosforilación de IκB lo que induce su degradación que libera el complejo NF-κB y permite su translocación al núcleo. En la vía de las MAPcinasas, TAK1 lleva a la activación de AP-1 y así la regulación de la expresión de citocinas proinflamatorias. Finalmente, la asociación de IRF5 con MyD88, su activación dimerización y translocación al núcleo. [6;21;70;83] (Figura 4).

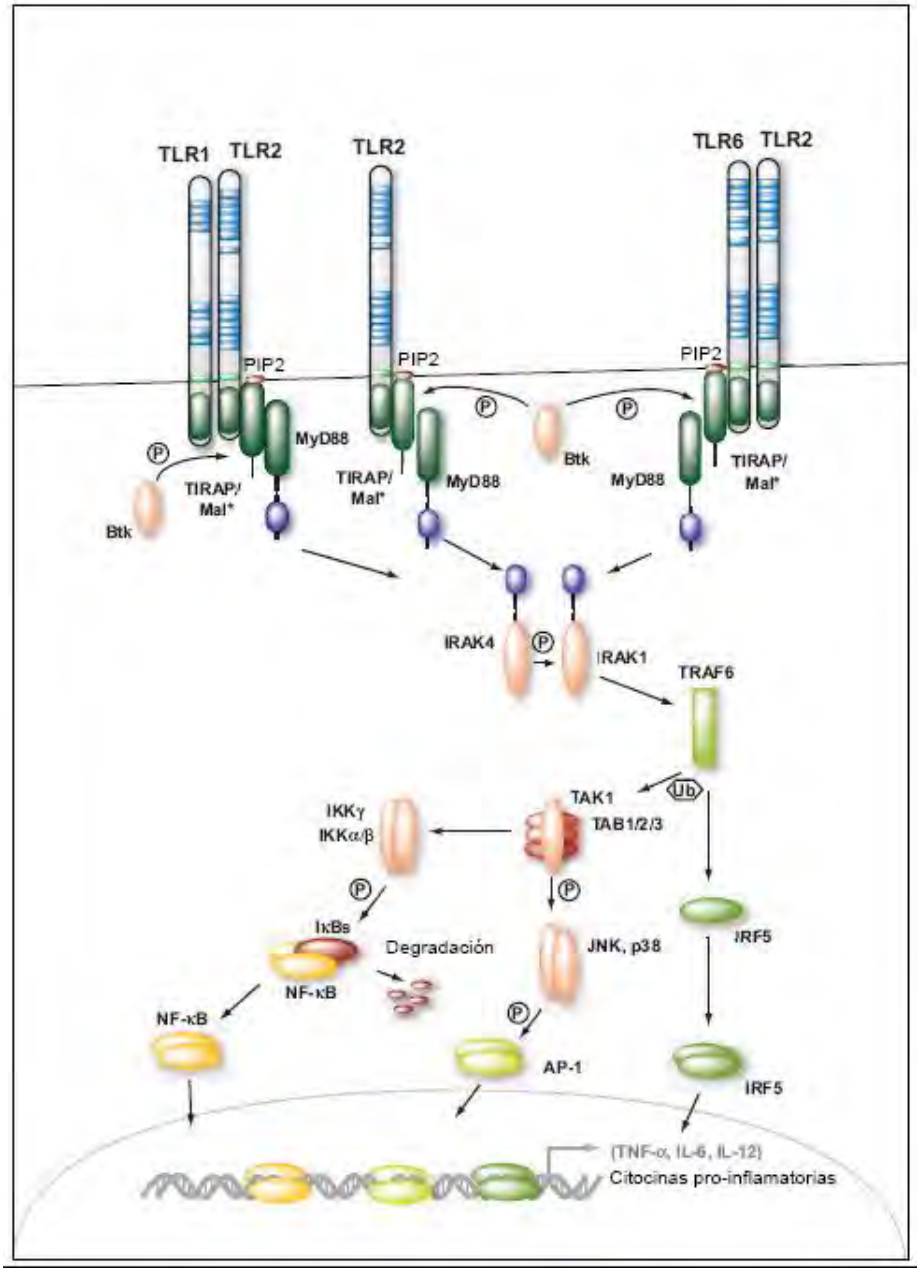


Figura 4. Vía de Señalización de TLR2. PIP2: fosfatidil inositol 4,5 difosfato (PtdIns(4,5)P2), **TIRAP**: Proteína adaptadora con dominio TIR, **BTK**: Tirosina cinasa de Bruton, **IRAK**: Cinasa asociada a IL-1R, **TRAF**: Factor asociado al receptor de TNF, **Ubc13**: Enzima conjugadora de ubiquitina 13, **Uev1A**: Enzima conjugadora de ubiquitina E2 variante 1 isoforma A, **TAK**: Cinasa activada por el factor de crecimiento transformante β (TGF-β), **TAB**: Proteína de unión a TAK1, **IKK**: Inhibidor de IκB cinasa, **JNK**: Cinasa N terminal de c-Jun, **IRF**: Factor regulador de interferón, **AP-1**: Activador proteína-1, **IκB**: Inhibidor del potenciador del gen de la cadena κ en linfocitos B, **NF-κB**: Factor nuclear de la cadena κ en linfocitos B, **TRIF**: Proteína adaptadora con dominio TIR inductora de IFN-β, **TNF-α**: Factor de necrosis tumoral α, **P**: Fosforilación, **Ub**: Ubiquitinación. Adaptado de Adaptado de Trinchieri G *et al.*, Kawai T *et al*., O'Neill LA *et al.*, Kenny EF *et al.* [6;21;70;83].

Vía de señalización de TLR7, TLR8 y TLR9

Los TLR7, 8 y 9 se encuentran localizados en los endosomas. Ahí interactúan con los ácidos nucleicos correspondientes (Tabla 1), iniciando su señalización a través de MyD88. MyD88 recluta a TRAF6 siguiendo las vías TRAF6-TAK1-MAPKK-AP1, TRAF6-TAK1-IKK-NF-κB y TRAF6-IRF5 previamente descritas. Sin embargo, en esta localización celular, el complejo MyD88-IRAK1-IRAK4, interactúa con TRAF3 [89] induciendo la fosforilación y dimerización de IRF7, su posterior translocación al núcleo y la producción de IFN-α [6;21;70;83]. Esta vía de señalización tiene importancia particularmente en células dendríticas plasmacitoides, que además de expresar TLR7 y TLR9, expresan IRF7 de manera constitutiva [90] (Figura 5).

Vía de Señalización de TLR3, TLR5, TLR11.

Previamente se describieron las vías de señalización de TLR4 a través de MyD88 en la membrana celular y TRIF en el compartimiento endosómico. TLR3, se une a TRIF directamente siguiendo la vía de señalización TRIF-TBK-IKKι-IRF3, induciendo la producción de IFN-β. TLR5 y TLR11 se unen directamente a MyD88 en la membrana celular siguiendo la vía de TRAF6 para inducir la producción de citocinas proinflamatorias a través de NF-κB, IRF5 y AP-1 [6;21;70;83] (Figura 5).

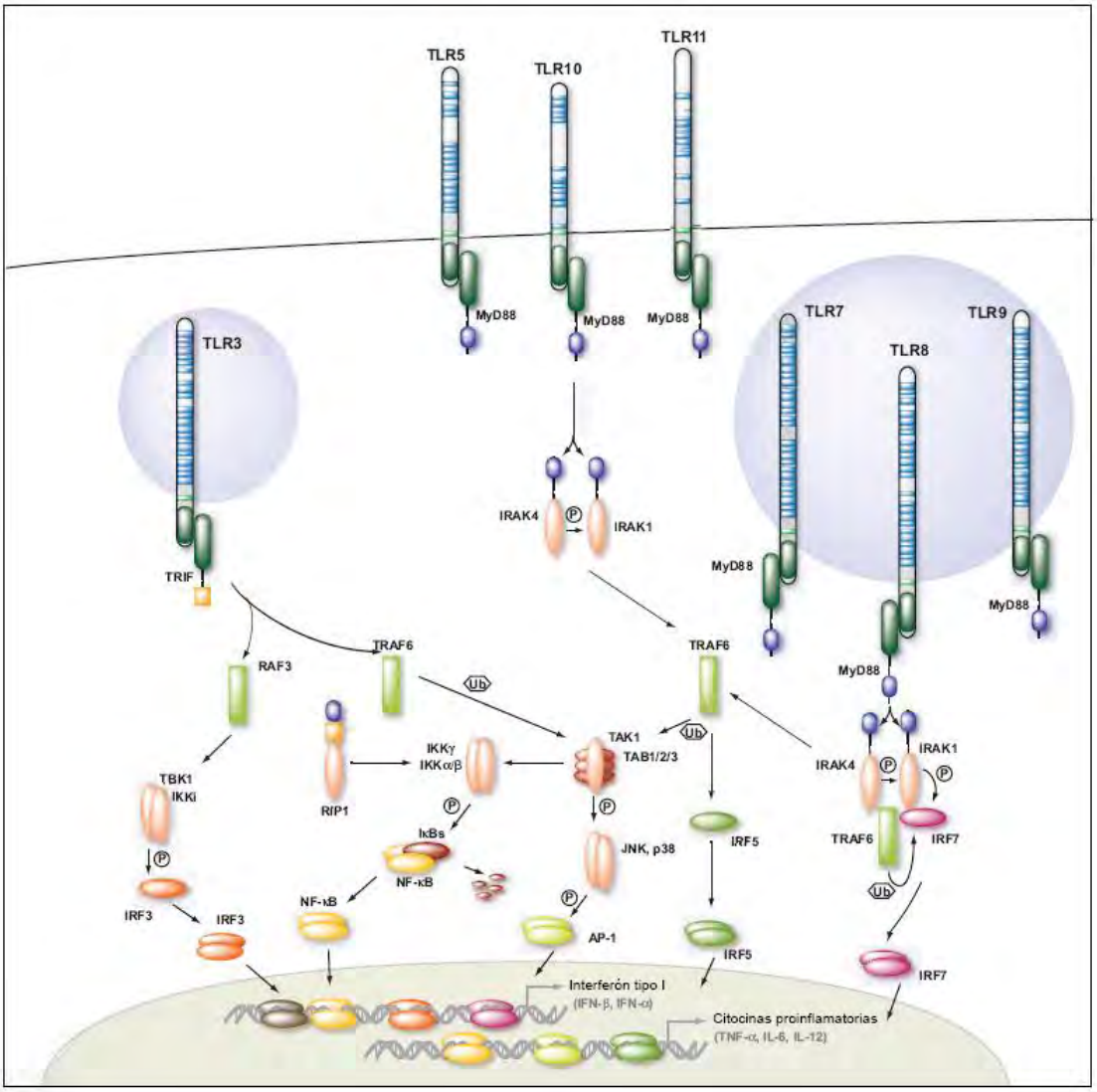


Figura 5. Vía de Señalización de TLR3, TLR5, TLR11, TLR7, 8 y 9. IRAK: Cinasa asociada a IL-1R, **TRAF**: Factor asociado al receptor de TNF, **TAK**: Cinasa activada por el factor de crecimiento transformante β (TGF-β), **TAB**: Proteína de unión a TAK1, **IKK**: Inhibidor de IκB cinasa, **JNK**: Cinasa N terminal de c-Jun, **IRF**: Factor regulador de interferón, **AP-1**: Activador proteína-1, **IκB**: Inhibidor del potenciador del gen de la cadena κ en linfocitos B, **NF-κB**: Factor nuclear de la cadena κ en linfocitos B, **TNF-α**: Factor de necrosis tumoral α, **IFN-β**: Interferón β, **P**: Fosforilación, **Ub**: Ubiquitinación. Adaptado de Adaptado de Trinchieri G *et al.*, Kawai T *et al*., O'Neill LA *et al.*, Kenny EF *et al.* [6;21;70;83].

RECEPTORES TIPO C-LECTINAS (CLRs)

Los receptores tipo c-lectinas son una amplia familia de receptores. El nombre proviene de proteínas de unión a carbohidratos y calcio, sin embrago a ella pertenecen también proteínas sin especificidad a estas moléculas pero que contienen un dominio de lectinas tipo C (CTLD) [5]. La mayoría de estos receptores median endocitosis o fagocitosis, aunque algunos de ellos, son capaces de iniciar señales tras el reconocimiento de algún PAMP. Probablemente el mejor ejemplo sea Dectina-1 que es capaz de reconocer zimosan a través de su CTLD e interactuar con TLR2 [91].

RECEPTORES TIPO NOD (NLRs)

Esta familia esta compuesta por receptores citoplasmáticos y contiene mas de 20 miembros en mamíferos. Están formados por tres dominios distintos: LRR en C terminal que permite el reconocimiento de sus ligandos, un dominio NACHT o NBD (dominio de unión a nucleótidos) que participa en su oligomerización en la parte central y una región N terminal efectora generalmente formada por un dominio de pirina (PYD) o un dominio de reclutamiento de caspasas (CARD). Los NLR se pueden subdividir en receptores NOD que comprenden NOD1 y NOD2 , y NALPs (NACHT, LRR y PYD 1) [4;5;92].

NOD1 reconoce el ácido diaminopimélico de la peptidoglicana [93], mientras que NOD2 reconoce muramilpéptidos [94] jugando ambos un papel central en el reconocimiento de bacterias intracelulares, y la consecuente producción de citocinas pro-inflamatorias.

La familia de los NALPs esta formada por aproximadamente 14 miembros con el dominio PYD característico. Aún cuando las funciones de muchos NALPs no se conocen, muchos de ellos al ser activados forman inflamasomas, capaces de activar las caspasas inflamatorias importantes en la producción de las formas activas de citocinas pro-inflamatorias como IL-1β

o IL-18 a partir de pro-IL-1β o pro-IL-18. Se han reportado una variedad de ligandos, de diversas estructuras y de origen tanto exógeno como endógeno para estas moléculas [7;95]. Una de las teorías mas aceptadas es la activación de los NALPs a través de fluctuaciones de K^+ [95;96], que son inducidas tras la unión de los diferentes ligandos, lo que explicaría la diversidad de los estímulos que los activan.

RECEPTORES TIPO RIG-I (RLRs)

Los receptores tipo RIG-I son helicasas citosólicas compuestas por un dominio de DExD/H box helicasa que reconoce RNA y un dominio CARD, que mediante interacciones CARD-CARD conlleva a la activación de IRF3 y la producción de IFN-β. Esta familia esta compuesta hasta la fecha por dos miembros: RIG-I y el gen asociado a la diferenciación de melanoma (MDA-5). Ambas moléculas son inducibles por IFN-I y se expresan en todas las células [4;7]. RIG-I reconoce 5'-trifosfato-ssRNA [97] y se asocia con el reconocimiento de virus como: Virus de la estomatitis vesicular (VSV) o virus de la enfermedad de New Castle (NDV) [7] mientras que MDA-5 reconoce dsRNA, es el receptor de poli(I:C) y reconoce picornavirus como el virus de la encefalomiocarditis (EMCV) [98]. Su papel es esencial en el reconocimiento de infecciones virales, como demuestra la profunda susceptibilidad a infecciones de los ratones deficientes de RIG-I [99].

LINFOCITOS B

Los linfocitos B son una población de leucocitos mononucleares, caracterizados por la expresión de inmunoglobulinas de superficie producto de rearreglos génicos y de distribución clonal [100] cuya función principal es la produción de anticuerpos. La generación de anticuerpos en forma clonal, se conocía desde los años 40s cuando Niels Jerne y Sir Mc Farlan Burnet propusieron la teoría de la "selección natural" [101] y de la "selección clonal" [102]. Sin embargo el descubrimiento y caracterización del linfocito B, inició hasta los años 60-70's cuando Max Cooper y Robert Good propusieron la división de labor entre las células encontradas en la bolsa de Fabricius de las aves, encargadas de la producción de anticuerpos y las células del timo, inductoras de la hipersensibilidad retardada (DTH) [103;104]. Evolutivamente, la producción de anticuerpos aparece en los peces mandibulados de la clase condrictios (peces cartilaginosos) gracias a la presencia de dos proteínas esenciales para la recombinación de la cadena pesada y ligera de los anticuerpos: Gen de activación de la recombinación 1 y 2 (RAG1 y RAG2) [105].

Receptor de células B (BCR) y vía de señalización

El BCR está formado por una inmunoglobulina de membrana constituida por dos cadenas pesadas y dos ligeras que se encuentran asociadas a heterodímeros de las proteínas accesorias Ig-α (CD79a) e Ig-β (CD79b) unidas por puentes disulfuro. Estas proteínas contienen motivos ricos en tirosinas asociados a la activación mediada por inmunoreceptores (ITAMs) que son responsables de la transducción de señales del BCR. La señalización del BCR empieza con la unión del receptor con el antígeno. Esta unión y la oligomerización de los receptores, provoca una fosforilación de las regiones ITAM de Ig-α e Ig-β, que reclutan a la tirosina-cinasa intracelular Syk y varias tirosina-cinasas de la familia de Src (Lyn, Fyn y Blk). Al unirse a los dos dominios ITAM, Syk se fosforila aumentando su actividad y activando la tirosina-cinasa de Bruton (Btk). Estos eventos provocan la hidrólisis de PIP_2 por la fosfolipasa C gamma 2 (PLCγ2) produciendo diacil-glicerol (DAG)

que activa la proteína-cinasa C (PKC) e inositol trifosfato (IP_3) que induce la liberación de calcio (Ca^{2+}) del retículo endoplasmático al citoplasma. También se activa la vía de RAS que actúa sobre las MAPKs, ERK1 y ERK2. La activación de la fosfatidil inositol 3 cinasa (PI-3K) lleva a la generación de fosfatidil inositol trifosfato (PIP_3) que puede servir como ligando para varias proteínas como Btk y c-Akt. Las concentraciones elevadas de Ca^{2+} se requieren para la activación de los factores de transcripción NF-κB y el factor nuclear de linfocitos T activados (NF-AT) a través de proteínacinasas atípicas y Ca^{2+}-calmodulina respectivamente. Existen además otros componentes que son fosforilados durante la señalización a través del BCR como por ejemplo Vav [106-110].

La estimulación del BCR, además de activar al linfocito B, provoca la proliferación celular, la producción de anticuerpos e induce la transcripción del MHC II (importante para la presentación de antígenos a linfocitos T), el aumento en la expresión de receptores para citocinas como el receptor de IL-4 e IL-2 que ayudan a la proliferación, moléculas de adhesión y coestimuladoras como B7-1, B7-2 CD40, CD70, etc. [111].

Subpoblaciones de linfocitos B

Los linfocitos B maduros *naive* se pueden caracterizar en 5 subpoblaciones con diferentes características, localización, repertorio de anticuerpos y funciones durante la respuesta inmune: Linfocitos B foliculares, linfocitos de zona marginal (MZB), linfocitos B1-a, linfocitos B1-b y linfocitos reguladores o B10.

Linfocitos B foliculares

Los linfocitos foliculares, cuyo fenotipo es $CD23^{high}$, $CD21^{int}$, $CD1^{int}$, IgM^{int}, IgD^{high} representan la población predominante en órganos linfoides secundarios, y sangre periférica. Presentan un repertorio amplio de anticuerpos, y se relacionan predominantemente con la respuesta humoral T-dependiente e intrafolicular.

Linfocitos de zona marginal (MZB)

Se localizan en la zona marginal del bazo, que es la zona donde la sangre es filtrada y por ende de muestreo de antígenos [112]. Requieren el receptor de esfingosina 1-fosfato ($S1P_1$) y parcialmente ($S1P_3$) para migrar hacia la zona marginal y permanecer ahí [113]. Presentan un fenotipo $CD23^{-/low}$, $CD21^{high}$, $CD1^{high}$, IgM^{high}, IgD^{low}, y comparten con los linfocitos B1 un marcador denominado NIM-R7 [114], tienen un tiempo de vida media largo (de 6-8 semanas) y a diferencia de los linfocitos foliculares no recirculan en la sangre o linfa. Estos linfocitos participan preferentemente en respuestas T-independientes [115] aunque también lo hacen en respuestas T-dependientes. Se les ha relacionado con los macrófagos de zona marginal en la fagocitosis para la formación de una primera línea de defensa rápida contra patógenos, por lo que se les considera parte de la respuesta inmune innata [116]. Cuando reconocen antígenos a través del BCR y receptores de complemento 1 y 2 (CR1/2) producen anticuerpos, sin embargo también son capaces de unir complejos antigénicos solo a través de CR1/2 migrando hacia los folículos y llevando estos complejo a las células dendríticas foliculares [117;118]. Otra característica importante es su capacidad para proliferar rápidamente ante estímulos como el de LPS, activándose y produciendo células plasmáticas mucho más rápido que los linfocitos B foliculares [119]. Finalmente, en comparación con los linfocitos B foliculares son mejores células presentadoras de antígeno y son capaces de activar linfocitos T $CD4^+$ naive eficientemente [120].

Linfocitos B1-a

Estos linfocitos de fenotipo $CD23^{-/low}$, IgM^{high}, IgD^{low}, $CD5^+$, se encuentran principalmente en la cavidad peritoneal y pleural y se diferencian de los linfocitos B1-b por la expresión de CD5. A diferencia de los otros linfocitos B, estos tienen su origen en el hígado fetal y poseen la capacidad de autorenovarse, manteniéndose hasta la vida adulta [121]. Tienen un repertorio de inmunoglobulinas restringido, son multivalentes, y en experimentos con ratones quiméricos donde las células progenitoras de linfocitos B1 producían IgM de alotipo a (IgM^a) y el resto IgM^b se demostró

que producen los llamados anticuerpos naturales [122;123] ya que pueden producir anticuerpos sin necesidad de un estímulo específico a través del BCR [121;124]. Muchos de ellos son autorreactivos [125] o poseen especificidad por carbohidratos de la pared bacterial como fosforilcolina [126]. En ratones estimulados con LPS, son los linfocitos B1-a peritoneales los que responden rápidamente migrando al bazo donde se dividen y diferencian a células plasmáticas productoras de IgM, mientras que los pocos B1-a residentes del bazo se diferencian a células plasmáticas de IgM sin previa división [127]. Por su rápida capacidad de producción de anticuerpos a partir de estímulos independientes del BCR se les considera también parte de la respuesta inmune innata [100].

Linfocitos B1-b

Estas células se encuentran también principalmente en la cavidad peritoneal y pleural. Es una población que se encuentra en roedores y no se ha encontrado un equivalente en humanos. Su origen a partir del hígado fetal o de la médula ósea adulta es aun controvertido [128]. Tienen un fenotipo $CD23^{-/low}$, IgM^{high}, IgD^{low}, $CD5^{-}$. Se relacionan con respuestas T-independientes y pueden originar células plasmáticas de corta vida productoras de IgM [129]. Sin embargo, uno de sus papeles principales en la respuesta inmune es su cambio de isotipo a IgA probablemente T-independiente y la producción de células plasmáticas de larga vida productoras de IgA [130;131].

Linfocitos B reguladores

Durante el estudio de las subpoblaciones de linfocitos B, se describió, que algunos linfocitos B1-a en la cavidad peritoneal producen IL-10, y especialmente algunos linfocitos con fenotipo de MZB producen IL-10 en respuesta a CpG. Su fenotipo característico propuesto es $CD1d^{high}CD5^{+}CD19^{+}$. Y su caracterización como una población de linfocitos B independiente se realizó en ratones deficientes de CD19 y transgénicos

expresando el hCD19 [132]. Su función se ha demostrado por ejemplo en modelos de encefalitis autoinmune experimental (EAE), donde la ausencia de linfocitos B agrava la enfermedad, y la transferencia de linfocitos B pero no de linfocitos B deficientes de IL-10 la disminuye [133-135].

Desarrollo del linfocito B

Los linfocitos B foliculares y MZB se originan inicialmente en el hígado fetal y durante la vida adulta en la médula ósea. El desarrollo de los linfocitos B, inicia con la expresión de los primeros marcadores de linaje B en la célula progenitora común linfoide (CLP) como Ig-α e Ig-β y el fragmento de la cadena ligera surrogada (LSC) λ5. Esta célula llamada ahora pro-B comienza el rearreglo de la cadena pesada de inmunoglobulina y su expresión asociada con la LSC, en la membrana citoplásmica. La célula pro-B que rearregla exitosamente la cadena pesada, es después llamada pre-B, que continua con el rearreglo de la cadena ligera la cual una vez rearreglada suple la LSC y se acopla a la cadena pesada formando el BCR, convirtiéndose la célula en un linfocito B inmaduro. Los linfocitos B inmaduros ($IgM^{+}B220^{lo}$) se caracterizan por ser propensos a morir por apoptosis y no a proliferar al encontrar su antígeno. Los linfocito B inmaduros abandonan la médula ósea donde pasan al estadio transicional 1 ($CD24^{hi}CD21^{-}B220^{+}$) siguiendo el T2 ($CD24^{hi}CD21^{+}B220^{+}$). Se postula que es en este punto cuando la intensidad de la señal del BCR define la maduración a linfocito B folicular o MZB. Estudios en ratones transgénicos de IgH para antígenos como fosforilcolina, (expresada ampliamente en la pared de bacterias encapsuladas) muestran que los linfocitos B se diferencian preferentemente a MZB, y sugieren que los linfocitos B T2, que por su especificidad reciben una señal débil, se convierten en linfocitos B maduros foliculares, mientras que los que reciben una señal intermedia autoantigénica, pueden evitar la selección negativa y colonizar la zona marginal o convertirse en B1-b. Además de la señal de BCR, los linfocitos B T2 necesitan la señal de Notch2 para diferenciarse a MZB ya que en ratones deficientes de esta molécula no se encuentra esta población [100;136;137].

Los linfocitos B1 se originan en el hígado fetal, donde rearreglan un repertorio de IgH restringido. En el estadio inmaduro, las células que reciben una señal intensa del BCR sobreviven formando linfocitos B1-a mientras que señales intermedias dan origen a linfocitos B1-b. Estudios en ratones con mutaciones en los reguladores positivos de la vía de señalización del BCR, tales como la CD19 [138], la proteína adaptadora BLNK/SLP-65 [139], CD21 [140], o Btk [141] muestran que una disminución en la intensidad de la señal, resulta en una reducción importante de células B1 confirmando la necesidad de una señal positiva a través del BCR. Además se ha caracterizado que la vía BCR/CD19/vav/PI3K también está involucrada tanto en la selección como en la supervivencia de las células B1 [142].

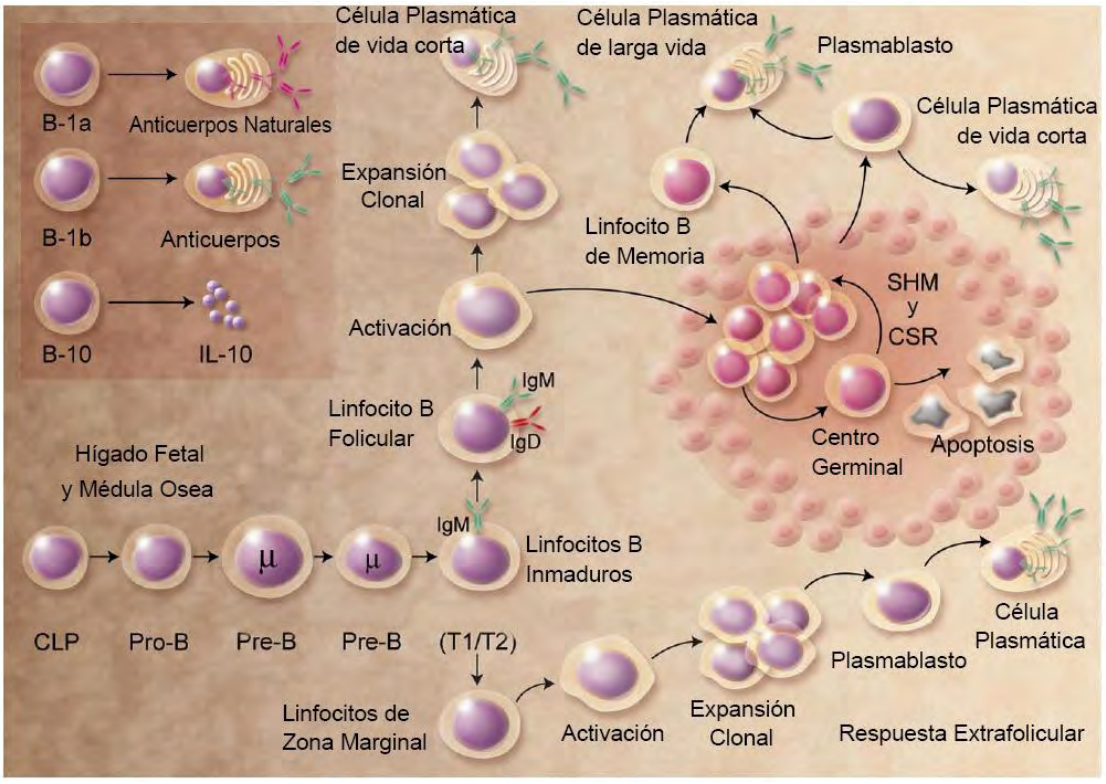


Figura 6. Subpoblaciones de linfocitos B. Esquema de las diferentes subpoblaciones de linfocitos B, su desarrollo y activación. **CLP**: Progenitor linfoide común, **T1/T2**: linfocito B transicional 1/2, **SHM:** Hipermutación somática, **CSR:** Recombinación de cambio de Isotipo. Adaptado de [100].

Activación de linfocitos B

Después de la activación antígeno específica, los linfocitos B pueden iniciar dos vías de diferenciación, una extrafolicular que lleva a la formación de células plasmáticas directamente y una intrafolicular que conlleva a la formación de centros germinales (GC)[111;143;144], esto depende principalmente del tipo de antígeno y el tipo de linfocito B involucrado.

Respuesta Extrafolicular (T-independiente)

Este tipo de reacción es inducida por una estimulación intensa del linfocito B. Los linfocitos B se activan y proliferan rápidamente formando dos o tres días después del estímulo, plasmablastos y células plasmáticas de corta vida productoras de IgM. Estas células aseguran una producción rápida de anticuerpos necesaria para el control inicial de patógenos. Aun cuando no necesitan la interacción con los linfocitos T, otras señales provistas a través de receptores expresados en los linfocitos B, que reconocen moléculas provenientes del exterior como PRRs, del sistema inmune como complemento o de otras células como ligandos de BAFF producido por las células dendríticas, colaboran para la correcta inducción de la producción de anticuerpos. Este tipo de respuesta se lleva a cabo principalmente por MZB o B1-b en gran medida por su localización estratégica para el encuentro con antígenos particulados y complejos [144].

Respuesta Intrafolicular (T-dependiente)

Este tipo de respuesta es inducida por ejemplo por antígenos proteicos, que no son capaces de entrecruzar los BCR para inducir una señal de suficiente intensidad. Los linfocitos B activados por su antígeno, migran de la zona de B en los folículos hacia la frontera de las zonas T y B (un fenómeno CCR7 dependiente [145]) donde interactúan con linfocitos T cooperadores (Th) activados, formando una sinapsis inmunológica en la cual se unen principalmente las moléculas del complejo principal de histocompatibilidad II

(MHC II) al receptor del linfocito T (TCR) y CD40 a CD154 entre otras muchas moléculas que participan en esta sinapsis. La señal de CD40 es necesaria para la inducción del cambio de isotipo (CSR) e hipermutación somática (SHM), como lo demuestran estudios en ratones deficientes de CD40, y pacientes que padecen el síndrome de hiper-IgM ligado a X (HIGM) los cuales presentan una mutación en el gen del ligando de CD40, CD154 [146-148]. Después de la interacción con los linfocitos Th, los linfocitos B siguen dos rutas de diferenciación, algunos forman plasmablastos y células plasmáticas de corta vida, mientras que otros regresan a la zona de B y forman centros pre-germinales. La expansión, agregación y reclutamiento de células a estos centros pre-germinales resulta en la formación de centros germinales, donde las estructuras se polarizan en zona luminosa y zona oscura del centro germinal. En la zona oscura los linfocitos B dejan de expresar su BCR y se convierten en centroblastos que proliferan e inician el proceso de hipermutación sómatica y cambio de isotipo. Durante la hipermutación somática, mediante la molécula citidin-deaminasa inducida por activación (AID) [149] y proteínas del sistema de reparación de DNA, se introducen mutaciones en los genes de las inmunoglobulinas, principalmente en las regiones variables, lo que puede aumentar o disminuir la afinidad por su antígeno. Durante el cambio de isotipo, también dirigido por AID, se reemplaza la región constante de la cadena pesada μ por $\gamma 1$, $\gamma 2\alpha$, $\gamma 2$b, $\gamma 3$, ε ó α, para producir anticuerpos IgG1, IgG2a, IgG2b, IgG3, IgE o IgA respectivamente. Los cetroblastos que salen del ciclo celular, expresan de nuevo el BCR “mutado”, y se encuentran en un estado pro-apoptótico se denominan centrocitos. El encuentro de éstos con su antígeno específico en la zona luminosa del centro germinal presentado por las células dendríticas foliculares, induce señales anti-apoptóticas que permiten la supervivencia de los linfocitos B con mayor afinidad al antígeno, fenómeno conocido como maduración de la afinidad. Algunos de estos linfocitos constituirán la fracción de linfocitos B de memoria, donde la interacción con linfocitos Th mediante CD40 proporciona señales que permiten su larga duración. Otros seguirán a la diferenciación terminal convirtiéndose en plasmablastos y células plasmáticas de larga vida que migran hacia la médula ósea donde reciben

continuamente señales de supervivencia por ejemplo mediante ligandos de BAFF [100;111;143]. Durante un segundo encuentro con el mismo antígeno, los linfocitos B de memoria, con mayor afinidad y de isotipo IgG o IgE o IgA, se activan mas rápidamente y se diferencian a célula plasmáticas productora de anticuerpos de alta afinidad [111;143].

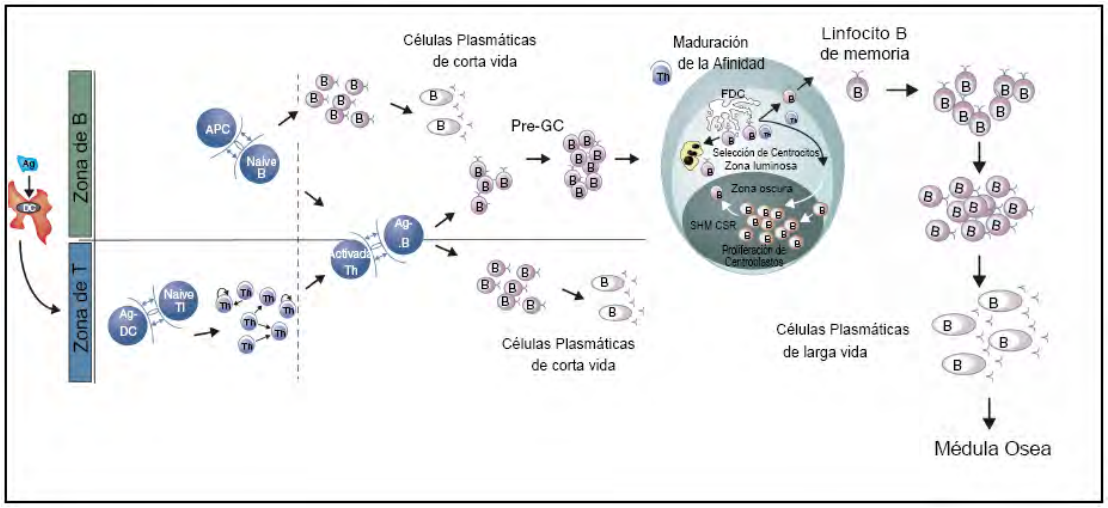


Figura 7. Respuestas intrafolicular y extrafolicular de linfocitos B. Ag-DC: Célula Dendrítica presentando antígenos**, Th:** Linfocito T cooperador, **APC:** Célula presentadora de antígeno, **Pre-GC:** Pre-centro germinal, **FDC:** Célula dendrítica folicular, **SHM:** Hipermutación somática, **CSR:** Cambio de isotipo. Adaptada de [111;143]

Anticuerpos y sus diversos isotipos.

Los anticuerpos, son proteínas solubles producidas por las células plasmáticas. Son producidos de forma clonal y tienen la misma especificidad que el BCR del linfocito B que los produce, ya que están formados por las mismas dos cadenas pesadas y ligeras, sin el dominio transmembranal e intracelular. Se pueden clasificar en diferentes isotipos de acuerdo a la región constante de sus cadenas pesadas, lo cual les confiere su actividad biológica. Cinco isotipos de anticuerpos existen en ratones y humanos: IgM, IgD, IgG, IgE e IgA. El isotipo IgG esta formado por cuatro subclases llamadas en el humano: IgG1, IgG2, IgG3 e IgG4 y en el ratón: IgG1, IgG2a o IgG2c, IgG2b, IgG3 e IgG4.

IgM

Es el primer isotipo de inmunoglobulina que se rearregla. Es producida durante la respuesta primaria, y por ello generalmente es de baja afinidad, lo que se compensa ya que su forma soluble se produce en pentámeros unidos por una cadena J, lo que aumenta su avidez. Debido a su estructura pentamérica es muy eficiente en la unión de complemento y participa también en mediar la opsonización y fagocitosis [150].

IgD

Se encuentra expresada en la membrana de los linfocitos B naive maduros. Se expresa conjuntamente con IgM por splicing alternativo, y es probablemente más eficiente que IgM para la transducción de señales. Su expresión se pierde en los linfocitos B de memoria. Su forma soluble no es muy abundante y su vida media en suero es de aproximadamente 2.8 días [150].

IgA

Se encuentra de forma mayoritaria en las mucosas, el calostro y la leche materna. Es producida en forma de monómeros o dímeros unidos por una cadena J. En el humano se encuentran dos isotipos IgA1 e IgA2. Después de su producción, el receptor polimérico de inmunglobulinas (PIgR) la transporta a través de la barrera epitelial. El PIgR es cortado después del transporte y una parte queda unida a la cadena J, llamado componente secretor. Por ello, y por su extensa glicosilación, los anticuerpos IgA secretores son particularmente resistentes a las proteasas. Su papel es principalmente neutralizante pudiendo unirse a virus, y evitando la adherencia de bacterias a las mucosas. Es muy poco opsonizante y solo algunos neutrófilos expresan el receptor de IgA. También unen pobremente al complemento y son poco inflamatorias, probablemente para mantener un ambiente anti-inflamatorio en las mucosas [150].

IgE

Es la menos abundante y tiene la vida media mas corta de todas las inmunoglobulinas en suero. La producen principalmente células plasmáticas en los pulmones y la piel y después de ser producida se une rápidamente al

receptor FcεRI de alta afinidad, donde puede permanecer durante semanas a meses en la superficie de basófilos y células cebadas. Cuando el complejo IgE-FcεRI es entrecruzado por su antígeno multivalente, se provoca la degranulación de estas células, liberando mediadores inflamatorios potentes como histamina. Aunque su producción y respuesta se encuentra relacionada con el control de parásitos, su inapropiada producción es una de las causas de alergia y anafilaxis [150].

IgG

Es la inmunoglobulina más abundante en el suero y característica de la respuesta secundaria de anticuerpos. Tiene una vida media larga en el suero, de aproximadamente 3 semanas. Esta subdividida en cuatro subclases de las cuales IgG1 e IgG3 son mejores en unión al complemento y para facilitar la fagocitosis [150].

Es muy importante mencionar que el cambio de isotipo, se encuentra en parte determinado por la presencia de citocinas en el medio. La presencia de IL-4 polariza hacia el isotipo IgE mientras que la presencia de IFN-γ induce la producción de IgG2a. TGF-β por su parte induce la producción de IgA [151].

INTERACCION DE SEÑALES CON EL BCR

Como se mencionó anteriormente, la señal del BCR es insustituible para el desarrollo y función de los linfocitos B, sin embargo, estos expresan otros receptores, que participan en la activación o regulación del linfocito B en forma conjunta con el BCR. A continuación se presenta algunos ejemplos de estos receptores.

CD40

La activación de linfocitos B durante una respuesta T dependiente requiere la interacción contacto dependiente con linfocitos Th activados. Las citocinas producidas por estos linfocitos, son importantes, sin embargo no son suficientes para suplir el contacto. La interacción a través del MHC II-TCR juega un papel importante en este proceso, sin embargo la estimulación eficiente de linfocitos B con membranas de linfocitos T que proporcionaban señales independientemente del TCR, llevaron al descubrimiento de CD154 o CD40L expresado en linfocitos T, que se une al receptor CD40 expresado en los linfocitos B [152]. Su importancia se hizo evidente cuando se relacionaron mutaciones del gen que codifica para CD154, con el síndrome de hiper-IgM ligado al X (HIGM) [146-148], cuyos síntomas incluyen deficiencias en el cambio de isotipo, la formación de centros germinales, respuesta de memoria y la recurrente infección con patógenos oportunistas. CD40 es miembro de la familia de receptores de TNF (TNFR), y aun cuando su estructura y vías de señalización no son similares a las del BCR, actúan de una manera sinérgica, a través de TRAF2 [153]. Ambos receptores activan cinasas como ERK, p38 e inducen la activación de los factores de transcripción NF-κB, NF-AT y AP-1. La señal de CD40 es insustituible para la inducción del cambio de isotipo en los linfocitos B, solo algunos linfocitos B1 en la cavidad peritoneal pueden producir anticuerpos de isotipo a IgA de manera T independiente [151].

CD19/ CD21

CD19 es una molécula expresada en la superficie de los linfocitos B desde etapas tempranas de su desarrollo y funciona modulando el umbral de la señal del BCR para la activación del linfocito B. El complejo CD19/CD21 esta compuesto por al menos 4 proteínas unidas no covalentemente: CD19, CD21, CD88 y CD255 [154]. Los fragmentos iC3b/C3d/g del complemento, se unen a antígenos, formando complejos que además de ser multivalentes y entrecruzar mejor el BCR, se unen a CD21 el cual señaliza a través de CD19. La capacidad de CD19 de modificar las respuestas de Ca^{2+} se relaciona con su actividad para amplificar la actividad de proteína cinasa de las cinasas src, en particular mediante la activación de Lyn [155]. Lo cual disminuye el umbral de activación de la señal del BCR cuando se presentan de manera conjunta.

BAFFR/BCMA/TACI

Las interacciones entre receptores y ligandos de la familia del Factor activador de linfocitos B de la familia de TNF (BAFF) y un ligando inductor de proliferación (APRIL), son probablemente de las señales mas importantes durante la respuesta de anticuerpos T-independiente, además de estar involucradas en la supervivencia de los linfocitos B. Son miembros de la familia de TNF. BAFF existe en forma soluble y de membrana, mientras que APRIL solo se expresa en forma soluble [156]. Ambos son expresados por monocitos, macrófagos y células dendríticas [157;158]. Los linfocitos B expresan tres diferentes receptores de BAFF/APRIL. El Activador transmembranal, modulador de calcio e interactor del ligando de ciclofilina (TACI), el antígeno de maduración de linfocitos B (BCMA) y el receptor de BAFF (BAFFR). BAFF se une específicamente a BAFFR, y APRIL a BCMA y TACI [159]. BAFF se une BAFFR induciendo señales de supervivencia, como se puede comprobar en ratones deficientes de BAFF o BAFFR donde el desarrollo de linfocitos B se detiene en el estadio T1 [160]. TACI por el contrario, aunque también induce señales de supervivencia, es una molécula mas bien reguladora de los linfocitos B. En los ratones deficientes de TACI los linfocitos B tienen un fenotipo hiperrespondedor y los ratones desarrollan

eventualmente enfermedades autoinmunes tipo lupus eritrematoso sistémico (SLE) y cáncer linfoide [161]. La señal de BAFFR induce la degradación de TRAF3, permitiendo la acumulación de la cinasa inductora de NF-κB (NIK) y el procesamiento alterno de NF-κB, promoviendo la translocación del complejo p52/RelB esencial para la supervivencia de los linfocitos B [156].

TLRs

Aun cuando los TLRs pertenecen a los recetores del sistema inmune innato, también se ha demostrado su expresión en las células del sistema inmune adaptativo como linfocitos B [162-164]. Además, su participación en la respuesta de anticuerpos se conoce empíricamente desde que se utilizan preparaciones de microorganismos como adyuvantes, para inducir la producción de anticuerpos contra moléculas poco inmunogénicas como proteínas solubles.

La contribución directa de los TLRs en la activación de los linfocitos B, fue descrita por primera vez por Leadbetter *et al* quienes mostraron que una activación eficiente de los linfocitos B factor reumatoide positivos (RF^+), se logra únicamente cuando estos reconocen complejos antigénicos de IgG-cromatina, proporcionando señales a través del BCR que reconoce los anticuerpos y posiblemente TLR9 reconociendo la cromatina [165]. Estudios posteriores mostraron que en efecto, TLR9 era el responsable del reconocimiento de la cromatina [166-168] y que además señales a través de TLR7 tienen el mismo efecto sobre los linfocitos B [169].

Los TLRs participan durante la activación de linfocitos B, particularmente MZB o B1 en respuestas T-independientes [170] y en su diferenciación a células plasmáticas productoras de IgM [171]. Sin embargo, la estimulación mediante TLR9 les confiere también mejores capacidades como APC [172]. Durante la respuesta T-dependiente, se ha propuesto, que las señales de TLRs junto con la estimulación del BCR y la cooperación del linfocito T mediante interacciones CD40-CD40L actúan de forma sinérgica para lograr la activación máxima de linfocitos B [173].

Los TLRs también se han relacionado con el mantenimiento de la memoria. Bernasconi y Lanzavecchia proponen, mediante experimentos con linfocitos B humanos *in vitro*, que la estimulación mediante ligandos de TLRs, sin la presencia de la señal de BCR, es capaz de inducir una proliferación moderada de los linfocitos B de memoria pero no de los naive, por lo que la presencia de microorganismos comensales o infecciones posteriores, aun con diferentes patógenos, pueden proporcionar los ligandos de TLRs necesarios para mantener las clonas de linfocitos B de memoria adquiridas previamente [174-176].

Las señales de TLRs en la inducción y modulación del cambio de isotipo también se han analizado. Uno de los primeros estudios abordando este tema, por Liu *et al* mostró que la estimulación de linfocitos B con CpG induce la expresión de T-bet, el cual inhibe el cambio de isotipo a IgG1 e IgE [177]. También se ha demostrado la necesidad de la señal de TLR9/MyD88 para el cambio de isotipo a IgG2a e IgG2b en la producción de autoanticuerpos durante el SLE [178].

Recientemente se mostró la importancia de las señales de TLR7 y TLR9 en la respuesta de anticuerpos contra antígenos virales. Heer *et al* demostraron que durante la infección con influenza, la señal de TLR7 activado por el RNA viral, juega un papel importante en la producción de anticuerpos. Actuando directamente sobre el linfocito B, induciendo la producción de IgG2a/c, e indirectamente, mediante la inducción de la producción de IFN I, probablemente en células dendríticas plasmacitoides, el cual aumenta la producción de IgG2a/c y reduce la expresión de IgG1, modulando de esta forma el cambio de isotipo [179]. La participación de la señal de TLR9 en el cambio de isotipo a IgG2a tras la inmunización con partículas similares a virus (VLPs), confirma la participación de las señales de los TLRs en la respuesta de anticuerpos antiviral [180].

Aun cuando la participación de los TLRs durante diferentes procesos de la respuesta de linfocitos B esta ampliamente documentada, la necesidad de las señales de TLRs para la inducción de la respuesta de anticuerpos sigue en controversia. En el 2005, Pasare y Medzhitov, publicaron que en ratones deficientes de MyD88, tras la inmunización con Ovoalbúmina (OVA) y LPS como adyuvante, la respuesta de anticuerpos se encontraba severamente afectada, por lo que los TLRs eran necesarios para la inducción de una respuesta eficiente de anticuerpos [181]. Sin embargo a finales del 2006, Gavin *et al* del laboratorio de Nemazee y Beutler publicaron lo contrario, inmunizando ratones deficientes de MyD88 y TRIF con el antígeno T-dependiente Trinitrofenol-hemocianina (TNP-Hy) en presencia de adyuvante completo de Freund o adyuvante incompleto de Freund. Estos ratones mostraron títulos similares de anticuerpos a los de los ratones silvestres, concluyendo que las señales de TLRs no son necesarias para la inducción de la respuesta de anticuerpos [182]. También, contrariamente a los artículos de Bernasconi, se publicó que las señales de TLR4 y TLR9 inducen la proliferación de linfocitos B de memoria *in vitro* pero no *in vivo* [183]. Recientemente se publicó, mediante transferencia de linfocitos B $MyD88^{-/-}$, que las señales intrínsecas de MyD88 en linfocitos B no es necesaria para la inducción, pero amplifica la respuesta de anticuerpos [184].

PLANTEAMIENTO DEL PROBLEMA

Las señales producidas por los TLRs activan la respuesta inmune innata, la cual inicia y modula la respuesta inmune adaptativa. Sin embargo, la expresión de TLRs en las células del sistema inmune adaptativo, como los linfocitos B, sugiere la posible interacción de señales a través de estos receptores y el BCR. Aun cuando la necesidad de las señales de TLRs para la inducción de anticuerpos se encuentra todavía en controversia, se ha demostrado la participación de los TLRs intracelulares en la respuesta de anticuerpos contra antígenos virales. Sin embargo, no se ha estudiado si los TLR2 y TLR4, participan directa o indirectamente en la activación de los linfocitos B antígeno específicos. Por ello, en el presente trabajo se estudió la participación de las señales directas e indirectas de los TLRs en el linfocito B durante la inducción de la respuesta de anticuerpos contra las porinas de *S. typhi*.

HIPÓTESIS

Las señales intrínsecas de TLR2 y TLR4 en el linfocito B cooperan con la activación vía BCR (específica) para la inducción de anticuerpos tanto IgM como las diferentes subclases de IgG contra antígenos bacteriales como las porinas de *S. typhi*.

OBJETIVO GENERAL

Determinar la importancia de las señales de TLRs en los linfocitos B durante la respuesta de anticuerpos contra las porinas de *S. typhi*.

OBJETIVOS PARTICULARES

- Determinar la expresión de TLRs en las principales subpoblaciones esplénicas de linfocitos B.
- Definir la importancia de las señales de TLRs durante la respuesta de anticuerpos anti-porinas.
- Determinar la participación del IFN-I en el cambio de isotipo a las diferentes subclases de IgG en la respuesta humoral anti-porinas.
- Analizar la capacidad de las porinas de *S. typhi* para inducir la activación de linfocitos B y células dendríticas.
- Comparar la importancia de las señales indirectas vs. directas en el linfocito B, de TLR2 y TLR4 para la producción de anticuerpos.

MODELO EXPERIMENTAL

Para analizar la participación de los TLRs en la respuesta de anticuerpos contra antígenos de origen bacteriano se utilizó como antígeno modelo las porinas de *S. typhi*.

Las porinas son proteínas de la membrana externa (OMPs) de las bacterias gram-negativas, mitocondrias y plástidos. El término porina fue utilizado por primera vez por Nakae para clasificar a las proteínas que formaban poros en la membrana externa de *Escherichia coli* [185]. *S. typhi* expresa en su superficie principalmente las porinas OmpC, OmpF [186] con un peso molecular de ~36 y 38 kDa respectivamente y las más recientemente descritas OmpS1 [187] y OmpS2 [188]. Sin embargo estas dos últimas han sido escasamente estudiadas. OmpC y OmpF pertenecen al grupo de proteínas principales según la clasificación de Di Rienzo, Nakamura e Inouye en 1978 [189] y Osborn y Wu en 1980 para OMPs [190]. Se incluyen en esta categoría por expresarse en aproximadamente 100,000 moléculas por bacteria [191]. Son un componente principal en la membrana externa, se ensamblan como trímeros y cada subunidad consiste en 16 β-plegadas antiparalelas unidas por asas con algunas α-hélices intercaladas. La forma cilíndrica que caracteriza a las porinas se forma al cerrarse las estructuras β-plegadas de forma pseudocíclica, mediante un enlace iónico entre el extremo carboxilo de la hoja β-16 y el extremo amino de la hoja β-1. Forman poros o canales que permiten la difusión inespecífica de pequeñas moléculas hidrofílicas, iones y nutrientes de aproximadamente 600 Da al interior de la bacteria y de desechos hacia el exterior [185;192], mientras que contribuyen a la exclusión de antibióticos, sales biliares, etc. [193;194]. Las estructuras de las porinas presentan una gran estabilidad al efecto de sales biliares y son altamente resistentes a la acción de algunas proteasas [192]. Entre las bacterias gram-negativas, las porinas son proteínas altamente conservadas. La gran homología de las porinas se confirmó a nivel inmunológico mediante la reactividad cruzada en el reconocimiento de diferentes porinas de *S. enterica* serovar Typhimurium y *E. coli* por anticuerpos monoclonales [195].

En *S. typhi*, OmpF es osmorregulada, reprimiéndose su expresión en medios con alta osmolaridad, mientras que la expresión de OmpC es constitutiva. La regulación es diferente en *E. coli* donde ambas porinas son osmoreguladas [196].

El descubrimiento de la capacidad inmunogénica de las porinas de *S. typhi* es el resultado de la búsqueda de antígenos para el desarrollo de una vacuna contra la fiebre tifoidea. En 1988 Isibasi *et al* purificaron las OMPs de *S. typhi*, y utilizando como modelo de infección ratones inoculados intraperitonealmente con *S. typhi* suspendida en mucina, demostraron que 30μg de OMPs eran capaces de inducir una protección del 100% contra $500DL_{50}$ de *S. typhi* [197]. Calderón *et al* en 1986 habían cuantificado por ELISA los anticuerpos de pacientes con fiebre tifoidea, encontrando que estos eran principalmente anti-porinas, anti-flagelina y en mucha menor cantidad anti-LPS [198]. Verdugo-Rodríguez *et al* por su parte, en 1993, también describieron la presencia de anticuerpos específicos para OMPs en pacientes con diagnóstico de fiebre tifoidea [199]. Estos datos, sumados a la creciente información sobre la estructura de la membrana externa, sugerían que los anticuerpos contra estas proteínas tenían un papel importante en la protección contra *S. typhi*. En 1989 Ortiz *et al* demostraron, que aún cuando la preparación de OMPs de *S. typhi* contiene más de 10 proteínas diferentes, los sueros de los pacientes con fiebre tifoidea, tanto en fase aguda como convaleciente reconocen principalmente 2 proteínas cuyos pesos moleculares correspondían a las porinas OmpC y OmpF [200]. Tras esta observación, Isibasi *et al*, purificaron las porinas de *S. typhi*, por el método de Nikaido modificado [193] y demostraron que la inmunización con 10μg de estas proteínas inducen una protección del 90% frente a $500DL_{50}$ de *S. typhi* [201] . Posteriormente se demostró que la inmunización de ratones con 10μg de porinas de *S. typhi*, inducen la producción de anticuerpos durante toda la vida del ratón y que además son bactericidas [202]. También se ha logrado llevar la preparación de porinas de *S. typhi* hasta estudios de fase clínica, donde se observó la producción de anticuerpos anti-porinas en voluntarios vacunados con 10μg de porinas de *S. typhi* [203].

Las porinas de *S. typhi* también inducen una respuesta de linfocitos T, González *et al*, inmunizaron con OMPs de *S. typhi* ratones de diferentes haplotipos de MHC y midieron la capacidad de proliferación de las células de bazo al ser estimuladas con porinas, encontrando que la mejor respuesta se obtiene en ratones H-2^k y H-2^a, después en H-2^b y la menor en H-2^d [204]. Más recientemente, Martín Orozco *et al* demostraron que macrófagos activados con IFN γ, durante la infección con *S. typhimurium*, producen péptidos capaces de unirse a moléculas del MHC I K^b [205]. Finalmente, Díaz Quiñónez reportó dos epitopos para linfocitos T $CD8^+$ de la porina OmpC (132-RNTDFFGL y 73-ENTNGRSL) de *S. typhimurium* restringidos a MHC I K^b capaces de inducir una respuesta citotóxica *in vitro.* Cabe mencionar que ambos epitopos se encuentran presentes también en la porina OmpC de *S. typhi* [206].

Tras el descubrimiento de la importancia de los TLRs en a inmunidad innata, Massari *et al* demostraron que la capacidad inmunoestimuladora de las porinas de *Neisseria* era mediada por TLR2 [27], recientemente Galdiero *et al* demostraron que lo mismo sucedía con las porinas de *H. influenza* [26] y Ray *et al* mostraron el aumento en la expresión de moléculas coestimuladoras, (CD80 y CD86), TLR2, TLR6 y la producción de IgA en presencia de IL-6, en linfocitos B1 (B1a y B1b) y el aumento en la expresión de TLR2, TLR6, IgM, IgG2a e IgA de membrana en linfocitos B2 de cavidad peritoneal después de la estimulación con porinas de *Shigella dysenteriae* [207-209]. Sin embargo, no existen datos concluyentes sobre la interacción de las porinas de *S. typhi* y los componentes del sistema inmune innato.

MATERIALES Y MÉTODOS

Bacterias

Para la producción de porinas se utilizó *Salmonella enterica* serovar Typhi (*S. typhi)* cepa ATCC No. 9993. Como control para la presencia de contaminantes se realizó una extracción similar a la utilizada para obtener porinas de una cepa isogénica de *S. typhi* mutante VALE39 *ΔompF* Km^R *ΔompC* Cm^R (*S. typhi ΔompFΔompC*) [202].

Animales de experimentación

Se utilizaron ratones silvestres cepa C57BL/6 (B6) de los laboratorios Charles River (Sulzfeld, Alemania). Los ratones $MyD88^{-/-}$ [210], $TRIF^{-/-}$[63], $TLR4^{-/-}$ [211], $TLR2^{-/-}$ [212], y μMT [213] con fondo génetico B6 fueron obtenidos del "Institut für Labortierkunde" (Universidad de Zürich, Suiza). Los ratones deficientes del receptor para IFN-I en fondo genético B6 ($IFNAR^{-/-}$) [214] fueron proporcionados por el Dr. Martin Bachmann (Cytos AG, Schlieren, Suiza). Todos los ratones fueron mantenidos en cajas individualmente ventiladas, en condiciones libres de patógenos específicos (SPF). Se utilizaron ratones entre 6 y 9 semanas de edad. Los experimentos fueron realizados según las buenas prácticas de manejo de animales.

RT-PCR

$1x10^6$ linfocitos B foliculares o MZB se aislaron por citometría de flujo. El RNA se obtuvo mediante RNAeasy microkit (Qiagen) se trató con DNAsa y se cuantificó mediante espectrofotómetría. Se utilizó 1μg para la sítesis de cDNA utilizando la enzima Superscript II (Invitrogen). La expresión de TLRs se analizó mediante RT-PCR utilizando Taq polimerasa (Sigma) y los siguientes oligonucleótidos [215]. **TLR1** 5' tct ctg aag gct ttg tcg ata ca, 5' gac aga gcc tgt aag cat att cg, **TLR2** 5'ctg caa gag ctc tat att tcc, 5'aac cag gat ttg agc cag agc, **TLR3** 5'ttg tct tct gca cga acc 5'cgc aac gca agg att tta tt **TLR4** 5'agc aga gga gaa agc atc tat gat gc, 5'ggt tta ggc ccc aga gtt ttg ttc tcc, **TLR5** 5'act gaa ttc ctt aag cga cgt a, 5'aga aga taa agc cgt gcg aaa, TLR6 5' aac agg ata cgg agc ctt ga, 5' cca gga aag tca gct tcg tc **TLR7** 5' ttc cga tac gat gaa tat gca cg,

5’tga gtt tgt cca gaa gcc gta at **TLR8** 5’ ggc aca act ccc ttg tga tt , 5’cat tg ggt gct gtt gtt tg **TLR9** 5’ccg caa gac tct att tgt gct gg, 5’tgt ccc tag tca ggg ctg tac tca. Como cotrol se amplifico el gen de **β-actina** 5’tca tga agt gtg acg ttg aca tcc gt, 5’cct aga agc att tgc ggt gca cga tg.

Purificación de porinas de S. typhi

Las porinas se purifican por el método de Nikaido modificado [203]. Se cultivó *S. typhi* ATCC 9993 o *S. typhi ΔompFΔompC* en 10 litros de medio mínimo A (70g K_2HPO_4, 30g KH_2PO_4, 10g $(NH_4)_2SO_4$, 5g citrato de sodio, 10g extracto de levadura, 50g glucosa, 0.1% $MgSO_4$) hasta obtener una densidad óptica de 1.0 a 540 nm que corresponde a la fase logarítmica tardía del crecimiento de la bacteria. Se cosechó centrifugando a 7,000 rpm 15 minutos a 4°C, y se lavó 2 veces con una solución de Tris-Cl 0.05M (pH 7.7). La pastilla bacteriana se pesó, se resuspendió en 50 ml de solución de Tris-Cl 0.05M (pH 7.7) y se lisaron las bacterias mediante ultrasonido. La suspensión se centrifugó a 7,000 rpm 30 minutos a 4°C para eliminar las bacterias no lisadas. El sobrenadante se trató con 25μl de DNAsa 10000 U/ml y 25μl de RNAsa 10000 U/ml y 2.77 ml de solución de $MgCl_2$ 1 M por cada 10g de biomasa húmeda obtenida de la cosecha, por 30 minutos a 37°C. Esta solución se ultracentrifugó a 45 000 rpm durante 45 min a 4°C. El sedimento se resuspendió en 100ml de solución de Tris HCl-dodecilsulfato de sodio (SDS) 2%, se incubó 30 minutos a 32°C en agitación y se ultracentrifugó a 40000 rpm 30 min a 20°C. El sedimento resultante se resuspendió en 25 ml de solución de Tris HCl-SDS 2%, se incubó nuevamente por 30 min a 32°C en agitación y se ultracentrifugó a 40000 rpm 30 minutos a 20°C. El sedimento se resuspendió en 20 ml de solución reguladora de Nikaido SDS 1% (Tris 0.05 M, NaCl 0.4 M, EDTA 0.005 M) pH 7.7. La mezcla se incubó 2 horas a 37°C en agitación y se ultracentrifugó a 40000 rpm 45 minutos a 20°C. En este último paso se recuperó el sobrenadante, el cual se purificó en una columna de Sephacryl S-200 (XK100 Pharmacia) a un flujo de 5ml/min con amortiguador de Nikaido SDS 0.5% pH 7.7. Se colectó el primer pico obtenido en el cromatograma que corresponde a las porinas OmpC y OmpF. En el caso de *S. typhi ΔompFΔompC* se colectaron las fracciones

correspondientes. La preparación se dializó en PBS pH 7.2 para eliminar el SDS. La concentración de proteínas se determinó por el método de Lowry y la identidad de las proteínas mediante electroforesis en gel de poliacrilamida y SDS (SDS-PAGE). El contenido de LPS se determinó mediante un ensayo de lisado de amebocitos de *Limulus* (LAL; Endosafe® KTA, Charles River Endosafe Laboratories, Charleston SC), cuyo límite de detección es 0.2ng LPS/ μg proteína.

Digestión de porinas con proteinasa K.

Se mezclaron las porinas de *S. typhi* con un volumen equivalente de 2x solución reguladora para proteinasa K. Se agregaron 10μg de proteinasa K (Roche) y se incubaron a 55°C por 4 horas, seguido de la inactivación de la proteinasa K a 85°C por 45 min. La digestión total de las porinas se comprobó por medio de SDS-PAGE.

Esquema de inmunización y toma de muestras

Los ratones fueron inmunizados intraperitonealmente el día 0 y reinmunizados el día 15 con 10 μg de porinas de *S. typhi*. Las muestras de sangre fueron colectadas de la cavidad retroorbital en los días indicados. Una vez separados los sueros por centrifugación, fueron congelados a −70° C hasta su análisis.

ELISA para detección de anticuerpos anti-porinas

Se recubrieron microplacas de 96 pozos (Costar®) con porinas de *S. typhi* en una concentración de 10μg/ml en solución reguladora de carbonatos y se incubó durante 1 hora a 37°C y durante la noche a 4°C. Se lavaron con una solución PBS-Tween 0.1% (PBS-T) y se bloquearon con PBS-leche 5% durante 1 hora a 37°C. Posteriormente, se lavaron 4 veces con la PBS. Se realizó una dilución inicial 1:40 de las muestras de suero y posteriormente se realizaron diluciones seriadas 1:2 en PBS – leche 5% que se incubaron durante 2 horas a 37°C. Nuevamente se lavaron las placas 4 veces con PBS-T. Los anticuerpos secundarios de conejo anti IgM, IgG, IgG1 e IgG2b (Zymed, San Francisco, CA), anti- IgG2a/c (Acris GmbH, Germany; Los

ratones B6 expresan el isotipo IgG2c que da una respuesta cruzada con anti-IgG2a) y anti IgG3 (Rockland, Gilbertsville, PA) de ratón conjugados a peroxidasa, se añadieron en una dilución 1:1000 en PBS – leche 5% y se incubaron durante 2 horas a 37°C. Por último se lavaron 4 veces con PBS-T y se revelaron agregando una solución de 0.5mg/ml de o-fenilendiamina y 0.83% H_2O_2 en solución amortiguadora de citratos, dejando incubar durante 10 minutos a temperatura ambiente y en la oscuridad. La reacción se detuvo agregando H_2SO_4 2.5 N. La absorbancia a 492 nm se determinó utilizando un lector de microplacas (Tecan). El título de anticuerpos se determinó como el número de diluciones consecutivas del suero antes de perder la señal de la reacción (triple del valor promedio de los controles negativos).

Producción de células dendríticas derivadas de médula ósea.

Se obtuvo una suspensión de células de médula ósea de tibias y fémures de ratones B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$. Se cultivaron en una densidad de 1x10^6/ml en RPMI 5% FCS y 5% de sobrenadante de células X63-GM-CSF (proporcionadas por el Dr. Antonius Rolink, Basel) durante 6 a 7 días. Se purificaron mediante un gradiente de densidad de Optiprep 20% (Sigma-Aldrich), centrifugándose a 70 x g 15 min, y colectándose la interfase que contiene >98% de células dendríticas derivadas de médula ósea (BMDCs) viables.

Purificación de linfocitos B y células dendríticas.

Se obtuvo una suspensión celular de bazos de ratones B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ mediante digestión con Colagenasa tipo II (Gibco). Para la obtención de linfocitos B o células dendríticas, las células fueron resuspendidas en una solución reguladora para separación magnética: PBS-MACs. Posteriormente se utilizó el anticuerpo monoclonal anti-CD45R (B220)-microbeads para linfocitos B o anti-CD11c de ratón (N418)-microbeads para células dendríticas (Miltenyi Biotec) para la separación magnética siguiendo el protocolo indicado por el proveedor. Para la separación de linfocitos B foliculares o MZB por citometría de flujo se tiñeron

$5x10^7$ células de bazo con anti-B220 APC, CD21/35-FITC y CD23 PE (BD biosciences) y se separaron con un FACsVantage cell sorter.

Transferencia de linfocitos B en ratones μMT.

Los linfocitos B de ratones B6, MyD88$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ fueron aislados como se describió previamente. Se verificó la pureza mediante la expresión de CD19 (>96% CD19$^+$ analizado por citometría de flujo). Las células se resuspendieron en una concentración de $1x10^8$ /ml en BSS, se filtraron para eliminar agregados celulares y $5x10^7$ células del fenotipo correspondiente fueron transferidas por vía intravenosa a los ratones μMT. Tres horas después de la transferencia se procedió con el esquema de inmunización de porinas.

Estimulación de linfocitos B o células dendríticas

$2x10^6$ linfocitos B o células dendríticas B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ fueron sembradas en RPMI 5%. Se estimularon con: medio, 1 μg/ml de porinas de *S. typhi*, 2 ng/ml o 100 ng/ml LPS de *S. typhi* (Proporcionado por el Dr. John S. Gunn, Ohio State University, Columbus, OH), 1 μg/ml de porinas de *S. typhi* digeridas con proteinasa K, o la preparación de *S. typhi ΔompFΔompC.* Después de 24 horas las células se cosecharon, se lavaron y se prosiguió con la tinción para citometría.

Anticuerpos y tinción para citometría de flujo

$1x10^6$ linfocitos B, células dendríticas, o células dendríticas derivadas de médula ósea fueron resuspendidos en PBS-FACs y teñidas 20 minutos a 4°C en la oscuridad con combinaciones de los siguientes anticuerpos monoclonales en 100μl de PBS-FACs: anti-CD19 PE (Biolegend), anti-CD11c PE , anti-CD40 APC, anti-I-A/I-E APC y anti- CD86 APC (BD Biosciences Pharmingen). Después de la incubación fueron lavados con 3 ml de PBS-FACs y resuspendidas en 300ul de una solución de 7-amino-actinomicina D (7AAD) (Sigma Aldrich) 2.5 μg/ml PBS-FACs. Se incubaron 20 min mas a 4°C para excluir las células muertas. Finalmente fueron analizados con un

citómetro de flujo FACS Calibur utilizando CellQuest software (BD Biosciences) para la adquisición de datos y el análisis.

Estimulación de celulas HEK 293-TLR.

2×10^5 células 293-TLR2, 293-TLR2/TLR6, 293-TLR4, o 293-TLR5 (Invivogen) se cultivaron en DMEM 10% +10 μg/ml de blasticidina. Se estimularon con: medio, 1 μg/ml de porinas de *S. typhi*, 1 μg/ml de porinas de *S. typhi* digeridas con proteinasa K (Roche) y como control se utilizó para 293-TLR2 y 293-TLR2/TLR6, 1μg/ml de zimosan, para 293-TLR4 2 ng/ml o 1μg/ml de LPS de *S. typhi* y para 293-TLR5 1μg/ml de flagelina. Después de 24 horas se recolectaron los sobrenadantes y la concentración de IL-8 se determinó por ELISA según las recomendaciones del proveedor.

Análisis estadístico

Los análisis estadísticos fueron realizados con la prueba t de Student cuando se compararon dos grupos, o ANOVA con prueba posterior de Bonferroni cuando se compararon más de dos grupos. Se utilizó Prism 5.0 (GraphPad Software) para el análisis. Un valor de P <0.05 se consideró significativo.

Soluciones y Medios de Cultivo

Solución salina balanceada (BSS)

KH_2PO_4	0.6 g	
$CaCl_2*2H_2O$	0.14 g	
$Na_2HPO_4*2H_2O$	0.24 g	
KCl	0.4 g	
D-Glucosa	1 g	
NaCl	8 g	
H_2O	1 L	pH 7.2. Esterilizada por filtración. (2μm)

Solución reguladora de fosfatos salina (PBS)

NaCl	8 g	
KCl	0.2 g	
Na_2HPO_4	1.15 g	
KH_2PO_4	0.2 g	
H_2O	1 L	pH 7.2.

Solución reguladora para citometría de flujo (PBS-FACS)
1L PBS
10 mM EDTA
2% Suero fetal bovino
0.05 % Azida de sodio

Solución reguladora para separación magnética (PBS-MACS)
1L PBS
2 mM EDTA
2% Suero fetal bovino

Solución reguladora de Carbonatos

$NaHCO_3$	7 g	
NaH_2CO_3	2.8 g	
H_2O	1 L	pH 9.5

Solución reguladora de Citratos

Acido citrico	4.1 g	
Citrato de Sodio	29 g	
H_2O	1 L	pH 5.6

2x solución reguladora para proteinasa K.

Tris-HCl 100mM
$CaCl_2$ 2mM pH 8.0

RPMI 5%

500 ml RPMI 1640 (Gibco)
5% Suero fetal bovino
100 U/ml penicilina
100 μg/ml estreptomicina

DMEM 10%

500 ml DMEM (Gibco)
10% Suero fetal bovino
100 U/ml penicilina
100 μg/ml estreptomicina

RESULTADOS

Expresión de TLRs en linfocitos B del bazo.

Como se mencionó anteriormente los linfocitos B expresan en su superficie, además del BCR, diversos receptores que contribuyen a la activación o la regulación de los linfocitos B, entre ellos los TLRs. Para estudiar la participación de los TLRs en la respuesta de linfocitos B contra antígenos bacteriales como las porinas de *S. typhi*, primero comprobamos la expresión de los TLRs en las principales poblaciones esplénicas de linfocitos B: linfocitos foliculares y MZB. Las poblaciones se separaron por citometría de flujo de suspensiones celulares de bazo, teñidas con los anticuerpos monoclonales anti-CD21, anti-CD23 y anti-B220. Las poblaciones se determinaron como linfocitos foliculares: $CD21^{low}CD23^{hi}B220^{+}$ y MZB: $CD21^{hi}CD23^{low}B220^{+}$. Posteriormente se analizó la expresión de TLRs mediante RT-PCR. Como se puede observar en la figura 8, los linfocitos B, tanto foliculares como MZB expresan todos los TLRs a excepción de TLR3, al menos a nivel de mRNA. Estos datos concuerdan con las publicaciones recientes, donde se demuestra la expresión de TLRs en las diferentes poblaciones de linfocitos B [163;164].

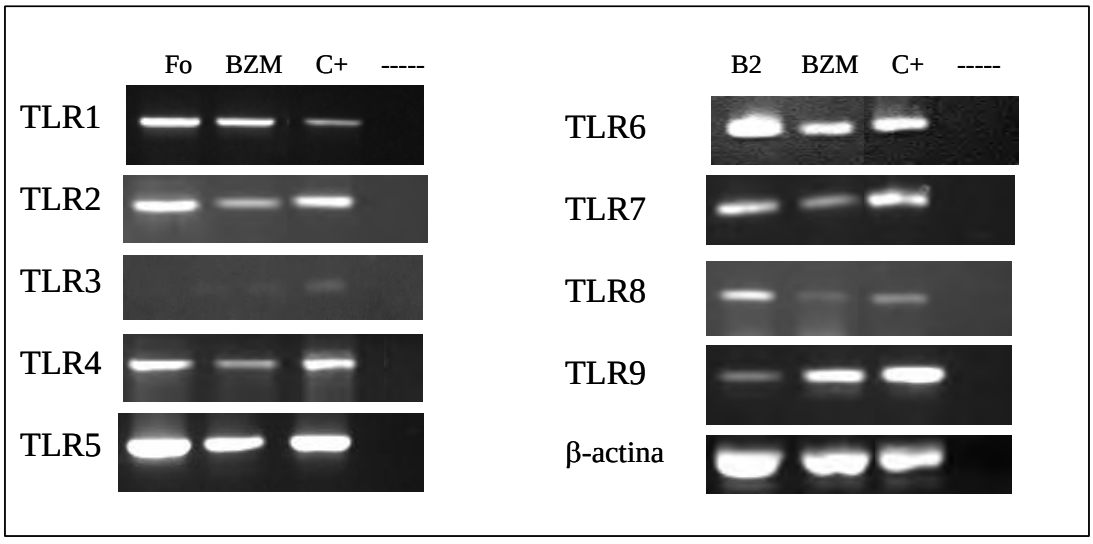


Figura 8. Expresión de TLRs en linfocitos B foliculares y MZB. La expresión de TLR1-9 se determinó por RT-PCR a partir de RNA de linfocitos B foliculares o MZB del bazo de ratones B6, aislados por citometría de flujo. Las poblaciones se determinaron como linfocitos B foliculares: $CD21^{low}CD23^{hi}B220^{+}$ y MZB $CD21^{hi}CD23^{low}B220^{+}$.

Papel de los TLRs en la respuesta de anticuerpos contra porinas.

La inmunización con porinas de *S. typhi*, induce eficientemente una respuesta de anticuerpos específica, aun sin la coadministración de adyuvantes [202;203]. Además, se ha reportado que las porinas de bacterias gram-negativas son capaces de activar la señal de TLR2 [27;28;216] induciendo el aumento de expression de moléculas co-estimuladoras y del MHC II en macrófagos, linfocitos B y células dendríticas [26;217;218], la producción de citocinas por macrófagos y el aumento en la expression del BCR en linfocitos B [208;209]. Para analizar la contribución de la señal de TLRs en la respuesta de anticuerpos anti-porinas específica, se analizó primeramente la inducción de anticuerpos IgM e IgG anti-porinas en ratones deficientes de las dos proteínas adaptadoras principales en la vía de señalización de TLRs. Como se mencionó en la introducción, 2 proteínas adaptadoras controlan las señales de los TLRs: MyD88 transduce las señales de todos los TLRs excepto TLR3, mientras que TRIF transduce la señal de TLR3 y de la vía de inducción de IFN-β de TLR4 cuando este se encuentra en el compartimiento endosomal [65;210;219]. Ratones B6, MyD88$^{-/-}$ y TRIF$^{-/-}$ fueron inmunizados con 10 μg de porinas de *S. typhi*, y los títulos de anticuerpos fueron determinados durante un periodo de 30 días. Tanto los ratones MyD88$^{-/-}$ como los TRIF$^{-/-}$ mostraron una respuesta de anticuerpos IgM e IgG anti-porinas disminuida, aun después de la reinmunización en el día 15 (Fig. 9A, B). El análisis de la producción de las difrentes subclases de IgG en el día 30 post inmunización mostró que los ratones MyD88$^{-/-}$ produjeron significativamente menos IgG1, IgG2a/c, IgG2b, e IgG3 anti-porinas (Fig. 9C). De la misma forma, los ratones TRIF$^{-/-}$ presentaron menores títulos anticuerpos anti-porinas de las subclases IgG2a/c, IgG2b e IgG3, aunque la producción de IgG3 no se encuentra tan afectada como las otras (Fig. 9C). Estos resultados indican que la señal de TLRs aumenta la respuesta de anticuerpos anti-porinas de *S. typhi* y que ambas vías de señalización, a través de MyD88$^{-/-}$ y de TRIF$^{-/-}$, contrabuyen al efecto adyuvante observado.

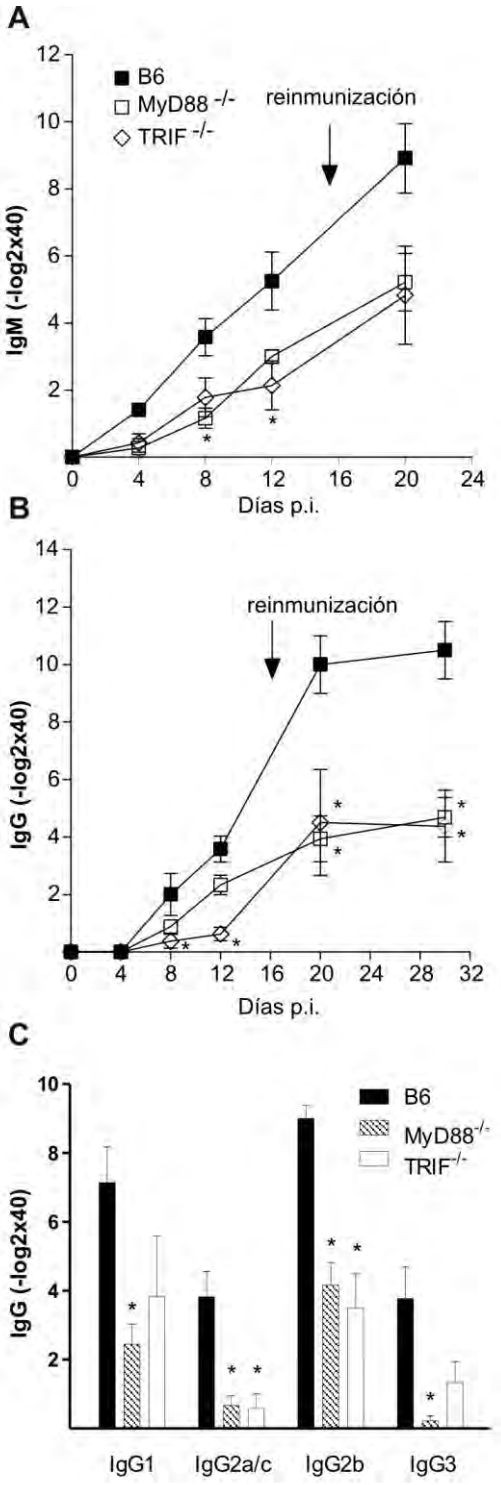


Figura 9. Respuesta de anticuerpos anti-porinas de *S. typhi* en ratones MyD88$^{-/-}$ y TRIF$^{-/-}$. Ratones MyD88$^{-/-}$, TRIF$^{-/-}$ y B6 fueron inmunizados con 10 μg de *S. typhi* el día 0 y 15. Los títulos de anticuerpos (A) IgM y (B) IgG anti-porinas se analizaron en los días indicados. (C) Las subclases de IgG anti-porinas: IgG1, IgG2a/c, IgG2b, e IgG3 se determinaron el día 30 post inmunización. El promedio ± SEM de 6 ratones por grupo se muestra en la gráfica. El análisis estadístico se realizó mediante ANOVA. Las diferencias significativas entre los ratones B6 y MyD88$^{-/-}$ o TRIF$^{-/-}$ en cada tiempo analizado estan indicadas. (*, P<0.05).

TLR2 y TLR4 modulan la respuesta de anticuerpos anti porinas de *S. typhi*

Como se mencionó anteriormente, las porinas de bacterias gram-negativas pueden unirse a TLR2 [27;28], por lo que las porinas de *S. typhi* podrían también inducir señales a través de TLR2. Además, la presencia de trazas de LPS por debajo del límite de detección en la preparación de porinas de *S. typhi* podrían proporcionar señales de estimulación vía TLR4 lo que explicaría porque los ratones TRIF$^{-/-}$ presentaron una respuesta deficiente de anticuerpos anti-porinas. Para esudiar la contribución de señales mediadas por TLR2 y TLR4 en la respuesta de anticuerpos anti-porinas, ratones B6, TLR2$^{-/-}$ y TLR4$^{-/-}$ fueron inmuniuzados con porinas de *S. typhi* siguiendo el mismo protocolo de inmunización mencionado anteriormente. Los ratones deficientes de TLR2$^{-/-}$ no presentaron ninguna deficiencia en la producción de IgM anti-porinas, sin embargo, los títulos de IgG anti-porinas eran 4 veces menores (Fig. 10A, B). Los ratones TLR4$^{-/-}$ presentaron una disminución más severa en la producción de anticuerpos IgM e IgG anti-porinas (Fig. 10A, B). El análisis de las subclases de IgG 30 días después de la inmunización mostró una ligera reducción en los titulos de IgG1 e IgG2b y una alteración mas drástica en la producción de IgG2a/c e IgG3 en los ratones TLR2$^{-/-}$ (Fig. 10C). Los ratones TLR4$^{-/-}$ mostraron una reducción general en los títulos de todas las subclases siendo particularmente afectada la producción de IgG2a/c e IgG2b (Fig. 10C). Estos datos demuestran que ambas señales, de TLR2 y TLR4 aumentan la producción de anticuerpos anti-porinas y ajustan o modulan la producción de las subclases de IgG.

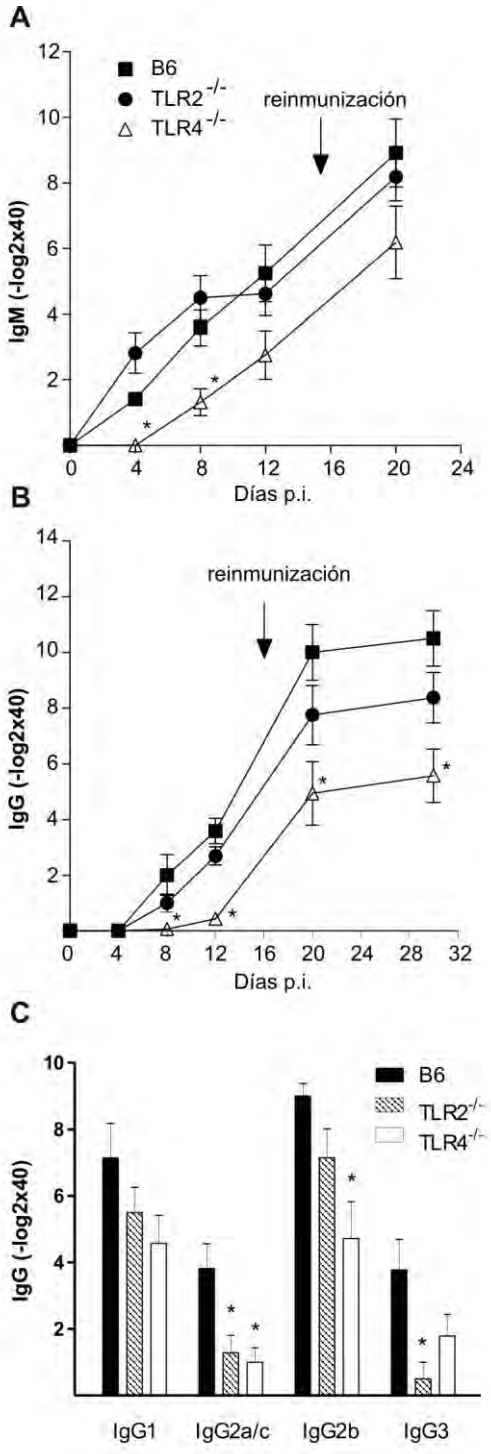


Figura 10. Respuesta de anticuerpos anti-porinas de *S. typhi* en ratones TLR4$^{-/-}$ y TLR2$^{-/-}$ Ratones TLR2$^{-/-}$, TLR4$^{-/-}$ y B6 fueron inmunizados con 10 μg de *S. typhi* el día 0 y 15. Los títulos de anticuerpos (A) IgM y (B) IgG anti-porinas se analizaron en los días indicados. (C) Las subclases de IgG anti-porinas: IgG1, IgG2a/c, IgG2b, e IgG3 se determinaron el día 30 post inmunización. El promedio ± SEM de 6 ratones por grupo se muestra en la gráfica. El análisis estadístico se realizó mediante ANOVA. Las diferencias significativas entre los ratones B6 y TLR2$^{-/-}$ o TLR4$^{-/-}$ en cada tiempo analizado estan indicadas. (*, P<0.05).

La respuesta de anticuerpos anti-porinas no depende del IFN I.

Estudios de la respuesta de anticuerpos contra antígenos virales han demostrado que el IFN I inducido por señales derivadas de TLRs juegan un papel importante en la inducción y el balance de las diferentes subclases de IgG [179;220]. Ya que las señales de TLR4 mediadas por TRIF llevan a la producción de IFN-β [65], se analizó en los siguientes experimentos la participación del IFN I en la respuesta humoral anti-porinas. Ratones B6 y ratones deficientes del receptor de IFN I ($IFNAR^{-/-}$) fueron inmunizados con 10 μg de porinas de *S. typhi* siguiendo el protocolo de inmunización establecido. Tanto la producción de IgM como de IgG anti-porinas fueron equivalentes en ratones $IFNAR^{-/-}$ y B6 (Fig. 11A, B). De forma similar el análisis de las subclases de IgG 30 días después de la inmunización, mostró ninguna diferencia entre los ratones $IFNAR^{-/-}$ y B6 (Fig. 11C). Por lo que concluimos que la respuesta de IFN I no es necesaria para la inducción de anticuerpos IgM, e IgG, ni participa en la producción de las diferentes subclases de IgG.

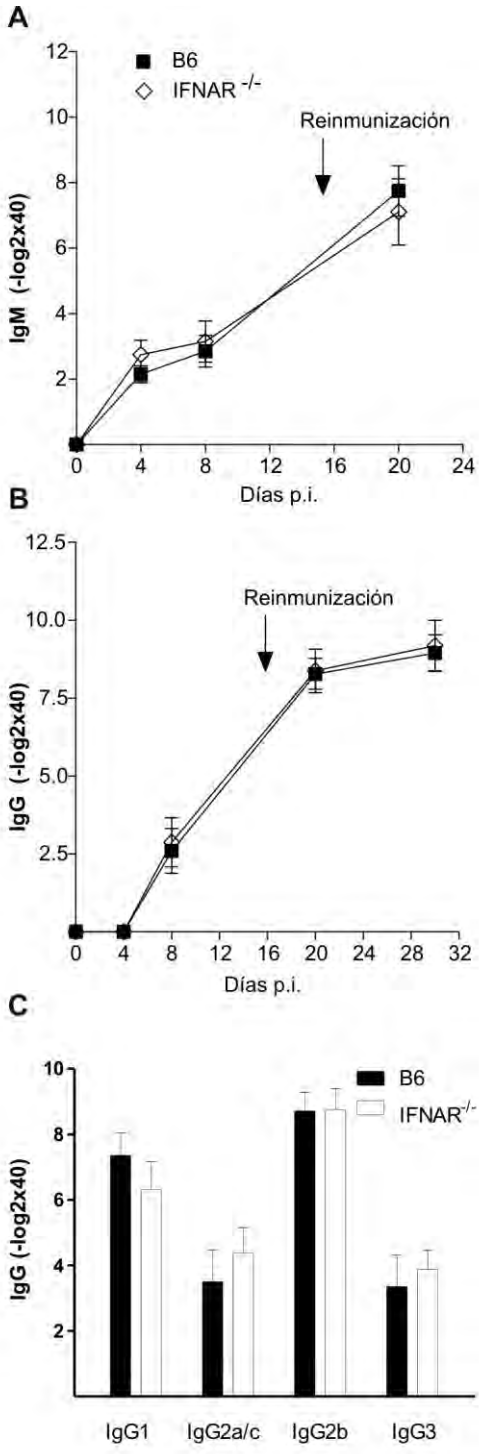


Figura 11. Respuesta de anticuerpos anti-porinas en ratones IFNAR$^{-/-}$.

Ratones IFNAR$^{-/-}$ y B6 fueron inmunizados con 10 μg de *S. typhi* el día 0 y 15. Los títulos de anticuerpos (A) IgM y (B) IgG anti-porinas se analizaron en los días indicados. (C) Las subclases de IgG anti-porinas: IgG1, IgG2a/c, IgG2b, e IgG3 se determinaron el día 30 post inmunización. El promedio ± SEM de 6 ratones por grupo se muestra en la gráfica. El análisis estadístico se realizó mediante la prueba de t de Student.

Las porinas de *S. typhi* activan los linfocitos B a través de TLR2 o TLR4.

Las señales de TLR2 y TLR4 pueden participar en diferentes procesos durante la respuesta inmune para la producción de anticuerpos anti-porinas. Una posibilidad es que los ligandos de TLR2 y TLR4 aumenten la producción de anticuerpos mediante la activación directa de los linfocitos B en cooperación con la señal del BCR. Para analizar si la preparación de porinas puede activar directamente a los linfocitos B, se aislaron linfocitos B de ratones B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ y se estimularon con 1 μg/ml de porinas de *S. typhi*. Después de 24 horas se analizó la expresión de marcadores de activación. Los linfocitos B TLR2$^{-/-}$, TLR4$^{-/-}$, y TRIF$^{-/-}$ aumentaron la expresión de CD86 y MHC II (Fig. 12A, B). Sin embargo, los linfocitos B MyD88$^{-/-}$ no se activaron (Fig. 12A, B), sugiriendo que las porinas pueden activar a los linfcitos B a través de TLR4 o TLR2, pero en ausencia de ambas señales los linfocitos B no se activan.

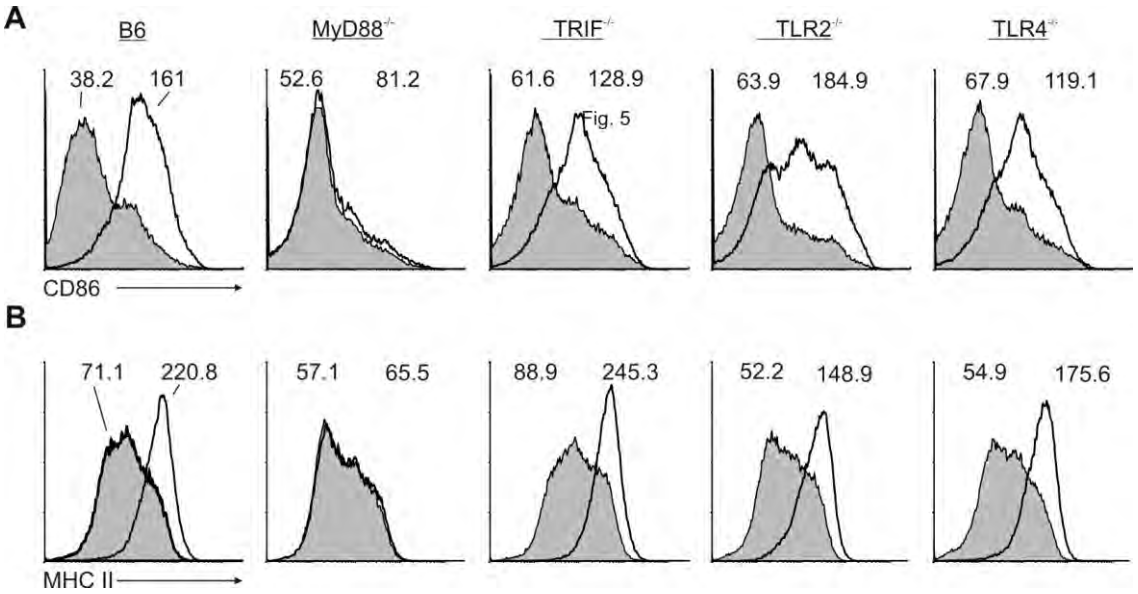


Figura 12. Activación de linfocitos B con porinas de *S. typhi*.

Linfocitos B de ratones B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ se estimularon con 1 μg/ml de porinas de *S. typhi* o medio de cultivo. Después de 24 horas, la expresión de (A) CD86 y (B) MHC II en células CD19+ (linfocitos B) fue analizada por citometría de flujo. Los histogramas representan 1 de 3 experimentos independientes. El histograma en gris representa las células incubadas solo con medio de cultivo y el histograma en blanco las células incubadas con porinas. Los valores en los histogramas representan la intensidad media de fluorescencia (IMF) de la respectiva población.

La preparación de porinas de *S. typhi* activan BMDCs a través de TLR4.

Las células dendríticas juegan un papel muy importante durante la respuesta humoral. No solo en la activación de linfocitos T cooperadores, sino también al interactuar directamente con los linfocitos B mediante la unión de receptores como BAFFR o TACI y la producción de citocinas, proporcionando señales de supervivencia o regulación [221]. Para analizar la capacidad de las porinas para estimular células dendríticas, se produjeron células dendríticas derivadas de medula ósea (BMDCs) de B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$. Y se estimularon con 1 μg/ml de porinas de *S. typhi*. El análisis de la expresión de CD86 y MHC II mostró que a diferencia de los linfcitos B, las BMDCs TLR4$^{-/-}$ no se activaron, mientras que las TLR2$^{-/-}$ presentaron una activación normal. (Fig.13A, B). Ya que las BMDCs se activan de forma TLR4 dependiente, es posible que trazas de LPS presentes en la preparación de porinas por debajo del límite de detección del ensayo de *Limulus* participen en su activación. Para analizar esto, se estimularon BMDCs con 100 y 2 ng/ml de LPS, siendo la segunda una concentración debajo del límite de detección del ensayo de *Limulus*. La estimulación aun con la concentración baja de LPS, fue capaz de inducir el aumento en la expresión de CD86 y MHC II en BMDCs B6 yTLR2$^{-/-}$ pero no TLR4$^{-/-}$ indicando la enorme sensibilidad de estas al estímulo con LPS.

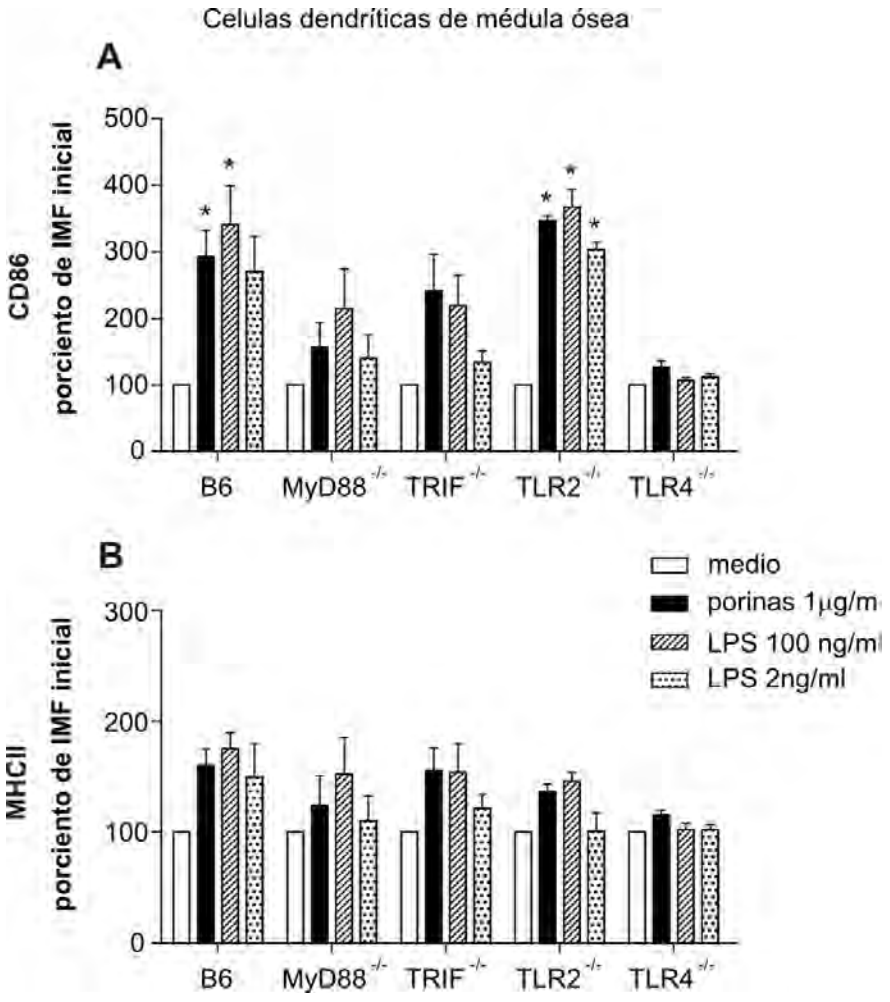


Figura 13. Activación de BMDC con porinas de *S. typhi.*

BMDCs de ratones B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ se estimularon con 1 μg/ml de porinas de *S. typhi*, 100 ng/ml de LPS, 2 ng/ml de LPS o medio de cultivo. Después de 24 horas, la expresión de (A) CD86 y (B) MHC II en BMDCs fue analizada por citometría de flujo. Los resultados se expresan como porcentaje de IMF inicial y representan el promedio ± SEM de 4 experimentos independientes. El análisis estadístico se realizó mediante ANOVA. Las diferencias significativas entre las células estimuladas com medio (control) y el estímulo respectivo del mismo fenotipo estan indicadas. (*, P<0.05).

Las porinas de *S. typhi* activan de forma diferente linfocitos B y células dendríticas del bazo.

Aun cuando los estudios con BMDCs proveen de información importante para el análisis de la activación de células dendríticas, para realizar una comparación en la respuesta y activación entre linfocitos B y células dendríticas se aislaron células dendríticas de bazo. Linfocitos B y células dendríticas de bazo de B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ se estimularon con 1 μg/ml de porinas de *S. typhi*. El análisis de la expresión de CD86 (Fig. 14A) y CD40 (Fig. 14C), mostraron que, a diferencia de las BMDCs, las células dendríticas TLR4$^{-/-}$ de bazo son estimuladas con porinas. Es importante mencionar que tanto los linfocitos B como las DCs de los ratones TLR2$^{-/-}$ fueron capaces de aumentar la expresión de CD86 (Fig. 14A, B), sin embargo los linfocitos B TLR2$^{-/-}$ no aumentaron la expresión de CD40 después de la estimulación con porinas (Fig. 14D). Para analizar si las diferencias observadas en la activación de linfocitos B y células dendríticas pueden deberse a la contribución de señales mediadas por trazas de LPS, linfocitos B y células dendríticas de bazo fueron estimulados con 100 y 2 ng/ml de LPS. Los linfocitos B de ratones B6, TLR2$^{-/-}$ y parcialmente de MyD88$^{-/-}$ y TRIF$^{-/-}$ fueron activados con la concentración alta de LPS, pero no respondieron al estímulo con la concentración baja. (Fig.14B, D). Contrariamente, aun esta baja concentración indujo el aumento en la expresión de CD86 y CD40 en células dendríticas de ratones B6 y TLR2$^{-/-}$ (Fig. 14A, C). Tanto los linfocitos B como las células dendríticas TLR4$^{-/-}$ no se activaron con la alta ni la baja concentración de LPS (Fig. 14). Estos resultados sugieren que la activación de las células dendríticas puede contribuir a la respuesta de anticuerpos anti-porinas y sobretodo sugieren que la presencia de ligandos de TLR2 y TLR4 en la preparación de porinas activa de manera diferente a linfocitos B y células dendríticas.

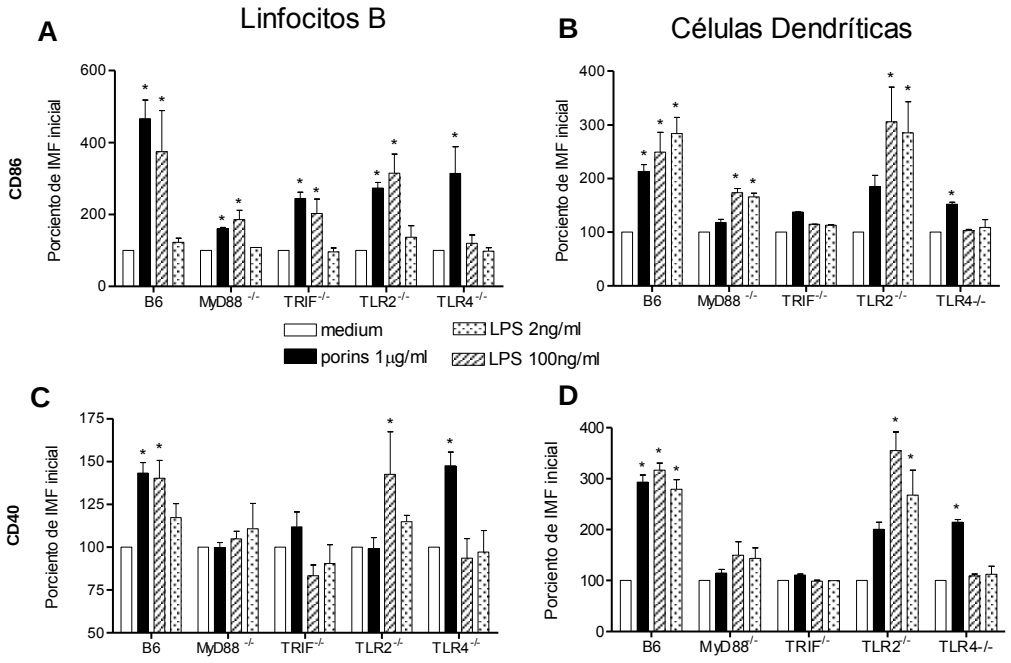


Figura 14. Comparación en la activación de linfocitos B y células dendríticas de bazo con porinas de *S. typhi*.

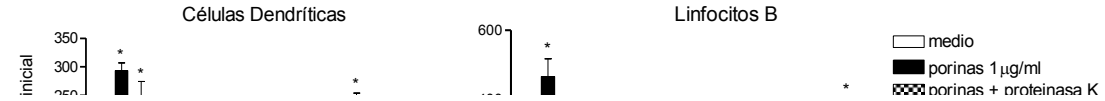
(A, C) Linfocitos B o (B, D) células dendríticas del bazo de ratones B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ se estimularon con 1 μg/ml de porinas de *S. typhi*, 100 ng/ml de LPS, 2 ng/ml de LPS o medio de cultivo. Después de 24 horas, la expresión de (A,C) CD86 y (B,D) CD40 fue analizada por citometría de flujo. Los resultados se expresan como porcentaje de IMF inicial y representan el promedio ± SEM de 4 experimentos independientes. El análisis estadístico se realizó mediante ANOVA. Las diferencias significativas entre las células estimuladas com medio (control) y el estímulo respectivo del mismo fenotipo estan indicadas. (*, P<0.05).

Las porinas de *S. typhi* son posibles ligandos de TLR2.

Como se mencionó anteriormente, las porinas de varias bacterias gram-negativas se han reportado como ligandos de TLR2, por lo que el estímulo mediado por TLR2 observado con la preparación de porinas, puede provenir directamente de las porinas de *S. typhi*. Sin embargo, un problema en la investigación de ligandos de TLRs de origen bacterial, es la contaminación con otros PAMPs durante la purificación de las moléculas de interés. Para analizar la capacidad de las porinas de activar al TLR2, se realizó la siguiente serie de experimentos.

Para analizar la presencia de otros ligandos de TLR2 como lipoproteinas o peptidoglicanas en la preparación de porinas, linfocitos B y células dendríticas de bazo de los diferentes ratones, se estimularon con porinas o porinas digeridas con proteinasa K. Mientras que las células dendríticas $TLR2^{-/-}$ aumentaron la expresión de CD86 con ambas preparaciones las dendríticas $TLR4^{-/-}$ no se activaron con las preparación de porinas digeridas con proteinasa K, sugiriendo la ausencia de otros ligandos no proteicos en la preparación de porinas (Fig. 15).

Para descartar la presencia de otros ligandos proteicos en la preparación, que pudieran inducir activación mediante TLR2 u otro TLR, como TLR5-flagelina, se estimularon los linfocitos B y células dendríticas de los diferentes ratones con una preparación obtenida siguiendo el mismo proceso para la purificación de porinas de *S. typhi*, de una cepa deficiente de los genes codificantes de OmpC y OmpF (*ΔOmpCΔOmpF)*. La estimulación de células dendriticas y linfocitos B deficientes de TLR4 con *ΔOmpCΔOmpF* no indujo la activación de las celulas, lo que indica que la preparación de porinas no contiene otros ligandos de TLR proteicos como flagelina (Fig. 16). Sin embargo cabe mencionar que la preparación de *ΔOmpCΔOmpF* contiene otras proteínas que no se encuentran normalmente en la preparación de porinas de *S. typhi* además de niveles detectables de LPS, probablemente por la diferencia en la organización de la membrana externa de las bacterias deficientes de porinas (Fig.16).



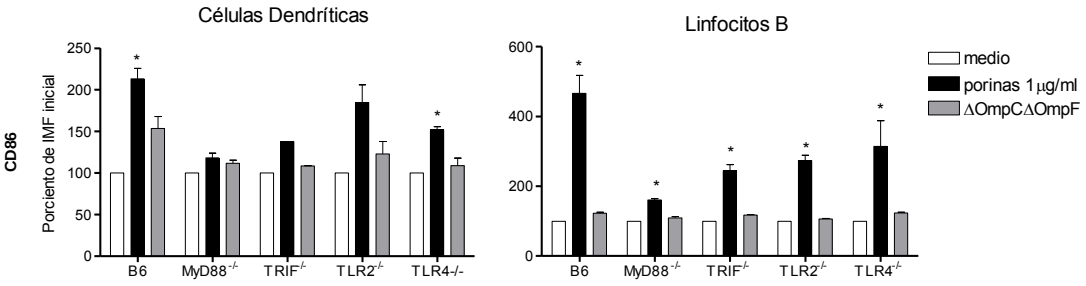


Figura 16. Comparación en la activación de linfocitos B y células dendríticas de bazo con la preparación de *S. typhi ΔOmpCΔOmpF.*

(A) Linfocitos B o (B) células dendríticas del bazo de ratones B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ y TLR4$^{-/-}$ se estimularon con 1 μg/ml de porinas de *S.* Typhi, con la preparación de *S. typhi ΔOmpCΔOmpF o medio de cultivo*. Después de 24 horas, la expresión de CD86 fue analizada por citometría de flujo. Los resultados se expresan como porcentaje de IMF inicial y representan el promedio ± SEM de 4 experimentos independientes. El análisis estadístico se realizo mediante ANOVA. Las diferencias significativas entre las células estimuladas con medio (control) y el estímulo respectivo del mismo fenotipo estan indicadas. (*, P<0.05).

Finalmente, para analizar si las porinas son capaces de activar al TLR2 se realizaron estimulaciones *in vitro* de líneas celulares HEK293 transfectadas establemente con TLR2, TLR2/6 y como control TLR4 y TLR5. Estas células, tras la activación de los receptores por un ligando, producen IL-8 que se secreta al sobrenadante. La estimulación de 293-TLR2 y 293 TLR2/6 con porinas pero no porinas tratadas con proteinasa K, induce la producción de IL-8. La estimulación de 293-TLR4 con la preparación de porinas sugiere la presencia de LPS en la preparación, sin embargo ya que la estimulación se pierde tras la digestión de las porinas, la presencia de LPS o el posible efecto acarreador de las porinas tiene que ser analizado en detalle en estudios futuros. La estimulación de 293-TLR5 con porinas no induce la producción de IL-8 lo que confirma la ausencia de flagelina en la preparación (Fig. 17).

Los resultados de estos experimentos sugieren fuertemente el papel de las porinas de *S. typhi* como ligandos de TLR2, y la ausencia de otros ligandos de TLRs en la preparación a excepción de trazas de LPS.

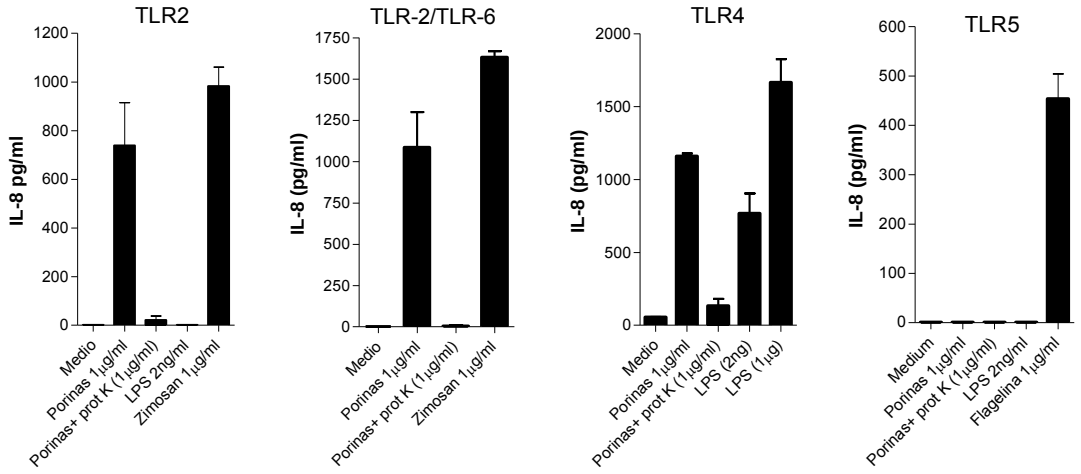


Figura 17. Estimulación de células HEK 293 transducidas con TLRs.
$2x10^5$ células 293-TLR2, 293-TLR2/TLR6, 293-TLR4, o 293-TLR5 (Invivogen) se cultivaron en DMEM 10% FCS y 10 μg/ml de blasticidina. Se adicionaron los estímulos indicados en las gráficas y 24 horas después se recolecto el sobrenadante. La concentración de IL-8 en los sobrenadantes se cuantificó por ELISA.

Las señales directas de TLR2 y TLR4 en los linfocitos B contribuyen a la respuesta de anticuerpos anti-porinas.

Como se mencionó anteriormente, las señales de TLR2 y TLR4 pueden actuar directamente sobre el linfocito B o indirectamente mediante la activación de células del sistema inmune innato como células dendríticas o macrófagos. Para determinar la contribución de las señales intrínsecas de TLR2 y TLR4 en el linfocito B durante la respuesta de anticuerpos anti-porinas, se aislaron linfocitos B del bazo de ratones B6, MyD88$^{-/-}$, TLR4$^{-/-}$, o TLR2$^{-/-}$ y se transfirieron a ratones μMT, que carecen de linfocitos B (solo mantienen una parte de los linfocitos B1-a de isotipo IgA). Después de tres horas, los ratones reconstituidos fueron inmunizados con 10 μg de porinas de *S. typhi* y reinmunizados el día 15. En la ausencia de TLR4 en los linfocitos B, la respuesta de anticuerpos IgM anti-porinas se encuentra reducida (Fig. 18A). Sin embargo después de la reinmunización, los títulos de IgG anti-porinas son comparables con los de ratones transferidos con linfocitos B de ratones B6 (Fig. 18B, C), indicando que la expresión de TLR4 en los linfocitos B juega un papel importante durante la inducción de la respuesta primaria de IgM. Por el contrario, los ratones que recibieron linfocitos B deficientes de TLR2 mostraron una respuesta normal de IgM (Fig. 18), y títulos reducidos de IgG, sugiriendo que las señales de TLR2 en los linfocitos B son importantes para la producción de IgG. Los ratones que recibieron linfocitos B MyD88$^{-/-}$ mostraron una producción deficiente de IgM e IgG (Fig. 18). De manera conjunta los experimentos realizados confirman la importancia de las señales de TLR2 y TLR4 en linfocitos B para la producción eficiente de anticuerpos anti-porinas y su contribución en el balance del cambio de isotipo a las diferentes subclases de IgG.

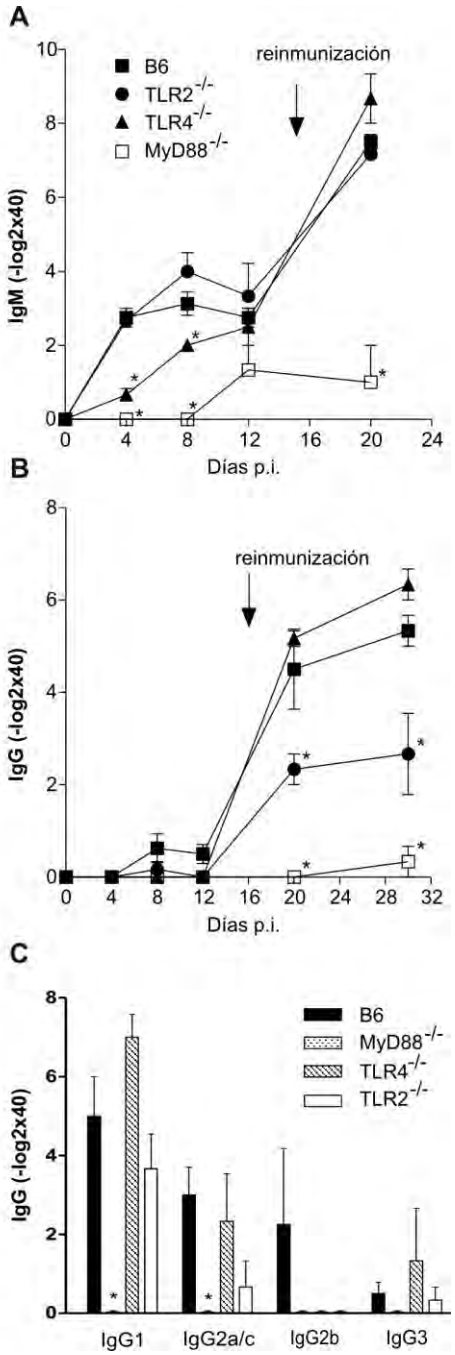


Figura 18. Contribución de las señales intrínsecas de TLR2 y TLR4 en linfocitos B durante la respuesta de anticuerpos anti-porinas. 5×10^7 linfocitos B de ratones B6, MyD88$^{-/-}$, TLR4$^{-/-}$ or TLR2$^{-/-}$ se transfirieron a ratones μMT por vía intravenosa. Tres horas después se inmunzaron los ratones con 10 μg de porinas de *S. typhi* y se reinmunizaron el día 15. Los títulos de (A) IgM y (B) IgG anti-porinas se analizaron en los días indicados. (C) Las subclases de IgG anti-porinas: IgG1, IgG2a/c, IgG2b, e IgG3 se determinaron el día 30 post inmunización. El promedio ± SEM de 4 ratones por grupo se muestra en la gráfica. El análisis estadístico se realizó mediante ANOVA. Las diferencias significativas entre los ratones transferidos con linfocitos B B6 y los transferidos con MyD88$^{-/-}$, TLR4$^{-/-}$ o TLR2 $^{-/-}$ en cada tiempo analizado estan indicadas. (*, P<0.05).

DISCUSIÓN

Los ligandos de TLRs se utilizan en muchas preparaciones vacunales como adyuvantes para aumentar la respuesta inmune específica, sin embargo, la necesidad de las señales de TLRs para la inducción y mantenimiento de la respuesta de anticuerpos se encuentra todavía en discusión. Se ha propuesto que la señal de TLRs es necesaria para la inducción de anticuerpos [181], sin embargo, ratones deficientes de cualquier señal de TLRs todavía son capaces de montar una respuesta inmune de anticuerpos específica [182]. Recientemente se publicó también que los TLRs son dispensables para la inducción de la respuesta de anticuerpos, no obstante, estas señales aumentan la intensidad de la respuesta [184]. Una desventaja en estos estudios ha sido el uso de antígenos modelo para las inmunizaciones, ya que se utilizaron OVA o TNP-Hy, los cuales pueden comportarse de manera diferente a las moléculas derivadas de patógenos. En el presente estudio, se analizó la participación de los TLRs en la respuesta de anticuerpos contra antígenos de origen bacteriano, mediante la inmunización con porinas de *S. typhi*, que se encuentran en estudio como vacuna candidata contra la fiebre tifoidea.

La inmunización de ratones MyD88$^{-/-}$, TRIF$^{-/-}$ o TLR4$^{-/-}$ con porinas de *S. typhi* indujo una respuesta de anticuerpos reducida tanto de IgM como de IgG. Mientras que la inmunización de ratones TLR2$^{-/-}$ solo mostró una reducción en los títulos de IgG anti-porinas, lo que indica que ambos TLRs participan, aunque probablemente de forma diferente, durante la respuesta de anticuerpos anti-porinas.

En los ratones TLR4$^{-/-}$ y TRIF$^{-/-}$, se presentó una reducción general de las subclases de IgG, mientras que en los TLR2$^{-/-}$ y MyD88$^{-/-}$ la reducción se presentó de forma más pronunciada en IgG2a/c e IgG3. Lo que indica el papel de estas moléculas en la modulación del cambio de isotipo, particularmente la vía TLR2/MyD88 para la producción de IgG2a/c e IgG3. Estos resultados corroboran estudios que mostraron que las señales a través de MyD88 son necesarias para el cambio de isotipo a IgG2a [178], y sugieren

un fenómeno similar para la producción de IgG3. Esto podría estar mediado por la inducción TLR dependiente de IFN-γ ya que se ha demostrado que este participa en el cambio de isotipo a IgG3 [222], sin embargo esto debe ser analizado a profundidad en estudios futuros.

La vía de señalización de TLR4 a través de TRIF induce la producción de IFN-β [65], y se ha demostrado que la presencia de IFN I durante una respuesta anti-viral modula el cambio de isotipo [179;180;220;223]. Aun cuando los ratones TRIF$^{-/-}$ mostraron una deficiencia en la producción de anticuerpos anti-porinas y una reducción general en el cambio de isotipo a las subclases de IgG, los ratones IFNAR$^{-/-}$ no presentaron deficiencias en la producción de anticuerpos, por lo que concluimos que el IFN I no participa en la regulación del cambio de isotipo en la respuesta anti-porinas. Esto muestra la diferencia en los mecanismos de acción de los TLRs involucrados en las respuestas anti-virales (endosómicos) y los que reconocen compuestos bacteriales (de membrana). También demuestran que las señales vía TLR4/TRIF participan en la producción de anticuerpos mediante un mecanismo diferente a la síntesis de IFN-β. Una posibilidad, es el aumento en la expresión de MHC II TRIF dependiente, la cual podría hacer más eficiente la cooperación con los linfocitos T.

Las señales de TLR2 y TLR4 pueden participar en diferentes etapas durante la respuesta de anticuerpos anti-porinas. Pueden participar indirectamente activando células del sistema inmune innato como células dendríticas, las cuales una vez activadas producen citocinas que pueden influir en el cambio de isotipo y proveer señales de supervivencia a los linfocitos B como BAFF [156;159], además de activar la respuesta de linfocitos T cooperadores [111;143]. Pero también pueden participar directamente mediante señales intrínsecas en el linfocito B, ya que éstos, como hemos mostrado y se ha publicado, expresan los receptores TLR2 y TLR4 [163;164]. Las señales de TLR podrían actuar por ejemplo, sinérgicamente con la señal del BCR para activar al linfocito B, aumentar sus capacidades de APC o mediar señales de supervivencia.

La estimulación de linfocitos B con porinas de *S. typhi* mostró su capacidad para activar directamente a los linfocitos B. La activación de los linfocitos B TLR2$^{-/-}$ y TLR4$^{-/-}$ pero no de los MyD88$^{-/-}$, mostró que la señal a través de TLR2 o TLR4, MyD88 dependiente, es suficiente para activar a los linfocitos B, pero que en ausencia de ambas, las porinas de *S. typhi* no pueden activar a los linfocitos B, al menos de una manera BCR independiente.

El estímulo de BMDCs con porinas también indujo su maduración, sin embargo, en ausencia de TLR4 no se aumentó la expresión de marcadores de activación, por lo que concluimos que el estímulo de BMDCs con porinas es mediado por TLR4. Es posible que las BMDCs expresen principalmente TLR4 y no otros TLRs, lo que explicaría porque son particularmente sensibles a dosis muy bajas de LPS, por ello se decidió aislar células dendríticas del bazo para realizar el análisis comparativo de células dendríticas con linfocitos B y para comprobar si las células dendríticas son activadas por las porinas exclusivamente vía TLR4.

Al comparar la activación de linfocitos B con células dendríticas se observó la maduración de ambas células con porinas de *S. typhi*, y contrariamente a las BMDCs, las células dendríticas del bazo TLR4$^{-/-}$ también mostraron activación, sugiriendo que estas células también pueden recibir otros estímulos probablemente a través de TLR2. El estímulo con dosis altas de LPS activó tanto a linfocitos B como a células dendríticas de forma TLR4-dependiente, sin embargo, dosis muy bajas como las que podrían estar presentes en las porinas, solo pueden activar a las células dendríticas ya que los linfocitos B no aumentaron su expresión de CD86 o CD40 después del estímulo con 2ng/ml de LPS. Además, las señales de activación de linfocitos B independientes de TLR2 inducen el aumento en la expresión de CD86, pero no de CD40, por lo que el aumento de CD40 podría estar mediado por las señales de TLR2. Todo lo anterior sugiere que las células dendríticas y linfocitos B se activan de manera diferente tras el estímulo con porinas de *S. typhi*.

Como se mencionó anteriormente, se ha demostrado la capacidad de las porinas de algunas bacterias gram-negativas para activar al TLR2 [26-28]. Lo que, en conjunto con la activación de linfocitos B y dendríticas con porinas de *S. typhi*, sugiere que estas sean un ligando de TLR2. Sin embargo, uno de los mayores problemas en la determinación de ligandos de TLRs es la contaminación de las preparaciones con trazas de otros ligandos de TLRs, lo que puede llevar a resultados erróneos. Por ello se realizaron una serie de experimentos para determinar la posible participación de las porinas como ligandos de TLR2. Las células dendríticas de bazo, se activaron tanto con las porinas de *S. typhi*, como con la preparación de porinas digeridas con proteinasa K. Sin embargo, las células dendríticas deficientes de TLR4 solo se activaron en presencia de las porinas, lo que demuestra la ausencia de ligandos de TLR2 no proteicos. Al estimular las células dendríticas con la preparación de la cepa de *S. typhi* deficiente de porinas, una vez más no se observó el estímulo de células dendríticas $TLR4^{-/-}$, lo que sugiere la ausencia tanto de otros ligandos de TLR2 de naturaleza proteica, como de ligandos de otros TLRs como flagelina, (TLR5). Finalmente, la activación de células HEK transfectadas con TLR2, TLR2/6 mediante porinas, pero no porinas digeridas con proteinasa K confirmó nuestras observaciones y sugieren fuertemente que las porinas de *S. typhi* son ligandos de TLR2. El estímulo de las células HEK293-TLR4 con la preparación de porinas, se debe probablemente a la presencia de trazas de LPS, sin embargo, las porinas digeridas con proteinasa K, inducen una menor activación. Esto podría deberse a una propiedad acarreadora de LPS de las porinas, o a un sinergismo de porinas-LPS, sin embargo esto debe ser estudiado con mayor detalle en el futuro.

Finalmente por medio de transferencias de linfocitos B de bazo deficientes de TLR2, TLR4, o MyD88 en ratones carentes de linfocitos B, se demostró que la señal de TLR4 es importante para la respuesta inicial de anticuerpos, en particular para la producción de IgM, mientras que después de la reinmunización las señales de TLR4 en los linfocitos B no son necesarias para la producción de IgG. Ya que los ratones $TLR4^{-/-}$ presentaron una producción deficiente de IgG tras la reinmunización, es posible que las señales de TLR4 en otras células, como células dendríticas o macrófagos

participen indirectamente para la inducción eficiente de la respuesta secundaria. También, ya que *in vitro* los linfocitos B no mostraron activación con las dosis bajas de LPS, es posible que el estímulo a través de TLR4 solo active los linfocitos B que también reciben señales del BCR y por ello no se observa una respuesta policlonal sino específica. A diferencia de TLR4, la señal de TLR2 no es necesaria para la respuesta primaria de IgM, como se puede observar en los títulos de IgM anti-porinas en los ratones que recibieron linfocitos B TLR2$^{-/-}$ que son similares a los títulos de ratones transferidos con linfocitos B B6, sin embargo, la producción de IgG se encuentra disminuida, lo que indica que la señal directa de TLR2 en linfocitos B participa en la respuesta secundaria para el cambio de isotipo. Es posible que mecanismos como el aumento en la expresión de CD40, la cual, como se observó en las estimulaciones de linfocitos B con porinas, depende de TLR2, permitan una mejor cooperación con el linfocito T para un cambio de isotipo mas eficiente, esto correlaciona con las inmunizaciones de los ratones TLR2$^{-/-}$, donde se encontró una disminución sólo en IgG, por lo que la señal de TLR2 participa principalmente directamente sobre el linfocito B.

El hecho de que los títulos de anticuerpos anti-porinas de la subclase IgG2b se encuentren disminuidos en todos los animales transferidos, incluyendo los que recibieron linfocitos B B6, sugiere que estos anticuerpos podrían provenir de una subpoblación de linfocitos B que no se encuentre en el bazo, como podrían ser linfocitos B de cavidad peritoneal. Experimentos donde se realicen transferencias de diferentes subpoblaciones de linfocitos B, podrían resolver esta pregunta.

Es importante señalar, que la estimulación conjunta de los linfocitos B a través del BCR y los TLRs mediante ligandos como las porinas que son a la vez antígenos y PAMPs (Pamptígenos) [224] permiten una producción eficiente de anticuerpos que como se ha observado en experimentos donde se realizaron inmunizaciones pasivas con anticuerpos anti-porinas [197], son protectores ante el reto con *S. typhi*. Por lo que en el futuro, la búsqueda de moléculas con estas características se pueda utilizar para el desarrollo de vacunas.

CONCLUSION

El trabajo realizado permite proponer que:

- Las señales intrínsecas de TLR4 en el linfocito B participan en la respuesta primaria de IgM anti-porinas, mientras que las señales en otras células colaboren para la producción de IgG.
- Las señales directas de TLR2 en el linfocito B inducidas por las porinas, son importantes para la respuesta de IgG y la modulación del cambio de isotipo a las diferentes subclases.
- Las señales de TLRs relacionados con el reconocimiento de PAMPs de origen bacteriano como TLR2 y TLR4 tienen un papel importante en la inducción y la regulación de la respuesta de anticuerpos contra antígenos bacterianos.

REFERENCIAS

1. **Janeway,C.A., Jr. and Medzhitov,R.,** Innate immune recognition. *Annu.Rev.Immunol.* 2002. **20**: 197-216.

2. **Beutler,B.,** Innate immunity: an overview. *Mol.Immunol.* 2004. **40**: 845-859.

3. **Janeway,C.A., Jr.,** Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb.Symp.Quant.Biol.* 1989. **54 Pt 1**: 1-13.

4. **Creagh,E.M. and O'Neill,L.A.,** TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol.* 2006. **27**: 352-357.

5. **Palsson-McDermott,E.M. and O'Neill,L.A.,** Building an immune system from nine domains. *Biochem.Soc.Trans.* 2007. **35**: 1437-1444.

6. **Trinchieri,G. and Sher,A.,** Cooperation of Toll-like receptor signals in innate immune defence. *Nat.Rev.Immunol.* 2007. **7**: 179-190.

7. **Lee,M.S. and Kim,Y.J.,** Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annu.Rev.Biochem.* 2007. **76**: 447-480.

8. **Anderson,K.V., Bokla,L., and Nusslein-Volhard,C.,** Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell* 1985. **42**: 791-798.

9. **Lemaitre,B., Nicolas,E., Michaut,L., Reichhart,J.M., and Hoffmann,J.A.,** The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 1996. **86**: 973-983.

10. **Chiang,C. and Beachy,P.A.,** Expression of a novel Toll-like gene spans the parasegment boundary and contributes to hedgehog function in the adult eye of Drosophila. *Mech.Dev.* 1994. **47**: 225-239.

11. **Taguchi,T., Mitcham,J.L., Dower,S.K., Sims,J.E., and Testa,J.R.,** Chromosomal localization of TIL, a gene encoding a protein related to the Drosophila transmembrane receptor Toll, to human chromosome 4p14. *Genomics* 1996. **32**: 486-488.

12. **Chaudhary,P.M., Ferguson,C., Nguyen,V., Nguyen,O., Massa,H.F., Eby,M., Jasmin,A., Trask,B.J., Hood,L., and Nelson,P.S.,** Cloning and characterization of two Toll/Interleukin-1 receptor-like genes TIL3 and TIL4: evidence for a multi-gene receptor family in humans. *Blood* 1998. **91**: 4020-4027.

13. **Medzhitov,R., Preston-Hurlburt,P., and Janeway,C.A., Jr.,** A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997. **388**: 394-397.

14. **Rock,F.L., Hardiman,G., Timans,J.C., Kastelein,R.A., and Bazan,J.F.,** A family of human receptors structurally related to Drosophila Toll. *Proc.Natl.Acad.Sci.U.S.A* 1998. **95**: 588-593.

15. **Poltorak,A., He,X., Smirnova,I., Liu,M.Y., Van,H.C., Du,X., Birdwell,D., Alejos,E., Silva,M., Galanos,C., Freudenberg,M., Ricciardi-Castagnoli,P., Layton,B., and Beutler,B.,** Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998. **282**: 2085-2088.

16. **Poltorak,A., Smirnova,I., He,X., Liu,M.Y., Van,H.C., McNally,O., Birdwell,D., Alejos,E., Silva,M., Du,X., Thompson,P., Chan,E.K., Ledesma,J., Roe,B., Clifton,S., Vogel,S.N., and Beutler,B.,** Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood Cells Mol.Dis.* 1998. **24**: 340-355.

17. **Coutinho,A., Forni,L., Melchers,F., and Watanabe,T.,** Genetic defect in responsiveness to the B cell mitogen lipopolysaccharide. *Eur.J.Immunol.* 1977. **7**: 325-328.

18. **Sultzer,B.M.,** Genetic control of leucocyte responses to endotoxin. *Nature* 1968. **219**: 1253-1254.

19. **Beutler,B.,** The Toll-like receptors: analysis by forward genetic methods. *Immunogenetics* 2005. **57**: 385-392.

20. **Akira,S., Uematsu,S., and Takeuchi,O.,** Pathogen recognition and innate immunity. *Cell* 2006. **124**: 783-801.

21. **Kawai,T. and Akira,S.,** TLR signaling. *Semin.Immunol.* 2007. **19**: 24-32.

22. **Means,T.K., Wang,S., Lien,E., Yoshimura,A., Golenbock,D.T., and Fenton,M.J.,** Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J.Immunol.* 1999. **163**: 3920-3927.

23. **Tapping,R.I. and Tobias,P.S.,** Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. *J.Endotoxin.Res.* 2003. **9**: 264-268.

24. **Takeuchi,O., Sato,S., Horiuchi,T., Hoshino,K., Takeda,K., Dong,Z., Modlin,R.L., and Akira,S.,** Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J.Immunol.* 2002. **169**: 10-14.

25. **Ozinsky,A., Underhill,D.M., Fontenot,J.D., Hajjar,A.M., Smith,K.D., Wilson,C.B., Schroeder,L., and Aderem,A.,** The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc.Natl.Acad.Sci.U.S.A* 2000. **97**: 13766-13771.

26. **Galdiero,M., Galdiero,M., Finamore,E., Rossano,F., Gambuzza,M., Catania,M.R., Teti,G., Midiri,A., and Mancuso,G.,** Haemophilus influenzae porin induces Toll-like receptor 2-mediated cytokine production in human

monocytes and mouse macrophages. *Infect.Immun.* 2004. **72**: 1204-1209.

27. **Massari,P., Henneke,P., Ho,Y., Latz,E., Golenbock,D.T., and Wetzler,L.M.,** Cutting edge: Immune stimulation by neisserial porins is toll-like receptor 2 and MyD88 dependent. *J.Immunol.* 2002. **168**: 1533-1537.

28. **Massari,P., Visintin,A., Gunawardana,J., Halmen,K.A., King,C.A., Golenbock,D.T., and Wetzler,L.M.,** Meningococcal porin PorB binds to TLR2 and requires TLR1 for signaling. *J.Immunol.* 2006. **176**: 2373-2380.

29. **Levitz,S.M.,** Interactions of Toll-like receptors with fungi. *Microbes.Infect.* 2004. **6**: 1351-1355.

30. **Becker,I., Salaiza,N., Aguirre,M., Delgado,J., Carrillo-Carrasco,N., Kobeh,L.G., Ruiz,A., Cervantes,R., Torres,A.P., Cabrera,N., Gonzalez,A., Maldonado,C., and Isibasi,A.,** Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. *Mol.Biochem.Parasitol.* 2003. **130**: 65-74.

31. **de Veer,M.J., Curtis,J.M., Baldwin,T.M., DiDonato,J.A., Sexton,A., McConville,M.J., Handman,E., and Schofield,L.,** MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. *Eur.J.Immunol.* 2003. **33**: 2822-2831.

32. **Maldonado-Bernal,C., Kirschning,C.J., Rosenstein,Y., Rocha,L.M., Rios-Sarabia,N., Espinosa-Cantellano,M., Becker,I., Estrada,I., Salazar-Gonzalez,R.M., Lopez-Macias,C., Wagner,H., Sanchez,J., and Isibasi,A.,** The innate immune response to Entamoeba histolytica lipopeptidophosphoglycan is mediated by toll-like receptors 2 and 4. *Parasite Immunol.* 2005. **27**: 127-137.

33. **Underhill,D.M., Ozinsky,A., Hajjar,A.M., Stevens,A., Wilson,C.B., Bassetti,M., and Aderem,A.,** The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* 1999. **401**: 811-815.

34. **Campos,M.A., Almeida,I.C., Takeuchi,O., Akira,S., Valente,E.P., Procopio,D.O., Travassos,L.R., Smith,J.A., Golenbock,D.T., and Gazzinelli,R.T.,** Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J.Immunol.* 2001. **167**: 416-423.

35. **van der,K.D., Latz,E., Brouwers,J.F., Kruize,Y.C., Schmitz,M., Kurt-Jones,E.A., Espevik,T., de Jong,E.C., Kapsenberg,M.L., Golenbock,D.T., Tielens,A.G., and Yazdanbakhsh,M.,** A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J.Biol.Chem.* 2002. **277**: 48122-48129.

36. **Dziarski,R. and Gupta,D.,** Staphylococcus aureus peptidoglycan is a toll-like receptor 2 activator: a reevaluation. *Infect.Immun.* 2005. **73**: 5212-5216.

37. **Schroder,N.W., Morath,S., Alexander,C., Hamann,L., Hartung,T.,**

Zahringer,U., Gobel,U.B., Weber,J.R., and Schumann,R.R., Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J.Biol.Chem.* 2003. **278**: 15587-15594.

38. **Compton,T., Kurt-Jones,E.A., Boehme,K.W., Belko,J., Latz,E., Golenbock,D.T., and Finberg,R.W.,** Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. *J.Virol.* 2003. **77**: 4588-4596.

39. **Hirschfeld,M., Weis,J.J., Toshchakov,V., Salkowski,C.A., Cody,M.J., Ward,D.C., Qureshi,N., Michalek,S.M., and Vogel,S.N.,** Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect.Immun.* 2001. **69**: 1477-1482.

40. **Morr,M., Takeuchi,O., Akira,S., Simon,M.M., and Muhlradt,P.F.,** Differential recognition of structural details of bacterial lipopeptides by toll-like receptors. *Eur.J.Immunol.* 2002. **32**: 3337-3347.

41. **Takeuchi,O., Kawai,T., Muhlradt,P.F., Morr,M., Radolf,J.D., Zychlinsky,A., Takeda,K., and Akira,S.,** Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int.Immunol.* 2001. **13**: 933-940.

42. **Bulut,Y., Faure,E., Thomas,L., Equils,O., and Arditi,M.,** Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *J.Immunol.* 2001. **167**: 987-994.

43. **Hirschfeld,M., Kirschning,C.J., Schwandner,R., Wesche,H., Weis,J.H., Wooten,R.M., and Weis,J.J.,** Cutting edge: inflammatory signaling by Borrelia burgdorferi lipoproteins is mediated by toll-like receptor 2. *J.Immunol.* 1999. **163**: 2382-2386.

44. **Hoebe,K., Georgel,P., Rutschmann,S., Du,X., Mudd,S., Crozat,K., Sovath,S., Shamel,L., Hartung,T., Zahringer,U., and Beutler,B.,** CD36 is a sensor of diacylglycerides. *Nature* 2005. **433**: 523-527.

45. **Alexopoulou,L., Holt,A.C., Medzhitov,R., and Flavell,R.A.,** Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001. **413**: 732-738.

46. **Oliveira,A.C., Peixoto,J.R., de Arruda,L.B., Campos,M.A., Gazzinelli,R.T., Golenbock,D.T., Akira,S., Previato,J.O., Mendonca-Previato,L., Nobrega,A., and Bellio,M.,** Expression of functional TLR4 confers proinflammatory responsiveness to Trypanosoma cruzi glycoinositolphospholipids and higher resistance to infection with T. cruzi. *J.Immunol.* 2004. **173**: 5688-5696.

47. **Kurt-Jones,E.A., Popova,L., Kwinn,L., Haynes,L.M., Jones,L.P.,**

Tripp,R.A., Walsh,E.E., Freeman,M.W., Golenbock,D.T., Anderson,L.J., and Finberg,R.W., Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat.Immunol.* 2000. **1**: 398-401.

48. **Hayashi,F., Smith,K.D., Ozinsky,A., Hawn,T.R., Yi,E.C., Goodlett,D.R., Eng,J.K., Akira,S., Underhill,D.M., and Aderem,A.,** The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001. **410**: 1099-1103.

49. **Diebold,S.S., Kaisho,T., Hemmi,H., Akira,S., and Reis e Sousa,** Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004. **303**: 1529-1531.

50. **Heil,F., Hemmi,H., Hochrein,H., Ampenberger,F., Kirschning,C., Akira,S., Lipford,G., Wagner,H., and Bauer,S.,** Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004. **303**: 1526-1529.

51. **Heil,F., hmad-Nejad,P., Hemmi,H., Hochrein,H., Ampenberger,F., Gellert,T., Dietrich,H., Lipford,G., Takeda,K., Akira,S., Wagner,H., and Bauer,S.,** The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Eur.J.Immunol.* 2003. **33**: 2987-2997.

52. **Hemmi,H., Kaisho,T., Takeuchi,O., Sato,S., Sanjo,H., Hoshino,K., Horiuchi,T., Tomizawa,H., Takeda,K., and Akira,S.,** Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat.Immunol.* 2002. **3**: 196-200.

53. **Hemmi,H., Takeuchi,O., Kawai,T., Kaisho,T., Sato,S., Sanjo,H., Matsumoto,M., Hoshino,K., Wagner,H., Takeda,K., and Akira,S.,** A Toll-like receptor recognizes bacterial DNA. *Nature* 2000. **408**: 740-745.

54. **Coban,C., Ishii,K.J., Kawai,T., Hemmi,H., Sato,S., Uematsu,S., Yamamoto,M., Takeuchi,O., Itagaki,S., Kumar,N., Horii,T., and Akira,S.,** Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J.Exp.Med.* 2005. **201**: 19-25.

55. **Zhang,D., Zhang,G., Hayden,M.S., Greenblatt,M.B., Bussey,C., Flavell,R.A., and Ghosh,S.,** A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 2004. **303**: 1522-1526.

56. **Yarovinsky,F., Zhang,D., Andersen,J.F., Bannenberg,G.L., Serhan,C.N., Hayden,M.S., Hieny,S., Sutterwala,F.S., Flavell,R.A., Ghosh,S., and Sher,A.,** TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 2005. **308**: 1626-1629.

57. **Matsushima,N., Tanaka,T., Enkhbayar,P., Mikami,T., Taga,M., Yamada,K., and Kuroki,Y.,** Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. *BMC.Genomics* 2007. **8**: 124.

58. **Bell,J.K., Mullen,G.E., Leifer,C.A., Mazzoni,A., Davies,D.R., and Segal,D.M.,** Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol.* 2003. **24**: 528-533.

59. **Jin,M.S. and Lee,J.O.,** Structures of TLR-ligand complexes. *Curr.Opin.Immunol.* 2008. **20**: 414-419.

60. **Medzhitov,R., Preston-Hurlburt,P., Kopp,E., Stadlen,A., Chen,C., Ghosh,S., and Janeway,C.A., Jr.,** MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol.Cell* 1998. **2**: 253-258.

61. **Fitzgerald,K.A., Palsson-McDermott,E.M., Bowie,A.G., Jefferies,C.A., Mansell,A.S., Brady,G., Brint,E., Dunne,A., Gray,P., Harte,M.T., McMurray,D., Smith,D.E., Sims,J.E., Bird,T.A., and O'Neill,L.A.,** Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* 2001. **413**: 78-83.

62. **Horng,T., Barton,G.M., and Medzhitov,R.,** TIRAP: an adapter molecule in the Toll signaling pathway. *Nat.Immunol.* 2001. **2**: 835-841.

63. **Hoebe,K., Du,X., Georgel,P., Janssen,E., Tabeta,K., Kim,S.O., Goode,J., Lin,P., Mann,N., Mudd,S., Crozat,K., Sovath,S., Han,J., and Beutler,B.,** Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 2003. **424**: 743-748.

64. **Oshiumi,H., Matsumoto,M., Funami,K., Akazawa,T., and Seya,T.,** TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat.Immunol.* 2003. **4**: 161-167.

65. **Yamamoto,M., Sato,S., Mori,K., Hoshino,K., Takeuchi,O., Takeda,K., and Akira,S.,** Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J.Immunol.* 2002. **169**: 6668-6672.

66. **Oshiumi,H., Sasai,M., Shida,K., Fujita,T., Matsumoto,M., and Seya,T.,** TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. *J.Biol.Chem.* 2003. **278**: 49751-49762.

67. **Yamamoto,M., Sato,S., Hemmi,H., Uematsu,S., Hoshino,K., Kaisho,T., Takeuchi,O., Takeda,K., and Akira,S.,** TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat.Immunol.* 2003. **4**: 1144-1150.

68. **Carty,M., Goodbody,R., Schroder,M., Stack,J., Moynagh,P.N., and Bowie,A.G.,** The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nat.Immunol.* 2006. **7**: 1074-1081.

69. **Lord,K.A., Hoffman-Liebermann,B., and Liebermann,D.A.,** Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL6. *Oncogene* 1990. **5**:

1095-1097.

70. **O'Neill,L.A. and Bowie,A.G.,** The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat.Rev.Immunol.* 2007. **7**: 353-364.

71. **Kawai,T., Adachi,O., Ogawa,T., Takeda,K., and Akira,S.,** Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity.* 1999. **11**: 115-122.

72. **Takeuchi,O., Hoshino,K., and Akira,S.,** Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J.Immunol.* 2000. **165**: 5392-5396.

73. **Scanga,C.A., Aliberti,J., Jankovic,D., Tilloy,F., Bennouna,S., Denkers,E.Y., Medzhitov,R., and Sher,A.,** Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. *J.Immunol.* 2002. **168**: 5997-6001.

74. **Edelson,B.T. and Unanue,E.R.,** MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity. *J.Immunol.* 2002. **169**: 3869-3875.

75. **Muraille,E., De,T.C., Brait,M., De,B.P., Leo,O., and Carlier,Y.,** Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to Leishmania major infection associated with a polarized Th2 response. *J.Immunol.* 2003. **170**: 4237-4241.

76. **Mansur,D.S., Kroon,E.G., Nogueira,M.L., Arantes,R.M., Rodrigues,S.C., Akira,S., Gazzinelli,R.T., and Campos,M.A.,** Lethal encephalitis in myeloid differentiation factor 88-deficient mice infected with herpes simplex virus 1. *Am.J.Pathol.* 2005. **166**: 1419-1426.

77. **Yamamoto,M., Sato,S., Hemmi,H., Sanjo,H., Uematsu,S., Kaisho,T., Hoshino,K., Takeuchi,O., Kobayashi,M., Fujita,T., Takeda,K., and Akira,S.,** Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 2002. **420**: 324-329.

78. **Kagan,J.C. and Medzhitov,R.,** Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 2006. **125**: 943-955.

79. **Jeyaseelan,S., Young,S.K., Yamamoto,M., Arndt,P.G., Akira,S., Kolls,J.K., and Worthen,G.S.,** Toll/IL-1R domain-containing adaptor protein (TIRAP) is a critical mediator of antibacterial defense in the lung against Klebsiella pneumoniae but not Pseudomonas aeruginosa. *J.Immunol.* 2006. **177**: 538-547.

80. **Hoebe,K., Janssen,E.M., Kim,S.O., Alexopoulou,L., Flavell,R.A., Han,J., and Beutler,B.,** Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. *Nat.Immunol.* 2003. **4**: 1223-1229.

81. **Kagan,J.C., Su,T., Horng,T., Chow,A., Akira,S., and Medzhitov,R.,**

TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat.Immunol.* 2008. **9**: 361-368.

82. **Kim,Y., Zhou,P., Qian,L., Chuang,J.Z., Lee,J., Li,C., Iadecola,C., Nathan,C., and Ding,A.,** MyD88-5 links mitochondria, microtubules, and JNK3 in neurons and regulates neuronal survival. *J.Exp.Med.* 2007. **204**: 2063-2074.

83. **Kenny,E.F. and O'Neill,L.A.,** Signalling adaptors used by Toll-like receptors: an update. *Cytokine* 2008. **43**: 342-349.

84. **Lu,Y.C., Yeh,W.C., and Ohashi,P.S.,** LPS/TLR4 signal transduction pathway. *Cytokine* 2008. **42**: 145-151.

85. **Triantafilou,M. and Triantafilou,K.,** The dynamics of LPS recognition: complex orchestration of multiple receptors. *J.Endotoxin.Res.* 2005. **11**: 5-11.

86. **Ouyang,X., Negishi,H., Takeda,R., Fujita,Y., Taniguchi,T., and Honda,K.,** Cooperation between MyD88 and TRIF pathways in TLR synergy via IRF5 activation. *Biochem.Biophys.Res.Commun.* 2007. **354**: 1045-1051.

87. **Hasan,U., Chaffois,C., Gaillard,C., Saulnier,V., Merck,E., Tancredi,S., Guiet,C., Briere,F., Vlach,J., Lebecque,S., Trinchieri,G., and Bates,E.E.,** Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J.Immunol.* 2005. **174**: 2942-2950.

88. **Triantafilou,M., Gamper,F.G., Haston,R.M., Mouratis,M.A., Morath,S., Hartung,T., and Triantafilou,K.,** Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. *J.Biol.Chem.* 2006. **281**: 31002-31011.

89. **Oganesyan,G., Saha,S.K., Guo,B., He,J.Q., Shahangian,A., Zarnegar,B., Perry,A., and Cheng,G.,** Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature* 2006. **439**: 208-211.

90. **Honda,K., Ohba,Y., Yanai,H., Negishi,H., Mizutani,T., Takaoka,A., Taya,C., and Taniguchi,T.,** Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* 2005. **434**: 1035-1040.

91. **Brown,G.D.,** Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat.Rev.Immunol.* 2006. **6**: 33-43.

92. **Ting,J.P., Willingham,S.B., and Bergstralh,D.T.,** NLRs at the intersection of cell death and immunity. *Nat.Rev.Immunol.* 2008. **8**: 372-379.

93. **Chamaillard,M., Hashimoto,M., Horie,Y., Masumoto,J., Qiu,S., Saab,L., Ogura,Y., Kawasaki,A., Fukase,K., Kusumoto,S., Valvano,M.A., Foster,S.J., Mak,T.W., Nunez,G., and Inohara,N.,** An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat.Immunol.* 2003. **4**: 702-707.

94. **Inohara,N., Ogura,Y., Fontalba,A., Gutierrez,O., Pons,F., Crespo,J., Fukase,K., Inamura,S., Kusumoto,S., Hashimoto,M., Foster,S.J., Moran,A.P., Fernandez-Luna,J.L., and Nunez,G.,** Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J.Biol.Chem.* 2003. **278**: 5509-5512.

95. **Mariathasan,S. and Monack,D.M.,** Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat.Rev.Immunol.* 2007. **7**: 31-40.

96. **Perregaux,D. and Gabel,C.A.,** Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J.Biol.Chem.* 1994. **269**: 15195-15203.

97. **Pichlmair,A., Schulz,O., Tan,C.P., Naslund,T.I., Liljestrom,P., Weber,F., and Reis e Sousa,** RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 2006. **314**: 997-1001.

98. **Gitlin,L., Barchet,W., Gilfillan,S., Cella,M., Beutler,B., Flavell,R.A., Diamond,M.S., and Colonna,M.,** Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc.Natl.Acad.Sci.U.S.A* 2006. **103**: 8459-8464.

99. **Kato,H., Takeuchi,O., Sato,S., Yoneyama,M., Yamamoto,M., Matsui,K., Uematsu,S., Jung,A., Kawai,T., Ishii,K.J., Yamaguchi,O., Otsu,K., Tsujimura,T., Koh,C.S., Reis e Sousa, Matsuura,Y., Fujita,T., and Akira,S.,** Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006. **441**: 101-105.

100. **LeBien,T.W. and Tedder,T.F.,** B lymphocytes: how they develop and function. *Blood* 2008. **112**: 1570-1580.

101. **Jerne,N.K.,** THE NATURAL-SELECTION THEORY OF ANTIBODY FORMATION. *Proc.Natl.Acad.Sci.U.S.A* 1955. **41**: 849-857.

102. **Burnet,F.M.,** A modification of Jerne's theory of antibody production using the concept of clonal selection. *CA Cancer J.Clin.* 1976. **26**: 119-121.

103. **COOPER,M.D., PETERSON,R.D., and GOOD,R.A.,** DELINEATION OF THE THYMIC AND BURSAL LYMPHOID SYSTEMS IN THE CHICKEN. *Nature* 1965. **205**: 143-146.

104. **COOPER,M.D., Raymond,D.A., PETERSON,R.D., South,M.A., and GOOD,R.A.,** The functions of the thymus system and the bursa system in the chicken. *J.Exp.Med.* 1966. **123**: 75-102.

105. **COOPER,M.D. and Alder,M.N.,** The evolution of adaptive immune systems. *Cell* 2006. **124**: 815-822.

106. **Kurosaki,T.,** Molecular mechanisms in B cell antigen receptor signaling. *Curr.Opin.Immunol.* 1997. **9**: 309-318.

107. **Kurosaki,T. and Tsukada,S.,** BLNK: connecting Syk and Btk to calcium signals. *Immunity.* 2000. **12**: 1-5.

108. **Mohamed,A.J., Nore,B.F., Christensson,B., and Smith,C.I.,** Signalling of Bruton's tyrosine kinase, Btk. *Scand.J.Immunol.* 1999. **49**: 113-118.

109. **Reth,M. and Wienands,J.,** Initiation and processing of signals from the B cell antigen receptor. *Annu.Rev.Immunol.* 1997. **15**: 453-479.

110. **Dal Porto,J.M., Gauld,S.B., Merrell,K.T., Mills,D., Pugh-Bernard,A.E., and Cambier,J.,** B cell antigen receptor signaling 101. *Mol.Immunol.* 2004. **41**: 599-613.

111. **Heyzer-Williams,L.J., Malherbe,L.P., and Heyzer-Williams,M.G.,** Checkpoints in memory B-cell evolution. *Immunol.Rev.* 2006. **211**: 255-268.

112. **Junt,T., Scandella,E., and Ludewig,B.,** Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. *Nat.Rev.Immunol.* 2008. **8**: 764-775.

113. **Cinamon,G., Matloubian,M., Lesneski,M.J., Xu,Y., Low,C., Lu,T., Proia,R.L., and Cyster,J.G.,** Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat.Immunol.* 2004. **5**: 713-720.

114. **Manjarrez-Orduno,N., Parkhouse,R.M., and Santos-Argumedo,L.,** NIM-R7, a novel marker for resting B1 and marginal-zone B lymphocytes, is also expressed on activated T and B cells. *Immunology* 2003. **109**: 232-237.

115. **Fagarasan,S. and Honjo,T.,** T-Independent immune response: new aspects of B cell biology. *Science* 2000. **290**: 89-92.

116. **Kraal,G.,** Cells in the marginal zone of the spleen. *Int.Rev.Cytol.* 1992. **132**: 31-74.

117. **Gray,D., Kumararatne,D.S., Lortan,J., Khan,M., and MacLennan,I.C.,** Relation of intra-splenic migration of marginal zone B cells to antigen localization on follicular dendritic cells. *Immunology* 1984. **52**: 659-669.

118. **Cinamon,G., Zachariah,M.A., Lam,O.M., Foss,F.W., Jr., and Cyster,J.G.,** Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat.Immunol.* 2008. **9**: 54-62.

119. **Oliver,A.M., Martin,F., and Kearney,J.F.,** IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J.Immunol.* 1999. **162**: 7198-7207.

120. **Attanavanich,K. and Kearney,J.F.,** Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *J.Immunol.* 2004. **172**: 803-811.

121. **Hardy,R.R. and Hayakawa,K.,** CD5 B cells, a fetal B cell lineage. *Adv.Immunol.* 1994. **55**: 297-339.

122. **Berland,R. and Wortis,H.H.,** Origins and functions of B-1 cells with notes on the role of CD5. *Annu.Rev.Immunol.* 2002. **20**: 253-300.

123. **Baumgarth,N., Herman,O.C., Jager,G.C., Brown,L.E., Herzenberg,L.A., and Chen,J.,** B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J.Exp.Med.* 2000. **192**: 271-280.

124. **Hardy,R.R. and Hayakawa,K.,** Developmental origins, specificities and immunoglobulin gene biases of murine Ly-1 B cells. *Int.Rev.Immunol.* 1992. **8**: 189-207.

125. **Hayakawa,K., Asano,M., Shinton,S.A., Gui,M., Wen,L.J., Dashoff,J., and Hardy,R.R.,** Positive selection of anti-thy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. *J.Exp.Med.* 2003. **197**: 87-99.

126. **Masmoudi,H., Mota-Santos,T., Huetz,F., Coutinho,A., and Cazenave,P.A.,** All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. *Int.Immunol.* 1990. **2**: 515-520.

127. **Yang,Y., Tung,J.W., Ghosn,E.E., Herzenberg,L.A., and Herzenberg,L.A.,** Division and differentiation of natural antibody-producing cells in mouse spleen. *Proc.Natl.Acad.Sci.U.S.A* 2007. **104**: 4542-4546.

128. **Dorshkind,K. and Montecino-Rodriguez,E.,** Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential. *Nat.Rev.Immunol.* 2007. **7**: 213-219.

129. **Martin,F., Oliver,A.M., and Kearney,J.F.,** Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity.* 2001. **14**: 617-629.

130. **Fagarasan,S., Shinkura,R., Kamata,T., Nogaki,F., Ikuta,K., and Honjo,T.,** Mechanism of B1 cell differentiation and migration in GALT. *Curr.Top.Microbiol.Immunol.* 2000. **252**: 221-229.

131. **Fagarasan,S., Kinoshita,K., Muramatsu,M., Ikuta,K., and Honjo,T.,** In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* 2001. **413**: 639-643.

132. **Yanaba,K., Bouaziz,J.D., Haas,K.M., Poe,J.C., Fujimoto,M., and Tedder,T.F.,** A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity.* 2008. **28**: 639-650.

133. **Bouaziz,J.D., Yanaba,K., and Tedder,T.F.,** Regulatory B cells as inhibitors of immune responses and inflammation. *Immunol.Rev.* 2008. **224**: 201-214.

134. **Fillatreau,S., Sweenie,C.H., McGeachy,M.J., Gray,D., and Anderton,S.M.,** B cells regulate autoimmunity by provision of IL-10. *Nat.Immunol.* 2002. **3**: 944-950.

135. **Matsushita,T., Yanaba,K., Bouaziz,J.D., Fujimoto,M., and Tedder,T.F.,** Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J.Clin.Invest* 2008. **118**: 3420-3430.

136. **Allman,D., Srivastava,B., and Lindsley,R.C.,** Alternative routes to maturity: branch points and pathways for generating follicular and marginal zone B cells. *Immunol.Rev.* 2004. **197**: 147-160.

137. **Allman,D. and Pillai,S.,** Peripheral B cell subsets. *Curr.Opin.Immunol.* 2008. **20**: 149-157.

138. **Inaoki,M., Sato,S., Weintraub,B.C., Goodnow,C.C., and Tedder,T.F.,** CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. *J.Exp.Med.* 1997. **186**: 1923-1931.

139. **Xu,S., Tan,J.E., Wong,E.P., Manickam,A., Ponniah,S., and Lam,K.P.,** B cell development and activation defects resulting in xid-like immunodeficiency in BLNK/SLP-65-deficient mice. *Int.Immunol.* 2000. **12**: 397-404.

140. **Carroll,M.C.,** The role of complement and complement receptors in induction and regulation of immunity. *Annu.Rev.Immunol.* 1998. **16**: 545-568.

141. **Khan,W.N., Alt,F.W., Gerstein,R.M., Malynn,B.A., Larsson,I., Rathbun,G., Davidson,L., Muller,S., Kantor,A.B., Herzenberg,L.A., and .,** Defective B cell development and function in Btk-deficient mice. *Immunity.* 1995. **3**: 283-299.

142. **O'Rourke,L.M., Tooze,R., Turner,M., Sandoval,D.M., Carter,R.H., Tybulewicz,V.L., and Fearon,D.T.,** CD19 as a membrane-anchored adaptor protein of B lymphocytes: costimulation of lipid and protein kinases by recruitment of Vav. *Immunity.* 1998. **8**: 635-645.

143. **Heyzer-Williams,L.J. and Heyzer-Williams,M.G.,** Antigen-specific memory B cell development. *Annu.Rev.Immunol.* 2005. **23**: 487-513.

144. **MacLennan,I.C., Toellner,K.M., Cunningham,A.F., Serre,K., Sze,D.M., Zuniga,E., Cook,M.C., and Vinuesa,C.G.,** Extrafollicular antibody responses. *Immunol.Rev.* 2003. **194**: 8-18.

145. **Scandella,E., Fink,K., Junt,T., Senn,B.M., Lattmann,E., Forster,R., Hengartner,H., and Ludewig,B.,** Dendritic cell-independent B cell activation during acute virus infection: a role for early CCR7-driven B-T helper cell collaboration. *J.Immunol.* 2007. **178**: 1468-1476.

146. **Callard,R.E., Armitage,R.J., Fanslow,W.C., and Spriggs,M.K.,** CD40 ligand and its role in X-linked hyper-IgM syndrome. *Immunol.Today* 1993. **14**: 559-564.

147. **DiSanto,J.P., Bonnefoy,J.Y., Gauchat,J.F., Fischer,A., and de Saint,B.G.,** CD40 ligand mutations in x-linked immunodeficiency with hyper-IgM. *Nature* 1993. **361**: 541-543.

148. **Fuleihan,R., Ramesh,N., Loh,R., Jabara,H., Rosen,R.S., Chatila,T., Fu,S.M., Stamenkovic,I., and Geha,R.S.,** Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc.Natl.Acad.Sci.U.S.A* 1993. **90**: 2170-2173.

149. **Nagaoka,H., Muramatsu,M., Yamamura,N., Kinoshita,K., and Honjo,T.,** Activation-induced deaminase (AID)-directed hypermutation in the immunoglobulin Smu region: implication of AID involvement in a common step of class switch recombination and somatic hypermutation. *J.Exp.Med.* 2002. **195**: 529-534.

150. Paul, W. E. Fundamental Immunology. Lippincott-Raven Publishers, Philadelphia. 1-2-2003.
Ref Type: Serial (Book,Monograph)

151. **Cerutti,A.,** The regulation of IgA class switching. *Nat.Rev.Immunol.* 2008. **8**: 421-434.

152. **Kehry,M.R., Yamashita,L.C., and Hodgkin,P.D.,** B-cell proliferation and differentiation mediated by Th-cell membranes and lymphokines. *Res.Immunol.* 1990. **141**: 421-423.

153. **Haxhinasto,S.A., Hostager,B.S., and Bishop,G.A.,** Cutting edge: molecular mechanisms of synergy between CD40 and the B cell antigen receptor: role for TNF receptor-associated factor 2 in receptor interaction. *J.Immunol.* 2002. **169**: 1145-1149.

154. **Fearon,D.T. and Carroll,M.C.,** Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu.Rev.Immunol.* 2000. **18**: 393-422.

155. **Tedder,T.F., Haas,K.M., and Poe,J.C.,** CD19-CD21 complex regulates an intrinsic Src family kinase amplification loop that links innate immunity with B-lymphocyte intracellular calcium responses. *Biochem.Soc.Trans.* 2002. **30**: 807-811.

156. **Mackay,F., Silveira,P.A., and Brink,R.,** B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling. *Curr.Opin.Immunol.* 2007. **19**: 327-336.

157. **Litinskiy,M.B., Nardelli,B., Hilbert,D.M., He,B., Schaffer,A., Casali,P., and Cerutti,A.,** DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat.Immunol.* 2002. **3**: 822-829.

158. **Nardelli,B., Belvedere,O., Roschke,V., Moore,P.A., Olsen,H.S., Migone,T.S., Sosnovtseva,S., Carrell,J.A., Feng,P., Giri,J.G., and Hilbert,D.M.,** Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood* 2001. **97**: 198-204.

159. **Mackay,F., Schneider,P., Rennert,P., and Browning,J.,** BAFF AND APRIL: a tutorial on B cell survival. *Annu.Rev.Immunol.* 2003. **21**: 231-264.

160. **Schiemann,B., Gommerman,J.L., Vora,K., Cachero,T.G., Shulga-Morskaya,S., Dobles,M., Frew,E., and Scott,M.L.,** An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 2001. **293**: 2111-2114.

161. **Yan,M., Wang,H., Chan,B., Roose-Girma,M., Erickson,S., Baker,T., Tumas,D., Grewal,I.S., and Dixit,V.M.,** Activation and accumulation of B cells in TACI-deficient mice. *Nat.Immunol.* 2001. **2**: 638-643.

162. **Dasari,P., Nicholson,I.C., Hodge,G., Dandie,G.W., and Zola,H.,** Expression of toll-like receptors on B lymphocytes. *Cell Immunol.* 2005. **236**: 140-145.

163. **Gururajan,M., Jacob,J., and Pulendran,B.,** Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. *PLoS.ONE.* 2007. **2**: e863.

164. **Hornung,V., Rothenfusser,S., Britsch,S., Krug,A., Jahrsdorfer,B., Giese,T., Endres,S., and Hartmann,G.,** Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J.Immunol.* 2002. **168**: 4531-4537.

165. **Leadbetter,E.A., Rifkin,I.R., Hohlbaum,A.M., Beaudette,B.C., Shlomchik,M.J., and Marshak-Rothstein,A.,** Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 2002. **416**: 603-607.

166. **Leadbetter,E.A., Rifkin,I.R., and Marshak-Rothstein,A.,** Toll-like receptors and activation of autoreactive B cells. *Curr.Dir.Autoimmun.* 2003. **6**: 105-122.

167. **Viglianti,G.A., Lau,C.M., Hanley,T.M., Miko,B.A., Shlomchik,M.J., and Marshak-Rothstein,A.,** Activation of autoreactive B cells by CpG dsDNA. *Immunity.* 2003. **19**: 837-847.

168. **Herlands,R.A., Christensen,S.R., Sweet,R.A., Hershberg,U., and Shlomchik,M.J.,** T Cell-Independent and Toll-like Receptor-Dependent Antigen-Driven Activation of Autoreactive B Cells. *Immunity.* 2008. **29**: 249-260.

169. **Lau,C.M., Broughton,C., Tabor,A.S., Akira,S., Flavell,R.A., Mamula,M.J., Christensen,S.R., Shlomchik,M.J., Viglianti,G.A., Rifkin,I.R., and Marshak-Rothstein,A.,** RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J.Exp.Med.* 2005. **202**: 1171-1177.

170. **Rubtsov,A.V., Swanson,C.L., Troy,S., Strauch,P., Pelanda,R., and Torres,R.M.,** TLR agonists promote marginal zone B cell activation and facilitate T-dependent IgM responses. *J.Immunol.* 2008. **180**: 3882-3888.

171. **Genestier,L., Taillardet,M., Mondiere,P., Gheit,H., Bella,C., and**

Defrance,T., TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. *J.Immunol.* 2007. **178**: 7779-7786.

172. **Jiang,W., Lederman,M.M., Harding,C.V., Rodriguez,B., Mohner,R.J., and Sieg,S.F.,** TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function. *Eur.J.Immunol.* 2007. **37**: 2205-2213.

173. **Ruprecht,C.R. and Lanzavecchia,A.,** Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *Eur.J.Immunol.* 2006. **36**: 810-816.

174. **Bernasconi,N.L., Traggiai,E., and Lanzavecchia,A.,** Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 2002. **298**: 2199-2202.

175. **Bernasconi,N.L., Onai,N., and Lanzavecchia,A.,** A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* 2003. **101**: 4500-4504.

176. **Lanzavecchia,A. and Sallusto,F.,** Toll-like receptors and innate immunity in B-cell activation and antibody responses. *Curr.Opin.Immunol.* 2007. **19**: 268-274.

177. **Liu,N., Ohnishi,N., Ni,L., Akira,S., and Bacon,K.B.,** CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nat.Immunol.* 2003. **4**: 687-693.

178. **Ehlers,M., Fukuyama,H., McGaha,T.L., Aderem,A., and Ravetch,J.V.,** TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *J.Exp.Med.* 2006. **203**: 553-561.

179. **Heer,A.K., Shamshiev,A., Donda,A., Uematsu,S., Akira,S., Kopf,M., and Marsland,B.J.,** TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. *J.Immunol.* 2007. **178**: 2182-2191.

180. **Jegerlehner,A., Maurer,P., Bessa,J., Hinton,H.J., Kopf,M., and Bachmann,M.F.,** TLR9 signaling in B cells determines class switch recombination to IgG2a. *J.Immunol.* 2007. **178**: 2415-2420.

181. **Iwasaki,A. and Medzhitov,R.,** Toll-like receptor control of the adaptive immune responses. *Nat.Immunol.* 2004. **5**: 987-995.

182. **Gavin,A.L., Hoebe,K., Duong,B., Ota,T., Martin,C., Beutler,B., and Nemazee,D.,** Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science* 2006. **314**: 1936-1938.

183. **Richard,K., Pierce,S.K., and Song,W.,** The agonists of TLR4 and 9 are sufficient to activate memory B cells to differentiate into plasma cells in vitro

but not in vivo. *J.Immunol.* 2008. **181**: 1746-1752.

184. **Meyer-Bahlburg,A., Khim,S., and Rawlings,D.J.,** B cell intrinsic TLR signals amplify but are not required for humoral immunity. *J.Exp.Med.* 2007. **204**: 3095-3101.

185. **Jap,B.K. and Walian,P.J.,** Structure and functional mechanism of porins. *Physiol Rev.* 1996. **76**: 1073-1088.

186. **Puente,J.L., Flores,V., Fernandez,M., Fuchs,Y., and Calva,E.,** Isolation of an ompC-like outer membrane protein gene from Salmonella typhi. *Gene* 1987. **61**: 75-83.

187. **Fernandez-Mora,M., Oropeza,R., Puente,J.L., and Calva,E.,** Isolation and characterization of ompS1, a novel Salmonella typhi outer membrane protein-encoding gene. *Gene* 1995. **158**: 67-72.

188. **Fernandez-Mora,M., Puente,J.L., and Calva,E.,** OmpR and LeuO positively regulate the Salmonella enterica serovar Typhi ompS2 porin gene. *J.Bacteriol.* 2004. **186**: 2909-2920.

189. **DiRienzo,J.M., Nakamura,K., and Inouye,M.,** The outer membrane proteins of Gram-negative bacteria: biosynthesis, assembly, and functions. *Annu.Rev.Biochem.* 1978. **47**: 481-532.

190. **Osborn,M.J. and Wu,H.C.,** Proteins of the outer membrane of gram-negative bacteria. *Annu.Rev.Microbiol.* 1980. **34**: 369-422.

191. **Nikaido,H.,** Outer membrane. In: Neidhard FC, Ingraham JL, Curtiss R III, et al., editors. Escherichia coli and Salmonella: Cellular and Molecular Biology. *Washington D.C.: ASM Press* 1996. 29-47.

192. **Nikaido,H.,** Porins and specific diffusion channels in bacterial outer membranes. *J.Biol.Chem.* 1994. **269**: 3905-3908.

193. **Nikaido,H., Rosenberg,E.Y., and Foulds,J.,** Porin channels in Escherichia coli: studies with beta-lactams in intact cells. *J.Bacteriol.* 1983. **153**: 232-240.

194. **Thanassi,D.G., Cheng,L.W., and Nikaido,H.,** Active efflux of bile salts by Escherichia coli. *J.Bacteriol.* 1997. **179**: 2512-2518.

195. **Singh,S.P., Upshaw,Y., Abdullah,T., Singh,S.R., and Klebba,P.E.,** Structural relatedness of enteric bacterial porins assessed with monoclonal antibodies to Salmonella typhimurium OmpD and OmpC. *J.Bacteriol.* 1992. **174**: 1965-1973.

196. **Martinez-Flores,I., Cano,R., Bustamante,V.H., Calva,E., and Puente,J.L.,** The ompB operon partially determines differential expression of OmpC in Salmonella typhi and Escherichia coli. *J.Bacteriol.* 1999. **181**: 556-562.

197. **Isibasi,A., Ortiz,V., Vargas,M., Paniagua,J., Gonzalez,C., Moreno,J., and Kumate,J.,** Protection against Salmonella typhi infection in mice after

immunization with outer membrane proteins isolated from Salmonella typhi 9,12,d, Vi. *Infect.Immun.* 1988. **56**: 2953-2959.

198. **Calderon,I., Lobos,S.R., Rojas,H.A., Palomino,C., Rodriguez,L.H., and Mora,G.C.,** Antibodies to porin antigens of Salmonella typhi induced during typhoid infection in humans. *Infect.Immun.* 1986. **52**: 209-212.

199. **Verdugo-Rodriguez,A., Gam,L.H., Devi,S., Koh,C.L., Puthucheary,S.D., Calva,E., and Pang,T.,** Detection of antibodies against Salmonella typhi outer membrane protein (OMP) preparation in typhoid fever patients. *Asian Pac.J.Allergy Immunol.* 1993. **11**: 45-52.

200. **Ortiz,V., Isibasi,A., Garcia-Ortigoza,E., and Kumate,J.,** Immunoblot detection of class-specific humoral immune response to outer membrane proteins isolated from Salmonella typhi in humans with typhoid fever. *J.Clin.Microbiol.* 1989. **27**: 1640-1645.

201. **Isibasi,A., Paniagua,J., Rojo,M.P., Martin,N., Ramirez,G., Gonzalez,C.R., Lopez-Macias,C., Sanchez,J., Kumate,J., and Ortiz-Navarrete,V.,** Role of porins from Salmonella typhi in the induction of protective immunity. *Ann.N.Y.Acad.Sci.* 1994. **730**: 350-352.

202. **Secundino,I., Lopez-Macias,C., Cervantes-Barragan,L., Gil-Cruz,C., Rios-Sarabia,N., Pastelin-Palacios,R., Villasis-Keever,M.A., Becker,I., Puente,J.L., Calva,E., and Isibasi,A.,** Salmonella porins induce a sustained, lifelong specific bactericidal antibody memory response. *Immunology* 2006. **117**: 59-70.

203. **Salazar-Gonzalez,R.M., Maldonado-Bernal,C., Ramirez-Cruz,N.E., Rios-Sarabia,N., Beltran-Nava,J., Castanon-Gonzalez,J., Castillo-Torres,N., Palma-Aguirre,J.A., Carrera-Camargo,M., Lopez-Macias,C., and Isibasi,A.,** Induction of cellular immune response and anti-Salmonella enterica serovar typhi bactericidal antibodies in healthy volunteers by immunization with a vaccine candidate against typhoid fever. *Immunol.Lett.* 2004. **93**: 115-122.

204. **Gonzalez,C.R., Mejia,M.V., Paniagua,J., Ortiz-Navarrete,V., Ramirez,G., and Isibasi,A.,** Immune response to porins isolated from Salmonella typhi in different mouse strains. *Arch.Med.Res.* 1995. **26 Spec No**: S99-103.

205. **Martin-Orozco,N., Isibasi,A., and Ortiz-Navarrete,V.,** Macrophages present exogenous antigens by class I major histocompatibility complex molecules via a secretory pathway as a consequence of interferon-gamma activation. *Immunology* 2001. **103**: 41-48.

206. **Diaz-Quinonez,A., Martin-Orozco,N., Isibasi,A., and Ortiz-Navarrete,V.,** Two Salmonella OmpC K(b)-restricted epitopes for CD8+-T-cell recognition. *Infect.Immun.* 2004. **72**: 3059-3062.

207. **Ray,A., Chatterjee,N.S., Bhattacharya,S.K., and Biswas,T.,** Porin of Shigella dysenteriae enhances mRNA levels for Toll-like receptor 2 and

MyD88, up-regulates CD80 of murine macrophage, and induces the release of interleukin-12. *FEMS Immunol.Med.Microbiol.* 2003. **39**: 213-219.

208. **Ray,A., Karmakar,P., and Biswas,T.,** Up-regulation of CD80-CD86 and IgA on mouse peritoneal B-1 cells by porin of Shigella dysenteriae is Toll-like receptors 2 and 6 dependent. *Mol.Immunol.* 2004. **41**: 1167-1175.

209. **Ray,A. and Biswas,T.,** Porin of Shigella dysenteriae enhances Toll-like receptors 2 and 6 of mouse peritoneal B-2 cells and induces the expression of immunoglobulin M, immunoglobulin G2a and immunoglobulin A. *Immunology* 2005. **114**: 94-100.

210. **Adachi,O., Kawai,T., Takeda,K., Matsumoto,M., Tsutsui,H., Sakagami,M., Nakanishi,K., and Akira,S.,** Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity.* 1998. **9**: 143-150.

211. **Hoshino,K., Takeuchi,O., Kawai,T., Sanjo,H., Ogawa,T., Takeda,Y., Takeda,K., and Akira,S.,** Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J.Immunol.* 1999. **162**: 3749-3752.

212. **Takeuchi,O., Hoshino,K., Kawai,T., Sanjo,H., Takada,H., Ogawa,T., Takeda,K., and Akira,S.,** Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 1999. **11**: 443-451.

213. **Kitamura,D., Roes,J., Kuhn,R., and Rajewsky,K.,** A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 1991. **350**: 423-426.

214. **Muller,U., Steinhoff,U., Reis,L.F., Hemmi,S., Pavlovic,J., Zinkernagel,R.M., and Aguet,M.,** Functional role of type I and type II interferons in antiviral defense. *Science* 1994. **264**: 1918-1921.

215. **Caramalho,I., Lopes-Carvalho,T., Ostler,D., Zelenay,S., Haury,M., and Demengeot,J.,** Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J.Exp.Med.* 2003. **197**: 403-411.

216. **Singleton,T.E., Massari,P., and Wetzler,L.M.,** Neisserial porin-induced dendritic cell activation is MyD88 and TLR2 dependent. *J.Immunol.* 2005. **174**: 3545-3550.

217. **Biswas,A., Banerjee,P., Mukherjee,G., and Biswas,T.,** Porin of Shigella dysenteriae activates mouse peritoneal macrophage through Toll-like receptors 2 and 6 to induce polarized type I response. *Mol.Immunol.* 2007. **44**: 812-820.

218. **Vega,M.I., Santos-Argumedo,L., Huerta-Yepez,S., Luria-Perez,R., Ortiz-Navarrete,V., Isibasi,A., and Gonzalez-Bonilla,C.R.,** A Salmonella typhi OmpC fusion protein expressing the CD154 Trp140-Ser149 amino acid strand binds CD40 and activates a lymphoma B-cell line. *Immunology* 2003. **110**: 206-216.

219. **Yamamoto,M., Sato,S., Hemmi,H., Hoshino,K., Kaisho,T., Sanjo,H., Takeuchi,O., Sugiyama,M., Okabe,M., Takeda,K., and Akira,S.,** Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003. **301**: 640-643.

220. **Fink,K., Lang,K.S., Manjarrez-Orduno,N., Junt,T., Senn,B.M., Holdener,M., Akira,S., Zinkernagel,R.M., and Hengartner,H.,** Early type I interferon-mediated signals on B cells specifically enhance antiviral humoral responses. *Eur.J.Immunol.* 2006. **36**: 2094-2105.

221. **Sornasse,T., Flamand,V., De,B.G., Bazin,H., Tielemans,F., Thielemans,K., Urbain,J., Leo,O., and Moser,M.,** Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo. *J.Exp.Med.* 1992. **175**: 15-21.

222. **Snapper,C.M., Rosas,F.R., Kehry,M.R., Mond,J.J., and Wetzler,L.M.,** Neisserial porins may provide critical second signals to polysaccharide-activated murine B cells for induction of immunoglobulin secretion. *Infect.Immun.* 1997. **65**: 3203-3208.

223. **Le Bon A., Thompson,C., Kamphuis,E., Durand,V., Rossmann,C., Kalinke,U., and Tough,D.F.,** Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J.Immunol.* 2006. **176**: 2074-2078.

224. **Acosta-Ramirez,E., Perez-Flores,R., Majeau,N., Pastelin-Palacios,R., Gil-Cruz,C., Ramirez-Saldana,M., Manjarrez-Orduno,N., Cervantes-Barragan,L., Santos-Argumedo,L., Flores-Romo,L., Becker,I., Isibasi,A., Leclerc,D., and Lopez-Macias,C.,** Translating innate response into long-lasting antibody response by the intrinsic antigen-adjuvant properties of papaya mosaic virus. *Immunology* 2008. **124**: 186-197.

PUBLICACIONES OBTENIDAS DURANTE EL DOCTORADO

1. Cervantes-Barragan, L., R. Züst, F. Weber, M. Spiegel, K.S. Lang, S. Akira, V. Thiel, and B. Ludewig. 2007. Control of coronavirus infection through plasmacytoid dendritic cell-derived type I interferon. *Blood* 109(3):1131-1137. Epub 2006 Sep 19

2. Cervantes-Barragan, L., U. Kalinke, R. Züst, B. Reizis, C. Lopez-Macias, V. Thiel, and B. Ludewig. Type I interferon-mediated protection of macrophages and dendritic cells secures control of murine coronavirus infection. Journal of Immunology (aceptado)

3. Cervantes-Barragán, L., Gil-Cruz, C., Pastelin-Palacios, R., Lang, KS. , Isibasi1, A., Ludewig, L. and López-Macías, C. TLR2 and TLR4 signaling shapes specific antibody responses to *Salmonella* Typhi antigens. European Journal of Immunology. (Aceptado)

4. Philipp A. Lang, Luisa Cervantes-Barragan, Admar Verschoor, Alexander A Navarini, Mike Recher, Marc Pellegrini, Lukas Flatz, Andreas Bergthaler, Kenya Honda, Burkhard Ludewig, Pamela S. Ohashi, Karl S. Lang. Hematopoietic cell derived interferon controls viral replication and virus induced disease. Blood. (aceptado)

5. Secundino I, C. Lopez-Macias, L. Cervantes-Barragan, C. Gil-Cruz, N. Ramos-Sarabia, R. Pastelin-Palacios, M.A. Villasis-Keever, I. Becker, J.L. Puente, E. Calva E, and A. Isibasi. 2006. Salmonella porins induce a sustained, lifelong specific bactericidal antibody memory response. Immunology 117(1):59-70.

6. Züst, R., L. Cervantes-Barragan, T. Kuri, G. Blakqori, F. Weber, B. Ludewig, and V. Thiel. 2007. Identification of Coronavirus Non-Structural Protein 1 as a Major Pathogenicity Factor – Implications for the Rational Design of Live Attenuated Coronavirus Vaccines. PLoS Pathog. 2007 Aug 10;3(8):e109.

7. Lang PA, Contaldo C, Georgiev P, El-Badry AM, Recher M, Kurrer M, Cervantes-Barragan L, Ludewig B, Calzascia T, Bolinger B, Merkler D, Odermatt B, Bader M, Graf R, Clavien PA, Hegazy AN, Löhning M, Harris NL, Ohashi PS, Hengartner H, Zinkernagel RM, Lang KS. Aggravation of viral hepatitis by platelet-derived serotonin. Nat Med. 2008 Jul;14(7):756-61. Epub 2008 May 30.

8. Acosta-Ramírez E, Pérez-Flores R, Majeau N, Pastelin-Palacios R, Gil-Cruz C, Ramírez-Saldaña M, Manjarrez-Orduño N, Cervantes-Barragán L, Santos-Argumedo L, Flores-Romo L, Becker I, Isibasi A, Leclerc D, López-Macías C.. Translating innate response into long-lasting antibody response by the intrinsic antigen-adjuvant properties of papaya mosaic virus. Immunology. 2008 Jun;124(2):186-97. Epub 2007 Dec 7.

9. Eriksson KK, Cervantes-Barragán L, Ludewig B, Thiel V.Mouse Hepatitis Virus liver pathology is dependent on ADP-ribose 1''-phosphatase, a viral function conserved in the alpha-like supergroup. J Virol. 2008 Oct 15. [Epub ahead of print]

10. Eriksson, K.K., D. Makia, R. Maier, L. Cervantes, B. Ludewig, and V. Thiel. 2006. Efficient transduction of dendritic cells using coronavirus-based vectors. *Adv. Exp. Med. Biol.* 581:203-6.
11. Tal Birnberg, Liat Bar-On, Anita Sapoznikov, Michele L. Caton, Luisa Cervantes-Barragán, Divine Makia, Rita Krauthgamer, Ori Brenner, Burkhard Ludewig, Damian Brockschnieder, Dieter Riethmacher, Boris Reizis and Steffen Jung. Lack of conventional Dendritic cells is compatible with normal development and T cell homeostasis, but causes Myeloid Proliferative Syndrome. Immunity (aceptado)

immunology

TLR2 and TLR4 signaling shapes specific antibody responses to *Salmonella typhi* antigens

Luisa Cervantes-Barragán[1,2,3], Cristina Gil-Cruz[1], Rodolfo Pastelin-Palacios[5,6], Karl S. Lang[5,6], Armando Isibasi[1], Burkhard Ludewig[2] and Constantino López-Macías[1]

[1] Medical Research Unit on Immunochemistry, Specialties Hospital of the National Medical Centre "Siglo XXI", Mexican Institute for Social Security, Mexico City, Mexico
[2] Research Department, Kantonal Hospital St. Gallen, St. Gallen, Switzerland
[3] Ph.D. Program on Biomedical Sciences, Universidad Nacional Autónoma de México, Mexico City, Mexico
[4] Departamento de Biología, Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, Mexico
[5] Institute of Experimental Immunology, University of Zürich, Zürich, Switzerland
[6] Campbell Family Institute for Breast Cancer Research, Ontario Cancer Institute, Toronto, Canada

TLR directly induce innate immune responses by sensing a variety of microbial components and are critical for the fine-tuning of subsequent adaptive immune responses. However, their impact and mechanism of action on antibody responses against bacterial antigens are not yet fully understood. *Salmonella enterica serovar typhi* (*S. typhi*) porins have been characterized as inducers of long-lasting specific antibody responses in mice. In this report, we show that immunization of TLR4-deficient ($TLR4^{-/-}$), myeloid differentiating gene 88-deficient and Toll/IL-R domain-containing adaptor-inducing IFN-β-deficient mice with *S. typhi* porins led to significantly reduced B-cell responses. $TLR2^{-/-}$ mice, as well, showed reduced IgG titers with a more pronounced impairment in the production of IgG3 anti-porin antibodies. Adoptive transfer of $TLR2^{-/-}$- or $TLR4^{-/-}$-B cells into B-cell-deficient mice revealed a direct effect of TLR4 on B cells for the primary IgM response, whereas stimulation of B cells *via* TLR2 was important for IgG production. Furthermore, *S. typhi* porins were found to efficiently elicit maturation of $CD11c^{+}$ conventional DC. Taken together, *S. typhi* porins represent not only a suitable B-cell antigen for vaccination, but exhibit potent TLR-dependent stimulatory functions on B cells and DC, which help to further enhance and shape the antibody response.

Key words: Antibody response · Porins · *S. typhi* · TLR

Introduction

Recognition of PAMP by TLR is critical for the induction of the innate immune response [1]. TLR can stimulate adaptive immune responses indirectly through the activation of innate immune cells that produce inflammatory cytokines and chemokines, and upregulate co-stimulatory molecules. Furthermore, TLR ligands participate in shaping adaptive immune responses by triggering their respective receptors on B and/or T cells [2–6]. The direct participation of TLR in B-cell activation was described by Leadbetter *et al.* [7], who showed that an effective activation of Rheumatoid $factor^{+}$ B cells requires a synergistic engagement of the BCR and a member of the TLR family. Subsequent studies revealed that TLR9 and TLR7 engagement contributes to the activation of autoreactive B cells [8, 9].

Correspondence: Dr. Constantino López-Macías
e-mail: constantino@sminmunologia.org;constantino.lopez@imss.gob.mx

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It has been proposed that TLR provide a third signal together with antigen recognition through the BCR and cognate T cells help to achieve the maximal activation of B cells and class switch recombination [10–12]. Likewise, during antiviral immune responses, TLR7 and TLR9 modulate the antibody response by regulating class switch recombination [13, 14]. These findings provided insight into the importance of particular TLR signals on antibody responses against viral antigens. During bacterial infections, particular TLR such as TLR4 and TLR2 are triggered [15]; however, their participation in the antibody response against bacterial antigens and their mechanism of action have remained largely unexplored.

Current protective vaccines against pathogens induce long-lasting neutralizing antibody responses [16]. Recent studies have shown that efficient B-cell immunity using vaccination with model antigens can only be achieved when the antigen is applied in conjunction with appropriate TLR stimulation [17]. However, this view has been challenged in a study by Nemazee and colleagues [18], who showed that antigen-induced antibody responses can be generated in the absence of TLR signaling. A major drawback in these previous studies is the use of typical model antigens such as trinitrophenol-hemocyanin, keyhole limpet hemocyanin or ovalbumin, which may not behave like vaccine preparations or pathogen-derived antigens. In the current study, we have analyzed the importance of TLR signaling in the antibody responses against a *Salmonella enterica serovar typhi* (*S. typhi*) porins' preparation. Porins are the most abundant proteins found in the outer membrane of Gram-negative bacteria; they assemble in trimers and achieve concentrations of up to 10^5 molecules *per* cell [19]. Porins purified from WT *S. typhi* (hereafter porins' preparation) consist of a mixture of two proteins: Outer membrane protein (Omp), C (OmpC) and F (OmpF), which assemble as homotrimers [20–22]. These antigens are highly immunogenic, both in mice and humans [20, 23, 24]. Immunization of mice with a preparation of *S. typhi* porins elicits a lifelong antibody response and protection against *S. typhi* challenge [22]. Furthermore, it has been shown that sera of patients in the acute and convalescent phases of typhoid fever contain IgM and IgG antibodies that mainly recognize porins [25, 26]. Healthy volunteers vaccinated with porins produced high titers of anti-porins antibodies without showing adverse effects. Therefore, these proteins have been proposed as vaccine candidates against typhoid fever [21].

We report here that TLR4-deficient ($TLR4^{-/-}$), myeloid differentiating gene 88-deficient ($MyD88^{-/-}$) and Toll/IL-R domain-containing adaptor-inducing IFN-β-deficient ($TRIF^{-/-}$) mice immunized with *S. typhi* porins showed a pronounced impairment in the generation of a specific antibody response against *S. typhi* porins. Likewise, $TLR2^{-/-}$ mice displayed impaired production of IgG anti-porins antibodies with a particularly pronounced alteration in the IgG3 subclass. Moreover, adoptive transfer experiments revealed that TLR4 expression on B cells impacts on the IgM response, whereas TLR2-mediated signaling in B cells was important for the generation of IgG. Furthermore, *S. typhi* porins elicited maturation of both B cells and DC. Overall, these results indicate that the recognition of the porins' preparation by TLR impacts on the quantity and quality of the specific antibody response. Moreover, these findings bear important implications for the rational design of vaccines against bacterial pathogens such as *S. typhi*, and help in the understanding of TLR-mediated adjuvant effects.

Results

Impaired antibody response against S. typhi porins in the absence of TLR signaling

Immunization with *S. typhi* porins induces an antibody response even in the absence of adjuvant co-administration [21, 22]. Porins from several Gram-negative bacteria bind to TLR2 [27–29], leading to upregulation of co-stimulatory molecules and MHC class II (MHC II) antigens on macrophages, B cells and DC [30–32], cytokine release by macrophages and upregulation of surface immunoglobulin molecules on B cells [33, 34]. To assess the contribution of TLR signaling to the antibody response against *S. typhi* porins, we first analyzed the induction of anti-porins IgM and IgG in mice deficient of the main adaptor proteins of the TLR signaling pathway. Two downstream adaptor proteins have been shown to control all TLR signals: MyD88 transduces signals from all TLR except TLR3, whereas TRIF transduces signals from TLR3 and partly from TLR4 [35–37]. B6, $MyD88^{-/-}$ and $TRIF^{-/-}$ mice were immunized with 10 μg of *S. typhi* porins, and antibody titers were determined during a period of 30 days. Both $MyD88^{-/-}$ and $TRIF^{-/-}$ mice showed impaired IgM and IgG responses, even after booster immunization on day 15 (Fig. 1A and B). Analysis of the different IgG subclasses on day 30 post immunization revealed that $MyD88^{-/-}$ mice displayed significantly reduced IgG1, IgG2a/c, IgG2b and IgG3 titers (Fig. 1C). Likewise, in $TRIF^{-/-}$ mice, IgG2a/c, IgG2b and IgG3 subclasses were reduced; however, IgG3 titers were only mildly affected (Fig. 1C). These results indicate that TLR signaling enhances the antibody response against *S. typhi* porins and that both $MyD88^{-/-}$ and $TRIF^{-/-}$ signals contribute to the observed adjuvant effect.

TLR2 and TLR4 shape S. typhi porins-specific B-cell immunity

As mentioned above, porins from different Gram-negative bacteria are TLR2 ligands [27, 28]. Moreover, the presence of undetectable trace amounts of LPS in the *S. typhi* porins' preparation could provide stimulation *via* TLR4, which might explain why $TRIF^{-/-}$ mice displayed reduced antibody responses. In order to study the contribution of TLR2 and TLR4 signaling in the antibody response against *S. typhi* porins, B6, $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were immunized with the *S. typhi* porins' preparation. $TLR2^{-/-}$ mice did not show impairment in anti-porins IgM, but roughly four times reduced anti-porins IgG titers (Fig. 2A and B). $TLR4^{-/-}$ mice displayed a more pronounced

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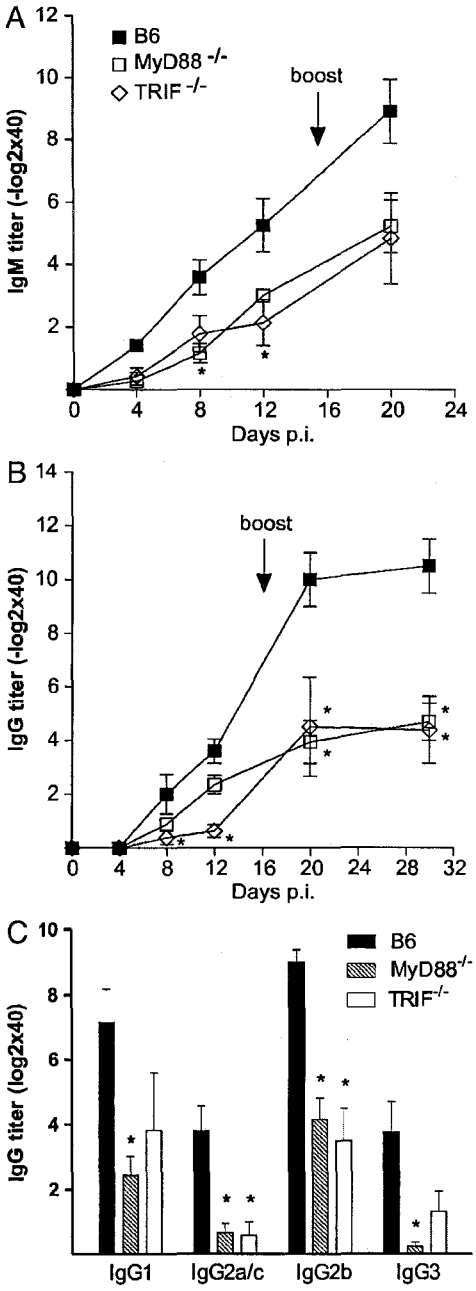

Figure 1. Antibody response against *S. typhi* porins is impaired in MyD88$^{-/-}$ and TRIF$^{-/-}$ mice. MyD88$^{-/-}$, TRIF$^{-/-}$ and B6 mice were immunized with 10 μg of *S. typhi* porins on days 0 and 15. (A) Anti-porins IgM and (B) anti-porins IgG antibody titers were measured by ELISA at the indicated time points. (C) Anti-porins IgG subclasses IgG1, IgG2a/c, IgG2b and IgG3 were determined on day 30 post immunization. Results are expressed as the mean ± SEM of six mice *per* group. Statistical analysis was performed using one-way ANOVA. Significant differences between the respective gene-deficient mice and the WT control at the same time point are indicated (*p<0.05).

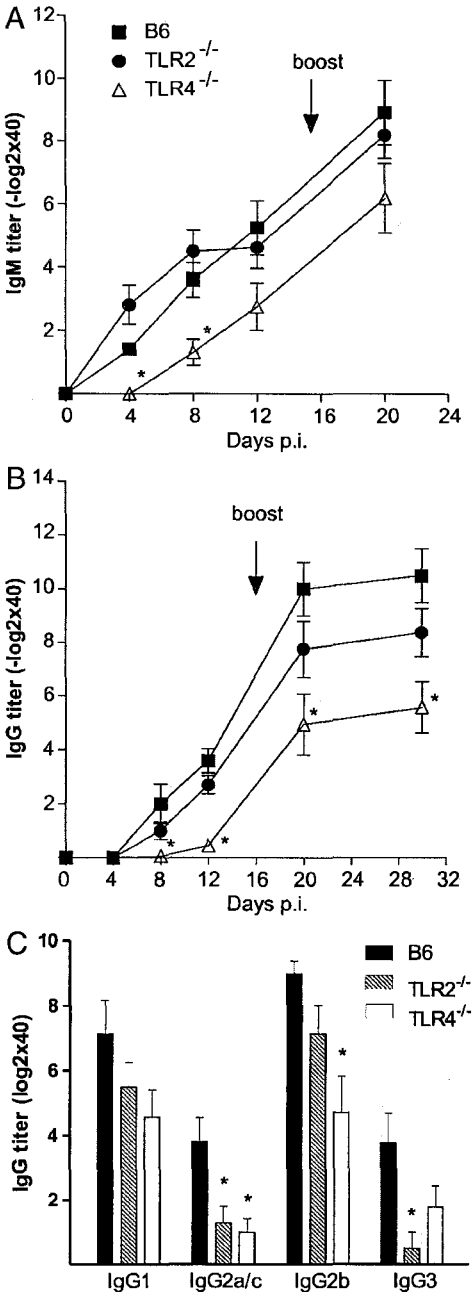

Figure 2. Antibody response against *S. typhi* porins is reduced in TLR4$^{-/-}$ and TLR2$^{-/-}$ mice. TLR4$^{-/-}$, TLR2$^{-/-}$ and B6 mice were immunized with 10 μg *S. typhi* porins on days 0 and 15. (A) Anti-porins IgM and (B) anti-porins IgG antibody titers were measured by ELISA at the indicated time points. (C) Anti-porins IgG subclasses IgG1, IgG2a/c, IgG2b and IgG3 were determined on day 30 post immunization. Results are expressed as the mean ± SEM of eight mice *per* group. Statistical analysis was performed using one-way ANOVA. Significant differences between the respective gene-deficient mice and the WT control at the same time point are indicated (*p<0.05).

decrease in both IgM and IgG titers (Fig. 2A and B). Analysis of IgG subclasses on day 30 post immunization revealed a small reduction in IgG1 and IgG2b titers, but a pronounced alteration in IgG2a/c and IgG3 titers in TLR2$^{-/-}$ mice (Fig. 2C). TLR4$^{-/-}$ mice showed a generally reduced production of IgG subclasses, with the decrease in titers of IgG2a/c and IgG2b being most

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dramatic (Fig. 2C). Thus, these data suggest that both TLR4 and TLR2 enhance the production of anti-porins antibodies and shape IgG subclass expression.

Type I IFN do not impact on the antibody response against S. *typhi* porins

Studies on the antibody response against viral antigens have shown that type I IFN induced through TLR receptor signals impact on the production of the different IgG subclasses [13, 38]. Because TLR4-mediated signals transmitted *via* the adaptor molecule TRIF lead to induction of IFN-β [36], we analyzed in the next experiments, the participation of type I IFN in the anti-porins antibody response. B6 and type I IFN receptor-deficient ($IFNAR^{-/-}$) mice were immunized with 10 μg of the *S. typhi* porins' preparation as described above. Both anti-porins IgM and IgG titers were unaltered in $IFNAR^{-/-}$ mice as compared with B6 mice (Fig. 3A and B). Moreover, the analysis of IgG subclasses on day 30, post immunization, revealed no differences between $IFNAR^{-/-}$ and B6 mice (Fig. 3C). Therefore, we conclude that type I IFN signals are neither necessary to elicit or enhance IgM or IgG antibody responses against *S. typhi* porins, nor do they participate in the IgG class switch.

Activation of B cells and DC *via* TLR2 or TLR4 ligands

TLR2 and TLR4 ligands could enhance the *S. typhi* porins-specific antibody response by directly activating B cells, and thereby augment the BCR-mediated stimulation. To address this question, B cells from B6, $MyD88^{-/-}$, $TRIF^{-/-}$, $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were isolated and stimulated with 1 μg/mL of *S. typhi* porins. $TLR2^{-/-}$, $TLR4^{-/-}$ and $TRIF^{-/-}$ B cells upregulated expression of the co-stimulatory molecules CD86 and of MHC II (Fig. 4A and B). In contrast, $MyD88^{-/-}$ B cells were not activated (Fig. 4A and B), suggesting that a stimulation of B cells through TLR4 or TLR2 induced by the *S. typhi* porins' preparation was sufficient for the activation of B cells, but that B cells were not activated in the absence of both signals.

DC are not only important for the activation of CD4 T cells that will later provide cognate T help to the B cells, but can also supply signals directly to B cells [39]. To assess the stimulation of DC by the *S. typhi* porins' preparation, primary splenic DC (DC) were stimulated with 1 μg/mL of *S. typhi* porins. Monitoring of CD86 (Fig. 5A), CD40 (Fig. 5C), CD80 and MHC II expression (data not shown) revealed that $TLR4^{-/-}$ DC were stimulated by porins. It is noteworthy that both B cells and DC from $TLR2^{-/-}$ mice upregulated the activation marker CD86 (Fig. 5A and B), whereas $TLR2^{-/-}$ B cells failed to respond with CD40 upregulation after porins stimulation (Fig. 5D). To test for the presence of other bacterial TLR2 ligands, such as lipoproteins and peptidoglycans in the *S. typhi* porins' preparation, the different B-cell and DC preparations were stimulated with proteinase K-digested porins. Although B6 and $TLR2^{-/-}$ DC and B cells were still activated by this preparation, $TLR4^{-/-}$ DC and B cells did not respond, indicating the absence of non-protein TLR2 ligands in the porins' preparation, and suggesting that *S. typhi* porins or other proteins in the preparation bind to TLR2. Stimulating

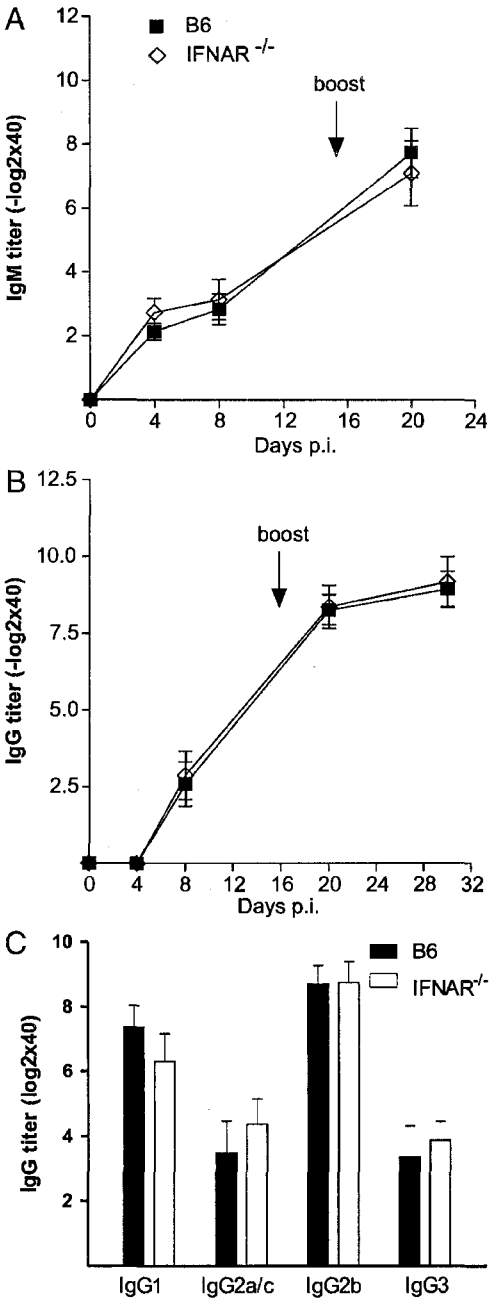

Figure 3. Antibody response against *S. typhi* porins is independent of type I IFN. $IFNAR^{-/-}$ and B6 mice were immunized with 10 μg porins at day 0 and 15. (A) Anti-porins IgM and (B) anti-porins IgG antibody titers were measured by ELISA at the indicated time points. (C) Anti-porins IgG subclasses IgG1, IgG2a/c, IgG2b and IgG3 were determined on day 30 post immunization. Results are expressed as the mean ± SEM of six mice *per* group. Statistical analysis was performed using Student *t*-test.

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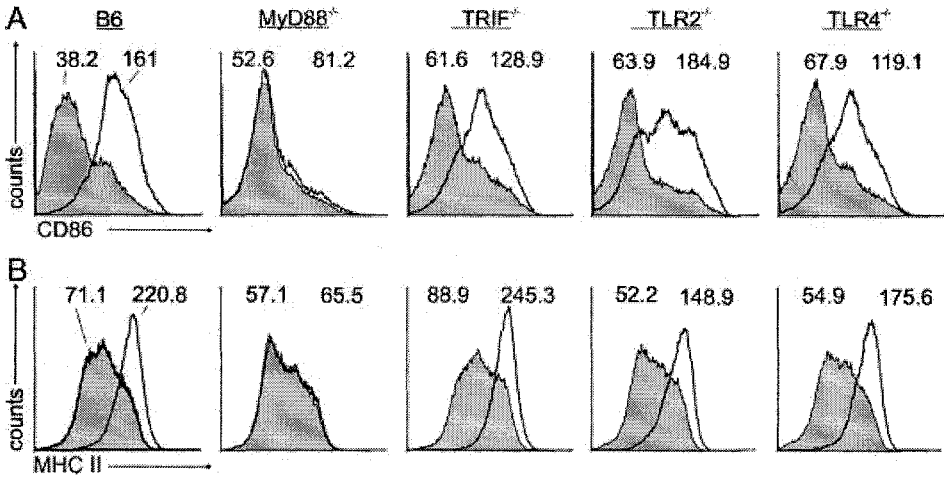

Figure 4. S. *typhi* porins induce B-cell activation. B cells from B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were stimulated with 1 μg/mL S. *typhi* porins. After 24 h, the expression of (A) CD86 and (B) MHC II on CD19^{+} B cells was analyzed by flow cytometry. Results are from one of three independent experiments with filled histograms representing B cells incubated in medium alone, and open histograms representing B cells incubated with porins. Values represent the MFI of the respective cell population.

TLR4$^{-/-}$ DC and B cells with a preparation from *ΔOmpCΔOmpF* (*i.e.* porins-deficient) *S. typhi* did not induce the activation of the cells, providing further support that the porins' preparation did not contain flagellin or another TLR2 binding protein (Fig. 5). However, this porins-deficient preparation contains other proteins that are normally not present in the *S. typhi* porins' preparation and detectable levels of LPS (data not shown) further indicating that such "contaminating" molecules are not responsible for TLR2 activation. These results could be confirmed using *in vitro* stimulation of HEK293 cells stably transfected with TLR2 and TLR2/6 (our unpublished data). Because the observed differences in the *S. typhi* porins-mediated activation of B cells and DC could be due to traces of LPS that were not detectable in our *Limulus* amoebocyte lysate assay, B cells and DC were stimulated with 100 and 2 ng/mL LPS (the latter being a concentration below the limit of detection of our Limulus assay). B6, TLR2$^{-/-}$ and to a lesser extent, MyD88$^{-/-}$ and TRIF$^{-/-}$ B cells were activated at a high concentration of LPS, but did not respond when 2 ng/mL were provided (Fig. 5B and D). In contrast, even this low concentration of LPS was able to induce upregulation of CD86, and CD40 in DC from B6 and TLR2$^{-/-}$ mice (Fig. 5A and C). As expected, TLR4$^{-/-}$ B cells and DC remained unstimulated both at high and low LPS concentrations (Fig. 5). Overall, these results suggest that DC activation through TLR signals could contribute to the antibody response against *S. typhi* porins. Furthermore, it appears that TLR2 and TLR4 signals provided by the *S. typhi* porins' preparation differentially affect the activation of DC and B cells.

TLR2 and TLR4 signals on B cells contribute in anti-S. typhi porins antibody responses

As outlined above, TLR2 and TLR4 signals could act directly on B cells, or through the activation of other APC such as DC. To determine the contribution of TLR2 and TLR4 signaling in B cells to the anti-porins response, splenic B cells from B6, MyD88$^{-/-}$, TLR4$^{-/-}$ or TLR2$^{-/-}$ mice were adoptively transferred into B-cell-deficient μMT mice. Reconstituted mice were immunized on days 0 and 15 with 10 μg of the *S. typhi* porins' preparation and antibody titers were analyzed as described above. In the absence of TLR4 on B cells, anti-porins IgM titers were reduced (Fig. 6A). However, booster immunization elicited an IgG response comparable with B6 B-cell recipient mice (Fig. 6B and C), indicating that TLR4 expression on B cells is most important during the induction of the primary IgM antibody response. It is noteworthy, that mice receiving TLR2$^{-/-}$ B cells showed a normal IgM response but a reduced IgG production (Fig. 6), suggesting that TLR2 signals on B cells are critical for anti-porins IgG production. Mice receiving MyD88$^{-/-}$ B cells exhibited both impaired IgM and IgG response. (Fig. 6). Overall, these experiments confirm the importance of TLR2 and TLR4 signals on B cells for the anti-porins antibody production, and their differential contribution in shaping the adaptive immune response.

Discussion

TLR ligands are used in several vaccine preparations as adjuvants to enhance specific immune responses; however, the contribution of TLR signaling to induction and maintenance of antibody responses is still controversial. For example, it has been proposed that TLR are required to elicit an antibody response [17]; on the other hand, mice with a total deficiency in TLR signaling were able to produce specific antibody responses when immunized with antigens in TLR ligand-free adjuvants [18]. Recently, it has been also proposed that intrinsic TLR signals in B cells enhance the specific antibody responses, but are not required for their induction [40]. Most of these studies have used model antigens

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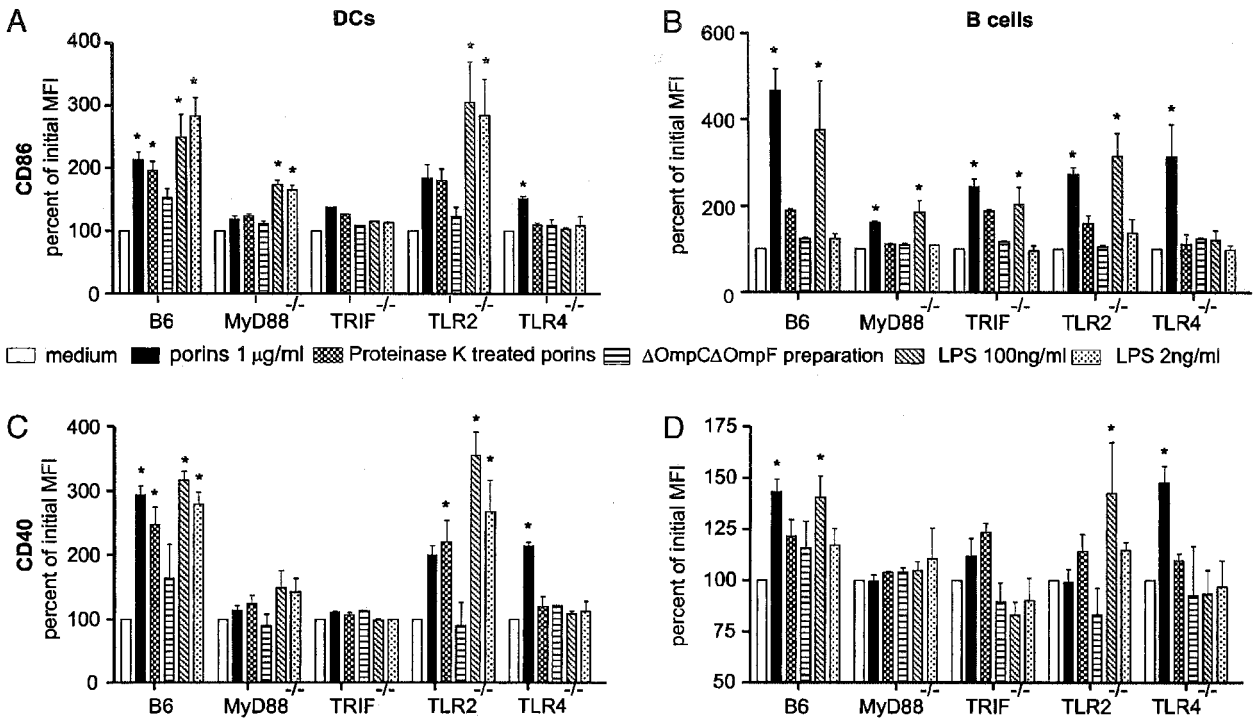

Figure 5. Activation of B cells and DC by *S. typhi* porins. B cells and DC from B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were stimulated with 1 µg/mL *S. typhi* porins, 1 µg/mL proteinase K-digested *S. typhi* porins, *ΔOmpCΔOmpF S. typhi* preparation, 100 ng/mL LPS, 2 ng/mL LPS or medium alone. After 24 h, the expression of CD86 (A, B) and CD40 (C, D) on CD11c^{+} DC (A, C), and CD19^{+} B cells (B, D) was analyzed by flow cytometry. Results are expressed as percentage of initial MFI and represent the mean ± SEM of four independent experiments. Statistical analysis was performed using one-way ANOVA. Significant differences between the MFI of the cells treated with the different stimuli and the MFI from the medium-treated cells of the corresponding genotype are indicated (*$p<0.05$).

that only partially reflect the situation in a response against bacterial antigens. Here, we show that TLR4- and TLR2-mediated signals are important for the enhancement of anti-porins antibody responses. Moreover, both TLR4- and TLR2-dependent signals contributed differentially in shaping the IgG antibody response against *S. typhi* porins. We decided to analyze the participation of TLR in the antibody response against bacterial antigens using immunization of *S. typhi* porins as a model because these proteins induce life-lasting bactericidal antibody responses [22]. In the present study, the porins-specific IgM and IgG antibody responses in MyD88$^{-/-}$ and TRIF$^{-/-}$, as well as TLR4$^{-/-}$ mice were reduced, whereas in TLR2$^{-/-}$ mice, a reduction in anti-porins IgG was observed. These results suggest that signaling through TLR participates in the enhancement of the anti-porins antibody response. The general reduction in the production of IgG subclasses in TLR4$^{-/-}$ and TRIF$^{-/-}$ mice, the pronounced reduction in IgG2a/c and IgG3 titers in TLR2$^{-/-}$ mice and, finally, the pronounced reduction in IgG2a/c and IgG3 in MyD88$^{-/-}$ mice indicated furthermore an important role of these molecules in shaping the IgG subclass expression. These results corroborate previous findings showing that MyD88 signaling is required for the production of IgG2a [14, 41]. Moreover, the similarities in the IgG subtype production between MyD88$^{-/-}$ and TLR2$^{-/-}$ also suggest that signaling through TLR2 and MyD88 impacts on the isotype class switch to IgG3. This could be mediated by the effect of TLR signals on the production of IFN-γ, because it has been shown that IFN-γ participates in the induction of IgG3 [42]; however, this issue remains to be addressed in future studies.

TLR signals could participate at different stages in the induction of antibody responses: through direct signals in the B cells enhancing their activation, proliferation and antibody production [12, 43, 44] or by the activation of DC to further trigger the adaptive immune response [45]. The presented *in vitro* and *in vivo* evidence shows that the *S. typhi* porins' preparation directly activates B cells *via* TLR2 and TLR4. It has been recently proposed that intrinsic TLR signals on B cells amplify the antibody response through MyD88, but are not required for initial triggering [40]. The present study supports this notion, and furthermore indicates that TLR2 and TLR4 signals act differentially during B-cell activation.

B cells can activate the T-dependent antibody responses by directly presenting antigen to Th cells [46]. However, other APC, in particular DC, contribute importantly to the antibody response by activating Th cells or by direct interaction with B cells [39]. DC from MyD88$^{-/-}$ and TRIF$^{-/-}$ mice showed no activation after

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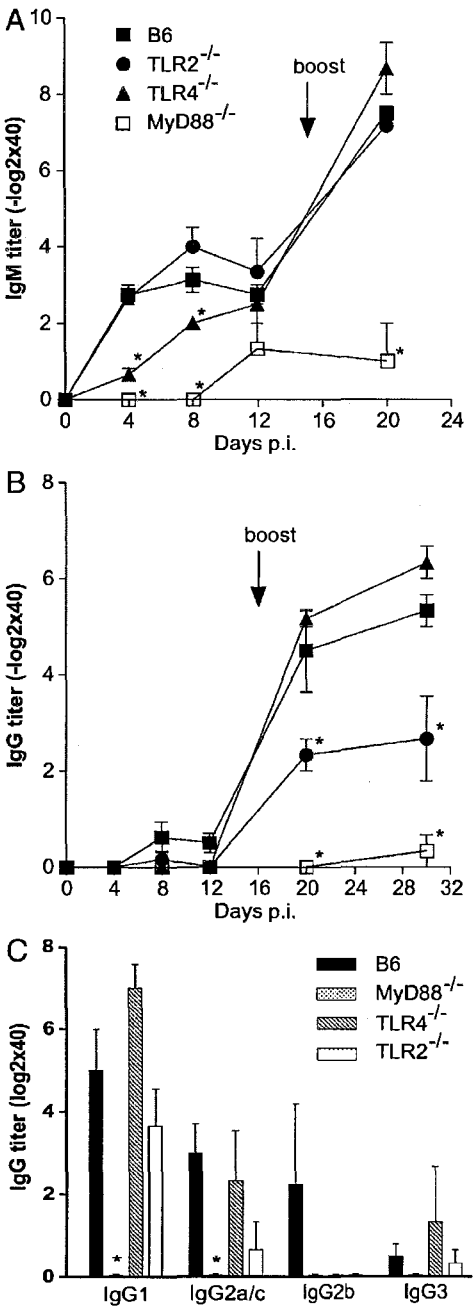

Figure 6. TLR2 and TLR4 signals on B cells contribute differentially to the antibody response. 5×10^7 B cells from B6, MyD88$^{-/-}$, TLR4$^{-/-}$ or TLR2$^{-/-}$ mice were adoptive transferred to μMT mice. Mice were immunized with 10 μg *S. typhi* porins on days 0 and 15. (A) Anti-porins IgM and (B) anti-porins IgG antibody titers were measured by ELISA at the indicated time points. (C) Anti-porins IgG subclasses IgG1, IgG2a/c, IgG2b and IgG3 were determined on day 30 post immunization. Results are expressed as the mean ± SEM, of at least three mice *per* group. Statistical analysis was performed using one-way ANOVA. Significant differences between the respective gene-deficient mice and the WT control at the same time point are indicated (*p<0.05).

S. typhi porins stimulation, whereas DC from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were activated. A possible difference between B cells and DC could be their activation through different TLR ligands, in particular to traces of LPS, which is supported by the fact that TLR4$^{-/-}$ mice showed a deficient antibody response. Although the low concentration of LPS probably present in the porins' preparation was not able to activate B cells, it was enough to activate DC. Importantly, the concerted stimulation of specific B cells through TLR2 and BCR by the porins' preparation, acting as a PAMP and an antigen (Pamptigen) [47], is probably sufficient in TLR4$^{-/-}$ mice to induce anti-porins IgM antibodies. Of note is that very small amounts of LPS, undetectable by conventional methods, can still participate in immune responses *in vivo*, which should be considered for the analysis of the immune response to isolated bacterial components.

Taken together, we propose that TLR4-mediated, B-cell intrinsic signals enhance the *S. typhi* porins-specific IgM production, whereas TLR2-mediated, B-cell intrinsic signals participate in the enhancement and shaping of the IgG response. These findings will help to guide further improvements in the development of vaccines against important human pathogens.

Materials and methods

Mice and bacteria

C57BL/6 (B6) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). MyD88$^{-/-}$ [35], TRIF$^{-/-}$ [48], TLR4$^{-/-}$ [49], TLR2$^{-/-}$ [50] and μMT [51] mice on the B6 background were bred in the Institut für Labortierkunde (University of Zurich, Switzerland). IFNAR$^{-/-}$ [52] on the B6 background were kindly provided by Martin Bachmann, Cytos AG, Schlieren, Switzerland, and bred in the Research Department Kantonal Hospital St Gallen. All mice were maintained in individually ventilated cages and were used between 6 and 9 weeks of age. All animal experiments were performed in accordance with the Swiss Federal legislation on animal protection. For porins' preparation the *S. typhi* strain from ATCC (No. 9993) and the isogenic *S. typhi* mutant strain VALE39*ΔompF* Km^R*ΔompC* Cm^R [22] were used.

Preparation of porins and immunization protocol

The porins (*i.e.* OmpC and OmpF) were purified from *S. typhi* or its isogenic mutant as previously described [21]. LPS content was determined by means of the *Limulus* amoebocyte lysate assay (LAL; Endosafe® KTA, Charles River Endosafe Laboratories, Charleston, SC, USA), detection limit 0.2 ng LPS/μg protein. Groups of mice were immunized i.p. on day 0 and boosted on day 15 with 10 μg of the *S. typhi* porins' preparation. Blood samples were collected at various times post immunization as indicated. Individual serum samples were stored at −20°C until analysis.

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Determination of antibody titers by ELISA

High-binding 96-well polystyrene plates (Corning®, New York, NY, USA) were coated with 10 μg/mL of porins in 0.1 M carbonate–bicarbonate buffer, pH 9.5. Plates were incubated for 1 h at 37° and then overnight at 4°. Before use, plates were washed three times in PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T) (Sigma-Aldrich). Non-specific binding was blocked with 5% non-fat dry milk diluted in PBS (PBS-M) for 2 h at 37°. After washing, sera were diluted 1:40 in PBS-M and twofold serial dilutions were added to the wells. Plates were incubated for 2 h at 37°, followed by four washes with PBS-T. The optimal dilution, 1:1000, of peroxidase-conjugated rabbit anti-mouse IgM, IgG, IgG1, IgG2b antibodies (Zymed, San Francisco, CA, USA), IgG2a/c (Acris GmbH, Germany; mice from the B6 express the subclass IgG2c that cross react with antibodies against the IgG2a subclass and therefore we refer to them as IgG2a/c [53, 54]), IgG3 (Rockland, Gilbertsville, PA, USA) in PBS-M was added, followed by 1 h of incubation at 37°C and four additional washes with PBS-T. Ortho-phenylenediamine (0.5 mg/mL; Sigma) in 0.1 M citrate buffer, pH 5.6, containing 0.08% H_2O_2 was used as the enzyme substrate. The reaction was stopped with 1.25 M H_2SO_4 and the optical densities were read at 492 nm using an automatic ELISA plate reader (Tecan). Antibody titers are given as $-\log_2$ dilution $\times 40$. Positive titers were defined as 3 SD above the mean values of the negative controls.

B lymphocyte and DC isolation

Single-cell splenocyte suspensions were obtained from B6, Myd88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice by digestion with collagenase (Sigma-Aldrich). The B-lymphocyte fraction was enriched using mouse CD45R (B220) microbeads (Miltenyi Biotec) and the CD11c^{+} DC fraction using mouse CD11c (N418) microbeads (Miltenyi Biotec) as recommended by the manufacturer. For adoptive transfer experiments, 5×10^7 B6, MyD88$^{-/-}$, TLR2$^{-/-}$ or TLR4$^{-/-}$ B cells (>96% purity as assessed by CD19 expression) were injected intravenously to μMT mice; reconstituted mice were immunized with porins 3 h later.

B lymphocyte and DC stimulation

B lymphocytes or DC from B6, Myd88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (2×10^6 cells/well) were seeded in RPMI 1640 (Gibco) 5% FCS (Gibco) and stimulated with 1 μg/mL of *S. typhi* porins' preparation, *S. typhi* porins' preparation previously digested with proteinase K (Roche), *ΔOmpCΔOmpF S. typhi* preparation, 2 or 100 ng/mL protein-free *S. typhi* LPS (provided by Dr. John S. Gunn, Ohio State University, Columbus, OH, USA) or medium alone. After 24 h, cells were harvested, stained with mAbs against CD19 PE (Immunotools), CD11c PE, CD40 APC, CD80 FITC, I-A/I-E APC and CD86 APC (BD Biosciences Pharmingen). Differential regulation of CD86 and CD40 was best suitable as read-out for DC *versus* B-cell activation because CD80 was only weakly expressed on B cells and MHC II was highly expressed on freshly isolated splenic DC and could not be further upregulated with the stimuli used here. 7-amino-actinomycin D (7AAD) (Sigma-Aldrich) was added at a concentration of 2.5 μg/mL 20 min prior to the analysis to exclude non-viable cells. Cells were analyzed with a FACS Calibur flow cytometer using the CellQuest software (BD Biosciences).

Statistical analysis

Statistical analyses were performed using Student *t*-test, or one-way ANOVA with Prism 5.0 (GraphPad Software). A *p*-value of <0.05 was considered significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 Kawai, T. and Akira, S., TLR signaling. *Semin. Immunol.* 2007. **19**: 24–32.

2 Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M. and Demengeot, J., Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med.* 2003. **197**: 403–411.

3 Dasari, P., Nicholson, I. C., Hodge, G., Dandie, G. W. and Zola, H., Expression of toll-like receptors on B lymphocytes. *Cell. Immunol.* 2005. **236**: 140–145.

4 Gururajan, M., Jacob, J. and Pulendran, B., Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. *PLoS ONE* 2007. **2**: e863.

Journal: EJI Issue Number 1 Manuscript Number 200838185 Color Figures nil

5 Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S. and Hartmann, G., Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 2002. **168**: 4531–4537.

6 Kabelitz, D., Expression and function of Toll-like receptors in T lymphocytes. *Curr. Opin. Immunol.* 2007. 19: 39–45.

7 Leadbetter, E. A., Rifkin, I. R., Hohlbaum, A. M., Beaudette, B. C., Shlomchik, M. J. and Marshak-Rothstein, A., Chromatin–IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 2002. **416**: 603–607.

8 Lau, C. M., Broughton, C., Tabor, A. S., Akira, S., Flavell, R. A., Mamula, M. J., Christensen, S. R. *et al.*, RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J. Exp. Med.* 2005. 202: 1171–1177.

9 Viglianti, G. A., Lau, C. M., Hanley, T. M., Miko, B. A., Shlomchik, M. J. and Marshak-Rothstein, A., Activation of autoreactive B cells by CpG dsDNA. *Immunity* 2003. 19: 837–847.

10 Bernasconi, N. L., Traggiai, E. and Lanzavecchia, A., Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 2002. **298**: 2199–2202.

11 Bernasconi, N. L., Onai, N. and Lanzavecchia, A., A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* 2003. **101**: 4500–4504.

12 Ruprecht, C. R. and Lanzavecchia, A., Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *Eur. J. Immunol.* 2006. **36**: 810–816.

13 Heer, A. K., Shamshiev, A., Donda, A., Uematsu, S., Akira, S., Kopf, M. and Marsland, B. J., TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. *J. Immunol.* 2007. 178: 2182–2191.

14 Jegerlehner, A., Maurer, P., Bessa, J., Hinton, H. J., Kopf, M. and Bachmann, M. F., TLR9 signaling in B cells determines class switch recombination to IgG2a. *J. Immunol.* 2007. 178: 2415–2420.

15 Nagai, Y., Kobayashi, T., Motoi, Y., Ishiguro, K., Akashi, S., Saitoh, S., Kusumoto, Y. *et al.*, The radioprotective 105/MD-1 complex links TLR2 and TLR4/MD-2 in antibody response to microbial membranes. *J. Immunol.* 2005. 174: 7043–7049.

16 Zinkernagel, R. M., Maternal antibodies, childhood infections, and autoimmune diseases. N. *Engl. J. Med.* 2001. **345**: 1331–1335.

17 Pasare, C. and Medzhitov, R., Control of B-cell responses by Toll-like receptors. *Nature* 2005. **438**: 364–368.

18 Gavin, A. L., Hoebe, K., Duong, B., Ota, T., Martin, C., Beutler, B. and Nemazee, D., Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science* 2006. **314**: 1936–1938.

19 Nikaido, H., Outer membrane, in: Neidhard, F. C., Ingraham, J. L., Curtiss, R., III *et al.* (Eds.), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, ASM Press, Washington, DC 1996, pp. 29–47.

20 Isibasi, A., Ortiz-Navarrete, V., Paniagua, J., Pelayo, R., Gonzalez, C. R., Garcia, J. A. and Kumate, J., Active protection of mice against *Salmonella typhi* by immunization with strain-specific porins. *Vaccine* 1992. 10: 811–813.

21 Salazar-Gonzalez, R. M., Maldonado-Bernal, C., Ramirez-Cruz, N. E., Rios-Sarabia, N., Beltran-Nava, J., Castanon-Gonzalez, J., Castillo-Torres, N. *et al.*, Induction of cellular immune response and anti-*Salmonella enterica serovar typhi* bactericidal antibodies in healthy volunteers by immunization with a vaccine candidate against typhoid fever. *Immunol. Lett.* 2004. 93: 115–122.

22 Secundino, I., Lopez-Macias, C., Cervantes-Barragan, L., Gil-Cruz, C., Rios-Sarabia, N., Pastelin-Palacios, R., Villasis-Keever, M. A. *et al.*, Salmonella porins induce a sustained, lifelong specific bactericidal antibody memory response. *Immunology* 2006. 117: 59–70.

23 Isibasi, A., Ortiz, V., Vargas, M., Paniagua, J., Gonzalez, C., Moreno, J. and Kumate, J., Protection against *Salmonella typhi* infection in mice after immunization with outer membrane proteins isolated from *Salmonella typhi* 9,12,d, Vi. *Infect. Immun.* 1988. 56: 2953–2959.

24 Isibasi, A., Paniagua, J., Rojo, M. P., Martin, N., Ramirez, G., Gonzalez, C. R., Lopez-Macias, C. *et al.*, Role of porins from *Salmonella typhi* in the induction of protective immunity. *Ann. N. Y. Acad. Sci.* 1994. 730: 350–352.

25 Calderon, I., Lobos, S. R., Rojas, H. A., Palomino, C., Rodriguez, L. H. and Mora, G. C., Antibodies to porin antigens of *Salmonella typhi* induced during typhoid infection in humans. *Infect. Immun.* 1986. 52: 209–212.

26 Ortiz, V., Isibasi, A., Garcia-Ortigoza, E. and Kumate, J., Immunoblot detection of class-specific humoral immune response to outer membrane proteins isolated from *Salmonella typhi* in humans with typhoid fever. *J. Clin. Microbiol.* 1989. 27: 1640–1645.

27 Massari, P., Henneke, P., Ho, Y., Latz, E., Golenbock, D. T. and Wetzler, L. M., Cutting edge: immune stimulation by neisserial porins is toll-like receptor 2 and MyD88 dependent. *J. Immunol.* 2002. **168**: 1533–1537.

28 Massari, P., Visintin, A., Gunawardana, J., Halmen, K. A., King, C. A., Golenbock, D. T. and Wetzler, L. M., Meningococcal porin PorB binds to TLR2 and requires TLR1 for signaling. *J. Immunol.* 2006. 176: 2373–2380.

29 Singleton, T. E., Massari, P. and Wetzler, L. M., Neisserial porin-induced dendritic cell activation is MyD88 and TLR2 dependent. *J. Immunol.* 2005. 174: 3545–3550.

30 Biswas, A., Banerjee, P., Mukherjee, G. and Biswas, T., Porin of *Shigella dysenteriae* activates mouse peritoneal macrophage through Toll-like receptors 2 and 6 to induce polarized type I response. *Mol. Immunol.* 2007. 44: 812–820.

31 Galdiero, M., Galdiero, M., Finamore, E., Rossano, F., Gambuzza, M., Catania, M. R., Teti, G. *et al.*, *Haemophilus influenzae* porin induces Toll-like receptor 2-mediated cytokine production in human monocytes and mouse macrophages. *Infect. Immun.* 2004. 72: 1204–1209.

32 Vega, M. I., Santos-Argumedo, L., Huerta-Yepez, S., Luria-Perez, R., Ortiz-Navarrete, V., Isibasi, A. and Gonzalez-Bonilla, C. R., A *Salmonella typhi* OmpC fusion protein expressing the CD154 Trp140-Ser149 amino acid strand binds CD40 and activates a lymphoma B-cell line. *Immunology* 2003. 110: 206–216.

33 Ray, A., Karmakar, P. and Biswas, T., Up-regulation of CD80-CD86 and IgA on mouse peritoneal B-1 cells by porin of *Shigella dysenteriae* is Toll-like receptors 2 and 6 dependent. *Mol. Immunol.* 2004. 41: 1167–1175.

34 Ray, A. and Biswas, T., Porin of *Shigella dysenteriae* enhances Toll-like receptors 2 and 6 of mouse peritoneal B-2 cells and induces the expression of immunoglobulin M, immunoglobulin G2a and immunoglobulin A. *Immunology* 2005. 114: 94–100.

35 Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K. and Akira, S., Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 1998. 9: 143–150.

36 Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K. and Akira, S., Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J. Immunol.* 2002. 169: 6668–6672.

37 Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O. *et al.*, Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003. **301**: 640–643.

Journal: EJI Issue Number 1 Manuscript Number 200838185 Color Figures nil

38 Fink, K., Lang, K. S., Manjarrez-Orduno, N., Junt, T., Senn, B. M., Holdener, M., Akira, S. *et al.*, Early type I interferon-mediated signals on B cells specifically enhance antiviral humoral responses. *Eur. J. Immunol.* 2006. 36: 2094–2105.

39 Somasse, T., Flamand, V., De, B. G., Bazin, H., Tielemans, F., Thielemans, K., Urbain, J. *et al.*, Antigen-pulsed dendritic cells can efficiently induce an antibody response *in vivo*. *J. Exp. Med.* 1992. 175: 15–21.

40 Meyer-Bahlburg, A., Khim, S. and Rawlings, D. J., B cell intrinsic TLR signals amplify but are not required for humoral immunity. *J. Exp. Med.* 2007. 204: 3095–3101.

41 Ehlers, M., Fukuyama, H., McGaha, T. L., Aderem, A. and Ravetch, J. V., TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *J. Exp. Med.* 2006. 203: 553–561.

42 Snapper, C. M., McIntyre, T. M., Mandler, R., Pecanha, L. M., Finkelman, F. D., Lees, A. and Mond, J. J., Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J. Exp. Med.* 1992. 175: 1367–1371.

43 Iwasaki, A. and Medzhitov, R., Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 2004. 5: 987–995.

44 Leadbetter, E. A., Rifkin, I. R. and Marshak-Rothstein, A., Toll-like receptors and activation of autoreactive B cells. *Curr. Dir. Autoimmun.* 2003. 6: 105–122.

45 Mackay, F., Silveira, P. A. and Brink, R., B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling. *Curr. Opin. Immunol.* 2007. 19: 327–336.

46 Scandella, E., Fink, K., Junt, T., Senn, B. M., Lattmann, E., Forster, R., Hengartner, H. and Ludewig, B., Dendritic cell-independent B cell activation during acute virus infection: a role for early CCR7-driven B-T helper cell collaboration. *J. Immunol.* 2007. 178: 1468–1476.

47 Acosta-Ramirez, E., Perez-Flores, R., Majeau, N., Pastelin-Palacios, R., Gil-Cruz, C., Ramirez-Saldana, M., Manjarrez-Orduno, N. *et al.*, Translating innate response into long-lasting antibody response by the intrinsic antigen-adjuvant properties of papaya mosaic virus. *Immunology* 2007.

48 Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J. *et al.*, Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 2003. 424: 743–748.

49 Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S., Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J.Immunol.* 1999. 162: 3749–3752.

50 Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S., Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999. 11: 443–451.

51 Kitamura, D., Roes, J., Kuhn, R. and Rajewsky, K., A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 1991. 350: 423–426.

52 Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. and Aguet, M., Functional role of type I and type II interferons in antiviral defense. *Science* 1994. 264: 1918–1921.

53 Jouvin-Marche, E., Morgado, M. G., LeGuern, C., Voegtle, D., Bonhomme, F. and Cazenave, P. A., The mouse Igh-1a and Igh-1b H chain constant regions are derived from two distinct isotypic genes. *Immunogenetics* 1989. 29: 92–97.

54 Morgado, M. G., Cam, P., Gris-Liebe, C., Cazenave, P. A. and Jouvin-Marche, E., Further evidence that BALB/c and C57BL/6 gamma 2a genes originate from two distinct isotypes. *EMBO J.* 1989. 8: 3245–3251.

Abbreviations: **IFNAR$^{-/-}$:** IFN receptor-deficient · **MHC II:** MHC class II · **MyD88$^{-/-}$:** myeloid differentiating gene 88-deficient · **Omp:** outer membrane protein · **S. typhi:** *Salmonella enterica serovar typhi* · **TLR4$^{-/-}$, TLR2$^{-/-}$:** TLR4-, TLR2-deficient · **TRIF$^{-/-}$:** Toll/IL-R domain-containing adaptor-inducing IFN-β-deficient

Full correspondence: Dr. Constantino López-Macías, UIMIQ, Coordinación de Investigación en Salud, Piso 4 Bloque B Unidad de Congresos Centro Médico Nacional Siglo XXI, Av. Cuauhtémoc 330, Col. Doctores, México D.F. C.P. 06020
Fax: +525557610952
e-mail: constantino@sminmunologia.org;constantino.lopez@imss.gob.mx

* Additional correspondence: Dr. Burkhard Ludewig
e-mail: burkhard.ludewig@kssg.ch

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Control of coronavirus infection through plasmacytoid dendritic-cell–derived type I interferon

Luisa Cervantes-Barragan,[1,2] Roland Züst,[1] Friedemann Weber,[3] Martin Spiegel,[3] Karl S. Lang,[4] Shizuo Akira,[5] Volker Thiel,[1] and Burkhard Ludewig[1]

[1]Research Department, Kantonal Hospital St Gallen, St Gallen, Switzerland; [2]Unidad de Investigación Médica en Inmunoquímica, Hospital de Especialidades, Mexico City, Mexico; [3]Abteilung Virologie, Institut fur Medizinische Mikrobiologie und Hygiene, Universitat Freiburg, Freiburg, Germany; [4]Institute of Experimental Immunology, University Hospital, Zurich, Switzerland; [5]Department of Host Defense, Osaka University, Osaka, Japan

This study demonstrates a unique and crucial role of plasmacytoid dendritic cells (pDCs) and pDC-derived type I interferons (IFNs) in the pathogenesis of mouse coronavirus infection. pDCs controlled the fast replicating mouse hepatitis virus (MHV) through the immediate production of type I IFNs. Recognition of MHV by pDCs was mediated via TLR7 ensuring a swift IFN-α production following encounter with this cytopathic RNA virus. Furthermore, the particular type I IFN response pattern was not restricted to the murine coronavirus, but was also found in infection with the highly cytopathic human severe acute respiratory syndrome (SARS) coronavirus. Taken together, our results suggest that rapid production of type I IFNs by pDCs is essential for the control of potentially lethal coronavirus infections. (Blood. 2007;109:1131-1137)

Introduction

Type I IFNs (IFNα/β) play a decisive role in shaping antiviral immune responses.[1] Signaling through the type I IFN receptor leads to the activation of a particular set of genes, including protein kinase R, and Mx proteins,[2] which exert potent direct antiviral effects. Other type I IFN–stimulated gene products, such as IFN-γ, activate downstream elements of the innate immune system that further promote rapid clearance of the viral pathogen.[3] Although almost all hematopoietic and nonhematopoietic cells are able to produce IFN-α/β after viral infections, plasmacytoid dendritic cells (pDCs) are the major source of IFN-α, both in humans and mice.[4-7] One key feature of pDCs is the expression of receptors for single-stranded RNA or DNA such as Toll-like receptor 7 (TLR7)[8,9] and TLR9,[10,11] respectively, which are essential to sense the viral pathogen and to trigger the innate immune response. In mouse cytomegalovirus (MCMV) infection, pDCs respond quickly and generate the first wave of IFN-α.[12,13] These previous studies have clearly established an important role of pDCs for the rapid production of type I IFNs in antiviral immune responses.

Coronaviruses are positive-stranded RNA viruses that are able to infect a broad range of vertebrates and are associated mainly with respiratory, enteric, and, sometimes, systemic diseases.[14] Human coronaviruses are known to generally cause mild upper-respiratory tract disease, including common cold, and occasional enteric infections. The emergence of a novel coronavirus (COV) causing severe acute respiratory syndrome (SARS) highlighted the potential of coronaviruses to seriously impact on human health.[14] Phylogenetic analyses revealed a close relationship of SARS-CoV to group II coronaviruses,[15] which is prototyped by the mouse hepatitis virus (MHV). MHV causes enteritis, pneumonia, hepatitis, and demyelinating encephalomyelitis in mice and is one of the most extensively studied coronaviruses in vitro and in vivo.[16] Both SARS-CoV and MHV have a similar genome organization and share common mechanisms and enzymes involved in genome expression.[15,17] Because of these similarities, it is conceivable that the pathogenesis of and the immune response against systemic MHV infection in mice recapitulates some of the essential features of SARS-CoV infection.

The immune response against MHV is characterized by a strong cytotoxic T-cell (CTL) response that mediates initial clearance of the virus,[18,19] whereas neutralizing antibodies appear to be required to prevent re-emergence of the persistent infection.[20,21] In contrast to the well-characterized defense mechanisms of the adaptive immune response against MHV, the innate immune response has not been sufficiently characterized. Similarly, the importance of innate immune mechanisms triggered by SARS-CoV has remained enigmatic. Several studies have shown that peripheral-blood mononuclear cells (PBMCs) from SARS-CoV–infected individuals produce high amounts of inflammatory cytokines and chemokines, but not type I IFNs.[22-24] Furthermore, in vitro studies have shown that neither macrophages nor monocyte-derived DCs respond to SARS-CoV infection with significant IFN-α production.[25-28] Nonetheless, there is clear evidence that treatment with recombinant IFN-β or IFN-α can inhibit SARS-CoV replication in vitro,[29-31] and, most importantly, diminish the severity of SARS-CoV infection in vivo.[32,33] Thus, although the antiviral activity of type I IFNs in SARS-CoV infections has been clearly demonstrated, it remains to be established whether a significant production of type I IFNs can be achieved upon coronavirus infection and how this may impact on virus replication and disease. The present study addressed this issue and identified pDCs as the major source of type I IFNs during cytopathic coronaviral replication. We show furthermore that pDCs are crucial during the initial phase of MHV

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infection since widespread replication in various nonlymphoid organs, and the associated organ damage, is efficiently kept in check through pDC-derived type I IFNs.

Materials and methods

Mice and viruses

C57BL/6 (B6) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). TLR3$^{-/-}$,[34] TLR7$^{-/-}$,[35] TLR3$^{-/-}$/TLR7$^{-/-}$, and MyD88$^{-/-}$[36] mice were bred in the Institut für Labortierkunde (University of Zurich, Switzerland). 129Sv and type I IFN receptor–deficient (IFNAR$^{-/-}$) mice[37] were obtained from the Institut für Labortierkunde (University of Zurich) and bred in our facilities. All mice were maintained in individually ventilated cages and were used between 6 and 9 weeks of age. All animal experiments were performed in accordance with the Swiss federal legislation on animal protection.

MHV A59 was generated from a molecularly cloned cDNA[38] based on the Albany strain of MHV A59 and propagated on L929 cells. SARS-CoV (isolate Frankfurt-1) was kindly provided by Stephan Becker (University of Marburg, Germany) and propagated on Vero cells. The Newcastle disease virus (NDV; strain H53) stock was grown on 10-day-old embryonated chicken eggs.

Isolation of dendritic cells and flow cytometry

Murine conventional DCs (cDCs) and pDCs were obtained from spleens of 129Sv or IFNAR$^{-/-}$ mice following digestion with collagenase type II (Invitrogen, Basel, Switzerland) for 20 minutes at 37°C. Cells were resuspended in PBS supplemented with 2% FCS and 2 mM EDTA and overlaid on 20% Optiprep density gradient medium (Sigma-Aldrich, Basel, Switzerland). After centrifugation at 700*g* for 15 minutes, low-density cells were depleted of CD3$^+$ and CD19$^+$ cells using DYNAL magnetic beads according to the instructions of the manufacturer (Invitrogen). The DC-enriched preparations were stained with α–PDCA-1, α-CD11b, and α-CD11c, and the distinct pDC and cDC populations were sorted using a fluorescence-activated cell sorter (FACS) ARIA (BD Biosciences, Allschwil, Switzerland). Purity of both cell preparations was always more than 98%.

Murine bone marrow–derived cDCs or pDCs were generated by 6 to 7 days of culture with either granulocyte-monocyte colony-stimulating factor (GM-CSF)–containing supernatant from the cell line X63-GM-CSF (kindly provided by Dr Antonius Rolink, University of Basel) or Flt3-L (R&D systems, Oxford, United Kingdom) at 20 ng/mL, respectively. Bone marrow–derived cDCs were further purified using Optiprep density gradient centrifugation. Bone marrow–derived pDCs were purified using the mouse pDC isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) adapted for the isolation of in vitro–derived pDCs by adding CD11b-biotin to the negative selection cocktail.

Human circulating conventional dendritic cells and plasmacytoid dendritic cells were isolated from healthy donors (obtained from the Blood Bank of the University Hospital Freiburg, Germany). PBMCs were obtained by Ficoll-Hypaque density gradient centrifugation. Human pDCs or cDCs were further purified by magnetic sorting with human plasmacytoid dendritic-cell isolation kit (Miltenyi Biotec) or human BDCA-1 dendritic-cell isolation kit (Miltenyi Biotec), respectively. Purity of both cell preparation was for all donors more than 90% (with B cells being the major contaminating cell type).

Cells were analyzed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). The antibodies CD123 biotin, CD11c PE, B220 APC, and CD11b FITC were purchased from Biolegend (San Diego, CA); and CD303 (BDCA-2)–PE, mPDCA-1-FITC, mPDCA-1-PE, and CD11c-APC, from Miltenyi Biotec.

Virus infections, determination of virus titers, and liver enzyme values

Human pDCs and cDCs were exposed to SARS-CoV or NDV for 1 hour at 37°C, washed, and plated at 1.5×10^5/mL. Murine pDCs or cDCs were infected with MHV A59 for 1 hour at 37°C, washed, and plated at 1 to 2×10^5/mL. CpG ODN 2216 was used as a positive control for IFN-α production as described previously.[39]

Mice were injected intraperitoneally with 5 pfu MHV A59 and killed at the indicated time points. For depletion of pDCs, mice were injected intraperitoneally with 0.5 mg α–mPDCA-1 (Miltenyi Biotec) or 0.5 mg rat IgG2b isotype control antibody (Biolegend) 12 hours prior to infection. Organs were stored at −70°C until further analysis. Blood was incubated at room temperature to coagulate and was then centrifuged, and serum was used for alanine 2-oxoglutarate-aminotransferase (ALT) measurements using a Hitachi 747 autoanalyzer (Tokyo, Japan). Virus titers in organs were determined from frozen organs after weighing and homogenization. Viral titers were determined by standard plaque assay using L929 cells.

Histology, IFN-α ELISA, and reverse-transcription–polymerase chain reaction (RT-PCR)

Organs were fixed in 4% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Human IFN-α and mouse IFN-α concentration in cell-culture supernatants or serum was measured by enzyme-linked immunosorbent assay (ELISA; PBL Biomedical Laboratories, Piscataway, NJ) according to the manufacturer's instructions. To detect cellular and viral mRNAs in pDCs and cDCs, total cellular RNA was prepared using the RNeasy Kit for murine DCs (Qiagen, Basel, Switzerland) or Trifast (PEQLAB, Erlangen, Germany) for human DCs. Reverse transcription was done with 100 ng total RNA using the SuperScript II First-Strand Synthesis System (Invitrogen). PCR was performed with Red Taq Polymerase (Sigma-Aldrich) using standard protocols. The following primers were used for amplification: *mIFN-β* forward, 5′-CATCAACTATAAGCAGCTCCA-3′; *mIFN-β* reverse, 5′TTCAAGTGGAGAGCAGTTGAG3′; *mIFN-α4* forward, 5′CTGGTCAGCCTGTTCTCTAGGATGT3′; *mIFN-α4* reverse, 5′TCAGAGGAGGTTCCTGCATCAC3′; *mIFN-α total* forward, 5′ATGGCTAGRCTC TGTGCTTTCCT3′; *mIFN-α total* reverse, 5′AGGGCTCTCCAGAYTTCTGCTCTG3′; *mGAPDH* forward, 5′CATCAAGAAGGTGGTGAAGC3′; *mGAPDH* reverse, 5′CCTGTTGCTGTAGCCGTATT3′; *MHV-N* forward, 5′TCCTGGTTTTCTGGCATTACCCAG3′; and *MHV-N* reverse, 5′CTGAGGCAATACCGTGCCGGGCGC3′. Primer sequences for amplifying human IFN-β were 5′CATACCCACGGAGAAAGGACATT3′ and 5′TGATAGACATTAGCCAGGAGGTTC3′; for ISG56, 5′AAGTGGACCCTGAAAACCCTGAAT3′ and 5′TGCCCTTTTGTAGCCTCCTTGAT3′; for MxA, 5′GTTGTTTCCGAAGTGGACATCGCAAAA3′ and 5′CGGGCATCTGGTCACGAT3′; and for γ-actin, 5′GCCGGTCGCAATGGAAGAAGA3′ and 5′CATGGCCGGGGTGTTGAAGGTC3′. SARS-CoV transcription was detected by using primer 5′TGTCTAGCAGCAATAGCGCGAGGGC3′ for reverse transcription and primers 5′GGAAAAGCCAACCAACCTCGATCTCT3′ and 5′AAGTTGTAGCACGGTGGCAGC3′ for PCR.

Results

Rapid type I IFN production in pDCs following MHV infection

In a first set of experiments, the type I IFN response of pDCs and cDCs following encounter with MHV was determined. To this end, CD11c^{low}B220$^+$PDCA-1$^+$ pDCs and CD11c$^+$B220$^-$ cDCs were sorted from spleen-cell suspensions (Figure 1A-B) and infected with MHV. Primary pDCs but not cDCs responded with rapid and significant production of IFN-α (Figure 1C). The high IFN-α production in pDCs correlated well with the control of the virus infection (Figure 1D). Bone marrow–derived pDCs and cDCs that were differentiated with the growth factors Flt-3L or GM-CSF, respectively, responded in a similar pattern: rapid and high production of IFN-α in pDCs but not in cDCs (Figure 2A-B) and a good containment of the viral replication by pDCs (Figure 2C-D). A time-course RT-PCR analysis confirmed that the type I IFN response is considerably slower in cDCs (Figure 2E) compared

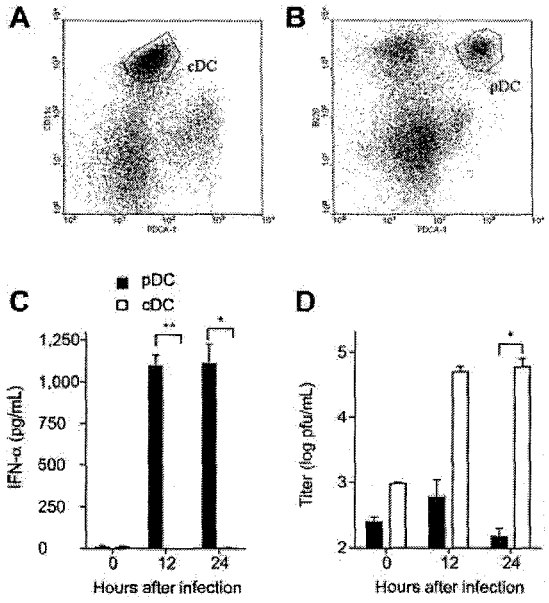

Figure 1. Type I IFN production and viral replication in MHV-infected splenic cDCs and pDCs. (A) Flow cytometric analysis of splenic cDCs ($CD11c^+PDCA\text{-}1^-$) and (B) splenic pDCs $CD11c^{low}$ $B220^+$ $PDCA\text{-}1^+$ before FACS sorting. Gates for sorting are indicated. (C) Primary FACS-purified murine splenic cDCs or pDCs were infected with MHV at a multiplicity of infection (moi) of 1. IFN-α secretion to culture supernatants was determined by ELISA at the indicated time points. (D) Virus titers in culture supernatants were determined by plaque assay. (C-D) Data represent mean values ± SD pooled from 2 experiments. Statistical analysis was performed using Student *t* test (*$P < .05$; **$P < .01$).

with pDCs (Figure 2F). Furthermore, pDCs lacking the type I IFN receptor were more susceptible to MHV infection than wild-type pDCs (Figure 2G-H). It appears therefore that pDCs are well equipped to respond efficiently against MHV with a strong type I IFN production, and that this early reaction exerts a potent protective effect against this cytopathic virus.

Type I IFN signaling is essential for the control of MHV infection

Signaling through the type I IFN receptor (IFNAR) is essential for the control of several viral infections.[40] To assess the importance of type I IFN signaling in the course of an MHV infection, IFNAR-deficient ($IFNAR^{-/-}$) and 129Sv wild-type (wt) mice were infected with 5 pfu MHV. MHV infection in $IFNAR^{-/-}$ mice was lethal within only 48 hours, while wt mice survived without showing signs of MHV infection–associated clinical disease (Figure 3A). Furthermore, $IFNAR^{-/-}$ but not wt mice showed rapidly rising liver enzyme values in serum (Figure 3B) and an acute hemorrhagic liver disease with massive hepatocyte necrosis (Figure 3C). The detailed time-course analysis of viral spread in both $IFNAR^{-/-}$ and wt mice indicated that a functional type I IFN system is essential to restrict the initial viral replication to the spleen and to prevent spread to nonhematopoietic organs such as the lung and the central nervous system (Figure 3D). Notably, the replication of the strongly hepatotropic MHV in the liver was efficiently reduced in the presence of a functional type I IFN system with a reduction of viral titers of 3 to 4 orders of magnitude (Figure 3D). It is thus most likely that the rapidly lethal disease in $IFNAR^{-/-}$ mice following MHV infection is a consequence of an insufficient initial control of the cytopathic virus in the spleen and the subsequent high-level replication in various organs, eventually causing an acute multiorgan failure.

Early control of MHV infection through pDC-derived type I IFN

The above results suggested that the initial control of MHV requires an efficient type I IFN response that might be generated by pDCs. It has been shown that pDCs use the TLR pathway rather than the RNA helicase RIG-I for recognition of RNA viruses and to produce type I IFN.[41] Therefore, to investigate how pDCs recognize MHV, bone marrow–derived pDCs from TLR3-deficient ($TLR3^{-/-}$), TLR3 and TLR7 double knock-out ($TLR3^{-/-}$/$TLR7^{-/-}$), TLR7-deficient ($TLR7^{-/-}$), and MyD88-deficient ($MyD88^{-/-}$) mice were infected with a low dose of MHV (moi = 1), and the production of IFN-α was determined after 24

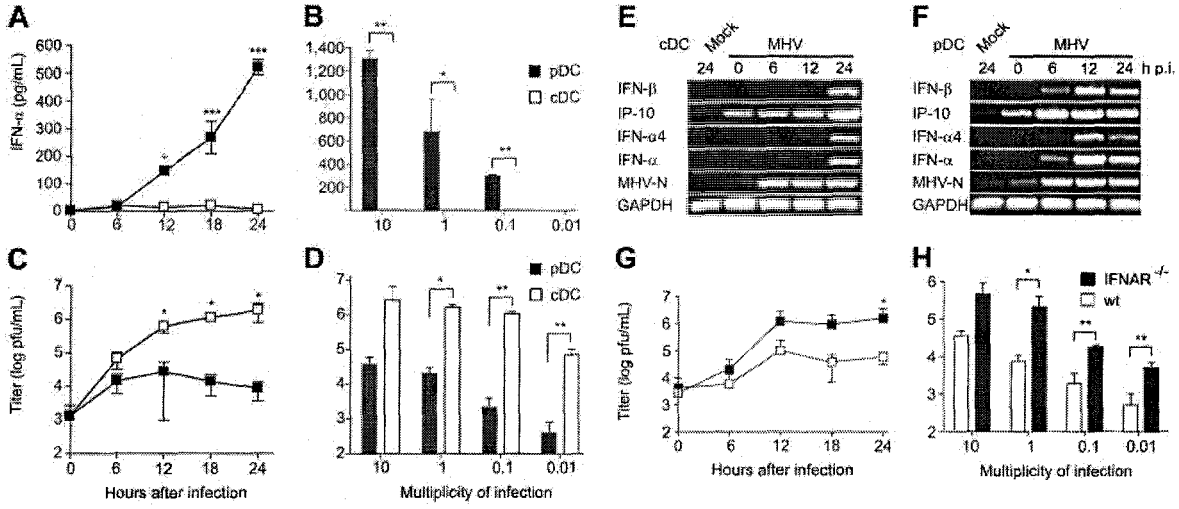

Figure 2. Type I IFN production and viral replication in MHV-infected in vitro–derived cDCs and pDCs. Infection of bone marrow–derived pDCs and cDCs with MHV. (A,C) IFN-α and virus titers in tissue culture supernatants were determined at the indicated times after MHV infection (moi = 1), or (B,D) at 24 hours after MHV infection with different moi as indicated. Values in panels A-D represent means ± SD from triplicate measurements. Experiments in panels A-D were repeated 3 times with comparable results. Expression of IFN-β, IP-10, IFN-α4, IFN-α , GAPDH, or MHV nucleoprotein (MHV-N) mRNAs was determined by RT-PCR using total RNA from bone marrow–derived (E) cDCs or (F) pDCs infected with MHV (moi = 1) or treated with PBS (mock). (G-H) Viral replication in MHV-infected wt or $IFNAR^{-/-}$ pDCs. Cells were infected with MHV A59 at (G) an moi of 1 or (H) at different moi as indicated. Virus titers in culture supernatants were determined by plaque assay at (G) the indicated times after MHV A59 infection (moi = 1) or (H) 24 hours after MHV infection. (A-D,G-H) Data represent mean values ± SD pooled from 2 experiments. Statistical analysis was performed using Student *t* test (*$P < .05$; **$P < .01$, ***$P < .001$).

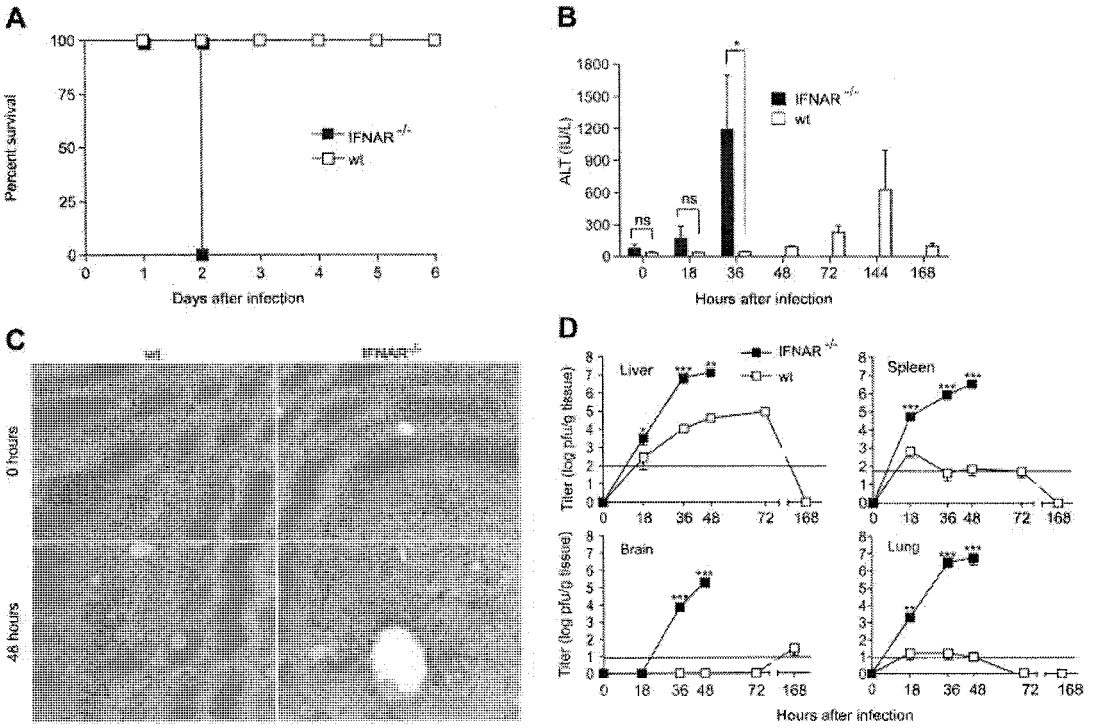

Figure 3. Impact of type I IFN signaling during MHV infection. IFNAR$^{-/-}$ or wt mice were injected intraperitoneally with 5 pfu MHV A59. (A) Health status of IFNAR$^{-/-}$ and wt mice was monitored twice daily after infection (n = 6). (B) ALT values were measured at the indicated time points after infection. (C) Liver pathology in IFNAR$^{-/-}$ and wt mice before or 48 hours after MHV A59 infection. Hematoxylin-eosin staining of 4% formaldehyde-fixed sections. Images were acquired using a Leica DMRA microscope (Leica, Heerbrugg, Switzerland) with a 25×/0.65 NA objective (total magnification, ×162). Images were processed using Adobe Photoshop (Adobe Systems, San Jose, CA). (D) Viral titers in liver, spleen, brain, and lung of MHV A59–infected IFNAR$^{-/-}$ or wt mice were determined at different time points after infection. Results represent the mean of 6 individual mice per time point. Solid horizontal lines in panel D represent limit of detection in the plaque assay. Data in panels B and D represent means ± SD from 2 experiments with a total of 3 or 6 mice evaluated per time point. Statistical analysis was performed using Student *t* test (ns, $P > .05$; $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$).

hours. Comparable amounts of IFN-α were found in the supernatants of TLR3$^{-/-}$ and wt control pDC cultures (Figure 4). In contrast, pDCs from neither TLR7$^{-/-}$, TLR3$^{-/-}$/TLR7$^{-/-}$, nor MyD88$^{-/-}$ mice responded with a significant IFN-α production to MHV infection (Figure 4), clearly indicating that MHV recognition by pDCs is triggered exclusively via the TLR7/MyD88 pathway.

To assess the importance of pDC-derived IFN-α during MHV infection in vivo, pDCs were ablated by the depleting antibody PDCA-1. As described for MCMV by Krug et al,[11] pDC depletion was accompanied by severely diminished IFN-α levels in serum following MHV infection (Figure 5A). The treatment with PDCA-1 resulted in an 80% depletion of splenic pDCs for roughly 48 hours (not shown). Although profound effects on viral titers could be observed (Figure 5B), transient pDC depletion did not lead to lethality following low-dose MHV infection. Nevertheless, initial viral titers in spleens were more than 1000-fold increased in pDC-depleted compared with isotype control antibody–treated mice, and virus was found in other organs such as lung or brain (Figure 5B). To exclude complement-mediated global changes in the status of the immune system, we evaluated the effect of natural killer (NK) cell depletion via anti–asialo GM1. Depletion of NK cells altered neither initial viral replication and distribution nor IFN-α levels in serum (not shown). Finally, ALT values in PDCA-1–depleted mice were elevated compared with control animals (Figure 5C), indicating significant liver damage. These data clearly show that pDCs are important for the early control of MHV infection and that the lack of pDCs not only leads to uncontrolled viral replication and spread to different organs but also impacts on the severity of viral disease.

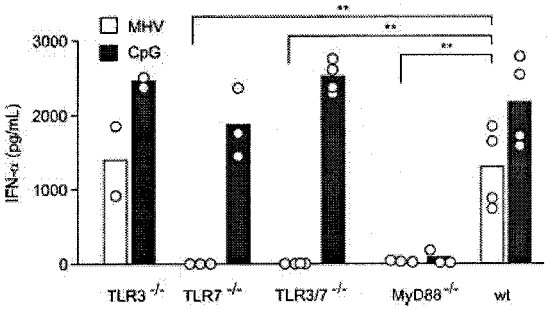

Figure 4. pDCs sense MHV via TLR7. Bone marrow–derived pDCs from TLR3$^{-/-}$, TLR7$^{-/-}$, TLR3$^{-/-}$/TLR7$^{-/-}$, MyD88$^{-/-}$, or wt mice were infected with MHV A59 (moi = 1) or treated with CpG oligonucleotides. IFN-α in tissue culture supernatants was determined 24 hours after infection by ELISA. Bars represent means with values from individual mice shown as open circles. Statistical analysis was performed using Student *t* test ($^{**}P < .01$).

Rapid induction of type I IFNs in pDCs following SARS-CoV infection

In order to relate the above findings to a pathogenic and potentially lethal human coronavirus infection, the ability of pDCs to produce IFN-α following encounter with SARS-CoV was assessed. Primary pDCs and cDCs were isolated from peripheral blood of healthy donors and infected with SARS-CoV. As described for monocyte-derived cDCs,[28] primary cDCs from healthy donors were also not able to produce significant amounts of IFN-α (Figure 6A) and did not up-regulate transcripts of IFN-β and IFN-stimulated genes such as ISG56 and MxA, located downstream in the type I IFN signaling pathway

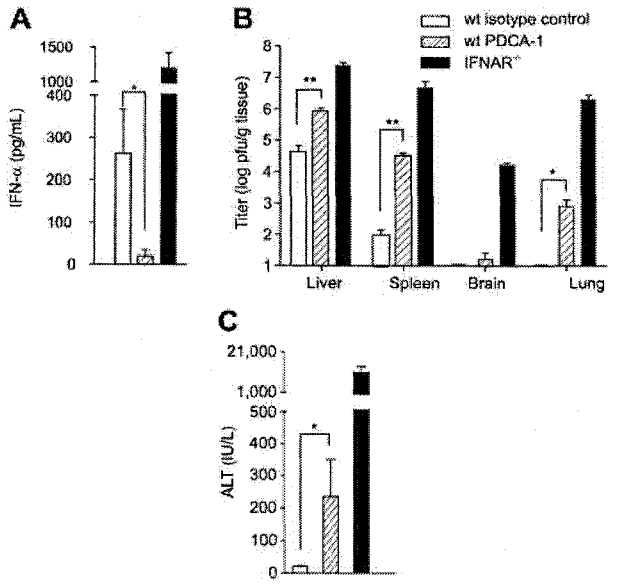

Figure 5. Effect of antibody-mediated pDC depletion on MHV infection. 129Sv mice were treated with rat IgG2b (wt isotype control) or α–mPDCA-1 (wt PDCA-1) and infected intraperitoneally with 5 pfu MHV A59 (n = 6). IFNAR$^{-/-}$ mice (n = 3) were used to demonstrate uncontrolled MHV spread in the absence of a functional IFN system. (A) IFN-α concentration in serum (means ± SD), (B) viral titers (means ± SD) in liver, spleen, brain, and lung, and (C) serum ALT values (means ± SD) were assessed at 48 hours after infection. (A-C) Statistical analysis was performed using Student *t* test (*$P < .05$; **$P < .01$).

(Figure 6B). In contrast and as expected from the MHV experiments, pDCs were able to produce IFN-α early after SARS-CoV infection (Figure 6A). Furthermore, IFN-β, MxA, or ISG56 mRNA expression was found in infected pDCs (Figure 6C). Based on this evidence and the unsuccessful efforts from previous studies to determine a cell type that produces IFN-α in response to SARS-CoV,[25-28] we conclude that pDCs are most likely the major source of type I IFNs in SARS-CoV infection.

Discussion

In this study, we demonstrate a unique and essential function for type I IFN–producing pDCs: protection against the rapidly replicating and cytopathic murine coronavirus. Furthermore, we have identified pDCs as the source of type I IFN in response to human SARS-CoV, suggesting an important biologic role of pDC-derived type I IFNs for highly pathogenic coronavirus infections in humans.

The expression of TLRs that recognize viral products such as CpG oligonucleotides[10,11] or ssRNA[9] has indicated that pDCs represent a highly specialized cell type that provides an early response against a particular set of infectious agents. A further characteristic of pDCs is the constitutively high expression of IFN regulatory factor-7 (IRF-7),[42,43] which directly stimulates IFN-α expression, independently of an IFN-β–mediated feedback loop.[44] In MHV infection, this efficient direct IFN-α induction appears to be not only essential for the regulation of the magnitude of the type I IFN response, but also important to restrict replication of this cytopathic virus within pDCs. Furthermore, pDC-derived type I IFNs provide an efficient "bystander" protection because the initial replication of MHV in lymphoid organs such as the spleen was diminished in the presence of pDCs. Notably, in MHV infection this function of pDCs cannot be substituted by other cells as demonstrated, for example, in MCMV infection.[11,13] It is possible that viruses that replicate rather slowly, such as MCMV, cannot reveal the full importance of pDCs in the control of cytopathic viruses that require a swift type I IFN response.

Within secondary lymphoid organs, macrophages are the major target cell of MHV,[14] and recent studies indicate that cDCs can also be readily infected with MHV A59[45] or MHV JHM.[46] It is important to note that the uncontrolled infection of cDCs by MHV is detrimental for the initiation of the adaptive antiviral immune response.[46] The rapid control of MHV through pDC-derived type I IFN in secondary lymphoid organs ensures therefore the subsequent induction of adaptive immune responses. Likewise, a recent study by Smit et al[47] indicates that pDCs not only help to minimize respiratory syncytial virus infection–associated immunopathologic damage, but also facilitate establishment of antiviral T-cell responses in the lung.

Fatal SARS-CoV infection–associated clinical disease is characterized by respiratory insufficiency and, eventually, respiratory failure. One of the reasons for this outcome may be the down-regulation of angiotensin-converting enzyme 2 by the viral spike protein leading to an exacerbation of the pulmonary damage.[48] Furthermore, it is well-documented that SARS-CoV infects macrophages and lymphocytes leading to a pronounced atrophy of lymphoid organs in those patients who succumbed to the infection.[49] Extrapolating from the data obtained in the MHV model, it is likely that these patients may have suffered from an insufficient early control of the virus within lymphoid organs that eventually led to the unrestrained replication in the respiratory tract. Because SARS-CoV is sensitive to type I IFNs both in vitro[30,31,50] and in vivo,[32,33] an early control of SARS-CoV by type I IFNs might have been a decisive advantage for those patients who have survived the

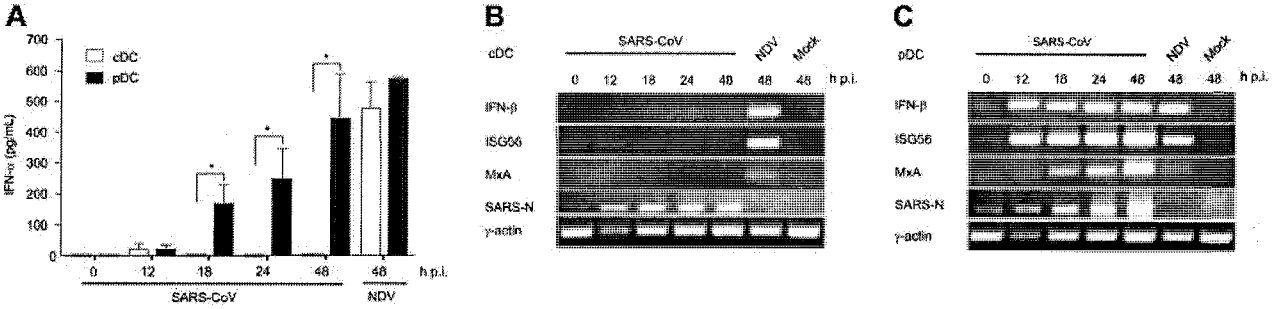

Figure 6. Infection of human pDCs and cDCs with SARS-CoV. Primary pDCs or cDCs were isolated from peripheral blood of healthy donors and infected with either SARS-CoV (moi = 1) or Newcastle disease virus (NDV) as positive control (moi = 5), or were left uninfected (mock). (A) IFN-α in culture supernatants was determined at the indicated time points. Results represent pooled data (means ± SD) using pDCs and cDCs from 4 healthy donors. Statistical analysis was performed using Student *t* test (*$P < .05$). (B-C) Expression of IFN-β, ISG56, MxA, SARS-CoV N protein, and γ-actin mRNAs in (B) cDCs and (C) pDCs was determined by RT-PCR. One representative result of 4 individual experiments is shown.

infection. It is noteworthy that neither macrophages, cDCs, fibroblasts, nor lung epithelial cells[28] are able to mount a significant type I IFN response against SARS-CoV. The lack of a significant type I IFN response in PBMCs of SARS-CoV–infected patients[23] might be due to a partial inhibition of type I IFN signaling not only in nonlymphoid cells,[51,52] but also in pDCs. Therefore, the potential of the various SARS-CoV nonstructural proteins that might inhibit and/or modulate type I IFN responses in pDCs and other important target cells should be addressed in future studies.

Taken together, the results of this study provide insight into the immunopathogenesis of coronavirus-associated diseases by demonstrating an exclusive role of pDC-derived type I IFNs for initial viral control. Triggering of this pathway, for example via specific TLR agonists, might open new avenues for the treatment of coronavirus infections. Indeed, stimulation of TLR3 at the vaginal mucosa can protect mice against herpes simplex virus-2 challenge via the mucosal route.[53] In a clinical setting, systemic administration of a TLR7 agonist elicited potent antiviral effects against hepatitis C virus with significant reduction of plasma viremia.[54] Our data provide the rationale that such a treatment approach might help to reduce initial viral load and eventually favor a mild course of SARS.

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Abbreviations: MHV indicates mouse hepatitis virus; SARS, severe acute respiratory syndrome; pDCs, plasmacytoid dendritic cells; and cDCs, conventional dendritic cells.

Authorship

Contribution: B.L. and V.T. designed the study and wrote the paper; L.C.-B. performed research and wrote the paper; R.Z., F.W., and M.S. performed research; K.S.L. and S.A. provided mice.

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Correspondence: Burkhard Ludewig, Research Department, Kantonsspital St Gallen, 9007 St Gallen, Switzerland; e-mail: burkhard.ludewig@kssg.ch; Volker Thiel, Research Department, Kantonsspital St Gallen, 9007 St Gallen, Switzerland; e-mail: volker.thiel@kssg.ch.

References

1. Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons (alpha/beta) in immunity and autoimmunity. Annu Rev Immunol. 2005;23: 307-336.
2. Der SD, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A. 1998; 95:15623-15628.
3. Nguyen KB, Watford WT, Salomon R, et al. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. Science. 2002;297:2063-2066.
4. Siegal FP, Kadowaki N, Shodell M, et al. The nature of the principal type 1 interferon-producing cells in human blood. Science. 1999;284:1835-1837.
5. Cella M, Jarrossay D, Facchetti F, et al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med. 1999;5:919-923.
6. Nakano H, Yanagita M, Gunn MD. CD11c(+) B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. J Exp Med. 2001;194:1171-1178.
7. Asselin-Paturel C, Boonstra A, Dalod M, et al. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat Immunol. 2001;2:1144-1150.
8. Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. 2004;303:1526-1529.
9. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004;303:1529-1531.
10. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med. 2003;198:513-520.
11. Krug A, French AR, Barchet W, et al. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity. 2004; 21:107-119.
12. Dalod M, Salazar-Mather TP, Malmgaard L, et al. Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. J Exp Med. 2002;195:517-528.
13. Delale T, Paquin A, Asselin-Paturel C, et al. MyD88-dependent and -independent murine cytomegalovirus sensing for IFN-alpha release and initiation of immune responses in vivo. J Immunol. 2005;175:6723-6732.
14. Perlman S, Dandekar AA. Immunopathogenesis of coronavirus infections: implications for SARS. Nat Rev Immunol. 2005;5:917-927.
15. Snijder EJ, Bredenbeek PJ, Dobbe JC, et al. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J Mol Biol. 2003;331:991-1004.
16. Bergmann CC, Lane TE, Stohlman SA. Coronavirus infection of the central nervous system: host-virus stand-off. Nat Rev Microbiol. 2006;4:121-132.
17. Thiel V, Ivanov KA, Putics A, et al. Mechanisms and enzymes involved in SARS coronavirus genome expression. J Gen Virol. 2003;84:2305-2315.
18. Marten NW, Stohlman SA, Zhou J, Bergmann CC. Kinetics of virus-specific CD8+ -T-cell expansion and trafficking following central nervous system infection. J Virol. 2003;77:2775-2778.
19. Bergmann CC, Parra B, Hinton DR, et al. Perforin and gamma interferon-mediated control of coronavirus central nervous system infection by CD8 T cells in the absence of CD4 T cells. J Virol. 2004;78:1739-1750.
20. Lin MT, Hinton DR, Marten NW, Bergmann CC, Stohlman SA. Antibody prevents virus reactivation within the central nervous system. J Immunol. 1999;162:7358-7368.
21. Ramakrishna C, Stohlman SA, Atkinson RD, Shlomchik MJ, Bergmann CC. Mechanisms of central nervous system viral persistence: the critical role of antibody and B cells. J Immunol. 2002; 168:1204-1211.
22. Jones BM, Ma ES, Peiris JS, et al. Prolonged disturbances of in vitro cytokine production in patients with severe acute respiratory syndrome (SARS) treated with ribavirin and steroids. Clin Exp Immunol. 2004;135:467-473.
23. Reghunathan R, Jayapal M, Hsu LY, et al. Expression profile of immune response genes in patients with severe acute respiratory syndrome. BMC Immunol. 2005;6:2.
24. Yu SY, Hu YW, Liu XY, et al. Gene expression profiles in peripheral blood mononuclear cells of SARS patients. World J Gastroenterol. 2005;11: 5037-5043.
25. Yilla M, Harcourt BH, Hickman CJ, et al. SARS-coronavirus replication in human peripheral monocytes/macrophages. Virus Res. 2005;107: 93-101.
26. Law HK, Cheung CY, Ng HY, et al. Chemokine up-regulation in SARS-coronavirus-infected, monocyte-derived human dendritic cells. Blood. 2005;106:2366-2374.
27. Cheung CY, Poon LL, Ng IH, et al. Cytokine responses in severe acute respiratory syndrome coronavirus-infected macrophages in vitro: possible relevance to pathogenesis. J Virol. 2005;79: 7819-7826.
28. Ziegler T, Matikainen S, Ronkko E, et al. Severe acute respiratory syndrome coronavirus fails to activate cytokine-mediated innate immune responses in cultured human monocyte-derived dendritic cells. J Virol. 2005;79:13800-13805.
29. Cinatl J, Morgenstern B, Bauer G, et al. Treatment of SARS with human interferons. Lancet. 2003;362:293-294.
30. Zheng B, He ML, Wong KL, et al. Potent inhibition of SARS-associated coronavirus (SCOV) infection and replication by type I interferons (IFN-alpha/beta) but not by type II interferon (IFN-gamma). J Interferon Cytokine Res. 2004;24: 388-390.

31. Spiegel M, Pichlmair A, Muhlberger E, Haller O, Weber F. The antiviral effect of interferon-beta against SARS-coronavirus is not mediated by MxA protein. J Clin Virol. 2004;30:211-213.
32. Loutfy MR, Blatt LM, Siminovitch KA, et al. Interferon alfacon-1 plus corticosteroids in severe acute respiratory syndrome: a preliminary study. JAMA. 2003;290:3222-3228.
33. Haagmans BL, Kuiken T, Martina BE, et al. Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques. Nat Med. 2004;10:290-293.
34. Honda K, Sakaguchi S, Nakajima C, et al. Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. Proc Natl Acad Sci U S A. 2003;100:10872-10877.
35. Hemmi H, Kaisho T, Takeuchi O, et al. Small antiviral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol. 2002;3:196-200.
36. Adachi O, Kawai T, Takeda K, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998;9: 143-150.
37. Muller U, Steinhoff U, Reis LF, et al. Functional role of type I and type II interferons in antiviral defense. Science. 1994;264:1918-1921.
38. Coley SE, Lavi E, Sawicki SG, et al. Recombinant mouse hepatitis virus strain A59 from cloned, full-length cDNA replicates to high titers in vitro and is fully pathogenic in vivo. J Virol. 2005;79:3097-3106.
39. Krug A, Rothenfusser S, Hornung V, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol. 2001;31:2154-2163.
40. van den Broek MF, Muller U, Huang S, Zinkernagel RM, Aguet M. Immune defence in mice lacking type I and/or type II interferon receptors. Immunol Rev. 1995;148:5-18.
41. Kato H, Sato S, Yoneyama M, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity. 2005;23:19-28.
42. Izaguirre A, Barnes BJ, Amrute S, et al. Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. J Leukoc Biol. 2003;74:1125-1138.
43. Honda K, Yanai H, Negishi H, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature. 2005;434:772-777.
44. Barchet W, Cella M, Odermatt B, et al. Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo. J Exp Med. 2002;195:507-516.
45. Turner BC, Hemmila EM, Beauchemin N, Holmes KV. Receptor-dependent coronavirus infection of dendritic cells. J Virol. 2004;78:5486-5490.
46. Zhou H, Perlman S. Preferential infection of mature dendritic cells by mouse hepatitis virus strain JHM. J Virol. 2006;80:2506-2514.
47. Smit JJ, Rudd BD, Lukacs NW. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. J Exp Med. 2006;203:1153-1159.
48. Kuba K, Imai Y, Rao S, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. Nat Med. 2005; 11:875-879.
49. Gu J, Gong E, Zhang B, et al. Multiple organ infection and the pathogenesis of SARS. J Exp Med. 2005;202:415-424.
50. Cinatl J Jr, Michaelis M, Scholz M, Doerr HW. Role of interferons in the treatment of severe acute respiratory syndrome. Expert Opin Biol Ther. 2004;4:827-836.
51. Spiegel M, Weber F. Inhibition of cytokine gene expression and induction of chemokine genes in non-lymphatic cells infected with SARS coronavirus. Virol J. 2006;3:17.
52. Spiegel M, Pichlmair A, Martinez-Sobrido L, et al. Inhibition of beta interferon induction by severe acute respiratory syndrome coronavirus suggests a two-step model for activation of interferon regulatory factor 3. J Virol. 2005;79:2079-2086.
53. Ashkar AA, Yao XD, Gill N, et al. Toll-like receptor (TLR)-3, but not TLR4, agonist protects against genital herpes infection in the absence of inflammation seen with CpG DNA. J Infect Dis. 2004; 190:1841-1849.
54. Horsmans Y, Berg T, Desager JP, et al. Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. Hepatology. 2005;42:724-731.

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Type I IFN-Mediated Protection of Macrophages and Dendritic Cells Secures Control of Murine Coronavirus Infection[1]

Luisa Cervantes-Barragán,*† Ulrich Kalinke,‡ Roland Züst,* Boris Reizis,§ Constantino López-Macías,† Volker Thiel,* and Burkhard Ludewig[2]*

The swift production of type I IFNs is one of the fundamental aspects of innate immune responses against viruses. Plasmacytoid dendritic cell-derived type I IFNs are of prime importance for the initial control of highly cytopathic viruses such as the mouse hepatitis virus (MHV). The aim of this study was to determine the major target cell populations of this first wave of type I IFNs. Generation of bone marrow-chimeric mice expressing the type I IFN receptor (IFNAR) on either hemopoietic or non-bone marrow-derived cells revealed that the early control of MHV depended mainly on IFNAR expression on hemopoietic cells. To establish which cell population responds most efficiently to type I IFNs, mice conditionally deficient for the IFNAR on different leukocyte subsets were infected with MHV. This genetic analysis revealed that IFNAR expression on LysM$^+$ macrophages and CD11c$^+$ dendritic cells was most important for the early containment of MHV within secondary lymphoid organs and to prevent lethal liver disease. This study identifies type I IFN-mediated cross-talk between plasmacytoid dendritic cells on one side and macrophages and conventional dendritic cells on the other, as an essential cellular pathway for the control of fatal cytopathic virus infection. *The Journal of Immunology,* **2009, 182: 0000–0000.**

For the control of fast replicating cytopathic virus infections, the immune system must act rapidly to control viral replication and dissemination before tissue damage and inflammation endanger survival of the host. Secretion of type I IFNs is an essential component of the innate immune response against viruses. These soluble factors induce an array of intracellular effectors including protein kinase R, 2′-5′-oligoadenylate synthetases and Mx proteins, which halt viral replication (1). Furthermore, type 1 IFNs exert proapoptotic activities that control viral spread by eliminating infected cells (2), and they deliver immunomodulatory stimuli that affect cell migration (3, 4), cross-presentation (5–8), B cell responses, and Ig isotype switch (9–11), CD4$^+$ T cell activation (12, 13), or CTL expansion (14, 15). However, chronic activation of the type I IFN system can be detrimental for the host because autoimmune responses might be aggravated (16, 17).

The fact that almost all cells are able to produce type I IFNs under certain conditions and also respond to it led to the initial idea of a general antiviral state. However, several in vitro studies have provided insight into the subtle differences of cell population-specific effects of type I IFNs which depend largely on the constitutive vs inducible expression of STAT proteins and IFN regulatory factors and the state of cellular differentiation (18, 19). It therefore appears that there is a cell type-specific, context-dependent differential requirement of type I IFN responsiveness that secures optimal protection against viral infection while reducing potential immunopathological side effects of these potent cytokines.

The murine hepatitis virus (MHV)[3] A59 is a group II coronavirus that causes hepatitis and demyelinating encephalomyelitis in mice. This natural mouse pathogen is one of the most extensively studied coronaviruses (20). A strong CTL response mediates clearance of the virus between days 6 and 8 postinfection (21, 22), and neutralizing Abs appear to be required to prevent re-emergence of persistent CNS infection (23, 24). Nonetheless, before effective adaptive immune responses are elicited, type I IFN-mediated innate immune responses are essential for the survival of the host in the early phase of infection. The first wave of type I IFNs is produced almost exclusively by plasmacytoid dendritic cells (pDC), leading to containment of the virus and prevention of disease (25). Thus, MHV infection represents a well-suited model to investigate whether a particular hierarchy exists in the dependency on pDC-derived type I IFNs which secure control of cytopathic viral infection and protect the host from severe disease. In this study, we have used type I IFNR$^-$ (*ifnar*$^{-/-}$) bone marrow-chimeric mice and conditionally gene-targeted mice with cell type-specific IFNAR deletion to elucidate whether type I IFN signaling is required on all nucleated cells. We found that during MHV infection, the presence of the IFNAR on LysM$^+$ macrophages and CD11c$^+$ conventional dendritic cells (cDC) is of utmost importance, whereas type I IFN responsiveness of other MHV target cells such as B cells appeared not to be critical for the control of the virus. Overall, our results indicate that cells from the hemopoietic system, and in particular, macrophages and cDCs are the prime target cells for type I IFNs during murine coronavirus infection.

*Research Department, Kantonal Hospital St. Gallen, St. Gallen, Switzerland; †Unidad de Investigación Médica en Inmunoquímica, Hospital de Especialidades, Centro Médico Nacional Siglo XXI, IMSS, Mexico City, Mexico; ‡Department of Immunology, Paul Ehrlich Institut, Langen, Germany; and §Department of Microbiology, Columbia University Medical Center, New York, NY 10032

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[2] Address correspondence and reprint requests to Dr. Burkhard Ludewig, Research Department, Kantonsspital St. Gallen, 9007 St. Gallen, Switzerland. E-mail address: burkhard.ludewig@kssg.ch

[3] Abbreviations used in this paper: MHV, mouse hepatitis virus; pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; IFNAR, *ifnar*$^{-/-}$, type I IFNR$^-$; ALT, alanine 2-oxoglutarate aminotransferase.

Materials and Methods

Mice and viruses

C57BL/6 (B6) mice were obtained from Charles River Laboratories. Type I IFNR$^-$ mice (*ifnar*$^{-/-}$; Ref. 26) on the B6 background were kindly provided by Martin Bachmann (Cytos, Schlieren, Switzerland) and bred in our facilities. R26-EYFP$^{+/-}$ mice (27) were kindly provided by Ari Waisman (University of Mainz, Mainz, Germany). R26-EYFP$^{+/-}$ mice and mice expressing a *loxP*-flanked *ifnar1* (*ifnar1*$^{fl/fl}$) (4) were bred with mice that express Cre recombinase specifically in B cells (CD19-Cre), T cells (CD4-Cre), T and B cells (CD19-CreCD4-Cre), macrophages, (LysM-Cre; Ref. 28), or CD11c$^+$ dendritic cells (CD11c-Cre; Ref. 29). For the generation of bone marrow-chimeric mice, recipients were lethally irradiated with 900 rad from a linear accelerator (Clinic of Radio-Oncology, Kantonal Hospital St. Gallen, St. Gallen, Switzerland) and injected i.v. 1 day later with 2×10^7 amounts of the indicated donor bone marrow cells. Chimeric mice were maintained on antibiotic water containing sulfadoxin and trimethoprim (Borgal; Veterinaria) for the following 3 wk. Mice were used for experiments 8–10 wk after bone marrow reconstitution. The degree of chimerism induced using this protocol has been routinely evaluated by reconstituting B6 mice expressing the congenic marker Thy1.2 with bone marrow cells derived from B6.Thy1.1 mice. Chimerism in these control animals was always >97%. MHV A59 was generated from a molecularly cloned cDNA (30) based on the Albany strain of MHV A59 and propagated on L929 cells. GFP-recombinant MHV was generated as previously described (31). Experiments were performed in accordance with Swiss Kantonal and Federal legislations.

Virus infections, determination of virus titers, liver enzyme values, liver histology, and IFN-α

Mice were injected i.p. with 50 PFU of MHV A59, representing a low dose infection with maximal liver disease around day 5 comparable with the kinetics of systemic infection as described previously (25). To achieve maximal target cell infection in B6 mice and minimal infection-associated death in *ifnar*$^{-/-}$ mice, a dose of 5×10^3 PFU GFP-recombinant MHV (31) was used. Intranasal infection was done with 5×10^4 PFU of MHV A59 because at this dose, 100% of the mice were reproducibly infected, and the virus did not spread systemically in B6 mice. Mice were sacrificed at the indicated time points, and organs were stored at −70°C until further analysis or disrupted for FACS analysis. Blood was incubated at room temperature to coagulate and then centrifuged; and serum was used for alanine 2-oxoglutarate aminotransferase (ALT) measurements using a Hitachi 747 autoanalyzer. Virus titers in organs were determined from frozen organs after weighing and homogenization. Viral titers were determined by standard plaque assay using L929 cells. Livers were fixed in 4% formalin and embedded in paraffin. Sections were stained with H&E. Mouse IFN-α concentration in serum or spleen homogenates was measured by ELISA (PBL Biomedical Laboratories) according to the manufacturers' instructions.

Splenocyte isolation, flow cytometry, and immunofluorescence

Splenocytes were obtained from spleens of B6, *ifnar*$^{-/-}$ or conditional EYFP mice following digestion with collagenase type II (Invitrogen) for 20 min at 37°C and resuspended in 5% RPMI. For isolation of the low-density-enriched population, cells were resuspended in PBS supplemented with 2% FCS, 2 mM EDTA and overlaid on 20% Optiprep density gradient medium (Sigma-Aldrich). After centrifugation at $700 \times g$ for 15 min, low-density cells were recovered from the interface and resuspended in 5% RPMI. Cells were stained with different lineage markers and analyzed for GFP expression with a FACSCalibur flow cytometer using the CellQuest software (BD Biosciences). Abs used in this study were purchased from BD Pharmingen (CD11c-PE, Ly6-G-PE (clone A8I), NK1.1-PE), Biolegend (CD4-PE, B220-allophycocyanin, CD3-allophycocyanin, CD11b-allophycocyanin), eBiosciences (F4/80-PE, CD8-PE), Miltenyi Biotec (mPDCA-1-allophycocyanin), and Immunotools (CD19-PE). For immunofluorescence analysis, spleens were immersed in HBSS and snap frozen. Five-micrometer tissue sections were fixed with acetone. Cryosections were blocked with the Fc-blocking Ab 2.4G2 and stained with the following Abs: B220 Alexa 488 (Biolegend); and F4/80 PE, CD11c-PE, and anti-MHV-N Alexa 647 (N556) kindly provided by Dr. Stuart Sidell (Department of Cellular and Molecular Medicine, University of Bristol, Bristol, U.K.). Images were acquired using a Leica DMRA microscope and processed using Adobe Photoshop (Adobe Systems).

Cell culture of primary cells and in vitro infections

Bone marrow-derived cDCs or pDCs were generated as described (25) with either GM-CSF-containing supernatant from the cell line X63-GM-CSF (kindly provided by Dr. Antonius Rolink, University of Basel, Basel, Switzerland) or Flt3-L (R&D Systems), at 20 ng/ml, respectively. cDCs were further purified using Optiprep density gradient centrifugation. pDCs were purified using the mouse pDC isolation kit (Miltenyi Biotec) adapted for the isolation of bone marrow-derived pDCs by adding CD11b-biotin (Biolegend) to the negative selection mixture. Thioglycolate-elicited macrophages were collected from the peritoneal cavity of mice and cultured overnight at 37°C. Nonadherent cells were removed by washing with ice-cold PBS. Type I IFN containing pDCs supernatant was produced by infecting pDCs with MHV at a MOI of 1 for 24 h. The supernatant was filtered with Amicon Ultra 100K centrifugal filter units (Millipore) to eliminate viral particles. Sterility was confirmed by incubating supernatant on L929 cells. IFN-α concentration was determined by ELISA, and the supernatant was diluted to the indicated IFN-α concentrations. Cell survival was determined with the Cell Proliferation MTS Assay (Celltiter 96 Aqueous one-solution cell proliferation assay) from Promega. MTS solution was added to the cells 24 h postinfection. The plate was incubated for 2 h at 37°C, and the optical density was measured at 492 nm. Macrophages and cDCs were infected with MHV A59 at the indicated MOI, incubated for 1 h at 37°C, and washed. pDCs were added to the cultures after washing. A transwell plate system (BD Falcon; pore size, 0.4 μm) was used to prevent cell-cell contact between pDCs and macrophages/cDCs.

Statistical analysis

Statistical analyses were performed with Graphpad Prism 5.0 using either a nonpaired, two-tailed Student *t* test or one-way ANOVA with Bonferroni posttest comparing the samples with their corresponding control group. Survival curves were generated using the Kaplan-Meier method and the significance of differences was calculated by the log rank test. Statistical significance was defined as a value of $p < 0.05$.

Results

Early control of MHV depends on type I IFN responsiveness of hemopoietic cells

To better define the cellular targets for the activity of type I IFNs, bone marrow chimeras were generated using *ifnar*$^{-/-}$ or B6 mice. The chimeric mice that expressed the IFNAR on either hemopoietic or nonhemopoietic cells were infected i.p. with 50 PFU of MHV A59. Because *ifnar*$^{-/-}$ mice succumb to MHV infection rapidly (25), mice were sacrificed after 48 h, and IFN-α production, severity of liver disease, and viral titers in spleens, livers, and lungs were determined. As shown in Fig. 1*A*, neither the lack of the IFNAR on hemopoietic nor that on nonhemopoietic cells precluded production of IFN-α. Furthermore, induction of IFN-β was not influenced by the absence of IFNAR on different cell subsets (data not shown). The lack of the IFNAR on radio-resistant parenchymal cells (B6→*ifnar*$^{-/-}$) did not lead to significantly elevated liver enzyme values, whereas the absence of the IFNAR on bone marrow-derived cells (*ifnar*$^{-/-}$→B6) resulted in severe liver disease (Fig. 1*C*). Moreover, viral titers in livers, spleens, and lungs (Fig. 1*B*) from these mice were significantly higher than in mice that expressed the IFNAR only on hemopoietic cells. Most importantly, the expression of IFNAR on the hemopoietic cells (B6→*ifnar*$^{-/-}$) secured significantly longer survival of the mice (Fig. 1*D*). These results indicate a clear hierarchy in the importance of the IFNAR expressed on hemopoietic vs nonhemopoietic cells; the presence of the IFNAR on hemopoietic cells appears to be important to contain the virus within secondary lymphoid organs and thereby contributes critically to the prevention of disease.

Target cells of MHV within the bone marrow-derived cell compartment

It is likely that those cells that are most easily infected by a cytopathic virus and therefore rapidly lost during the infection are most dependent on the protection provided by the type I IFN system. Working along this assumption, we first determined which cell

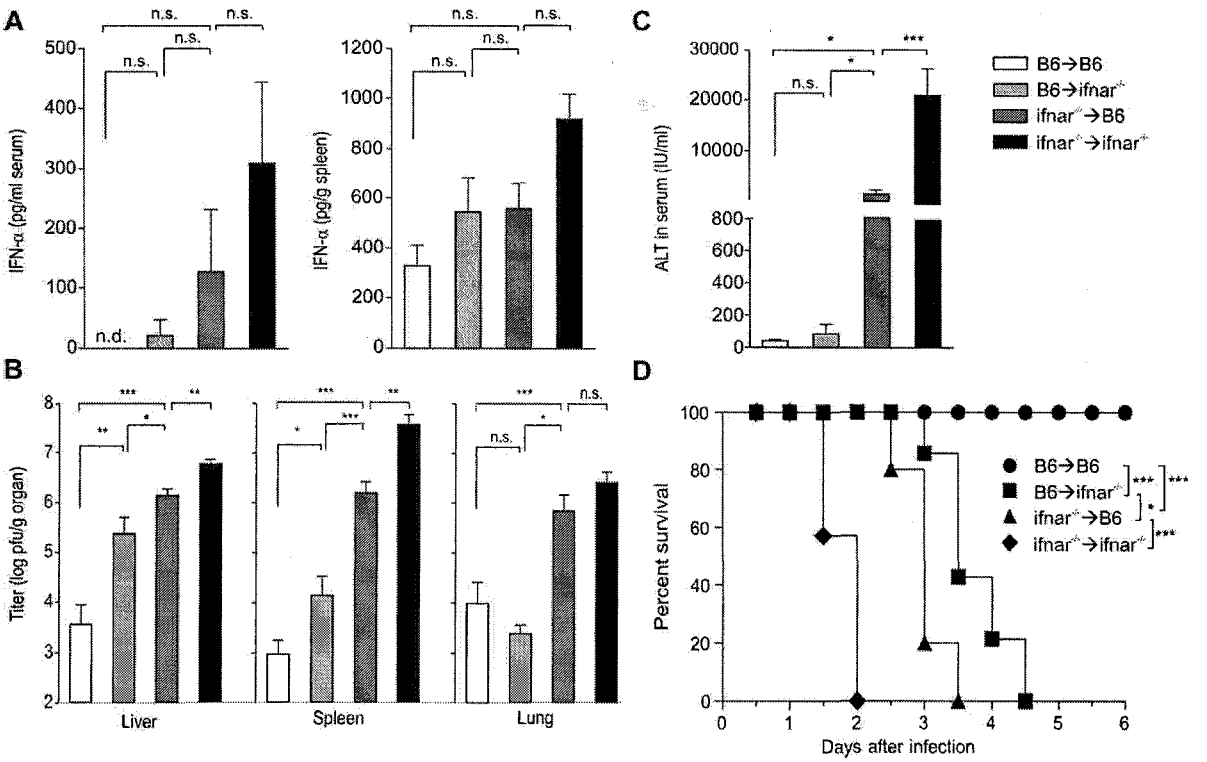

FIGURE 1. Type I IFN responsiveness of bone marrow-derived cells is essential for early control of MHV infection. Bone marrow-chimeric mice (B6→*ifnar*$^{-/-}$, *ifnar*$^{-/-}$→B6, B6→B6, *ifnar*$^{-/-}$→*ifnar*$^{-/-}$) were infected i.p. with 50 PFU of MHV A59. After 48 h, IFN-α concentration in serum and spleens (*A*); viral titers in livers, spleens, and lungs (*B*); ALT values in serum (*C*) were determined. Results represent means ± SD of five to six mice per group. *D*, Survival of bone marrow-chimeric mice. Health status was monitored twice daily, and moribund animals were euthanized (n = 5–6 mice per group). Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, n.s., $p > 0.05$). n.d., Not detected. Survival curves were generated using the Kaplan-Meier method, and the significance of differences was calculated by the log-rank test. Statistical significance was defined as ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, n.s., $p > 0.05$.

populations within the hemopoietic compartment support MHV infection. In a first set of experiments, splenocytes from B6 or *ifnar*$^{-/-}$ mice were infected in vitro with GFP-recombinant MHV at a MOI of 1. After 12 h, MHV replication in macrophages (F4/80$^+$CD11b$^+$), neutrophils (Ly6G$^+$CD11b$^+$), cDCs (CD11c$^+$B220$^-$), B cells (CD19$^+$), CD4$^+$ T cells (CD3$^+$CD4$^+$), and CD8$^+$ T cells (CD3$^+$CD8$^+$) was determined by flow cytometry (Fig. 2*A*). This analysis revealed that primary macrophages, cDCs, neutrophils, and B cells could be infected with MHV and that the lack of IFNAR on these cells slightly increased their susceptibility. To confirm whether this target cell tropism of MHV for particular leukocyte subsets can be reproduced in vivo, B6 and *ifnar*$^{-/-}$ mice were infected with 5×10^3 PFU of GFP-recombinant MHV i.p., and the different spleen cell populations were probed for GFP expression 36 h postinfection using flow cytometric analysis as described previously. We could not detect GFP-positive cells in the different splenocyte fractions derived from infected B6 mice (Fig. 2*B, top row*), suggesting that the intact type I IFN system in these mice had efficiently blocked viral replication below the level of detection. Indeed, macrophages, cDCs, B cells, and neutrophils from infected *ifnar*$^{-/-}$ mice showed significant GFP expression (Fig. 2*B, bottom row*). Other leukocyte populations such as CD4$^+$ and CD8$^+$ T lymphocytes (Fig. 2*B*) and NK cells (not shown) did not exhibit significant GFP expression. Furthermore, B6 and *ifnar*$^{-/-}$ mice were infected with 5×10^3 PFU of MHV, and fluorescence microscopic analysis was performed using anti-MHV nucleoprotein Ab to identify infected cells in situ. Whereas MHV-infected F4/80$^+$ cells in the red pulp (Fig. 2*C*) and CD11c$^+$ in the white pulp (Fig. 2*D*) could be readily detected in spleens of *ifnar*$^{-/-}$ mice, colocalization of the MHV nucleoprotein with the B cell marker B220 (Fig. 2, *C* and *D*) and with the neutrophil marker Ly6G (not shown) was rare. As expected, only very few MHV-infected cells were found in B6 mice (not shown), thus confirming the high susceptibility of cDCs and macrophages to MHV infection in the absence of a functional type I IFN system.

Requirement of IFNAR expression on different leukocyte populations

To assess the differential requirement of type I IFN responsiveness of the MHV target populations, we used a set of conditionally gene-targeted mice. Crossing of mice with a *loxP*-flanked *ifnar1* (*ifnar1*$^{fl/fl}$) with mice that express the Cre recombinase in a cell type-specific manner resulted in deletion of the IFNAR in T cells (CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$) (4), in B cells (CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$) (4), in T and B cells (CD4-Cre$^{+/-}$CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$), in macrophages, neutrophils, and some dendritic cells (LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$; Ref. 28), and specifically in CD11c$^+$ cDCs (CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$; Ref. 29). These mice were infected with MHV, and survival was monitored for 2 wk. As shown in Fig. 3*A*, the expression of the IFNAR on the surface of LysM$^+$ or CD11c$^+$ cells was essential for survival, since LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice succumbed to the infection. LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ developed a more severe phenotype with lethal disease after 4 days of infection. Likewise, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice showed the most severe liver pathology with significantly elevated ALT values as early as day 2 postinfection (Fig. 3*B*) and a massive damage of liver tissue (Fig. 3*C*). Because neutrophils can be infected with MHV in vivo (Fig. 2*B*), we determined next whether the presence of neutrophils in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$

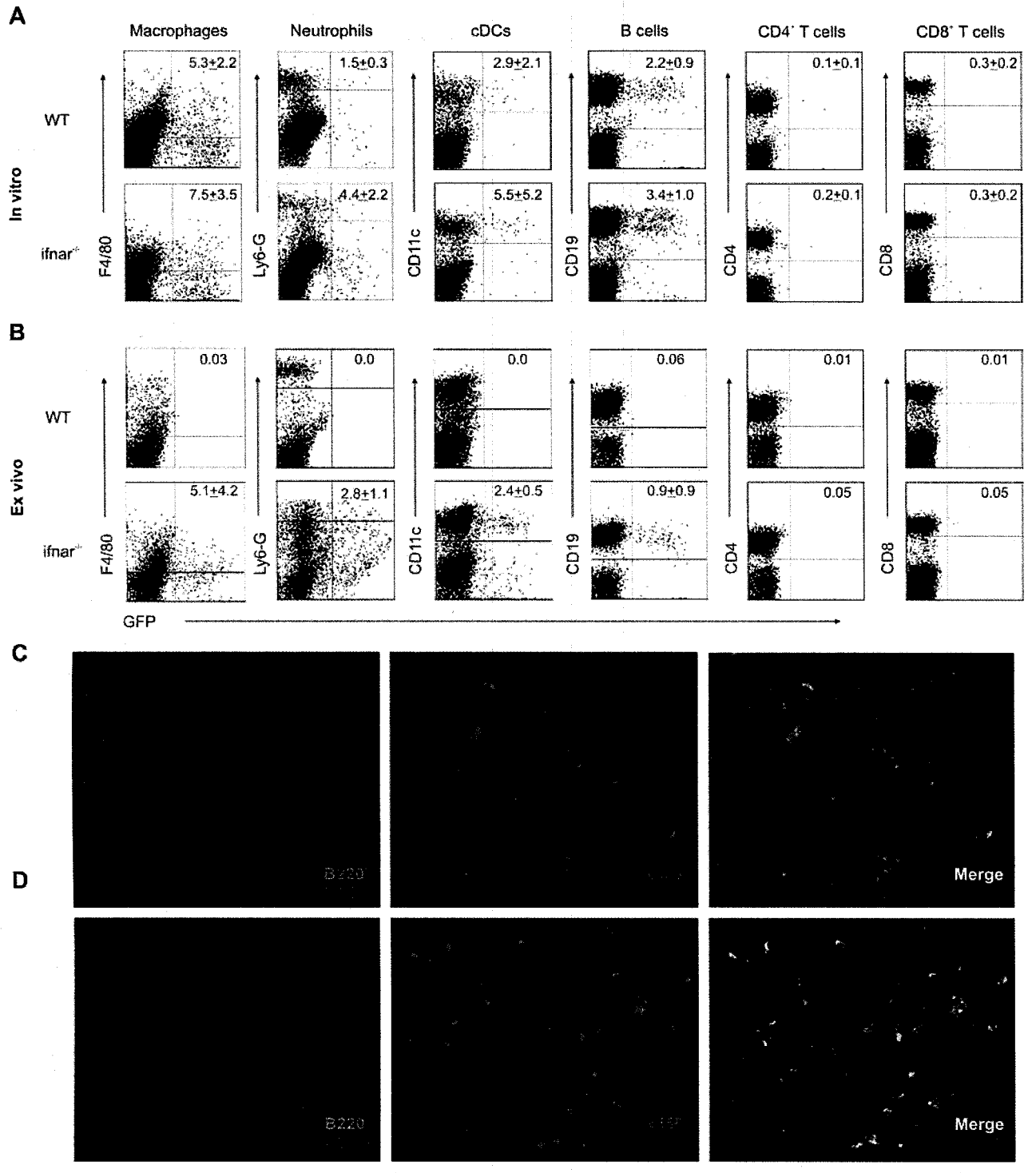

FIGURE 2. MHV target cells in vitro and in vivo. *A*, 10^6 splenocytes or low-density cell-enriched fractions (for cDC analysis) from *ifnar*$^{-/-}$ or B6 mice were infected with GFP-recombinant MHV (MOI 1). Cells were harvested 12 h later and stained for the indicated surface molecules (macrophages, F4/80$^+$, CD11b$^+$; neutrophils, Ly6G$^+$CD11b$^+$; cDCs, B220$^-$CD11c$^+$; B cells, CD19$^+$; CD4 T cells, CD4$^+$CD3$^+$; CD8 T cells, CD8$^+$CD3$^+$. *B*, *ifnar*$^{-/-}$ or B6 mice were infected i.p. with 5×10^3 PFU of GFP-recombinant MHV. Spleens were collected after 36 h and digested with collagenase, and splenocytes or low-density cells (for cDCs analysis) were stained for the indicated cell population as in *A*. Dot plots are representative of five individual mice. Numbers in the upper right quadrant indicate mean percentages ± SD of GFP$^+$ cells for each population. *C* and *D*, Representative sections from the spleens of *ifnar*$^{-/-}$ mice infected with 5×10^3 PFU of MHV A59 24 h postinfection. Staining: B220, blue; MHV-N, green; F4/80, red in *C*; CD11c, red in *D*. WT, Wild type. Original magnification, ×400).

could affect viral distribution and virus-mediated disease. To this end, neutrophils were depleted in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and B6 mice using the NIMP-R14 Ab (32). NIMP-R14-mediated depletion of neutrophils in B6 mice had no significant effect on MHV replication in the major target organs (data not shown). Likewise, MHV replication and infection-associated hepatitis was not affected by the absence of neutrophils in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice (Fig. 3*D*), indicating that in these mice it is the absence of the IFNAR on macrophages, not on neutrophils, that determines the high susceptibility to MHV infection. CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$

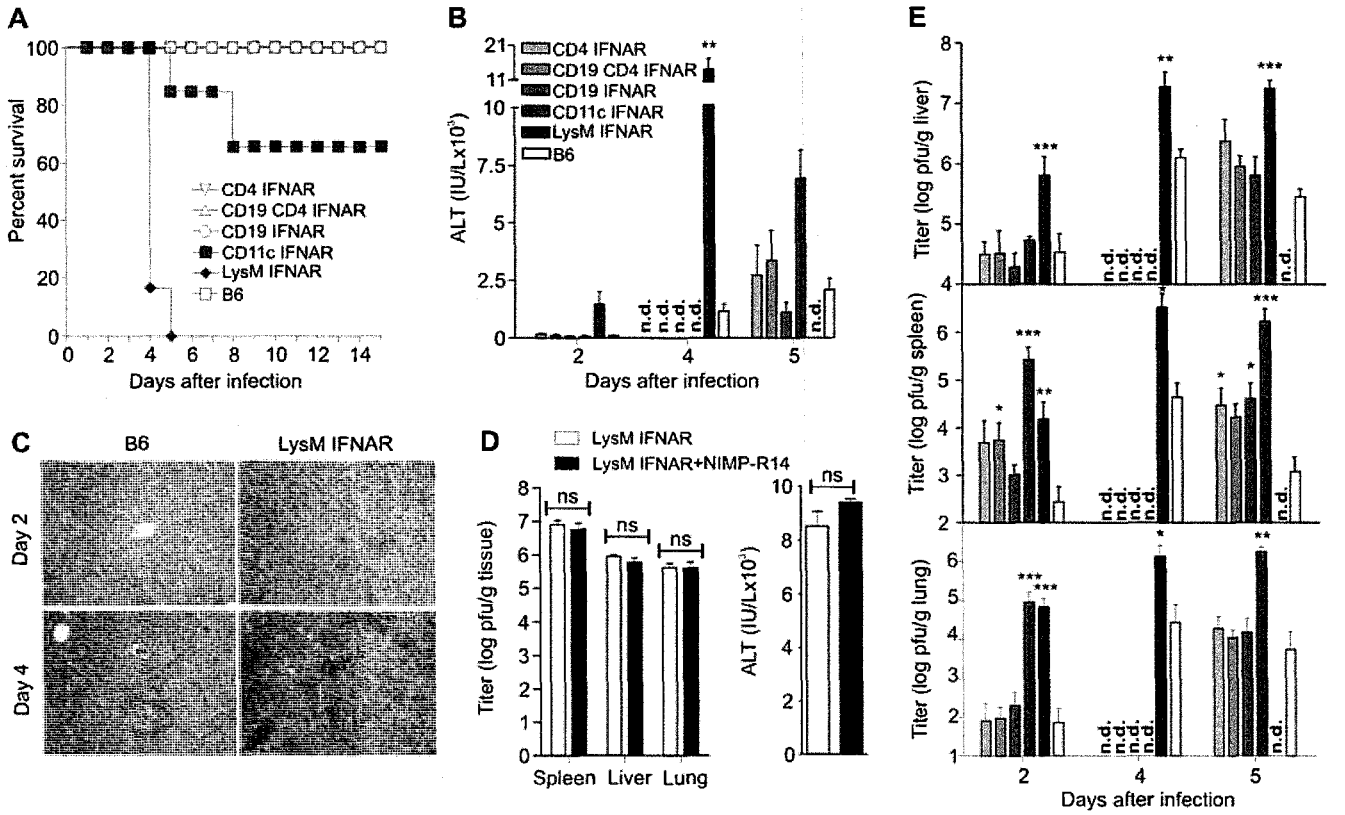

FIGURE 3. Requirement for IFNAR expression on different leukocyte subsets. CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD4-Cre$^{+/-}$CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, and B6 mice were infected i.p. with 50 PFU of MHV A59. *A*, Survival of conditionally IFNAR$^-$ mice. Health status was monitored twice daily, and moribund animals were euthanized (n = 6–7 mice per group). *B*, ALT values in serum were determined at the indicated time points postinfection. *C*, Liver pathology in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and B6 mice on days 2 and 4 postinfection. H&E staining. *D*, MHV replication in neutrophil-depleted LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice. LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice were injected with 250 μg of NIMP-R14 Ab and 24 h later were infected with 50 PFU of MHV A59. At day 2 postinfection, a second NIMP-R14 injection was administered. Neutrophil depletion (>95%) was confirmed by flow cytometry. Viral titers in livers, spleens, and lungs (*left*) and ALT values in serum (*right*) were determined at day 4 postinfection. Results represent means ± SD of three mice per group. Statistical analysis was performed using Student's t test. *E*, Viral titers in livers, spleens, and lungs were determined at the indicated time points postinfection. Results represent means ± SD of five mice per group. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ comparing the values from the corresponding conditional IFNAR$^-$ mice with B6 values at the same day. n.d., not determined.

mice exhibited a slightly delayed onset of liver disease with peak values at about day 5 postinfection (Fig. 3*B*). Mice lacking the IFNAR on T and/or B cells showed no exacerbation of MHV-induced liver disease (Fig. 3*B*). The clear hierarchy of cell type-dependent, type I IFN-mediated protection from disease correlated well with viral replication observed in livers, spleens, and lungs (Fig. 3*E*). Clearly, mice lacking the IFNAR on macrophages in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice were most susceptible to MHV infection resulting in uncontrolled spread through all organs. CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice were, as well, highly susceptible with particularly strong replication in spleens supporting the notion that splenic cDCs represent a major target cell population of MHV within this organ. Although B cells could be infected with MHV in vitro (Fig. 2*A*) and were found to be infectable in *ifnar*$^{-/-}$ mice in vivo (Fig. 2*B*), the specific IFNAR deficiency on B cells only moderately influenced viral replication (Fig. 3*E*), which is probably related to the poor capacity of MHV-infected B cells to produce viral particles in comparison with cDCs or macrophages (data not shown). Likewise, CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and CD4-Cre$^{+/-}$CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice showed only mildly increased susceptibility to MHV infection. All mice that survived until day 15 postinfection had cleared the virus, including the remaining CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice (data not shown), suggesting that the adaptive immune system had successfully contained the viral infection.

To evaluate the importance of IFN-α/β production for different target cell populations following a peripheral route of infection, CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, and B6 mice were infected intranasally with 5×10^4 PFU of MHV, and the severity of the disease, viral distribution, and viral titers were determined on day 6 postinfection. As shown in Fig. 4*A*, mice lacking the IFNAR on LysM$^+$ and CD11c$^+$ cells developed severe liver disease, whereas the absence of the IFNAR on T and B cells did not precipitate an elevation in liver enzyme values. Comparable with the results from the systemic (i.p.) infection, type I IFN responsiveness by macrophages was most important for the control of the virus (Fig. 4*B*). Also, the lack of the IFNAR on B or T cells was of importance for the systemic spread of the virus which was still detectable in the liver at substantial titers (Fig. 4*B*). The finding that the presence or absence of this receptor on different cell populations did not influence the ability of the virus to enter the CNS (Fig. 4*B*) illustrates the context-dependent and organ-specific importance of IFNAR expression.

Conditional targeting using the Cre/*loxP* system permits functional assessment of particular molecules in certain cell types. However, absolute cell type specificity can usually not be achieved

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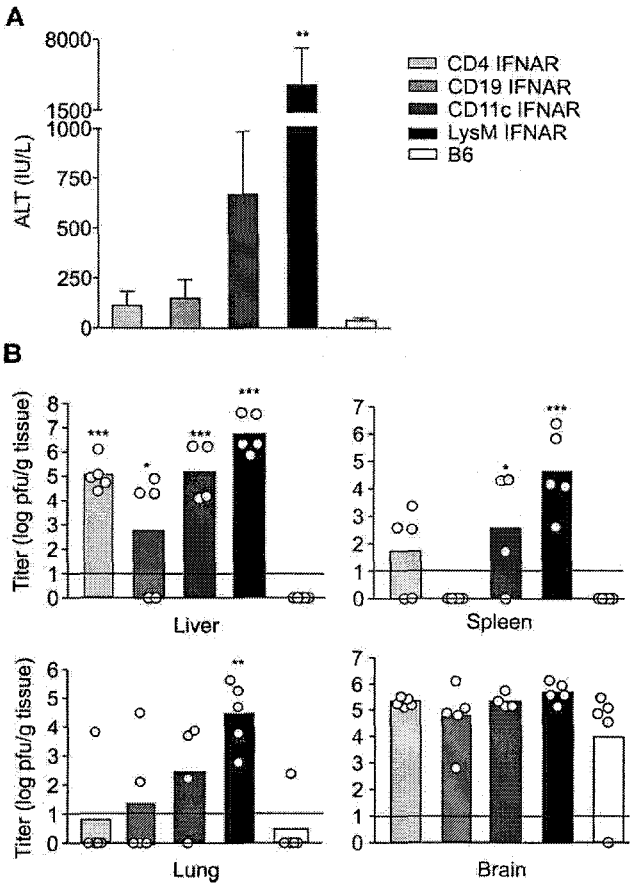

FIGURE 4. Type I IFN-dependent control of MHV following intranasal infection. CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and B6 mice were infected with 5×10^4 PFU MHV A59. Six days postinfection, ALT values in serum (*A*) and viral titers in livers, spleens, lungs, and the CNS (*B*) were determined. Bars, means; ○, values from individual mice. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ comparing the values from the corresponding conditional IFNAR$^-$ mice with B6 values at the same day).

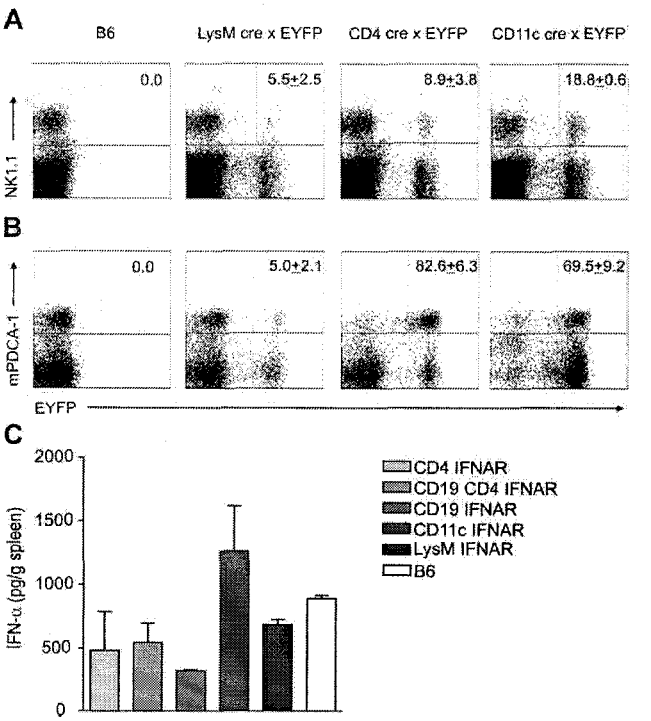

FIGURE 5. Cre recombinase-driven gene recombination in NK cells, pDCs, and type I IFN responsiveness. Cre recombinase activity in CD3$^-$NK1.1$^+$ NK cells (*A*) and CD11c$^+$mPDCA-1$^+$ pDCs (*B*) was analyzed by the expression of the *EYFP* reporter gene in B6, LysM-Cre$^{+/-}$R26-EYFP$^{+/-}$, CD4-Cre$^{+/-}$R26-EYFP$^{+/-}$, and CD11c-Cre$^{+/-}$R26-EYFP$^{+/-}$ mice. Dot plots show analysis of one representative of six individual mice. Numbers in the upper right quadrant indicate mean percentages ± SD of EYFP$^+$ cells for each population. *C*, IFN-α production in the different conditionally IFNAR$^-$ mice. CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD4-Cre$^{+/-}$CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, and B6 mice were infected i.p. with 50 PFU of MHV A59. Forth-eight hours after infection, IFN-α concentration in spleen homogenates was determined by ELISA (mean ± SD, $n = 2$–4 mice).

by this approach. We thus performed a side-by-side comparison of different Cre driver mice which allowed us to extract the relevant information from a more complex data set. NK cells and pDCs are critical during the early phase of a viral infection. To analyze the Cre recombinase activity in these cell populations, we crossed CD4-Cre, CD11c-Cre, and LysM-Cre mice with the R26-EYFP strain which permits detection of the *EYFP* reporter gene in those cells with active Cre recombinase (27). Moreover, to address the question of whether deletion of the IFNAR on pDCs in the conditionally gene-targeted mice might have influenced the overall type I IFN responsiveness to MHV infection, we determined IFN-α production in the conditionally IFNAR$^-$ mice. Cre recombinase was active in only a few NK1.1$^+$ cells from LysMCre$^{+/-}$R26-EYFP$^{+/-}$ or CD11c-Cre$^{+/-}$R26-EYFP$^{+/-}$ mice (Fig. 5*A*). Furthermore, given that 10% of NK1.1$^+$ cells in CD4-Cre$^{+/-}$R26-EYFP$^{+/-}$ mice were EYFP$^+$ and CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice did not show a significant impairment in their susceptibility to MHV (Fig. 3), such a small proportion of IFNAR$^-$ NK cells did most likely not influence susceptibility to MHV infection. Cre recombinase activity could be readily detected in mPDCA-1$^+$ pDCs from CD11c-Cre$^{+/-}$R26-EYFP$^{+/-}$ (Fig. 5*B*) as described previously (29). The finding that CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice responded with vigorous IFN-α production to MHV infection (Fig. 5*C*) suggested that the lack of the IFNAR on pDCs had no significant impact on the early type I IFN response. This notion is supported by the findings that >82% of the pDCs were EYFP$^+$ in CD4-Cre$^{+/-}$R26-EYFP$^{+/-}$ mice (Fig. 5*B*), which controlled MHV infection efficiently (Fig. 3), and that IFN-α production was not impaired in these mice (Fig. 5*C*). Taken together, these data indicate that the absence of the IFNAR on pDCs did not affect the resistance to MHV infection in conditionally IFNAR$^-$ mice.

Protection of macrophages and dendritic cells by pDC-derived IFN-α

Taken together, the data presented above indicated that macrophages and cDCs are most dependent on the protection provided by the type I IFN system and that this stimulation is necessary to secure control of systemic MHV infection. To provide insight into the mechanisms underlying the type I IFN-induced antiviral state in these two important target cell populations, a series of in vitro experiments were performed. Virus-free cell culture supernatant from MHV-infected pDCs containing defined amounts of IFN-α was used to estimate the protective capacity of pDC-derived IFN-α for both cDCs or macrophages. pDC-derived type I IFN significantly reduced cell death of cDCs (Fig. 6*A*) and macrophages (Fig. 6*B*) when the cells were exposed to high doses of virus (MOI 1).

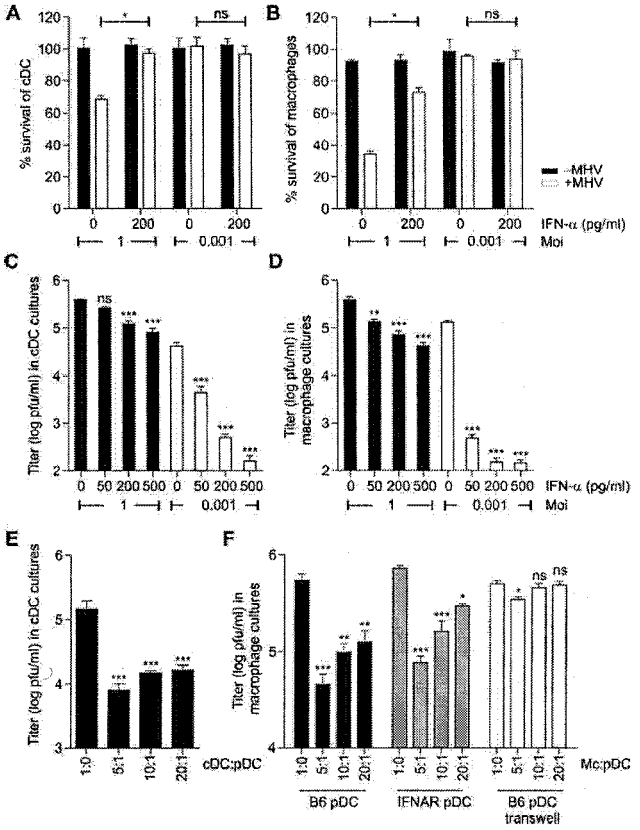

FIGURE 6. Effect of pDC-derived type I IFN on cDC and macrophage (Mc) survival and control of MHV replication. Five $\times$ 10^4 cDCs (*A*) or macrophages (*B*) were preincubated for 5 h with pDC supernatant containing 0 or 200 pg/ml IFN-α and infected with MHV A59 at a MOI of 1 or 0.001. Survival was analyzed after 24 h using the MTS assay. Statistical analysis was performed using Student's *t* test (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, n.s., $p > 0.05$). Five $\times$ 10^5 cDCs (*C*) or macrophages (*D*) were preincubated with pDC supernatant containing the indicated concentrations of IFN-α and infected with MHV A59 at a MOI of 1 or 0.001. Five $\times$ 10^5 cDCs (*E*) or macrophages (*F*) were infected with MHV A59 at a MOI of 0.001. B6 or *ifnar*$^{-/-}$ pDCs were added directly or in a transwell to the infected cells at the indicated ratio. Viral titers in the supernatants were analyzed after 24 h. Bars, Means $\pm$ SD of quadruplicate samples from one of three independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, n.s., $p > 0.05$ comparing the values from the corresponding column with the unpretreated control (*C* and *D*) or the cells without addition of pDCs (*E* and *F*).

Likewise, pDC-derived type I IFN significantly reduced MHV production in both target cell populations (Fig. 6, *C* and *D*). Incubation of *ifnar*$^{-/-}$ macrophages and cDCs with pDC-derived supernatant did neither reduce MHV-induced cell death nor MHV replication (data not shown) indicating that the observed protective effect depended solely on signals transmitted via the IFNAR. Furthermore, cocultures of uninfected pDCs with infected cDCs (Fig. 6*E*) and macrophages (Fig. 6*F*) revealed that only few pDCs were sufficient to protect the adjacent target cells. pDC-mediated target cell protection was not dependent on the presence of the IFNAR on pDCs (Fig. 6*F*, *middle*). However, viral replication in IFNAR-competent cells was not halted when pDCs and macrophages were separated in transwell chambers, indicating that infected target cells and type I IFN-producing pDCs had to be in close vicinity to permit access of pDCs to viral particles or viral compounds that could trigger the protective IFN response in pDCs. (Fig. 6*F*, *right*). Overall, these data revealed that the IFNAR on macrophages and cDCs together with its stimulation by pDC-derived type I IFN is essential to prevent excessive viral replication in the target cells and to secure survival of these important APCs.

Discussion

A major function of both macrophages and cDCs during viral infections is their instructive role for the developing adaptive immune response. Macrophages in the marginal sinuses of lymph nodes, for example, are able to collect Ag from the incoming lymph stream and present Ag to follicular B cells (33, 34). Marginal zone macrophages in spleen can bind viruses decorated by complement and natural Abs and reduce thereby dissemination of viruses to peripheral organs (35). This trapping of viral particles on macrophages is important to enhance the induction of protective T cell responses (36). It has been shown that the enhanced binding of viral particles to macrophages also fosters their infection (37). Likewise, cDCs can be infected with essentially all viruses irrespective of their tissue tropism. The high susceptibility of cDCs to viral infection appears to be important for the efficient direct priming of CTL (38). The results of this study emphasize the importance of type I IFN-mediated protection of both macrophages and cDCs. The lack of the IFNAR on macrophages in LysM-Cre$^{+/-}$ *ifnar1*$^{fl/fl}$ mice led to completely uncontrolled viral replication and death in only 4 days. Furthermore, the absence of the IFNAR on cDCs resulted in death of ~40% of the animals between days 6 and 8. At this time, the CTL response is supposed to clear the virus infection. Thus, type I IFNs provide protection of two highly vulnerable cell populations and therefore facilitate 1) removal of the virus from the circulation by macrophages and 2) preservation of cDC integrity for the priming of adaptive immune responses.

MHV is a rapidly replicating virus exhibiting a high cytopathicity that leads to severe inflammation in several organs (39). Systemic virus infection with dissemination via the bloodstream into visceral organs has been mimicked in this study by i.p. application. Our results show that type I IFN responsiveness in macrophages and cDCs is necessary to prevent severe liver disease and to secure survival of the host. However, MHV may escape immunosurveillance and establish chronic infection in the CNS, leading to progressive demyelinating disease (20). MHV can enter the CNS via the olfactory nerve system (40) and spreads transneuronally, leading to infection of distinct parts of the brain and the spinal cord. CTLs control viral replication within the CNS but cannot completely eliminate the virus (21), whereas neutralizing Abs are essential to prevent viral recrudescence (24). Thus, MHV is well adapted to use the CNS as an immunoprivileged site to escape complete clearance from the system. Part of that escape strategy may be the inability of the type I IFN system to prevent spread of the virus to the CNS, as shown in this study. Intranasal inoculation which permits direct access of the virus to olfactory nerve endings, facilitated efficient neuroinvasion of the virus irrespective of the presence of absence of the IFNAR. Direct intracranial application of a gliatropic strain of MHV results in a severely accelerated lethal CNS disease even in the presence of fully function antiviral CD8$^+$ T cells (41), indicating that the type I IFN system also contributes to the control of viral dissemination within the CNS. It will be important in future studies to determine which cell type (neuron, glia, or hemopoietic) is critical for the type I IFN-mediated containment of MHV in this immunoprivileged site.

Viruses have developed a remarkable array of countermeasures to interfere with the type I IFN system. Coronaviruses, despite generation of significant amounts of type I IFN inducing dsRNA, are able to suppress early IFN-β induction (42, 43). Furthermore, immunomodulatory nonstructural proteins (Nsp) such as Nsp1 are

able to inhibit IFN-α responsiveness in a cell type-specific manner (31). Thus, cDCs and macrophages, which fail to raise significant IFNα responses following coronavirus infection (25), are particularly dependent on the external supply of protective type I IFNs. It appears that during coronavirus infections, it is the pDC-derived type I IFN (25) that provides protection for those infected cells that are otherwise incapacitated by particular viral proteins. Taken together, our study provides insight into the context-dependent regulation of the type I IFN system and highlights the importance of type I IFN-mediated cross-talk between pDCs and cDCs/macrophages which most likely represents an essential cellular pathway for the protection against cytopathic virus infections.

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Disclosures

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References

1. Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber. 1998. How cells respond to interferons. *Annu. Rev. Biochem.* 67: 227–264.
2. Balachandran, S., C. N. Kim, W. C. Yeh, T. W. Mak, K. Bhalla, and G. N. Barber. 1998. Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling. *EMBO J.* 17: 6888–6902.
3. Salazar-Mather, T. P., C. A. Lewis, and C. A. Biron. 2002. Type I interferons regulate inflammatory cell trafficking and macrophage inflammatory protein 1α delivery to the liver. *J. Clin. Invest.* 110: 321–330.
4. Kamphuis, E., T. Junt, Z. Waibler, R. Forster, and U. Kalinke. 2006. Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia. *Blood* 108: 3253–3261.
5. Lapenta, C., S. M. Santini, M. Spada, S. Donati, F. Urbani, D. Accapezzato, D. Franceschini, M. Andreotti, V. Barnaba, and F. Belardelli. 2006. IFN-α-conditioned dendritic cells are highly efficient in inducing cross-priming $CD8^+$ T cells against exogenous viral antigens. *Eur. J. Immunol.* 36: 2046–2060.
6. Longman, R. S., D. Braun, S. Pellegrini, C. M. Rice, R. B. Darnell, and M. L. Albert. 2007. Dendritic-cell maturation alters intracellular signaling networks, enabling differential effects of IFN-αβ on antigen cross-presentation. *Blood* 109: 1113–1122.
7. Le Bon, A., V. Durand, E. Kamphuis, C. Thompson, S. Bulfone-Paus, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Direct stimulation of T cells by type I IFN enhances the $CD8^+$ T cell response during cross-priming. *J. Immunol.* 176: 4682–4689.
8. Schulz, O., S. S. Diebold, M. Chen, T. I. Naslund, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljestrom, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433: 887–892.
9. Fink, K., K. S. Lang, N. Manjarrez-Orduno, T. Junt, B. M. Senn, M. Holdener, S. Akira, R. M. Zinkernagel, and H. Hengartner. 2006. Early type I interferon-mediated signals on B cells specifically enhance antiviral humoral responses. *Eur. J. Immunol.* 36: 2094–2105.
10. Heer, A. K., A. Shamshiev, A. Donda, S. Uematsu, S. Akira, M. Kopf, and B. J. Marsland. 2007. TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. *J. Immunol.* 178: 2182–2191.
11. Le Bon, A., G. Schiavoni, G. D'Agostino, I. Gresser, F. Belardelli, and D. F. Tough. 2001. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* 14: 461–470.
12. Havenar-Daughton, C., G. A. Kolumam, and K. Murali-Krishna. 2006. Cutting edge: the direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection. *J. Immunol.* 176: 3315–3319.
13. Le, B. A., C. Thompson, E. Kamphuis, V. Durand, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J. Immunol.* 176: 2074–2078.
14. Kolumam, G. A., S. Thomas, L. J. Thompson, J. Sprent, and K. Murali-Krishna. 2005. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J. Exp. Med.* 202: 637–650.
15. Aichele, P., H. Unsoeld, M. Koschella, O. Schweier, U. Kalinke, and S. Vucikuja. 2006. CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. *J. Immunol.* 176: 4525–4529.
16. Banchereau, J., and V. Pascual. 2006. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity* 25: 383–392.
17. Theofilopoulos, A. N., R. Baccala, B. Beutler, and D. H. Kono. 2005. Type I interferons (αβ) in immunity and autoimmunity. *Annu. Rev. Immunol.* 23: 307–336.
18. Honda, K., Y. Ohba, H. Yanai, H. Negishi, T. Mizutani, A. Takaoka, C. Taya, and T. Taniguchi. 2005. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* 434: 1035–1040.
19. Boxel-Dezaire, A. H., M. R. Rani, and G. R. Stark. 2006. Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* 25: 361–372.
20. Bergmann, C. C., T. E. Lane, and S. A. Stohlman. 2006. Coronavirus infection of the central nervous system: host-virus stand-off. *Nat. Rev. Microbiol.* 4: 121–132.
21. Marten, N. W., S. A. Stohlman, J. Zhou, and C. C. Bergmann. 2003. Kinetics of virus-specific $CD8^+$-T-cell expansion and trafficking following central nervous system infection. *J. Virol.* 77: 2775–2778.
22. Bergmann, C. C., B. Parra, D. R. Hinton, C. Ramakrishna, K. C. Dowdell, and S. A. Stohlman. 2004. Perforin and γ interferon-mediated control of coronavirus central nervous system infection by CD8 T cells in the absence of CD4 T cells. *J. Virol.* 78: 1739–1750.
23. Lin, M. T., D. R. Hinton, N. W. Marten, C. C. Bergmann, and S. A. Stohlman. 1999. Antibody prevents virus reactivation within the central nervous system. *J. Immunol.* 162: 7358–7368.
24. Ramakrishna, C., S. A. Stohlman, R. D. Atkinson, M. J. Shlomchik, and C. C. Bergmann. 2002. Mechanisms of central nervous system viral persistence: the critical role of antibody and B cells. *J. Immunol.* 168: 1204–1211.
25. Cervantes-Barragan, L., R. Zust, F. Weber, M. Spiegel, K. S. Lang, S. Akira, V. Thiel, and B. Ludewig. 2007. Control of coronavirus infection through plasmacytoid dendritic-cell-derived type I interferon. *Blood* 109: 1131–1137.
26. Muller, U., U. Steinhoff, L. F. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264: 1918–1921.
27. Srinivas, S., T. Watanabe, C. S. Lin, C. M. William, Y. Tanabe, T. M. Jessell, and F. Costantini. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the *ROSA26* locus. *BMC Dev. Biol.* 1: 4.
28. Clausen, B. E., C. Burkhardt, W. Reith, R. Renkawitz, and I. Forster. 1999. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 8: 265–277.
29. Caton, M. L., M. R. Smith-Raska, and B. Reizis. 2007. Notch-RBP-J signaling controls the homeostasis of $CD8^-$ dendritic cells in the spleen. *J. Exp. Med.* 204: 1653–1664.
30. Coley, S. E., E. Lavi, S. G. Sawicki, L. Fu, B. Schelle, N. Karl, S. G. Siddell, and V. Thiel. 2005. Recombinant mouse hepatitis virus strain A59 from cloned, full-length cDNA replicates to high titers in vitro and is fully pathogenic in vivo. *J. Virol.* 79: 3097–3106.
31. Züst, R., L. Cervantes-Barragan, T. Kuri, G. Blakqori, F. Weber, B. Ludewig, and V. Thiel. 2007. Identification of coronavirus non-structural protein 1 as a major pathogenicity factor-implications for the rational design of live attenuated coronavirus vaccines. *PLoS Pathog.* 3: E109.
32. Lopez, A. F., M. Strath, and C. J. Sanderson. 1984. Differentiation antigens on mouse eosinophils and neutrophils identified by monoclonal antibodies. *Br. J. Haematol.* 57: 489–494.
33. Phan, T. G., I. Grigorova, T. Okada, and J. G. Cyster. 2007. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat. Immunol.* 8: 992–1000.
34. Junt, T., E. A. Moseman, M. Iannacone, S. Massberg, P. A. Lang, M. Boes, K. Fink, S. E. Henrickson, D. M. Shayakhmetov, N. C. Di Paolo, et al. 2007. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* 450: 110–114.
35. Ochsenbein, A. F., D. D. Pinschewer, B. Odermatt, M. C. Carroll, H. Hengartner, and R. M. Zinkernagel. 1999. Protective T cell-independent antiviral antibody responses are dependent on complement. *J. Exp. Med.* 190: 1165–1174.
36. Oehen, S., B. Odermatt, U. Karrer, H. Hengartner, R. Zinkernagel, and C. Lopez-Macias. 2002. Marginal zone macrophages and immune responses against viruses. *J. Immunol.* 169: 1453–1458.
37. Halstead, S. B., E. J. O'Rourke, and A. C. Allison. 1977. Dengue viruses and mononuclear phagocytes. II: identity of blood and tissue leukocytes supporting in vitro infection. *J. Exp. Med.* 146: 218–229.
38. Freigang, S., H. C. Probst, and M. van den Broek. 2005. DC infection promotes antiviral CTL priming: the 'Winkelried' strategy. *Trends Immunol.* 26: 13–18.
39. Perlman, S., and A. A. Dandekar. 2005. Immunopathogenesis of coronavirus infections: implications for SARS. *Nat. Rev. Immunol.* 5: 917–927.
40. Sun, N., and S. Perlman. 1995. Spread of a neurotropic coronavirus to spinal cord white matter via neurons and astrocytes. *J. Virol.* 69: 633–641.
41. Ireland, D. D., S. A. Stohlman, D. R. Hinton, R. Atkinson, and C. C. Bergmann. 2008. Type I interferons are essential in controlling neurotropic coronavirus infection irrespective of functional CD8 T cells. *J. Virol.* 82: 300–310.
42. Spiegel, M., A. Pichlmair, L. Martinez-Sobrido, J. Cros, A. Garcia-Sastre, O. Haller, and F. Weber. 2005. Inhibition of β interferon induction by severe acute respiratory syndrome coronavirus suggests a two-step model for activation of interferon regulatory factor 3. *J. Virol.* 79: 2079–2086.
43. Versteeg, G. A., P. J. Bredenbeek, S. H. van den Worm, and W. J. Spaan. 2007. Group 2 coronaviruses prevent immediate early interferon induction by protection of viral RNA from host cell recognition. *Virology* 361: 18–26.

Hematopoietic cell derived interferon controls viral replication and virus induced disease

Philipp A. Lang[1,2,3,*], Luisa Cervantes-Barragan[4,*], Admar Verschoor[1], Alexander A Navarini[1,5], Mike Recher[1, 6], Marc Pellegrini[3], Lukas Flatz[1], Andreas Bergthaler[1], Kenya Honda[7], Burkhard Ludewig[4], Pamela S. Ohashi[3], Karl S. Lang[1,3]

[1]: Institute of Experimental Immunology, University Hospital of Zurich, Schmelzbergstrasse 12, CH-8091 Zurich, Switzerland.

[2]: Institute of Physiology, University of Tubingen, Gmelinstr. 5, D-72076, Tubingen

[3]: Campbell Family Institute for Breast Cancer Research, Ontario Cancer Institute, Departments of Medical Biophysics and Immunology, 620 University Ave, Toronto, Ontario Canada M5G 2C1

[4]: Research Department, Kantonal Hospital St Gallen, St Gallen, Switzerland;

[5]: Department of Dermatology, University Hospital of Zurich, Gloriastrasse 31, 8091 Zurich, Switzerland.

[6]: Division of Clinical Immunology, University Hospital of Zurich, Haeldeliweg 4, 8044 Zurich, Switzerland

[7]: Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

*: Philipp A. Lang and Luisa Cervantes-Barragan contributed equally to this work

Address correspondence to Philipp A. Lang, Campbell Family Institute for Breast Cancer Research, Ontario Cancer Institute, Toronto

620 University Ave, Ontario Canada M5G 2C1, phone (+1) 416 946 4501 ex4993; E-mail: philipp.lang@gmx.net

Key words: interferon, virus, plasmacytoid dendritic cells, innate immunity

Abstract:

Type I interferon (IFN-I) strongly inhibits viral replication and is a crucial factor in the control of a range of virus infections and diseases. Cellular activation through pattern recognition receptors induces interferon-production in a wide variety of hematopoietic and non-hematopoietic cell types, including dendritic cells, fibroblasts, hepatocytes and cells of neuronal origin. The relative contribution of hematopoietic and non hematopoietic cells to the overall interferon response is an important issue which has not been fully addressed. Using *irf7*$^{-/-}$ and WT bone marrow chimeras we analyzed the contribution of IFN-I from bone marrow derived sources in the control of viral infections and immunopathology in mice. We found that during systemic cytopathic virus infection, hematopoietic cells were essential for production of IFN-I, inhibition of viral spread to peripheral organs and in limiting cell damage. In a model of type I diabetes induced by non-cytopathic virus infection, hematopoietic cell derived IFN-I was essential for $CD8^+$ T cell dependent cytotoxicity in pancreatic β-islet cells and induction of diabetes. These data suggest that during systemic viral infection primarily hematopoietic derived IFN-I controls viral replication and viral induced disease.

Introduction:

Interferon is a polypeptide which has strong antiviral capacity[1-3]. Mice lacking the type I interferon receptor suffer from widespread of several mouse pathogenic viruses[4], which hinds to a strong antiviral activity in vivo. Interferons are produced by a wide variety of cell-types that are capable of responding to specific microbial elements (for example double and single stranded RNA or DNA of viral origin) via pattern recognition receptors (PRR). While some PRRs are widely expressed, others are limited to specific cell populations. For instance, RIG-I[5,6], TLR3[7-9] and TLR9[10-13] induce IFN-I in a wide variety of hematopoietic and non-hematopoietic cell types as diverse as macrophages, fibroblasts[14,15], endothelial cells, hepatocytes[16-18], microglia and even neurons[19]. In contrast, interferon inducing toll like receptor 7 (TLR7) has only been reported on murine bone marrow derived plasmacytoid dendritic cells[20,21]. Two main pathways have been characterized to induce IFN I production. One through cytoplasmic PRRs, mainly the helicases RIG-I or MDA-5[5], and the second through transmembrane PRRs, namely TLR3, TLR4, TLR7 and TLR9[22-25]. After virus recognition by the cytosolic PRRs or TLRs, Tank-binding kinase 1 (TBK1) is activated and induces phosphorylation of the transcription factors IRF3 and IRF7 which form homo and heterodimers and migrate into the nucleus[26,27]. IRF3 homodimers induce IFN-β production, but are not essential. In contrast IRF7 (homo and heterodimers with IRF3) are essential for the production of IFN-α via both cytosolic and transmembrane PRR pathways[23]. While *irf3*$^{-/-}$ mice are still able to produce IFN-I, mice deficient in IRF7 are severely impaired in the production of IFN-I. Therefore IRF7 is called the master regulator of interferon[28,29].

Although many non-immune cells possess the molecular elements that may allow them to produce IFN-I, the extent to which they participate in an effective IFN-I response during viral infection is not clear. In this report we analyzed the contribution of hematopoietic versus non-hematopoietic cells in the production of IFN-I. As expression of IRF7 is essential for production of IFN-I, we used bone marrow chimeras generated from *irf7*$^{-/-}$ and WT mice. We found that during systemic viral infections, IFN-I from a bone marrow derived source was essential for the control of virus replication and inhibition of viral spread to peripheral organs. In addition hematopoietic derived interferon controlled disease onset and the extent of pathology.

Material and Methods

Mice and viruses: Vesicular Stomatitis virus, Indiana strain (VSV-IND, Mudd-Summers isolate) was originally obtained from Prof. D. Kolakofsky (University of Geneva, Switzerland). Viruses were propagated on BHK-21 cells at a MOI of 0.01 and plaqued on Vero cells. LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated in L929 cells. Virus titers were measured using a focus forming assay as previously described[30]. MHV A59 was generated from a molecularly cloned cDNA [31] based on the Albany strain of MHV A59 and propagated on L929 cells. HSV was used as described [29]. For generation of bone marrow chimeras mice were lethally irradiated (1050 rad) on day –1. On day 0, 8 x 10^6 BM cells, generated by flushing the femur and tibia of naive WT (C57BL/6) mice or *irf7*$^{-/-}$ mice, were injected intravenously. *Irf7*$^{-/-}$ mice, RIP-GP mice and GFP transgenic mice were maintained on the C57BL/6 genetic background. All experiments were performed in single ventilated cages. Animal experiments were carried out with authorization of the Veterinäramt of the Kanton Zurich and in accordance with the Swiss law for animal protection and according to institutional guidelines at the Ontario Cancer Institute.

In vitro treatment with IFN-α: The fibroblast cell line MC57 was seeded in 24 well plates. After 24 hours cells were treated with 1000, 100 and 10 units of IFN-α (PBL, Biomedical Laboratories) and in parallel infected with VSV (MOI 0.001) and LCMV (MOI 0.001). After 6, 12 and 24 hours virus in supernatant was analyzed for infectious virus by using focus forming assay.

Depletion of NK cells, CD8$^+$ T cells: NK cells were depleted by using the commercially available α-asialo GM1 antibody (Wako pure chemical industries) according to the manufactures suggestions. CD8$^+$ T cells were depleted by intraperitoneal or intravenous injection of a rat monoclonal antibody specific for mouse CD8 (clone YTS169.4) on days -3 and -1 before infection.

mRNA gene-profiling by quantitative RT-PCR: RNA extraction and production of cDNA was performed as described[32]. Gene expression analysis of collagen and 18S RNA was performed using kits from Applied Biosystems. For analysis, the expression levels of all target genes were normalized against 18S rRNA (ΔCt). Gene expression values were then calculated based on the ΔΔCt method, using the mean of 3 untreated mice as a standard to which all other samples were compared. Relative quantities (RQ) were determined using the equation: RQ=2^-ΔΔCt.

IFN-α ELISA: was performed according to the manufacturers suggestions (Research diagnostics RDI, Flanders, NJ).

Histology: Histological analysis was performed on snap frozen tissue as described[32]. Antibodies against mouse CD8 (clone YTS169.4) , LCMV-NP (clone VL4) and MHC I (M1/42) were used. Histology was developed using an alkaline phosphatase or a peroxidase system. Pictures were made with an Olympus BX50 (San Diego) and a Photometrics (Pleasanton) Coolsnap HQ2 camera using a 200x magnification (20x objective). Image Pro analyzer Software (Media Cybernetics, Bethesda) was used.

FACS analysis: Tetramer production, surface and intracellular FACS staining was performed as described previously[32]. In brief cells were stained for 15 minutes with tetramers at 37°C followed by incubation with the BD Biosciences antibodies anti-CD8, anti-CD69, anti-CTLA4, anti-CD62L, anti-CD44 or the eBiosciences antibodies anti-PD-1 and anti-IL-7Rα. To measure intracellular IFN-γ production, splenocytes were incubated with peptide for 6 hours at 37°C, for the last 5 hours with 1mg/l Brefeldin A (Sigma), then stained for CD8 at 4°C. After 30 minutes cells were fixed and permeabilized and then stained with anti-IFN-γ antibody (BD Biosciences).

ALT measurement: serum was used for alanine 2-oxoglutarate-aminotransferase (ALT) measurements using a Hitachi 747 autoanalyzer (Tokio, Japan).

Blood glucose was measured using glucometer (Bayer).

Statistical analysis: Data are expressed as mean ± S.E.M. For statistical analysis a one way ANOVA or a two way ANOVA (repeated measurements) with additional Bonferoni test was used to deterimine significant differences.

Results:

Systemic Interferon is produced by hematopoietic cells

Since IRF7 is the master regulator of type I interferon (IFN-I), cells derived from *irf7*$^{-/-}$ mice were unable to produce IFN-α. To evaluate if the lack of IRF7 led to insufficient production of IFN-α, *irf7*$^{-/-}$ mice and control wildtype (WT) mice were infected with the cytopathic vesicular stomatitis virus (VSV) or the non-cytopathic lymphocytic choriomeningitis virus (LCMV), which are well known to induce a strong systemic interferon response. As previously described, we found that infection of mice with VSV or LCMV led to a strong IRF7 dependent production of IFN-α (Figure 1A+B)[29,33]. Increased IFN-α correlated with enhanced production of *ifna4, ifnb1*, but not *ifnk* in the spleen (Figure 1C+D). In parallel the interferon induced genes *irf7*, *usp18* and *isg15* were up-regulated in response to viral infection in

our models (Figure 1E+F). To evaluate the contribution of hematopoietic versus non-hematopoietic cells in production of systemic IFN-I, we used bone marrow chimeras where *irf7*$^{-/-}$ BM was transferred into WT mice and vice versa. Bone marrow development and reconstitution efficiency was comparable between different sets of chimeras suggesting that development of bone marrow cells was independent of IRF7 (Supplementary Figure 1). To analyze the contribution of bone marrow derived cells in the production of systemic IFN-I, we infected chimeric mice with VSV, LCMV and herpes simplex virus (HSV). We found that during infection, in all three cases, systemic IFN-I was dependent on IRF7 expressed on bone marrow derived cells (Figure 1G-I).

Hematopoietic-cell derived interferon is essential for the control of murine hepatitis virus, and to limit hepatitis

We found that the induction of high concentrations of IFN-I is dependent on bone marrow derived cells during systemic viral infections. Therefore we continued to analyze the influence of hematopoietic cell derived IFN-I on the control of viral replication and disease outcome. *Irf7*$^{-/-}$ mice, WT mice and bone marrow chimeric mice were infected with the cytopathic mouse hepatitis virus A59 (MHV A59). Although it is known that plasmacytoid dendritic cell derived IFN-I is important for MHV control[34], the possible contribution of small amounts of non-hematopoietic cell derived interferon in viral control has not yet been analyzed. While *irf7*$^{-/-}$ mice were overwhelmed with MHV infection, transplantation of WT bone marrow restored the host's capacity to control the virus (Figure 2A). However, WT mice transplanted with IRF7 deficient bone marrow were unable to control the infection, since high viral titers in liver and spleen were detected and virus was found also in other organs such as the lungs (Figure 2A). The lack of viral control in these mice was similar to that observed in mice with a total deficiency in IRF7, suggesting that IFN from hematopoietic origin was essential for controlling MHV infection.

To analyze the effect of interferon derived from distinct sources on MHV induced hepatitis, we determined blood alanine aminotransferase (ALT) levels. ALT levels were elevated during MHV infection in mice with general IRF7 deficiency (Figure 2B). While transplantation of WT bone marrow into *irf7*$^{-/-}$ mice prevented hepatitis, WT mice with *irf7*$^{-/-}$ bone marrow had severe hepatitis suggesting that liver damage in MHV-induced hepatitis can be reduced only by IFN-I from hematopoietic origin (Figure 2B). Taken together our results show that only IFN-α from hematopoietic origin can control MHV infection and that non-hematopoietic cells are not able to produce IFN-α in sufficient amounts to prevent MHV induced pathology.

Replication of LCMV is inhibited by IFN-I produced by hematopoietic cells

Since systemic replication of cytopathic MHV was controlled by interferon derived from hematopoietic cells, we wanted to determine whether this was also true for the non-cytopathic virus LCMV. Consistent with the MHV model, we found that in the absence of IRF7 in bone marrow derived cells, LCMV spread into liver, lung and kidney (Figure 3A). Interestingly, we found that viral titers in the spleen were independent of IRF7 (Figure 3A). LCMV did not replicate in the lungs of WT mice. If such mice were transplanted with WT bone marrow there was viral replication detectable (Figure 3A), which suggests that there may be a form of innate immunodeficiency in the lung after bone marrow transplantation. Interferon signaling can directly suppress virus replication due to induction of molecules like RNAse L, Mx1, Protein kinase R[35,36]. In addition interferons influence the T cells, B cells and NK cells which also might contribute to the suppression of virus replication[32,37,38]. To analyze if activation of adaptive immune cells rather then a direct antiviral effect was responsible for early LCMV suppression we depleted *Rag*$^{-/-}$ mice (lacking B and T cells) of NK cells and infected them with LCMV. *Rag*$^{-/-}$ mice showed a slightly reduced replication of LCMV likely due to their splenic architecture[39]. However, the interferon induced suppression of virus replication in peripheral organs was not dependent on lymphocytes (Figure 3B). This suggests that early inhibition of virus replication in vivo is due to a direct antiviral effect. This was supported by in vitro data which indeed showed that replication of both, VSV and LCMV, in MC57 cells can be inhibited strongly by interferons (Figure 3C).

Hematopoietic cell derived interferon influenced CD8$^+$ T cell expansion

We found that production of IFN-I by bone marrow derived cells reduces early viral replication. Next we wanted to examine the role of bone marrow derived IFN-I in the adaptive immune response and the induction of virus induced immunopathology. To address this we utilized the RIP-GP model. RIP-GP mice transgenically express the glycoprotein of LCMV under the rat insulin promoter. Infection with LCMV leads to activation of LCMV-GP specific CD8$^+$ T cells that recognize the LCMV-GP expressed on pancreatic β-islet cells. This leads to the destruction of the β-islet cells and the induction of diabetes[40]. Chimeras were generated using bone marrow from WT or *irf7*$^{-/-}$ mice, and after reconstitution the animals were infected with LCMV. The frequencies of virus specific tet-gp33$^+$ CD8$^+$ T cells were not significantly different between RIP-GP mice reconstituted with WT or *irf7*$^{-/-}$ BM (Figure 4A). Expression of CD44, CD62L, IL-7Rα, PD-1 was not obviously affected by systemic IFN-I (Figure 4B). Expression of CD69 and CTLA4 seemed to be slightly higher in WT > WT mice, however the difference was not statistically significant under the settings of our experiment (Figure 4B). The

expansion of $CD8^+$ T cells was significantly reduced in mice lacking IRF7 in bone marrow derived cells (Figure 4C). Generation of $IL7R\alpha^+$ memory $CD8^+$ T cells was also significantly reduced in mice with IRF7 deficient bone marrow correlating with persistence of antigen (Figure 4D&E). Thus, we found a reduced expansion of total $CD8^+$ T cells when IRF7 was deficient in bone marrow derived cells which was probably due to a lack of interferon signaling directly in $CD8^+$ T cells[37,41]. Therefore, in response to LCMV challenge, bone marrow derived IFN influence $CD8^+$ T cell expansion, the memory T cell population and consequently virus persistence.

Hematopoietic cell derived interferon influenced type I diabetes

Recently it was shown that induction of type I interferon by pathogen recognition receptor ligands can enhance antigen presentation and production of chemokines in the target organ[42,43]. We therefore attempted to address the role of IRF7 in these processes. Indeed we found that poly(I:C) induced up-regulation of MHC I was reduced in *irf7*$^{-/-}$ mice (Figure 5A). In keeping with the described interferon dependent chemokine production, minimal $CD8^+$ T cells infiltration was observed 8 days after infection in RIP-GP mice receiving *irf7*$^{-/-}$ BM (Figure 5B). Infection with LCMV led to up-regulation of MHC I in WT mice receiving WT BM and this was reduced in WT mice receiving *irf7*$^{-/-}$ BM (Figure 5B). Interferon type I act on the β-islet cells probably synergistically with other cytokines including TNF-α and Interferon-γ[44,45]. Lack of IRF on bone marrow derived cells reduced $CD8^+$ T cell dependent cytotoxicity in pancreatic islets and therefore was essential for induction of type I diabetes in this mouse model (Figure 5C).

Discussion:

Several cell-types are potentially able to produce IFN-I[6-13]. We found here that IFN-I produced by hematopoietic cells was essential to control systemic viral infections. Mice lacking IRF7 on bone marrow derived cells were unable to control and limit MHV infection and viral induced hepatitis. Lack of IRF7 on hematopoietic cells resulted in virus dissemination to non-lymphoid organs. This enhanced virus widespread correlated with a subsequent reduction of T cell frequencies, especially at later stages of viral disease. Systemically produced IFN-I did directly influence infiltration of auto-reactive $CD8^+$ T cells into pancreatic β-islet cells. In our mouse model, lack of IFN-I therefore prevented induction of type I diabetes. This stands in line with a general role of interferons in autoimmune diseases[46]. In humans, diseases like systemic Lupus erythematodes[47-50], type I diabetes[51,52] and psoriasis [53] are typically linked with IFN-I. Those diseases require local action of IFN-I. From our data

we would suggest that not only the local production of IFN-I[53], but also a strong systemic induction of IFN-I can trigger such autoimmune diseases. Therefore a systemic infection with virus could potentially trigger type I diabetes, without any cross reactivity or any replication of the virus in the pancreatic β-islet cells[54]. The induction of diabetes by type I interferon was confirmed in other mouse models[42,55,56]. Infection of mice with coxsackievirus however led to induction of diabetes if IFN-I is not signalling in β-islet cells[57]. Although this model is contradictory to our RIP-GP model from the phenotype, it is in line with our data as lacking interferon response in β-islet cells led to direct replication of coxsackievirus in the target organ. Therefore the β-islet cells become targets of the virus cytotoxicity and the immune response against the virus[57]. Those data are in consistent with our MHV data, were lack of systemic IFN-I led to enhanced replication of MHV in the liver, which turns hepatocytes into target cells of virus cytotoxicity.

It is difficult to interpret the role of systemic interferon in multiple sclerosis. While multiple sclerosis is certainly an autoimmune disease involving the innate and the adaptive immune system, interferons are beneficial for the outcome of disease[58]. This was first shown by local treatment with IFN-I[59], but is also able with systemically action of IFN-I[60,61]. This would suggest that although neurons can produce type I interferon[19], systemic interferon can influence relapses of multiple sclerosis. This role of systemic interferon could theoretically be explained by enhanced replication of virus in the CNS. Although there is a some evidence of lack of virus control in MS patients[62,63] the link between systemic IFN-I and multiple sclerosis remains unclear.

The role of systemically induced interferon in humans is not clear defined. First, the route of infection is usually not intravenously, therefore local replication in endothelial cells or lymphnodes might induce type I interferon before virus spreads into the blood. It was shown that in the SARS-coronavirus there is not induction of interferon in several cell types[64,65]. This could hinds to the importance of specialised interferon producing cells during this disease[34]. In fact treatment with interferon can prevent SARS[66]. The human pathogenic hepatitis C virus can suppress the production of IFN-I in hepatocytes[67]. This could mean that lack of a strong systemic interferon response leads to persistence of virus in the liver[68]. Also during this type of infection systemic treatment with IFN-I can inhibit replication of virus in hepatocytes[69,70]. Special is the role of systemic interferon during HIV infection. During HIV infection there is only little beneficial effect of IFN-I treatment[71], indeed recent evidence suggest that over-activation of TLR signalling leads to faster progression of Aids[72].

In summary, although a great variety of cell types are able to produce IFN-I, hematopoietic cell derived IFN-I is essential to control systemic viral infection. Along with its strong anti-viral effects, hematopoietic cell derived IFN-I influences the progression of diabetes induced in our LCMV model. These data suggest that a systemic rather than a local treatment (depletion or addition) of IFN-I is required to influence interferon-dependent diseases.

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Author contributions

P.A.L. and L.C.B. designed and performed research. A.V. and A.A.N. performed research. M.R. analyzed and discussed data. L.F. and A.B. provided reagents and analyzed data. K.H. provided mice and performed research. B.L. discussed data and manuscript. M.P. and P.S.O. discussed data and wrote the paper. K.S.L. performed some experiments and wrote the paper.

References:

1. Isaacs A, Lindenmann J. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci. 1957;147:258-267.
2. Nagata S, Taira H, Hall A, et al. Synthesis in E. coli of a polypeptide with human leukocyte interferon activity. 1980. Biotechnology. 1992;24:299-303.
3. Weissmann C, Nagata S, Boll W, et al. Structure and expression of human IFN-alpha genes. Philos Trans R Soc Lond B Biol Sci. 1982;299:7-28.
4. Muller U, Steinhoff U, Reis LF, et al. Functional role of type I and type II interferons in antiviral defense. Science. 1994;264:1918-1921.
5. Yoneyama M, Kikuchi M, Natsukawa T, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol. 2004;5:730-737.
6. Kato H, Sato S, Yoneyama M, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity. 2005;23:19-28.
7. Furrie E, Macfarlane S, Thomson G, Macfarlane GT. Toll-like receptors-2, -3 and -4 expression patterns on human colon and their regulation by mucosal-associated bacteria. Immunology. 2005;115:565-574.
8. Scumpia PO, Kelly KM, Reeves WH, Stevens BR. Double-stranded RNA signals antiviral and inflammatory programs and dysfunctional glutamate transport in TLR3-expressing astrocytes. Glia. 2005;52:153-162.
9. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun. 2000;68:7010-7017.
10. Dalpke AH, Schafer MK, Frey M, et al. Immunostimulatory CpG-DNA activates murine microglia. J Immunol. 2002;168:4854-4863.
11. Liu L, Zhou X, Shi J, Xie X, Yuan Z. Toll-like receptor-9 induced by physical trauma mediates release of cytokines following exposure to CpG motif in mouse skin. Immunology. 2003;110:341-347.
12. Shimosato T, Tohno M, Kitazawa H, et al. Toll-like receptor 9 is expressed on follicle-associated epithelia containing M cells in swine Peyer's patches. Immunol Lett. 2005;98:83-89.
13. Rodriguez-Martinez S, Cancino-Diaz ME, Jimenez-Zamudio L, Garcia-Latorre E, Cancino-Diaz JC. TLRs and NODs mRNA expression pattern in healthy mouse eye. Br J Ophthalmol. 2005;89:904-910.
14. Higuchi A, Takanashi Y, Tsuzuki N, et al. Production of interferon-beta by fibroblast cells on membranes prepared with RGD-containing peptides. J Biomed Mater Res A. 2003;65:369-378.
15. Erlandsson L, Blumenthal R, Eloranta ML, et al. Interferon-beta is required for interferon-alpha production in mouse fibroblasts. Curr Biol. 1998;8:223-226.
16. Gale M, Jr., Foy EM. Evasion of intracellular host defence by hepatitis C virus. Nature. 2005;436:939-945.
17. Zhang T, Lin RT, Li Y, et al. Hepatitis C virus inhibits intracellular interferon alpha expression in human hepatic cell lines. Hepatology. 2005;42:819-827.
18. Takii Y, Nakamura M, Ito M, et al. Enhanced expression of type I interferon and toll-like receptor-3 in primary biliary cirrhosis. Lab Invest. 2005;85:908-920.
19. Delhaye S, Paul S, Blakqori G, et al. Neurons produce type I interferon during viral encephalitis. Proc Natl Acad Sci U S A. 2006;103:7835-7840.
20. Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med. 2001;194:863-869.
21. Cella M, Jarrossay D, Facchetti F, et al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med. 1999;5:919-923.
22. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001;413:732-738.
23. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000;408:740-745.
24. Hemmi H, Kaisho T, Takeuchi O, et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol. 2002;3:196-200.
25. Toshchakov V, Jones BW, Perera PY, et al. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat Immunol. 2002;3:392-398.

26. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. Science. 2003;300:1148-1151.
27. Fitzgerald KA, McWhirter SM, Faia KL, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol. 2003;4:491-496.
28. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol. 2006;6:644-658.
29. Honda K, Ohba Y, Yanai H, et al. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature. 2005;434:1035-1040.
30. Junt T, Nakano H, Dumrese T, et al. Antiviral immune responses in the absence of organized lymphoid T cell zones in plt/plt mice. J Immunol. 2002;168:6032-6040.
31. Coley SE, Lavi E, Sawicki SG, et al. Recombinant mouse hepatitis virus strain A59 from cloned, full-length cDNA replicates to high titers in vitro and is fully pathogenic in vivo. J Virol. 2005;79:3097-3106.
32. Fink K, Lang KS, Manjarrez-Orduno N, et al. Early type I interferon-mediated signals on B cells specifically enhance antiviral humoral responses. Eur J Immunol. 2006;36:2094-2105.
33. Dalod M, Salazar-Mather TP, Malmgaard L, et al. Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. J Exp Med. 2002;195:517-528.
34. Cervantes-Barragan L, Zust R, Weber F, et al. Control of coronavirus infection through plasmacytoid dendritic-cell-derived type I interferon. Blood. 2007;109:1131-1137.
35. Balachandran S, Roberts PC, Brown LE, et al. Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. Immunity. 2000;13:129-141.
36. Yang YL, Reis LF, Pavlovic J, et al. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. Embo J. 1995;14:6095-6106.
37. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med. 2005;202:637-650.
38. Matikainen S, Paananen A, Miettinen M, et al. IFN-alpha and IL-18 synergistically enhance IFN-gamma production in human NK cells: differential regulation of Stat4 activation and IFN-gamma gene expression by IFN-alpha and IL-12. Eur J Immunol. 2001;31:2236-2245.
39. Muller S, Hunziker L, Enzler S, et al. Role of an intact splenic microarchitecture in early lymphocytic choriomeningitis virus production. J Virol. 2002;76:2375-2383.
40. Ohashi PS, Oehen S, Buerki K, et al. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell. 1991;65:305-317.
41. Aichele P, Unsoeld H, Koschella M, Schweier O, Kalinke U, Vucikuja S. CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. J Immunol. 2006;176:4525-4529.
42. Lang KS, Recher M, Junt T, et al. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med. 2005;11:138-145.
43. Lang KS, Georgiev P, Recher M, et al. Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling. J Clin Invest. 2006;116:2456-2463.
44. von Herrath MG, Oldstone MB. Interferon-gamma is essential for destruction of beta cells and development of insulin-dependent diabetes mellitus. J Exp Med. 1997;185:531-539.
45. Ohashi PS, Oehen S, Aichele P, et al. Induction of diabetes is influenced by the infectious virus and local expression of MHC class I and tumor necrosis factor-alpha. J Immunol. 1993;150:5185-5194.
46. Lang KS, Burow A, Kurrer M, Lang PA, Recher M. The role of the innate immune response in autoimmune disease. J Autoimmun. 2007;29:206-212.
47. Crow MK, Kirou KA. Interferon-alpha in systemic lupus erythematosus. Curr Opin Rheumatol. 2004;16:541-547.
48. Sibbitt WL, Jr., Mathews PM, Bankhurst AD. Natural killer cell in systemic lupus erythematosus. Defects in effector lytic activity and response to interferon and interferon inducers. J Clin Invest. 1983;71:1230-1239.
49. Ytterberg SR, Schnitzer TJ. Serum interferon levels in patients with systemic lupus erythematosus. Arthritis Rheum. 1982;25:401-406.
50. Preble OT, Black RJ, Friedman RM, Klippel JH, Vilcek J. Systemic lupus erythematosus: presence in human serum of an unusual acid-labile leukocyte interferon. Science. 1982;216:429-431.
51. Foulis AK, Farquharson MA, Meager A. Immunoreactive alpha-interferon in insulin-secreting beta cells in type 1 diabetes mellitus. Lancet. 1987;2:1423-1427.

52. Betterle C, Fabris P, Zanchetta R, et al. Autoimmunity against pancreatic islets and other tissues before and after interferon-alpha therapy in patients with hepatitis C virus chronic infection. Diabetes Care. 2000;23:1177-1181.
53. Nestle FO, Conrad C, Tun-Kyi A, et al. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med. 2005;202:135-143.
54. Richer MJ, Horwitz MS. Viral infections in the pathogenesis of autoimmune diseases: focus on type 1 diabetes. Front Biosci. 2008;13:4241-4257.
55. Alba A, Puertas MC, Carrillo J, et al. IFN beta accelerates autoimmune type 1 diabetes in nonobese diabetic mice and breaks the tolerance to beta cells in nondiabetes-prone mice. J Immunol. 2004;173:6667-6675.
56. Ewel CH, Sobel DO, Zeligs BJ, Bellanti JA. Poly I:C accelerates development of diabetes mellitus in diabetes-prone BB rat. Diabetes. 1992;41:1016-1021.
57. Flodstrom M, Maday A, Balakrishna D, Cleary MM, Yoshimura A, Sarvetnick N. Target cell defense prevents the development of diabetes after viral infection. Nat Immunol. 2002;3:373-382.
58. Weinstock-Guttman B, Ramanathan M, Zivadinov R. Interferon-beta treatment for relapsing multiple sclerosis. Expert Opin Biol Ther. 2008;8:1435-1447.
59. Jacobs L, O'Malley J, Freeman A, Ekes R. Intrathecal interferon reduces exacerbations of multiple sclerosis. Science. 1981;214:1026-1028.
60. Knobler RL, Panitch HS, Braheny SL, et al. Systemic alpha-interferon therapy of multiple sclerosis. Neurology. 1984;34:1273-1279.
61. Knobler RL, Panitch HS, Braheny SL, et al. Clinical trial of natural alpha interferon in multiple sclerosis. Ann N Y Acad Sci. 1984;436:382-388.
62. Kurtzke JF. Epidemiologic evidence for multiple sclerosis as an infection. Clin Microbiol Rev. 1993;6:382-427.
63. Sibley WA, Bamford CR, Clark K. Clinical viral infections and multiple sclerosis. Lancet. 1985;1:1313-1315.
64. Cheung CY, Poon LL, Ng IH, et al. Cytokine responses in severe acute respiratory syndrome coronavirus-infected macrophages in vitro: possible relevance to pathogenesis. J Virol. 2005;79:7819-7826.
65. Spiegel M, Pichlmair A, Martinez-Sobrido L, et al. Inhibition of Beta interferon induction by severe acute respiratory syndrome coronavirus suggests a two-step model for activation of interferon regulatory factor 3. J Virol. 2005;79:2079-2086.
66. Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr HW. Treatment of SARS with human interferons. Lancet. 2003;362:293-294.
67. Meylan E, Curran J, Hofmann K, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature. 2005;437:1167-1172.
68. Zachoval R, Abb J, Zachoval V, et al. Interferon alpha in hepatitis type B and non-A, non-B. Defective production by peripheral blood mononuclear cells in chronic infection and development of serum interferon in acute disease. J Hepatol. 1988;6:364-368.
69. Hoofnagle JH, Mullen KD, Jones DB, et al. Treatment of chronic non-A,non-B hepatitis with recombinant human alpha interferon. A preliminary report. N Engl J Med. 1986;315:1575-1578.
70. Thomson BJ, Doran M, Lever AM, Webster AD. Alpha-interferon therapy for non-A, non-B hepatitis transmitted by gammaglobulin replacement therapy. Lancet. 1987;1:539-541.
71. Miles SA, Wang HJ, Cortes E, Carden J, Marcus S, Mitsuyasu RT. Beta-interferon therapy in patients with poor-prognosis Kaposi sarcoma related to the acquired immunodeficiency syndrome (AIDS). A phase II trial with preliminary evidence of antiviral activity and low incidence of opportunistic infections. Ann Intern Med. 1990;112:582-589.
72. Mandl JN, Barry AP, Vanderford TH, et al. Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections. Nat Med. 2008.

Figure Legend:

Figure 1: Systemic Interferon is produced by hematopoietic cells

A: *Irf7*$^{-/-}$ mice and WT mice were infected with vesicular stomatitis virus (VSV). (n=2). **B:** *Irf7*$^{-/-}$ mice and WT mice were infected with lymphocytic choriomeningitis virus (LCMV). Interferon alpha serum levels are shown (n=2). **C:** WT mice were infected with VSV. After 10hours IFNa4, IFNb and IFNk transcript levels were quantitated by RT-PCR (n=3). **D:** WT mice were infected with LCMV. After 10hours IFNa4, IFNb and IFNk transcript levels were assessed by RT-PCR (n=3). **E:** WT mice were infected with VSV. After 10hours IRF7, USP18 and ISG15 transcript levels were assessed in the spleen by RT-PCR (n=3). **F:** WT mice were infected with LCMV. After 10hours IRF7, USP18 and ISG15 transcript levels were assessed in the spleen by RT-PCR (n=3). **G-I:** Bone marrow chimeras (WT-BM>WT, WT-BM>*irf7*$^{-/-}$, *irf7*$^{-/-}$-BM>WT and *irf7*$^{-/-}$-BM>*irf7*$^{-/-}$ as described in Material and Methods) were infected with Vesicular stomatitis virus (n=3-4, C), lymphocytic choriomeningitis virus (n=4, D) or Herpes simplex virus (n=5, E). Interferon-α was measured in the serum.

Figure 2: Heamatopoietic-derived interferon is essential for the control of murine hepatitis virus, and to limit hepatitis

WT mice, *irf7*$^{-/-}$ mice and bone marrow chimeras (WT-BM>WT, WT-BM>*irf7*$^{-/-}$, *irf7*$^{-/-}$-BM>WT and *irf7*$^{-/-}$-BM>*irf7*$^{-/-}$) were infected with 50 PFU MHV i.p.. **A:** After 2 days viral titers were measured in the spleen, liver, lung and blood (n=4-5). **B:** ALT levels were measured in the serum (n=4-5).

Figure 3: Systemic replication of LCMV is inhibited by IFN-I produced by hematopoietic cells

A: WT mice, *irf7*$^{-/-}$ mice, and bone marrow chimeras (WT-BM>WT, WT-BM>*irf7*$^{-/-}$, *irf7*$^{-/-}$-BM>WT and *irf7*$^{-/-}$-BM>*irf7*$^{-/-}$) were infected with 200 PFU LCMV. After 4 days viral titers were analyzed (n=4-5). **B:** WT mice, WT mice depleted of CD8^{+} T and NK cells and *Rag*$^{-/-}$ mice depleted of NK cells were infected with 200PFU of LCMV-WE intravenously. Virus titers were analyzed in spleen liver, lung and kidney 3 days after infection. **C:** MC57 cells were treated with different concentrations of IFN-α and then infected with VSV or LCMV (MOI 0.001). After 6, 12 and 24 hours infectious virus was analyzed in the supernatant.

Figure 4: Hematopoietic cell derived interferon influenced CD8 T cell expansion

Bone marrow chimeras (WT-BM>RIP-GP, *irf7*$^{-/-}$-BM>RIP-GP) were infected with 200 PFU of LCMV-WE. **A:** Frequencies of LCMV specific CD8^{+} T cells were analyzed on day 8 in the blood (n = 4-5). **B:**

Splenic $CD8^+$ T cells were analyzed for tet-$gp33^+$ cells (LCMV-specific cells) and phenotype. One representative dot plot is shown. Cells are gated on $CD8^+$ T cells. **C:** Expansion of total $CD8^+$ T cells was analyzed in the blood using tetramers (n=4-5). **D:** Expression of IL-7Rα on tet-gp33 positive cells was analyzed in blood derived $CD8^+$ T cells. 1 out of 4-5 representative stainings is shown. **E:** On day 30 pancreatic islet cells were analyzed for replication of LCMV within the islets. 1 out of 3 representative slides is shown.

Figure 5: Hematopoietic cell derived interferon influenced type I diabetes

A: WT and *irf7*$^{-/-}$ mice were treated with 500μg of poly(I:C) intravenously. After 24 hours MHC I expression was analyzed in the pancreas. One of three representative stainings is shown. **B:** Bone marrow chimeras (WT-BM>RIP-GP, *irf7*$^{-/-}$-BM>RIP-GP) were infected with 200 PFU of LCMV-WE. On day 8 pancreatic β-islets were analyzed for expression of MHC I and infiltration of $CD8^+$ T cells. One of 3 representative stainings is shown. **C:** Bone marrow chimeras (WT-BM>RIP-GP, *irf7*$^{-/-}$-BM>RIP-GP) were infected with 200 PFU of LCMV-WE. Blood glucose levels were analyzed in the blood (n=4-5).

Supplementary Figure 1: Bone marrow reconstiution of immune cells

A-B: Bone marrow was taken form both femurs and tibiae of WT mice, *irf7*$^{-/-}$ mice and reconstituted animals (WT-BM>WT, WT-BM>*irf7*$^{-/-}$, *irf7*$^{-/-}$-BM>WT and *irf7*$^{-/-}$-BM>*irf7*$^{-/-}$). Numbers of total bone marrow cells were analyzed by FACS (A, n=2-3). Different lineages and $sca1^+$ $ckit^+$ lin^- stem cells were analyzed in the bone marrow (B, n=2-3). **C-D:** Single cell suspensions were generated from spleens of WT mice, *irf7*$^{-/-}$ mice and bone marrow chimeras (WT-BM>WT, WT-BM>*irf7*$^{-/-}$, *irf7*$^{-/-}$-BM>WT and *irf7*$^{-/-}$ BM>*irf7*$^{-/-}$). Numbers of total red cells and leukocytes per spleen were analyzed by the FACS (C, n=2-3). Frequencies of T cells (CD3), B cells (B220) and Granolocytes (Gr1) were analyzed (D, n=2-3). **E:** WT mice were irradiated with 1050rad on day -1 and on day 0 mice were reconstituted with $8x10^6$ bone marrow cells from GFP transgenic mice. After 6 weeks spleens were analyzed for GFP macrophages ($CD11b^+Gr1^-$), conventional dendritic cells ($CD11c^+$), plasmacytoid dendritic cells (PDCA-1^+), granulocytes (GR-1^+), B cells ($B220^+$) and $CD8^+$ T cells ($CD8^+$). GFP expression of 1 out of 3 mice is shown in original histogram plot. Staining of GFP^- naïve WT mice is shown as grey area.

Figure 1

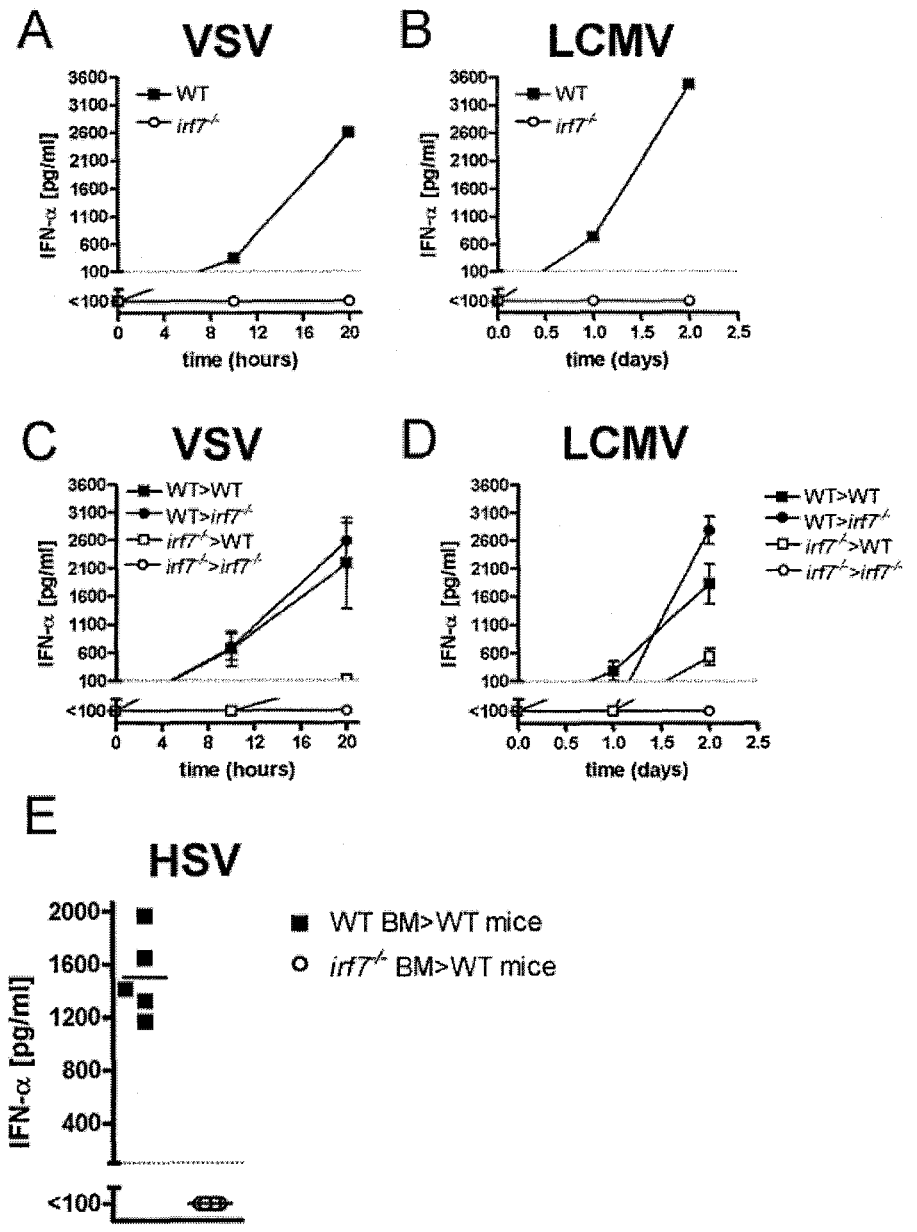

A
VSV
B
LCMV
C
VSV
D
LCMV
E
HSV
IFN-α [pg/ml]
3600
3100
2600
2100
1600
1100
600
100
<100
WT
irf7-/-
WT>WT
WT>irf7-/-
irf7-/->WT
irf7-/->irf7-/-
0 4 8 12 16 20
time (hours)
0.0 0.5 1.0 1.5 2.0 2.5
time (days)
2000
1600
1200
800
400
WT BM>WT mice
irf7-/- BM>WT mice

Figure 2

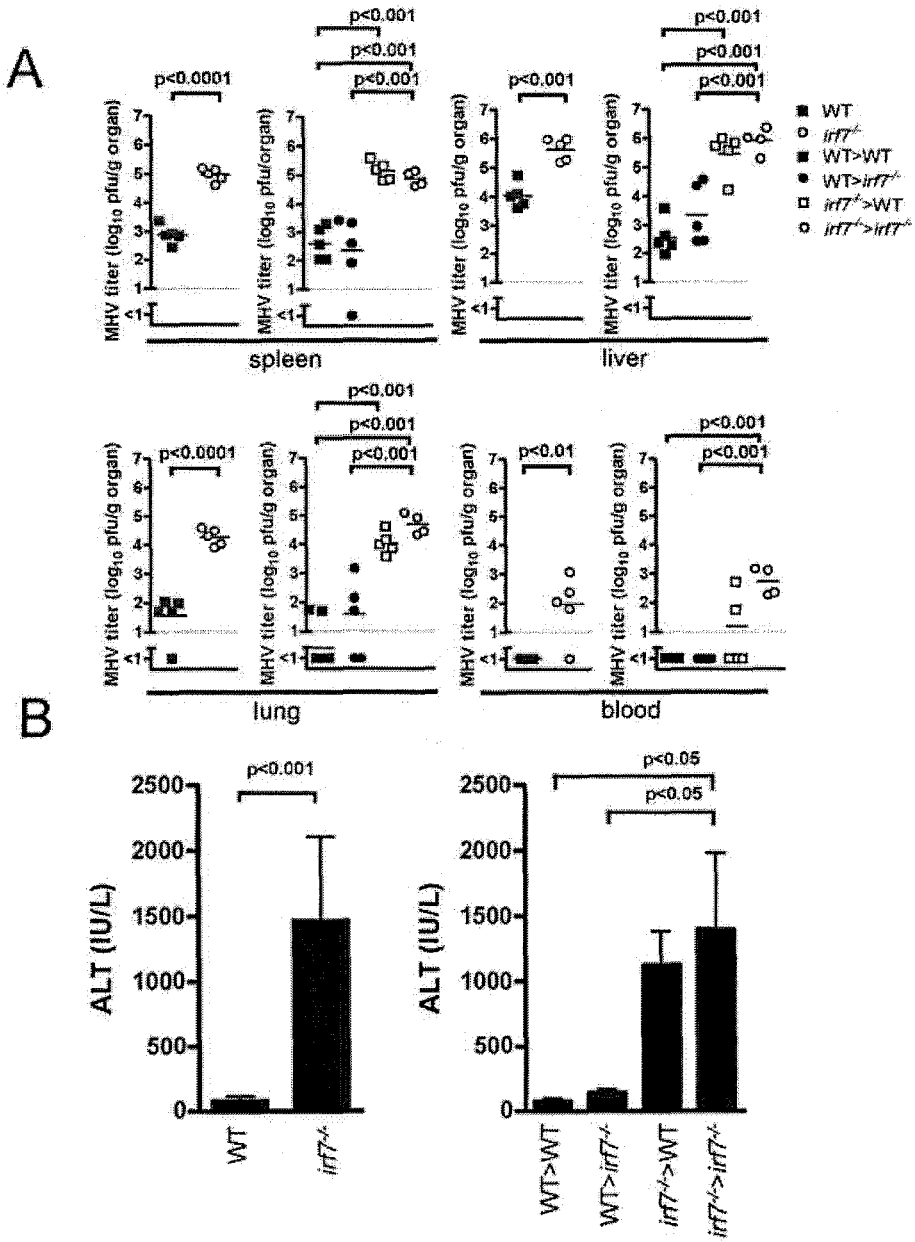

A
B
MHV titer (log10 pfu/g organ)
MHV titer (log10 pfu/organ)
p<0.0001
p<0.001
p<0.01
spleen
liver
lung
blood
WT
irf7-/-
WT>WT
WT>irf7-/-
irf7-/->WT
irf7-/->irf7-/-
ALT (IU/L)
p<0.05
WT
irf7-/-
WT>WT
WT>irf7-/-
irf7-/->WT
irf7-/->irf7-/-

Figure 3

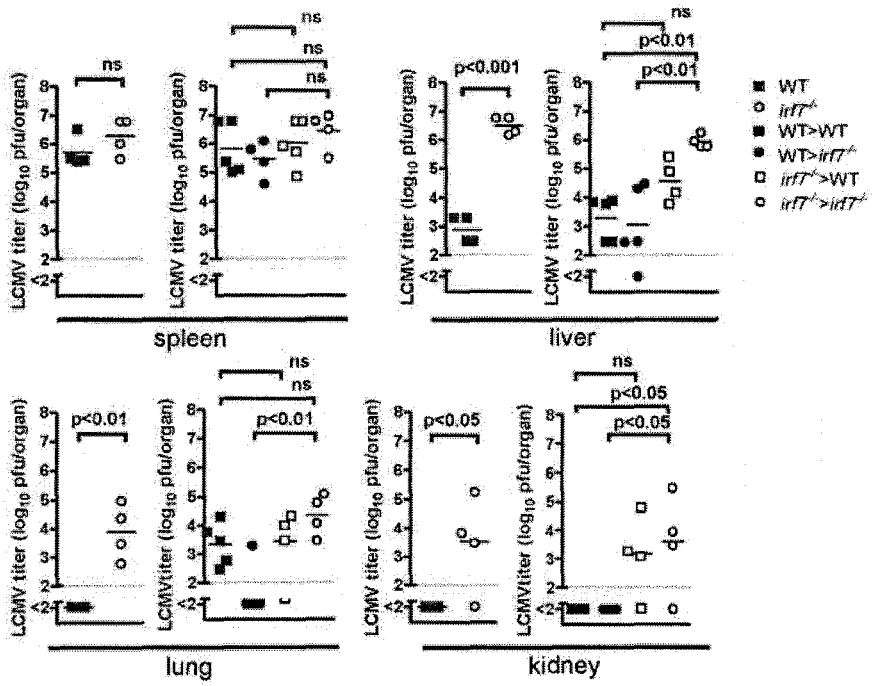

LCMV titer (log10 pfu/organ)
ns
ns
ns
spleen
p<0.001
p<0.01
p<0.01
liver
p<0.01
ns
p<0.01
lung
p<0.05
p<0.05
kidney
WT
irf7-/-
WT>WT
WT>irf7-/-
irf7-/->WT
irf7-/->irf7-/-

Figure 4

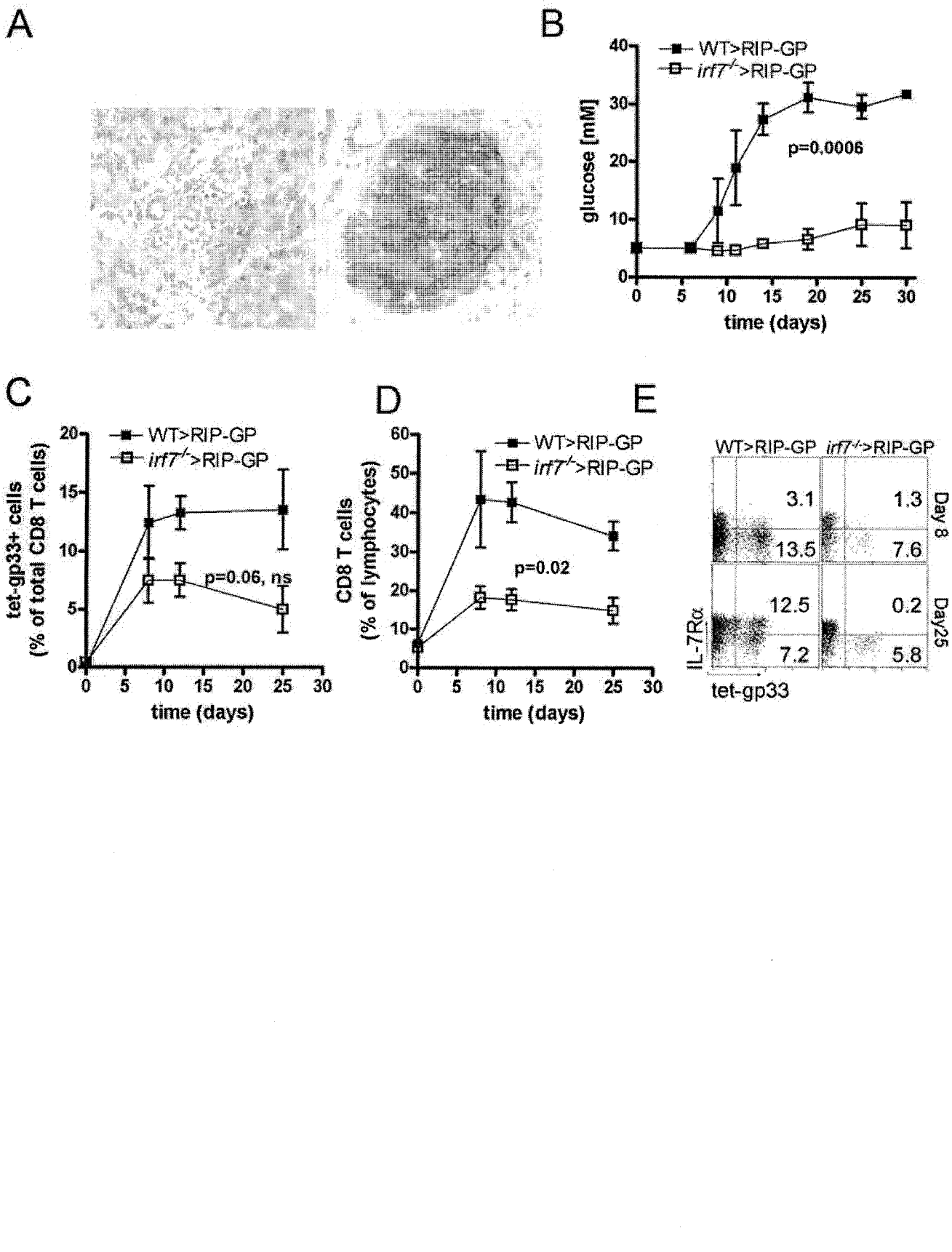
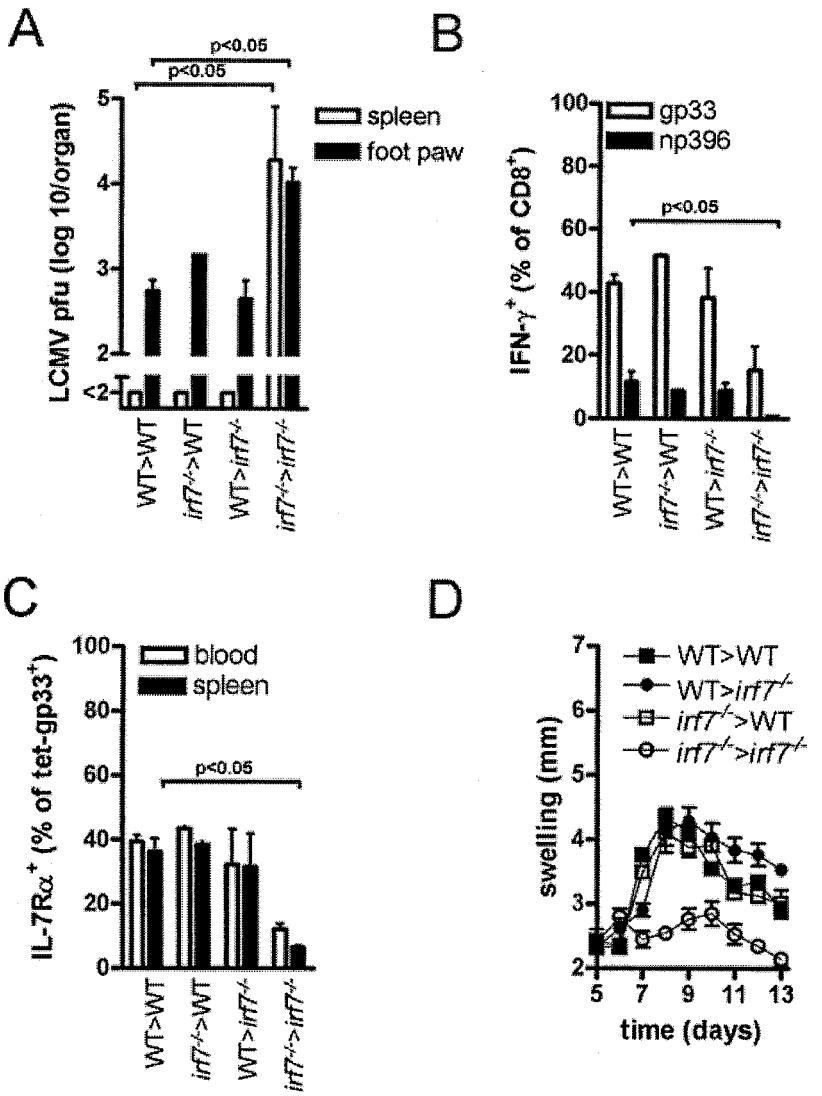
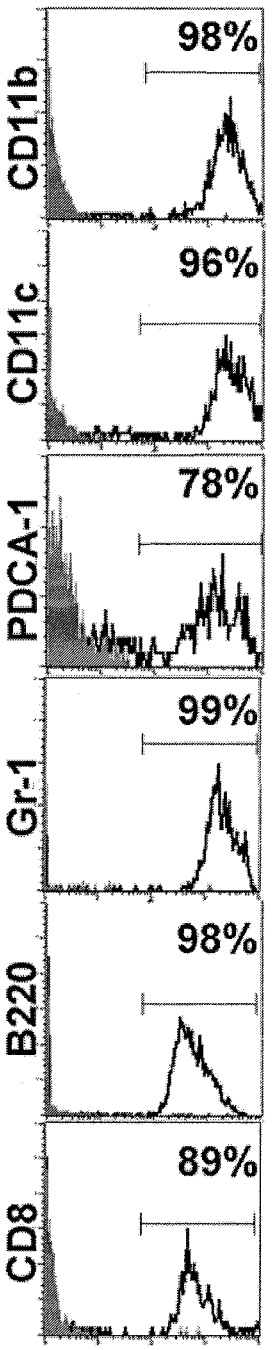

A
B
WT>RIP-GP
irf7-/->RIP-GP
glucose [mM]
p=0.0006
time (days)
C
tet-gp33+ cells
(% of total CD8 T cells)
p=0.06, ns
time (days)
D
CD8 T cells
(% of lymphocytes)
p=0.02
time (days)
E
WT>RIP-GP
irf7-/->RIP-GP
3.1
1.3
13.5
7.6
Day 8
12.5
0.2
7.2
5.8
Day25
IL-7Rα
tet-gp33

Figure 5


A
p<0.05
p<0.05
LCMV pfu (log 10/organ)
spleen
foot paw
WT>WT
irf7-/->WT
WT>irf7-/-
irf7-/->irf7-/-
B
gp33
np396
p<0.05
IFN-γ+ (% of CD8+)
C
blood
spleen
p<0.05
IL-7Rα+ (% of tet-gp33+)
D
WT>WT
WT>irf7-/-
irf7-/->WT
irf7-/->irf7-/-
swelling (mm)
time (days)

Supplementary Figure 1



macrophages

conventional dendritic cells

plasmacytoid dendritic cells

Granulocytes

B cells

Cytotoxic T cells

Coronavirus Non-Structural Protein 1 Is a Major Pathogenicity Factor: Implications for the Rational Design of Coronavirus Vaccines

Roland Züst[1], Luisa Cervantes-Barragán[1,2], Thomas Kuri[3], Gjon Blakqori[3¤], Friedemann Weber[3], Burkhard Ludewig[1], Volker Thiel[1*]

1 Research Department, Kantonal Hospital St. Gallen, St. Gallen, Switzerland, **2** Unidad de Investigación Médica en Inmunoquímica, Hospital de Especialidades, Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, México City, México, **3** Department of Virology, University of Freiburg, Freiburg, Germany

Attenuated viral vaccines can be generated by targeting essential pathogenicity factors. We report here the rational design of an attenuated recombinant coronavirus vaccine based on a deletion in the coding sequence of the non-structural protein 1 (nsp1). In cell culture, nsp1 of mouse hepatitis virus (MHV), like its SARS-coronavirus homolog, strongly reduced cellular gene expression. The effect of nsp1 on MHV replication in vitro and in vivo was analyzed using a recombinant MHV encoding a deletion in the nsp1-coding sequence. The recombinant MHV nsp1 mutant grew normally in tissue culture, but was severely attenuated in vivo. Replication and spread of the nsp1 mutant virus was restored almost to wild-type levels in type I interferon (IFN) receptor-deficient mice, indicating that nsp1 interferes efficiently with the type I IFN system. Importantly, replication of nsp1 mutant virus in professional antigen-presenting cells such as conventional dendritic cells and macrophages, and induction of type I IFN in plasmacytoid dendritic cells, was not impaired. Furthermore, even low doses of nsp1 mutant MHV elicited potent cytotoxic T cell responses and protected mice against homologous and heterologous virus challenge. Taken together, the presented attenuation strategy provides a paradigm for the development of highly efficient coronavirus vaccines.

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Introduction

Coronaviruses are vertebrate pathogens mainly associated with respiratory and enteric diseases [1]. They can cause severe diseases in livestock animals and lead thereby to high economic losses. In humans, coronavirus infections manifest usually as mild respiratory tract disease (common cold) that may cause more severe symptoms in elderly or immune-compromised individuals [2,3]. In 2002–2003, the appearance of severe acute respiratory syndrome (SARS), caused by a formerly unknown coronavirus (SARS-CoV), exemplified the potential of coronaviruses to seriously affect human health [4–7]. The frequent detection of SARS-like coronaviruses in horseshoe bats (*Rhinolophus* sp.) and the broad range of mammalian hosts that are susceptible to SARS-CoV infection may facilitate a potential reintroduction into the human population [8]. Therefore, the development of efficacious coronavirus vaccines is of high medical and veterinary importance.

Effective vaccines controlling virus spread and disease are available for a number of infections, such as smallpox, poliomyelitis, measles, mumps, rubella, influenza, hepatitis A, and hepatitis B [9,10]. Some of these vaccines consist of virus subunits or inactivated virus preparations that mainly induce the production of pathogen-specific antibodies. In contrast, live attenuated vaccines consist of replication-competent viruses that induce broad cellular and humoral immune responses without causing disease [10]. The most prominent live attenuated vaccines are vaccinia virus [11], poliovirus [12], and yellow fever virus (YF-17D) [13]. Despite their documented efficacy, it is still not fully understood why and how successful vaccines work [10,14]. However, recent concepts in immunology provide a link between innate and adaptive immune responses and suggest that the quality, quantity, and longevity of adaptive immune responses is determined very early after infection or vaccination [14]. Of major importance are professional antigen-presenting cells (pAPCs) such as dendritic cells (DCs) and macrophages, which play a major role in (i) sensing pathogen-associated molecular patterns, (ii) inducing innate immune responses, and (iii) shaping the upcoming adaptive immune response. Efficient live attenuated vaccines should therefore not only lack significant pathogenicity, but should also deliver antigens to pAPCs and activate the innate immune system.

Notably, the majority of currently available attenuated vaccines have been derived empirically. Given the recent proceedings in the areas of virus reverse genetics and virus-

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Abbreviations: ALT, alanine 2-oxoglutarate-aminotransferase; cDC, conventional dendritic cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; IFN, interferon; ISRE, interferon-stimulated response element; LCMV, lymphocytic choriomeningitis virus; MHV, mouse hepatitis virus; nsp1, non-structural protein 1; nt, nucleotide; pAPC, professional antigen-presenting cell; pDC, plasmacytoid dendritic cell; p.i., post infection

* To whom correspondence should be addressed. E-mail: volker.thiel@kssg.ch

¤ Current address: Centre for Biomolecular Sciences, School of Biology, University of St. Andrews, North Haugh, St. Andrews, Scotland, United Kingdom

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0022-538X/08/$08.00+0 doi:10.1128/JVI.02082-08

Mouse Hepatitis Virus Liver Pathology Is Dependent on ADP-Ribose-1″-Phosphatase, a Viral Function Conserved in the Alpha-Like Supergroup▽

Klara Kristin Eriksson,[1] Luisa Cervantes-Barragán,[1] Burkhard Ludewig,[1] and Volker Thiel[1,2]*

Research Department,[1] Institute of Pathology,[2] Kantonal Hospital St. Gallen, St. Gallen, Switzerland

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Viral infection of the liver can lead to severe tissue damage when high levels of viral replication and spread in the organ are coupled with strong induction of inflammatory responses. Here we report an unexpected correlation between the expression of a functional X domain encoded by the hepatotropic mouse hepatitis virus strain A59 (MHV-A59), the high-level production of inflammatory cytokines, and the induction of acute viral hepatitis in mice. X-domain (also called macro domain) proteins possess poly-ADP-ribose binding and/or ADP-ribose-1″-phosphatase (ADRP) activity. They are conserved in coronaviruses and in members of the "alpha-like supergroup" of phylogenetically related positive-strand RNA viruses that includes viruses of medical importance, such as rubella virus and hepatitis E virus. By using reverse genetics, we constructed a recombinant murine coronavirus MHV-A59 mutant encoding a single-amino-acid substitution of a strictly conserved residue that is essential for coronaviral ADRP activity. We found that the mutant virus replicated to slightly reduced titers in livers but, strikingly, did not induce liver disease. In vitro, the mutant virus induced only low levels of the inflammatory cytokines tumor necrosis factor alpha and interleukin-6 (IL-6). In vivo, we found that IL-6 production, in particular, was reduced in the spleens and livers of mutant virus-infected mice. Collectively, our data demonstrate that the MHV X domain exacerbates MHV-induced liver pathology, most likely through the induction of excessive inflammatory cytokine expression.

Acute viral hepatitis is accompanied by strong host innate immune responses that include the expression of type I interferons (IFNs) and the release of proinflammatory cytokines (31, 37). These responses are considered essential to control early virus replication in the liver (14, 30). Type I IFNs induce the expression of antiviral effector molecules (12, 16) but also shape the upcoming adaptive immune response (29). Proinflammatory cytokines and chemokines mediate the migration of cells with antiviral effector functions into the liver and thereby promote viral clearance (23). This process can, when excessive activation occurs, also result in severe tissue damage (4). Although the main producer cells of type I IFNs and proinflammatory cytokines have been identified as plasmacytoid dendritic cells (pDCs) and activated macrophages, respectively (25, 31), very little is known about if and how viruses may affect their expression during acute viral hepatitis and how these virus-host interactions may impact the course of the infection and disease.

Mouse hepatitis virus (MHV) is a positive-strand RNA virus of the *Coronaviridae* family. Its natural host is the mouse, and MHV has been extensively studied in the context of various disease models and host innate and adoptive immune responses (3, 38). MHV strain A59 (MHV-A59) is both hepatotropic and neurotropic and can infect hepatocytes, macrophages, conventional DCs (cDCs), and pDCs. Although virus replication is controlled by pDC-mediated alpha IFN (IFN-α) production during the early phase of infection (5), acute viral hepatitis and markedly elevated serum alanine aminotransferase (ALT) levels become apparent at day 5 postinfection (p.i.). Clearly, the coronavirus spike protein, as the major determinant of virus target cell tropism (39), is responsible for the pronounced MHV-A59 liver tropism. However, our knowledge of other coronaviral factors that may be involved in the induction of acute hepatitis is limited.

There is accumulating evidence that coronavirus replicase gene products impact virus pathogenicity (7, 28, 33, 41). Replicase gene expression involves the translation of large polyproteins that undergo extensive proteolytic processing by viral proteinases to give rise to 15 to 16 nonstructural proteins (nsps) (40). They assemble to form the viral replicase-transcriptase complex at endoplasmic reticulum-derived double-membrane vesicles (17). Interestingly, the ADP-ribose-1″-phosphatase (ADRP) activity (26) encoded in nsp3 appears to be dispensable for virus RNA synthesis (20), suggesting a possible role in vivo. The ADRP domain, also called the X domain, is strictly conserved among coronaviruses, and moreover, a homologous domain can be found in viruses belonging to the "alpha-like supergroup" of positive-strand RNA viruses (10, 11). This group of phylogenetically related viruses includes alphaviruses such as Semliki Forest virus (SFV), a number of plant viruses, and viruses of medical importance, such as rubella virus and hepatitis E virus (HEV). Viral X domains are related to a large family of macro domain proteins found in many cellular organisms (15, 26). It is generally accepted that macro domains are associated with ADP-ribose binding or with the processing of ADP-ribose derivatives such as ADP-ribose-1″-phosphate (Appr-1″-p) (9, 15). The X domains of human coronavirus 229E (HCoV-229E), severe acute respiratory syndrome coronavirus (SARS-CoV), transmissible gastroenteritis virus, and HEV pos-

* Corresponding author. Mailing address: Kantonal Hospital St. Gallen, Research Department and Institute of Pathology, 9007 St. Gallen, Switzerland. Phone: 41 71 4942843. Fax: 41 71 4946321. E-mail: Volker.Thiel@kssg.ch.

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Salmonella porins induce a sustained, lifelong specific bactericidal antibody memory response

Ismael Secundino,[1] Constantino López-Macías,[1] Luisa Cervantes-Barragán,[1] Cristina Gil-Cruz,[1] Nora Ríos-Sarabia,[1] Rodolfo Pastelin-Palacios,[2] Miguel Angel Villasis-Keever,[3] Ingeborg Becker,[4] José Luis Puente,[5] Edmundo Calva[5] and Armando Isibasi[1]

[1]*Unidad de Investigación Médica en Inmunoquímica, Hospital de Especialidades del Centro Médico Nacional (CMN) Siglo XXI, Instituto Mexicano del Seguro Social (IMSS), México,* [2]*Facultad de Química, Universidad Nacional Autónoma de México (UNAM) México,* [3]*Unidad de Investigación en Epidemiología Clínica, Hospital de Pediatría, CMN Siglo XXI, IMSS, México,* [4]*Departmento de Medicina Experimental, Facultad de Medicina, UNAM, México, and* [5]*Departamento de Microbiología Molecular, Instituto de Biotecnología, UNAM, Cuernavaca, Morelos, México*

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Ismael Secundino and Constantino López-Macias contributed equally to this work.
Correspondence: Dr Constantino López-Macías, Unidad de Investigación Médica en Inmunoquímica, Coordinación de Investigación en Salud, Instituto Mexicano del Seguro Social, PO Box A-047, C.P. 06703 Mexico City, Mexico.
Email: constantino@sminmunologia.org
Senior author: Armando Isibasi, email: isibasi@prodigy.net.mx

Summary

We examined the ability of porins from *Salmonella enterica* serovar *typhi* to induce a long-term antibody response in BALB/c mice. These porins triggered a strong lifelong production of immunoglobulin G (IgG) antibody in the absence of exogenous adjuvant. Analysis of the IgG subclasses produced during this antibody response revealed the presence of the subclasses IgG2b, IgG1, IgG2a and weak IgG3. Despite the high homology of porins, the long-lasting anti-*S. typhi* porin sera did not cross-react with *S. typhimurium*. Notably, the antiporin sera showed a sustained lifelong bactericidal-binding activity to the wild-type *S. typhi* strain, whereas porin-specific antibody titres measured by enzyme-linked immunosorbent assay (ELISA) decreased with time. Because our porin preparations contained the outer membrane proteins C and F (OmpC and OmpF), we evaluated the individual contribution of each porin to the long-lasting antibody response. OmpC and OmpF induced long-lasting antibody titres, measured by ELISA, which were sustained for 300 days. In contrast, although OmpC induced sustained high bactericidal antibody titres for 300 days, postimmunization, the bactericidal antibody titre induced by OmpF was not detected at day 180. These results indicate that OmpC is the main protein responsible for the antibody-mediated memory bactericidal response induced by porins. Taken together, our results show that porins are strong immunogens that confer lifelong specific bactericidal antibody responses in the absence of added adjuvant.

Keywords: bactericidal antibodies; memory B-cell response; OmpC; OmpF; porins; *Salmonella typhi*

Introduction

Upon antigen contact in the presence of cognate T-cell help, naive B cells differentiate into antibody-secreting plasma cells and memory B cells. During this process, which occurs in germinal centres, B cells undergo affinity maturation and class-switch recombination, giving rise to different subclasses of immunoglobulin G (IgG) memory B cells, depending on the cytokine environment.[1,2] Following re-exposure to the same antigen, memory B cells undergo rapid proliferation, culminating in differentiation into plasma cells and in the secretion of high-affinity IgG, which might persist in the circulation for years, thus providing long-term antibody production.[1,3] However,

nature medicine

Aggravation of viral hepatitis by platelet-derived serotonin

Philipp A Lang[1,2,10], Claudio Contaldo[3,10], Panco Georgiev[4,10], Ashraf Mohammad El-Badry[4], Mike Recher[1], Michael Kurrer[5], Luisa Cervantes-Barragan[6], Burkhard Ludewig[6], Thomas Calzascia[2], Beatrice Bolinger[6], Doron Merkler[1,7], Bernhard Odermatt[5], Michael Bader[8], Rolf Graf[4], Pierre-Alain Clavien[4], Ahmed N Hegazy[1,9], Max Löhning[1,9], Nicola L Harris[1], Pamela S Ohashi[2], Hans Hengartner[1], Rolf M Zinkernagel[1,11] & Karl S Lang[1,2,11]

More than 500 million people worldwide are persistently infected with hepatitis B virus or hepatitis C virus[1]. Although both viruses are poorly cytopathic, persistence of either virus carries a risk of chronic liver inflammation, potentially resulting in liver steatosis, liver cirrhosis, end-stage liver failure or hepatocellular carcinoma. Virus-specific T cells are a major determinant of the outcome of hepatitis, as they contribute to the early control of chronic hepatitis viruses, but they also mediate immunopathology during persistent virus infection[1–4]. We have analyzed the role of platelet-derived vasoactive serotonin during virus-induced CD8$^+$ T cell–dependent immunopathological hepatitis in mice infected with the noncytopathic lymphocytic choriomeningitis virus. After virus infection, platelets were recruited to the liver, and their activation correlated with severely reduced sinusoidal microcirculation, delayed virus elimination and increased immunopathological liver cell damage. Lack of platelet-derived serotonin in serotonin-deficient mice normalized hepatic microcirculatory dysfunction, accelerated virus clearance in the liver and reduced CD8$^+$ T cell–dependent liver cell damage. In keeping with these observations, serotonin treatment of infected mice delayed entry of activated CD8$^+$ T cells into the liver, delayed virus control and aggravated immunopathological hepatitis. Thus, vasoactive serotonin supports virus persistence in the liver and aggravates virus-induced immunopathology.

Infection of mice with the noncytopathic lymphocytic choriomeningitis virus (LCMV) caused hepatocyte damage, as revealed by elevated serum alanin-aminotransferase (ALT) activities and serum bilirubin concentrations[5] (**Fig. 1a**). Virus-specific T cells are a major determinant of the outcome of hepatitis—these cells contribute to the early control of chronic hepatitis viruses and mediate immunopathology during persistent virus infection[1–4]. In a model of LCMV-induced hepatitis, complete depletion of CD8$^+$ T cells enhanced virus replication, but strongly reduced hepatocyte damage (**Fig. 1a**), showing that the activation of virus-specific CD8$^+$ T cells by viral antigen and not LCMV replication alone is responsible for liver cell damage, confirming earlier results[5,6]. To induce a delayed appearance of CD8$^+$ T cells within the liver, we treated C57BL/6 mice with a 1:10 dilution of a CD8 cell–depleting antibody, which leads to a transient absence of CD8$^+$ T cells. After 8–12 days, CD8$^+$ T cells re-emerged (**Fig. 1b**). The delayed CD8$^+$ T cell response within the liver was associated with prolonged viral replication and enhanced ALT levels in the serum when CD8$^+$ T cells re-emerged (**Fig. 1b**).

Therefore, similarly to the situation in human hepatitis[7–11], delayed control of LCMV in the liver enhanced the overall immunopathology in the liver.

Infection and virus-induced hepatitis have been linked to platelet activation[12–14]. To study the role of platelet activation in LCMV-triggered CD8$^+$ T cell–dependent hepatitis, we histologically stained liver infiltrates with antibodies specific for the platelet markers CD61 or von Willebrand factor (vWF). Periportal infiltrates of CD8$^+$ T cells contained platelets (**Fig. 1c**). Serum concentrations of soluble P-selectin increased during infection (**Fig. 1d**), indicative of extensive platelet–endothelial cell interactions[15]. CD8$^+$ T cells were necessary for virus-induced platelet activation and recruitment, as shown in *Cd8a*$^{-/-}$ mice that had attenuated platelet infiltration into the liver together with reduced serum P-selectin concentrations (**Fig. 1d,e**). Because activated platelets physiologically reduce blood flow, we wanted to determine whether LCMV-induced platelet activation influences hepatic microcirculation[16]. For this purpose, we injected soluble sodium fluorescein into the portal vein of LCMV-infected mice and monitored

[1]Institute of Experimental Immunology, University Hospital of Zurich, Schmelzberstrasse 12, CH-8091 Zurich, Switzerland. [2]Campbell Family Institute for Breast Cancer Research, Ontario Cancer Institute, UHN, 620 University Avenue, Toronto, Ontario M5G 2C1, Canada. [3]Division of Plastic and Reconstructive Surgery, [4]Swiss Hepato-Pancreato-Biliary Center, Department of Surgery and [5]Department of Surgical Pathology, University Hospital of Zurich, Schmelzberstrasse 12, CH-8091 Zurich, Switzerland. [6]Research Department, Kantonal Hospital St Gallen, Rorschacher Strasse 95, CH-9007 St Gallen, Switzerland. [7]Institute of Neuropathology, Georg August University Göttingen, Robert-Koch-Strasse 40, D-37075 Göttingen, Germany. [8]Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin-Buch, Germany. [9]Department of Rheumatology and Clinical Immunology, Charité–University Medicine Berlin and Deutsches Rheuma-Forschungszentrum Berlin, Schumannstrasse 21-22, 10117 Berlin, Germany. [10]These authors contributed equally to this work. [11]These authors contributed equally to this work. Correspondence should be addressed to R.M.Z. (rolf.zinkernagel@usz.ch) or K.S.L. (karlslang@gmail.com).

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Translating innate response into long-lasting antibody response by the intrinsic antigen-adjuvant properties of papaya mosaic virus

Elizabeth Acosta-Ramírez,[1,†] Rebeca Pérez-Flores,[1,†] Nathalie Majeau,[2] Rodolfo Pastelin-Palacios,[3] Cristina Gil-Cruz,[1,‡] Maricela Ramírez-Saldaña,[1,‡] Nataly Manjarrez-Orduño,[4] Luisa Cervantes-Barragán,[1,†] Leopoldo Santos-Argumedo,[4] Leopoldo Flores-Romo,[5] Ingeborg Becker,[6] Armando Isibasi,[1] Denis Leclerc[2] and Constantino López-Macías[1]

[1]*Medical Research Unit on Immunochemistry, Specialties Hospital, National Medical Centre 'Siglo XXI'. Mexican Institute for Social Security (IMSS), Mexico City,* [2]*Infectology Research Centre, Laval University, Quebec, Canada,* [3]*Faculty of Chemistry, National Autonomous University of Mexico (UNAM), Mexico City,* [4]*Molecular Biomedicine Department, Center for Advance Studies and Research (CINVESTAV) of the National Polythechnic Institute (IPN), Mexico City,* [5]*Department of Cell Biology, CINVESTAV-IPN, Mexico City, and* [6]*Department of Experimental Medicine, Faculty of Medicine, UNAM, Mexico City*

Summary

Identifying the properties of a molecule involved in the efficient activation of the innate and adaptive immune responses that lead to long-lasting immunity is crucial for vaccine and adjuvant development. Here we show that the papaya mosaic virus (PapMV) is recognized by the immune system as a pathogen-associated molecular pattern (PAMP) and as an antigen in mice (Pamptigen). A single immunization of PapMV without added adjuvant efficiently induced both cellular and specific long-lasting antibody responses. PapMV also efficiently activated innate immune responses, as shown by the induction of lipid raft aggregation, secretion of pro-inflammatory cytokines, up-regulation of co-stimulatory molecules on dendritic cells and macrophages, and long-lasting adjuvant effects upon the specific antibody responses to model antigens. PapMV mixed with *Salmonella enterica* serovar Typhi (*S. typhi*) outer membrane protein C increased its protective capacity against challenge with *S. typhi*, revealing the intrinsic adjuvant properties of PapMV in the induction of immunity. Antigen-presenting cells loaded with PapMV efficiently induced antibody responses *in vivo*, which may link the innate and adaptive responses observed. PapMV recognition as a Pamptigen might be translated into long-lasting antibody responses and protection observed. These properties could be used in the development of new vaccine platforms.

Keywords: adjuvants; antigens; memory; papaya mosaic virus; vaccination

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†PhD program on Biomedical Sciences, UNAM, Mexico City.
‡PhD program on Immunology, National School of Biological Sciences (ENCB) IPN, Mexico City.
Correspondence: Dr C. López-Macías, UIM en Inmunoquímica, Coordinación de Investigación en Salud, PO Box A047, C.P. 06703, México D.F.
Email: constantino@sminmunologia.org; constantino.lopez@imss.gob.mx
Senior author: Constantino López-Macías

Abbreviations: APC, antigen-presenting cell; BCR, B-cell receptor; BMDC, bone marrow-derived dendritic cells; BMDM, bone marrow-derived macrophages; CFA, complete Freund's adjuvant; CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester; CP, coat protein; CTB, cholera toxin B subunit; DC, dendritic cell; DMEM, Dubecco's modified Eagle's minimal essential medium; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HEL, hen egg- white lysozyme; i.p., intraperitoneal; IFA, incomplete Freund's adjuvant; IL, interleukin; LD_{50}, 50% lethal dose; LPS, lipopolysaccharide; MHC class II, major histocompatibility complex class II; OmpC, outer membrane protein C; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; Pamptigen, pathogen-associated molecular pattern and antigen; PapMV, papaya mosaic virus; PBS, phosphate-buffered saline; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PRR, pattern recognition receptor; s.c., subcutaneous; SD, standard deviation; TCR, T-cell receptor; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α.

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Lack of Conventional Dendritic Cells Is Compatible with Normal Development and T Cell Homeostasis, but Causes Myeloid Proliferative Syndrome

Tal Birnberg,[1,7,8] Liat Bar-On,[1,7] Anita Sapoznikov,[1] Michele L. Caton,[2] Luisa Cervantes-Barragán,[6] Divine Makia,[5] Rita Krauthgamer,[1] Ori Brenner,[4] Burkhard Ludewig,[6] Damian Brockschnieder,[3] Dieter Riethmacher,[5] Boris Reizis,[2,*] and Steffen Jung[1,*]

[1]Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel
[2]Department of Microbiology, Columbia University Medical Center, New York, NY 10032, USA
[3]Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel
[4]Department of Veterinary Resources, The Weizmann Institute of Science, Rehovot 76100, Israel
[5]Human Genetics Division, University of Southampton, SGH, Tremona Road, Southampton SO16 6YD, UK
[6]Research Department, Kantonal Hospital St. Gallen, CH-9007 St. Gallen, Switzerland
[7]These authors contributed equally to this work
[8]Present address: Bayer Schering Pharma AG, 13342 Berlin, Germany
*Correspondence: bvr2101@columbia.edu (B.R.), s.jung@weizmann.ac.il (S.J.)
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SUMMARY

Dendritic cells are critically involved in the promotion and regulation of T cell responses. Here, we report a mouse strain that lacks conventional CD11c^{hi} dendritic cells (cDCs) because of constitutive cell-type specific expression of a suicide gene. As expected, cDC-less mice failed to mount effective T cell responses resulting in impaired viral clearance. In contrast, neither thymic negative selection nor T regulatory cell generation or T cell homeostasis were markedly affected. Unexpectedly, cDC-less mice developed a progressive myeloproliferative disorder characterized by prominent extramedullary hematopoiesis and increased serum amounts of the cytokine Flt3 ligand. Our data identify a critical role of cDCs in the control of steady-state hematopoiesis, revealing a feedback loop that links peripheral cDCs to myelogenesis through soluble growth factors, such as Flt3 ligand.

INTRODUCTION

Antigens have to be processed and presented in the form of peptides bound to major histocompatibility complex (MHC) molecules to be recognized by T cells. Antigen-presenting cells (APCs) thus play a central role in the activation and control of T cell immunity. Dendritic cells (DCs), a morphologically distinct APC described by Steinman and colleagues (Steinman and Witmer, 1978), belong to the body-wide network of mononuclear phagocytes (van Furth and Cohn, 1968) and seem to have coevolved with adaptive T cell immunity. Beyond their unique potential to stimulate naive T cells in vitro (Steinman and Witmer, 1978), in vivo antigen targeting to DCs elicits strong T cell priming and long-lived T cell help for antibody responses (Bonifaz et al., 2002; Boscardin et al., 2006). Furthermore, vaccination with antigen-pulsed DCs proved to be a potent way to stimulate T cell responses both in mouse and man with respective protocols being in clinical trials (Gilboa, 2007; Palucka et al., 2007). Finally, conditional in vivo DC ablation established that splenic conventional DCs (cDCs) are required for the initiation of naive CD4$^+$ and CD8$^+$ T cell responses to protein antigens and pathogens (Jung et al., 2002; Probst and van den Broek, 2005; Sapoznikov et al., 2007).

Beyond their role in T cell stimulation, DCs are also involved in controlling the inherent autoreactivity of the T cell compartment. DCs were reported to play a critical role in the establishment of central T cell tolerance (Brocker et al., 1997), although more recent studies highlight the contribution of medullary thymic epithelial cells (mTECs) that promiscuously express tissue-restricted self-antigens (Kyewski and Klein, 2006). Immature or resting DCs that did not encounter pathogen signatures and hence lack expression of costimulatory molecules were shown to induce peripheral tolerance, both of CD4$^+$ and CD8$^+$ T cells (Hawiger et al., 2001; Probst et al., 2005). In addition, thymic and peripheral DCs were proposed to play a critical role in the generation of T regulatory (Treg) cells that suppress effector T cell responses (Coombes et al., 2007; Fehervari and Sakaguchi, 2004; Mahnke and Enk, 2005; Yamazaki et al., 2007). Finally, peripheral DCs were reported to support homeostatic proliferation and survival of T cells (Brocker et al., 1997; Gruber and Brocker, 2005). Although the role of DCs in T cell activation has been well established through transient DC depletion and/or DC-specific antigen targeting, the study of DC functions in T cell development and homeostasis requires long-term DC elimination in the steady state. Similarly, any potential DC functions outside of bona fide antigen presentation may be revealed only after early-onset DC deletion during development. Thus, an experimental model of constitutive DC deletion is required so that the immunological and developmental in vivo functions of this critical immune cell type are fully understood.

Here, we report the generation and characterization of a binary transgenic mouse model that constitutively lacked conventional CD11c^{hi} DCs. cDC-less mice were born at normal Mendelian frequencies and showed unimpaired development. The lack of