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MECANISMOS DE POLARIZACION EN LA RESPUESTA INMUNE: PAPEL DEL ESTADO DE ACTIVACION DE LOS MACROFAGOS Y SUS MOLECULAS DE MEMBRANA

T E S I S

QUE PARA OBTENER EL GRADO ACADEMICO DE

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P R E S E N T A :

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Ing. Leopoldo Silva Gutiérrez
Director General de Administración Escolar, UNAM
P r e s e n t e

Por medio de la presente me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 30 de septiembre de 2002, se acordó poner a su consideración el siguiente jurado para el examen de grado del Doctorado en Ciencias del alumno(a) **Miriam Rodríguez Sosa**, con número de cuenta 96808553 y número de expediente 3961668, con la tesis titulada: **"Mecanismos de polarización en la respuesta inmune: Papel del estado de activación de los macrófagos y sus moléculas de membrana"**, bajo la dirección del (la) **Dr. Rafael Bojalil Parra.**

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Sin otro particular, quedo de usted.

A t e n t a m e n t e
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Este trabajo de tesis se realizó en el departamento de inmunología del Instituto Nacional de Cardiología "Ignacio Chávez". La dirección estuvo a cargo del Dr. Rafael Bojalil Parra y se contó con el asesoramiento técnico y científico del Dr. Luis Ignacio Terrazas Valdés.

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APENDICE I: Artículos relacionados con los mecanismos de polarización de la respuesta inmune en la infección murina por *Taenia crassiceps*.

ABSTRACT

After encountering antigen, T helper (Th) cells undergo differentiation to effector cells, which can induce Th1- (high IFN-γ) or Th2- type immune responses (high IL-4, IL-5, IL-10). How Th cells acquire and retain new patterns of gene expression is not fully understood. In early stages of experimental murine cysticercosis by *Taenia crassiceps*, there is a clear but transient Th1-type immune response (IL-2, IFN-γ and IgG2a antibodies), but as time of infection progresses an energic and more permanent Th2-type response follows (IL-4, IL-6, IL-10 and IgG1 antibodies). In this work we take advantage of this murine model to study the mechanisms involved in the polarization from Th1 to Th2 immune response developed in this parasitosis.

Helminth infections are frequently strong inductors of Th2-type cytokines. This implies that infection by such parasites could alter the susceptibility to subsequent infections by other pathogens, particularly intracellular parasites. In this thesis is explored whether a persistent infection, caused by *Taenia crassiceps cysticerci*, in BALB/c mice could affect susceptibility to a later infection by *Trypanosoma cruzi*. The presence of the cysticerci indeed modified the immune response and the susceptibility to *T. cruzi*, and these modifications depended on the time-course evolution of the initial infection. Coinfection with the protozoan at 2 weeks post helminth infection induced both, a delay on the onset of parasitemia and, an early specific production of IFN-γ. A significant increase in susceptibility to *T. cruzi* was observed only when mice were coinfected at 8 or 12 weeks post-helminth infection, when the helminth load is greater and a Th2 type response against it is predominant. These findings suggest that chronic helminth infections could potentially have a significant influence over the immune response and hence susceptibility to other pathogens.

Initial findings showed resistance and susceptibility to experimental murine cysticercosis could be associated with the predominance of Th1 or Th2-type immune responses, respectively. However, this was partially demonstrated using monoclonal antibody anti-cytokines or with recombinant murine cytokines during early stages of infection. STAT6-mediated IL-4/IL-13 signaling pathway is critical for Th2 differentiation. Therefore, we used $STAT6^{-/-}$ and $STAT6^{+/+}$ BALB/c mice to determine the role of Th2 response in this parasitosis. At 8 wk postinfection, $STAT6^{-/-}$ mice mounted a strong Th1-like response (high IgG2a, IL-12, IFN-γ, as well as nitric oxide) and efficiently controlled *T. crassiceps* infection. These findings demonstrate that Th2-like response induced via STAT6-mediated signaling pathway mediates susceptibility to *T. crassiceps* and, furthermore, that unlike the case in most helminths, immunity against *T. crassiceps* is mediated by a Th1-like rather than Th2-like response. The importance of this observation was confirmed using mice IL-12p35 deficient ($IL\text{-}12p35^{-/-}$). Here is shown that mice $IL\text{-}12p35^{-/-}$ following *T. crassiceps* infection mounted a strong Th2-type biased response, produced higher levels of IgG1, IgE, IL-4, IL-5 as well as IL-13 than wild type mice, but remained highly susceptible to the larval stage of this cestode. In contrast, similarly infected CD40 deficient mice ($CD40^{-/-}$) had an impaired Th2-type response with extremely low levels

of IgE, IL-4 and IL-5 and efficiently controlled *T. crassiceps* infection. Together, these findings demonstrate a detrimental role for Th2-biased responses during the larval stage of *T. crassiceps* infection. Furthermore, they also suggest a pivotal role for CD40 in developing Th2-type responses.

Finally, BALB/c mice lacking a proinflamatory cytokine MIF ($MIF^{-/-}$) were highly susceptible to *T. crassiceps* and developed significantly higher parasite loads as compared to similarly infected $MIF^{+/+}$ mice. Non-significant differences were observed in cytokines TcAg-stimulated spleen cells from $MIF^{+/+}$ and $MIF^{-/-}$mice, suggesting that the susceptibility of $MIF^{-/-}$ mice to *T. crassiceps* was not due to the lack of IFN-γ production. Interestingly, peritoneal macrophages obtained from *T. crassiceps*-infected $MIF^{-/-}$ mice at different time points failed to respond efficiently to LPS+IFN-γ-stimulation *in vitro* and produced significantly lower levels of IL-12, TNF-α and NO as compared to those from $MIF^{+/+}$ mice. These findings demonstrate that MIF plays a critical role in mediating protection against *T. crassiceps in vivo*. Moreover, they also suggest that impaired macrophage function besides of lack of Th1 development may be responsible for mediating susceptibility to *T. crassiceps*.

Together, these findings demonstrate that Th2-type immune response renders susceptibility to *T. crassiceps* infection, while Th1-type immune response and macrophage activation play a fundamental role in the protection against this helminth. This is contrary to the dogma in the immuno-parasitology field that establishes that Th2 biased responses evolved to eliminate helminths, while Th1-type responses eliminate intracellular parasites.

Currently has been suggested that antigen-presenting cells (APC) could play an important role to induce polarization of the immune response. Therefore in this thesis, we used peritoneal macrophages (F4/80+) recruited at different times after challenge with *T. crassiceps* as APC and tested their ability to regulate Th1/Th2 differentiation. Macrophages from acute infections produced high levels of interleukin-12 (IL-12) and nitric oxide (NO), paralleled with low levels of IL-6 and prostaglandin E_2 (PGE_2) and with the ability to induce strong antigen-specific $CD4^+$ T-cell proliferation in response to nonrelated antigens. In contrast, macrophages from chronic infections produced higher levels of IL-6 and PGE_2 and had suppressed production of IL-12 and NO, associated with a poor ability to induce antigen-specific proliferation in $CD4^+$ T cells. These macrophages from chronic infections were able to bias $CD4^+$ T cells to produce IL-4 but not IFN-γ, contrary to macrophages from acute infections. Blockade of B7-2 and IL-6 and inhibition of PGE_2 failed to restore the proliferative response in $CD4^+$ T cells. Furthermore, studies using $STAT6^{-/-}$ mice revealed that STAT6-mediated signaling was essential for the expansion of these "alternatively activated macrophages".

In conclusion, our findings show that: the larval stage of *T. crassiceps* infection can induce different macrophage populations that have Th2-biasing properties. The strong Th2-biased immune developed in this infection can affect the susceptibility to a subsequent non-related infection. Moreover, they also demonstrate that Th2-like response induced via STAT6-mediated signaling pathway mediates susceptibility to *T. crassiceps* and, furthermore, that unlike the case in most helminths, immunity against *T. crassiceps* is mediated by a Th1-like rather than Th2-like response.

RESUMEN

Después del encuentro con un antígeno, las células T $CD4^+$ se diferencian en células efectoras, que pueden desarrollar respuestas inmunes tipo Th1 (IFN-γ alto) o tipo Th2 (IL-4, IL-5 e IL-10 altos). Cómo las células T adquieren y mantienen su nuevo patrón de expresión de genes es actualmente un área de intensa investigación. En el modelo experimental de cisticercosis murina causada por el helminto *Taenia crassiceps* se ha descrito que al inicio de la infección se genera una respuesta inmune tipo Th1 (IFN-γ, IL-2 y anticuerpos IgG2a) y que conforme la infección se hace crónica se da una respuesta de tipo Th2 (IL-4, IL-6, IL-10 y anticuerpos IgG1). En esta tesis se utilizó este modelo para estudiar varios de los factores que pudieran estar involucrados en la polarización de la respuesta inmune de Th1 hacia Th2 que se observa tras la infección por este parásito.

En principio se analizó si una respuesta inmune preestablecida tipo Th1 o tipo Th2 generada por la infección con el cisticerco de *T. crassiceps* era capaz de afectar diferencialmente la susceptibilidad a un nuevo reto con otro parásito no relacionado, *Trypanosoma cruzi*. Se demostró que si la infección con *T. cruzi* se realizaba 2 semanas posteriores la infección con *T. crassiceps* había reducción en el número de tripomastigotes, con una producción temprana de IFN-γ. Si la coinfección se iniciaba a las 8 o 12 semanas distantes a la infección con el metacéstodo de *T. crassiceps* había un marcado decremento en los niveles de IFN-γ y la susceptibilidad a la infección por *T. cruzi* se incrementaba significativamente. Esto demostró que la infección con *T. crassiceps* es capaz de modular de alguna forma la respuesta inmune de su huésped de manera que ante nuevos retos antigénicos montan respuestas polarizadas hacia Th2 que facilitan el establecimiento del segundo reto.

La resistencia y susceptibilidad en la cisticercosis experimental murina ha sido asociada con el predominio de las respuestas inmunes tipo Th1 o Th2 respectivamente; esto fue parcialmente demostrado con el uso de anticuerpos monoclonales anti-citocinas y con citocinas recombinantes como tratamiento en las fases tempranas la infección. Para establecer más claramente la participación de la respuesta inmune tipo Th2 en esta parasitosis se infectaron con metacéstodos de *T. crassiceps* ratones genéticamente deficientes (Knock-out) en la molécula STAT6. Esta molécula funciona como un mediador en la vía de señalización interna para IL-4/IL-13 y es crítica para la diferenciación a Th2. Los ratones BALB/c deficientes en STAT6 fueron capaces de desarrollar una fuerte respuesta tipo Th1 (niveles altos de IFN-γ, IL-12, y óxido nítrico) que resolvió la infección, en contraste con los ratones $STAT6^{+/+}$ que desarrollaron una respuesta tipo Th2 (niveles altos de IL-4, IL-13, IgG1, IgE y eosinofilia) con una alta carga parasitaria. Estos hallazgos apoyan la hipótesis de que la vía de señalización mediada por STAT6 es crítica para suprimir la respuesta tipo Th1 requerida para controlar la cisticercosis murina y también sugiere que las citocinas tipo Th2 favorecen el establecimiento del parásito vía la activación de STAT6.

La importancia de este hallazgo se confirmó al infectar animales deficientes en IL-12 (citocina primordial para sostener una respuesta tipo Th1), los cuales

tuvieron un incremento significativo (100%) en la carga parasitaria con respecto a los animales IL-12 $^{+/+}$. Finalmente, ratones BALB/c deficientes en MIF (MIF-/-), una citocina pro-inflamatoria, mostraron una susceptibilidad 3 veces mayor, con altos niveles de IL-6 e IL-13 y una disminución en la actividad de los macrófagos. Estos datos en su conjunto demuestran que una respuesta inmune tipo Th2 favorece el establecimiento de *T. crassiceps*, un parásito helminto, mientras que una tipo Th1 esta fuertemente asociada a su eliminación. Esto contrasta con el dogma de que una respuesta tipo Th2 es protectora en las infecciones por helmintos.

Por otro lado, recientemente se ha sugerido que una posible vía común que podrían utilizar los helmintos para polarizar la respuesta inmune ante nuevos retos antigénicos es la activación diferencial de los macrófagos. En este trabajo se caracterizaron los macrófagos peritoneales (F4/80$^+$) reclutados en diferentes tiempos después de la infección con *T. crassiceps* y se probó su habilidad para regular la diferenciación de las células T CD4$^+$ hacia Th1 ó Th2. Los macrófagos de tiempos cortos de infección produjeron altos niveles de IL-12 y NO, paralelamente con bajos niveles de IL-6 y prostaglandina E_2 (PGE_2), con la capacidad para inducir una fuerte proliferación antígeno-específica de células T CD4$^+$ en respuesta a antígenos no relacionados (OVA y KLH). En contraste, los macrófagos provenientes de infecciones crónicas produjeron mayores niveles de IL-6, PGE_2 y tuvieron niveles disminuidos de IL-12 y NO, asociados con poca habilidad para inducir proliferación antígeno-especifica de células T CD4$^+$. Estos macrófagos fueron caracterizados como "alternativamente activados", capaces de inducir a las células T CD4$^+$ a producir niveles altos de IL-4, pero no de IFN-γ, contrario a los macrófagos de infecciones agudas. Por otro lado, el bloqueo de B7-2 e IL-6 y la inhibición de PGE2 fallaron para restaurar la respuesta proliferativa en las células T CD4$^+$. Estudios posteriores usando ratones deficientes en STAT6 e infectados con *T. crassiceps* revelaron que los macrófagos de estos animales expresaron niveles normales de CD23 y CCR5, una mayor producción de IL-12 que indujo una mejor proliferación en las células T CD4$^+$ y mayor producción de IFN-γ, lo que reveló que la señal a través de STAT6 es esencial para la expansión de los "macrófagos alternativamente activados" .

Tomando en conjunto todos los resultados descritos anteriormente se puede concluir que: la infección por *T. crassiceps* en su estado de cisticerco puede inducir diferentes poblaciones de macrófagos con la habilidad de dirigir la diferenciación de las células T hacia el tipo Th2. Además la fuerte inducción de respuesta tipo Th2 generada por esta infección puede modular la respuesta inmune a antígenos o infecciones no relacionados con el parásito. Por otro lado se estableció con mayor claridad que una respuesta inmune tipo Th1 esta involucrada en la protección y que la vía de señalización mediada por STAT6 es crítica para suprimir esta respuesta, lo que sugiere que las citocinas tipo Th2 favorecen el establecimiento de *T. crassiceps* vía la activación de STAT6.

INTRODUCCIÓN

La generación de una respuesta inmune dependiente de células T a agentes infecciosos es un proceso complejo en el cual algunas citocinas, moléculas co-estimuladoras y células presentadoras de antígeno proveen señales imprescindibles para dirigir el desarrollo de la inmunidad adaptativa (Mossman et al, 1986; Jankovic et al, 2001). El comprender los eventos que influyen en la generación inicial de la respuesta inmune, así como en la expansión de células efectoras y el mantenimiento de la memoria inmunológica es crítico para el diseño racional de intervenciones inmunológicas (vacunas, tratamientos terapéuticos).

Después de la unión del receptor de la célula $TCD4^+$ con su apropiado complejo peptídico-MHC, las células $TCD4^+$ entran rápidamente a un programa de diferenciación. En el caso de infecciones crónicas, como son las infecciones por parásitos, este proceso de diferenciación puede resultar en una respuesta inmune altamente polarizada, no así en el caso de las infecciones agudas donde es común observar una respuesta inmune más heterogénea (Murphy y Reiner, 2002).

En 1986, Mosmann y colaboradores establecieron que las células $TCD4^+$ vírgenes cooperadoras (Th), podían dividirse en dos clases de células funcionales, según el perfil de citocinas que secretaban. Así se estableció que la respuesta inmune tipo Th1 se caracteriza por la producción de citocinas IL-2, IFN-γ, y TNF-β, las cuales se les ha reconocido como las responsables de la inmunidad mediada por células, es decir, de la activación de macrófagos, de promover las reacciones de hipersensibilidad retardada (DTH), y de la activación de células $TCD8^+$ citotóxicas y asesinas naturales (NK). Por otro lado, la respuesta inmune de tipo Th2 se caracteriza por la producción de IL-4, IL-5, IL-6, IL-10 e IL-13, las cuales son las responsables de generar y mantener gran parte de la respuesta inmune de tipo humoral induciendo la proliferación y diferenciación de linfocitos B, la producción de anticuerpos, principalmente de la clase IgG1 e IgE, además, favorece la inmunidad de las mucosas por células cebadas y la inducción de la diferenciación y proliferación de los eosinófilos.

Así, el control de agentes infecciosos requiere de la maduración de células $TCD4^+$ a células efectoras que secreten diferentes tipos de citocinas importantes para la regulación y coordinación de una apropiada respuesta inmune. En términos del control de parásitos, de acuerdo con uno de los dogmas centrales de la inmunología actual, se considera que diferentes patógenos requieren diferentes respuestas inmunes para ser eliminados: protozoarios intracelulares (como algunas especies de leishmanias y *Toxoplasma gondii*) son controlados por células $TCD4^+$ que se diferencian para secretar citocinas de tipo Th1, mientras que los organismos extracelulares tales como los helmintos son controlados por células $TCD4^+$ que secretan citocinas tipo Th2 (Mossman et al, 1989). Además de su papel protector en la defensa del hospedero ambas subpoblaciones de células T han sido relacionadas en respuestas patológicas. Las células Th1 median la autoinmunidad órgano-especifica (Kapsenberg et al., 1998) y las células Th2 han sido relacionadas en la patología de asma y alergías (Mojtabavi et al., 2002). Es claro que la composición final de las células Th en respuesta a un antígeno puede determinar el curso final de una infección parasitaria o de otra patología donde estén involucradas respuestas inmunes altamente polarizadas. Así, el establecimiento de los mecanismos que condicionan el estado final de diferenciación hacia el subtipo celular Th1 o Th2 *in vivo* puede ayudar a comprender cómo los parásitos regulan la respuesta inmune de su hospedero, lo cual facilitaría el establecimiento de estrategias para el futuro desarrollo de vacunas antiparasitarias o de tratamientos terapéuticos.

El proceso por el cual una célula $TCD4^+$ no diferenciada (Th0) se desarrolla hacia Th1 o Th2 es un modelo ampliamente utilizado para tratar de entender los mecanismos de regulación y expresión de los genes involucrados en la polarización de la respuesta inmune (Gajewski et al., 1994). Existen muchas evidencias que indican que este proceso de diferenciación es altamente plástico. Además de las características genéticas del individuo (Lindsay y Vijay, 1996), muchos factores pueden influir en la decisión final para que las células Th0 se conviertan en células efectoras tipo Th1 o Th2. Las citocinas como la IL-12 e IL-4, que actúan a través del transductor de señales y activador de la trascripción 4 (signal transducer and activator of transcription-STAT4) y STAT6, respectivamente, son consideradas como

uno de los elementos esenciales que dirigen el curso de la diferenciación (Magram et al. 1996; Afonso, et al. 1994; Pearlman, 1993; Nicholson y Kuchroo 1996). Igualmente se ha propuesto que la naturaleza del antígeno (Wraith et al. 1989; Wauben et al., 1992; De Magistris et al., 1992; Evavold, 1991), la vía de entrada del antígeno (Van den Eertweg et al., 1992; Nabors et al., 1995), la dosis administrada del antígeno (Nicholson y Kuchroo 1996), la cronicidad del estímulo (Pearlman, 1993), y las células presentadoras de antígeno (Gajewski et al.,1990, 1991), influyen en el curso de la diferenciación.

Respecto a cómo las células presentadoras de antígeno (CPA) podrían estar participando en los procesos de diferenciación hacia Th1 o Th2, se ha propuesto que sería a través de la interacción de señales asociadas al receptor antigénico de las células $TCD4^+$, así como a través de las señales coestimuladoras (Gause et al, 1997). La posibilidad de que las moléculas coestimuladoras estén involucradas en la diferenciación de las células $TCD4^+$ está fuertemente apoyada por diversos estudios que demuestran que el bloqueo o ausencia de B7-1, B7-2, CD4, CD30 y CD2 puede afectar el curso de ciertas enfermedades autoinmunes e incluso modificar la susceptibilidad a algunas infecciones, como una consecuencia de la alteración en el fenotipo de las células efectoras Th1 o Th2 (Kuchroo et al, 1995; Lenschow et al, 1996). Existen varias evidencias de que existe una respuesta diferencial dependiente de las señales coestimuladoras; por ejemplo se ha reportado que la producción de IL-2 por las células T es más dependiente de B7 que la IL-4 (Dubey et al, 1996). Esta observación es consistente con el estudio donde usando clonas de células Th1 y Th2 se comprobó que las células Th1 eran mas dependientes de B7 que las Th2 (McArthur et al, 1993; McKnight et al, 1994; Harding et al, 1992; Lichtman et al, 1988). Sin embargo estas observaciones no fueron contundentes ya que no se hizo una distinción entre B7-1 y B7-2, lo cual es importante considerar debido a que existen datos que apoyan que la sobreexpresión de B7-2 puede inducir a las células $TCD4^+$ a que se diferencien hacia Th2. Por otro lado, los ligandos para B7, CD28 y su homologo CTLA-4, parecen participar de manera importante en la activación y proliferación de las células T. Se ha demostrado que la interacción entre B7 y CD28 generalmente resulta en la diferenciación tanto de Th1 como de Th2. Sin

embargo la interacción entre B7 y CTLA-4, parece tener un papel inhibitorio para el desarrollo de ambas subpoblaciones (Alegre et al, 1998).

Todas estas evidencias apuntan a que las células presentadoras de Ag podrían estar participando activamente en los procesos de polarización de la respuesta inmune a través de sus moléculas coestimuladoras. Sin embargo aun no queda claro cómo éstas células, así como los otros factores (naturaleza del antígeno, dosis de antígeno, vía de entra del antígeno, cronicidad del estimulo) podrían estar influyendo en el proceso de diferenciación.

Evidencias de los últimos años sugieren que todos estos elementos de diferenciación participan influyendo sobre ciertos elementos de transcripción involucrados en la expresión de los genes para que se dé la diferenciación de las células Th1 o Th2. Por ejemplo, se ha demostrado que existe un factor de transcripción, conocido como T-bet, que juega un papel importante en la diferenciación de las células Th1, induciendo tanto la trascripción del gene que codifica para el IFN-γ como la respuesta selectiva al factor de crecimiento IL-12 (Mullen et al., 2001). De igual manera STAT4 parece ser un elemento esencial en los eventos tempranos de diferenciación hacia la respuesta Th1 (Nishikomori et al., 2002). En contraste, el factor de transcripción GATA3 parece ser crucial para inducir algunas de los factores de diferenciación hacia Th2, en particular compitiendo por la transcripción de las citocinas tipo Th2, a través de los genes que codifican para IL-13, IL-4 e IL-5 (Takemoto et al., 2002). De igual manera, STAT6 parece ser esencial para el desarrollo de las células tipo Th2 (Kaplan, M. H. et al., 1996).

PARTICIPACIÓN DE LAS SUBPOBLACIONES Th1 y Th2 EN INFECCIONES POR HELMINTOS

Actualmente es ampliamente conocido y aceptado que las infecciones causadas por helmintos inducen respuestas inmunes altamente polarizadas, caracterizadas por elevados niveles de citocinas tipo Th2, particularmente IL-4, IL-5, IL-6, IL-10 e IL-13. La sobre-expresión de estas citocinas dan como resultado altos niveles de IgE en suero, eosinofilia e hiperplasia de los mastocitos de la mucosa intestinal (Finkelman. et al., 1997; Maizels et. Al., 1993; Urban et. al., 2000).

Varios autores han sugerido que este tipo de respuesta (Th2) es protectora en las infecciones por helmintos. Particularmente, se ha reconocido ampliamente la participación crítica de la IL-4 e IL-13 en los mecanismos de eliminación de los helmintos intestinales (Capron, 1989; Maizels y Allen, 1997). Una respuesta completa del hospedero contra infecciones entéricas incluye el incremento de fluidos en el lumen intestinal a efecto de facilitar la expulsión, acceso limitado a la superficie epitelial, y un lavado potencial del agente patógeno (Cooke, 1998). Recientemente se ha propuesto que tanto la IL-4, como la IL-13 causan alteraciones importantes en la fisiología de las células epiteliales del intestino, como es el incremento de la permeabilidad, la disminución de la absorción de la glucosa, y disminución en la secreción del HCl a nivel intestinal, y estos efectos son dependientes de la señal STAT6 (Madden et al., 2002). Este fenómeno está fuertemente apoyado por Shea-Donohue y colaboradores (2001), quienes demostraron que la expulsión del nematodo intestinal *Heligmosomoides polygyrus* está mediada por IL-4, la cual induce la disminución de la absorción de la glucosa y el incremento de la secreción de líquidos, en respuesta a diversos mediadores solubles, incluyendo histamina y prostaglandina E2 (PGE2) que se sabe afectan la función celular del epitelio (Madden et al,. 1991). De igual manera, se ha demostrado que la IL-13 tiene un papel relevante en la expulsión del helminto gastrointestinal *Nippostrongylus brasiliensis* (Finkelman et al, 2000). De modo que el binomio IL-4/IL-13 se considera uno de los factores más importantes para eliminar a los parásitos helmintos. Lo anterior ha tomado más fuerza al demostrarse que animales deficientes en STAT6 (molécula de señalización interna

del receptor compartido por IL-4/IL-13) son incapaces de expulsar a los helmintos intraintestinales (Urban et al, 2000; Finkelman et al, 2000). Sin embargo, esto es plenamente cierto sólo para aquellos helmintos gastrointestinales, dado que este fenómeno es mucho más complejo en las infecciones donde el helminto es extraintestinal (Fallon, 2000; Maizels y Lawrence, 1995). Por ejemplo, hasta ahora se han reportado en la literatura dos infecciones por parásitos helmintos que no siguen este patrón: *Schistosoma mansoni* y *Taenia crassiceps*, que a pesar de generar una fuerte respuesta tipo Th2 en sus huéspedes, éstos no eliminan al parásito (Fallon, 2000; Chiaramonte et al, 1999; Terrazas et al, 1998). En ambos casos, diferentes grupos de investigadores han demostrado que al inducir respuestas tipo Th1, se observa una reducción significativa de la carga parasitaria, así como una disminución en la patología asociada a la respuesta Th2 en la infección por *S. mansoni* (Terrazas et al, 1999; Hernández et al, 1999).

Por otro lado, es indudable que la fuerte inducción de respuestas tipo Th2 generadas por helmintos (donde predominan IL-4, IL-13 e IL-10) pueden modular la respuesta inmune a antígenos o infecciones no relacionados con el parásito, ya que existen sólidas evidencias de que ésto ocurre con una variedad de helmintos (Actor et al 1993; Kullberg et al 1986; Cox, 2001). Por ejemplo, se conoce que la infección con *S. mansoni* retarda la eliminación del virus de la vaccinia y que altera la respuesta al antígeno soluble, mioglobina de ballena (Actor et al., 1993; Curry et al, 1995); *Brugia malayi* o sus extractos modulan la respuesta Th1 a antígenos de micobacteria (Pearlman et al, 1993; Maizels, 1993). *Nippostrongylus brasiliensis*, que también es conocido por inducir respuestas tipo Th2, retarda el rechazo de injerto de riñón en ratas mediante la inhibición de la respuesta Th1 (Cox, 2001). Antígenos solubles de *Toxocara canis* han sido descritos recientemente como inductores de respuestas tipo Th2, al mismo tiempo que disminuyen las Th1 (Hollan et al, 2000). Por otro lado, en el modelo de cisticercosis experimental causada por *T. crassiceps*, se ha observado que aumenta la respuesta tipo Th2, lo cual incrementa la susceptibilidad a un parásito no relacionado (*Trypanosoma cruzi*), cuya eliminación en muchos de los casos se ha reportado Th1-dependiente (Apéndice I/Artículo 1/Rodríguez-Sosa et al, 1999).

No obstante que es conocido que los helmintos, tanto gastrointestinales como extraintestinales, inducen respuestas tipo Th2, hasta ahora no se ha determinado cuál es el mecanismo que usan estos parásitos para polarizar de esa forma la respuesta inmune (Murphy y Reiner; 2002), no sólo a sus propios antígenos sino también a antígenos no relacionados con el parásito. Recientemente, se ha iniciado la búsqueda de factores del parásito que tengan un papel importante en esta polarización; existen hasta ahora sólo dos trabajos proponiendo una posible vía de inducción de respuestas Th2 en helmintos: uno de ellos ha demostrado que la inyección de proteínas de secreción/excreción de diferentes helmintos puede inducir en animales sanos una respuesta polarizada hacia la producción de citocinas tipo Th2 y a la producción de IgE (Hollan et al, 2000). El otro, en cambio, propone que los carbohidratos presentes en los antígenos del helminto pueden polarizar la respuesta hacia Th2 (Okano et al., 1999). Sin embargo, en ambos casos, no se conoce la posible célula blanco de estas moléculas parasitarias que pudiera estar participando en los eventos de polarización.

Los eventos involucrados en la polarización de la respuesta inmune hacia el tipo Th1 o Th2 son muy diversos que van desde la dosis antigénica, el sitio de entrada del antígeno, la función de las células accesorias y de las moléculas co-estimuladoras, hasta el perfil de citocinas en el microambiente al momento de la estimulación antigénica (Gajewsky et al, 1991; Van den Eertweg et al, 1992; Constant y Bottomly, 1997; Hunter y Reiner 2000; Ashnagly et al, 2001). Recientemente, se ha dado gran importancia al tipo de células presentadoras de antígeno en la inducción de respuestas inmunes altamente polarizadas (Gajewsky et al 1991, Jankovic et al., 2001). Básicamente, los estudios en este nivel se han centrado en las células dendríticas estimuladas por antígenos de patógenos intracelulares como bacterias y protozoarios (Bliss et al, 1999; Aliberti et al, 2000) y su papel en la polarización temprana hacia Th1 observada en esas infecciones. En el caso de las infecciones por helmintos queda aún por determinar si las células presentadoras de antígeno (como célula blanco de los factores solubles o constitutivos del parásito) están involucradas en la polarización de la respuesta inmune Th2 observada en estas parasitosis.

LOS MODELOS EXPERIMENTALES (cisticercosis y leishmaniosis): GENERALIDADES

Taenia crassiceps

La cisticercosis es una enfermedad parasitaria causada por el estado larvario del céstodo de *Taenia solium* y afecta la salud humana seriamente. Los humanos adquieren la larva por ingestión de huevos liberados por un gusano adulto. Esta enfermedad es común en México y en muchos de los países subdesarrollados, aunque recientemente se ha detectado en el sur de los Estados Unidos (Money et al., 2000). La infección del sistema nervioso central por este céstodo, es reconocida como neurocisticercosis (Bale, 2000).

El papel esencial de los cerdos como hospederos intermediarios obligados en el ciclo de vida del parásito ofrece la oportunidad de intervenir en la transmisión de esta enfermedad a través de inducir la inmunidad adquirida con vacunas o por manipulación genética (Fragoso et al., 1998). Sin embargo, la exploración sistemática del papel de los factores biológicos, genéticos, hormonales e inmunológicos involucrados en la resistencia y susceptibilidad del hospedero son muy costosos. Así, para sistematizar, bajar los costos de los estudios y explorar hipótesis que serían imposibles probar en seres humanos, en los últimos años se ha utilizado el modelo experimental murino de cisticercosis causada por *Taenia crassiceps*, el cual presenta una gran reactividad antigénica e inmunidad protectora cruzada con *T. solium* (Sciutto et al., 1990). Hasta ahora, muchas de las observaciones resultantes de la investigación con este modelo han sido extrapoladas exitosamente a la cisticercosis generada por la larva *T. solium*, (Larralde et al., 1990; Sciutto et al., 1990; Valdez et al., 1994; Sciutto et al., 1995; Morales et al., 2002).

En este modelo experimental, los roedores son los intermediarios naturales para el parásito *T. crassiceps*, y el hospedero definitivo es el zorro rojo (Fig.1). La infección de ratones BALB/c con metacéstodos de *T. crassiceps* presenta algunas ventajas, como la particularidad de que en su fase de cisticerco puede reproducirse asexualmente por gemación, de manera incontrolada en la cavidad peritoneal del

ratón, lo que ha permitido mantenerlo fácilmente por pases intraperitoneales de un ratón a otro durante varios años (Freeman, 1964).

Hasta ahora se ha establecido que diversos factores, como los genéticos, hormonales e inmunológicos, están involucrados en los mecanismos de resistencia y susceptibilidad a la infección por este parásito. Dentro de los factores hormonales que participan en esta parasitosis están los relacionados con las hormonas sexuales. Inicialmente se observó que las hembras presentan una mayor carga parasitaria que los machos (Larralde et al.,1989). La gonadectomía hace a las hembras más resistentes y a los machos más susceptibles a esta parasitosis, eliminándose así las diferencias de susceptibilidad entre sexos (Huerta et al., 1992). Los mecanismos de acción de las hormonas sexuales hasta ahora no han sido bien establecidos, pero hay suficientes evidencias que indican que la acción de estas hormonas es a través del sistema inmune (Huerta et al., 1992), probablemente influyendo a nivel del timo y en células T periféricas (Bojalil et al., 1993). La hormona sexual más importante en esta parasitosis es el 17 β-estradiol, cuyo nivel influye directamente en una mayor susceptibilidad en ambos sexos y afecta negativamente la respuesta de hipersensibilidad tardía a antígenos específicos del parásito (Terrazas et al., 1994).

Los factores genéticos inherentes a las cepas de los ratones también marcan diferencias significativas en la carga de los metacéstodos de *T. crassiceps*. Así, los ratones BALB/cAnN y DBA/2 son los más susceptibles, mientras que los BALB/b, C57BL/6J, C57BL/10J, BALB/k, C3H/Hej y C3H/FeJ son comparativamente más resistentes. Al parecer tal variabilidad está relacionada con el haplotipo $H\text{-}2^d$ para las cepas mas susceptibles, $H\text{-}2^b$ y $H\text{-}2^k$ para las más resistentes (Fragoso et al., 1996). Estudios posteriores, demostraron que en particular la región Qa2 (molécula del complejo principal de histocompatibilidad clase I no clásica) es la que participa en la resistencia a la cisticercosis por *T. crassiceps*. Ratones BALB/cAnN transgénicos para Qa-2 se convierten en altamente resistentes a la infección por *T. crassiceps* comparados con su control de ratones silvestres. Los mecanismos por lo cuales los ratones que sobreexpresan Qa-2 soluble GP1 controlan esta parasitosis no han sido definidos, sin embargo se ha sugerido que podría depender de cómo se da la presentación de los antígenos del cisticerco a las células T, lo que probablemente

favoreciera la respuesta de aquellas células que expresan receptores αβ, o probablemente a otra subclase única la cual podría participar a través de la producción de citocinas (Fragoso et al., 1998).

Con respecto a los factores inmunológicos, se sabe que la respuesta de tipo humoral no parece participar de manera importante en el control de la parasitosis, debido a que los títulos de anticuerpos específicos no correlacionan con la resistencia al mismo (Hermánek y Prokopic, 1989; Sciutto, 1989), mientras que la respuesta de tipo celular es la que está involucrada en su eliminación. Esto fue demostrado con ratones neonatales que fueron timectomizados, los cuales se hicieron más susceptibles, mientras que la transferencia pasiva de células T les confirió resistencia (Bojalil et al., 1993). Además, hay una correlación positiva entre una mayor intensidad de la respuesta de hipersensibilidad retardada y el bajo número de parásitos instalados (Terrazas et al., 1994), lo que confirma que la respuesta de tipo celular está involucrada en la resistencia a esta parasitosis.

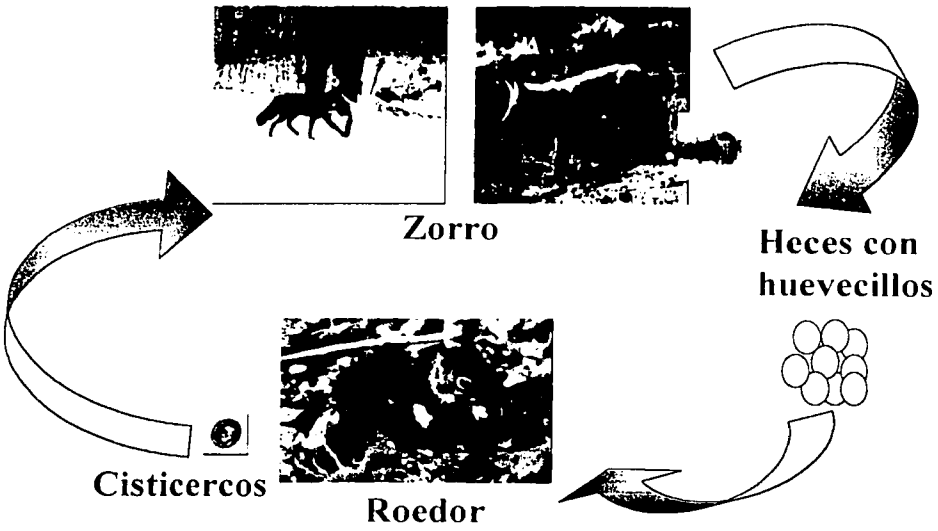
La respuesta celular T, responsable de la eliminación del parásito, fue caracterizada por Terrazas y colaboradores en 1998. Ellos demostraron que al inicio de la infección con *T. crassiceps* se desarrolla una respuesta de tipo Th1, compuesta principalmente por niveles altos de IFN-γ, IL-2 y anticuerpos IgG2a. Sin embargo, cuando la infección se hace crónica se da una respuesta de tipo Th2 con niveles altos de IL-4, IL-6, IL-10 y anticuerpos IgG1 e IgG2b (Terrazas et al., 1998). Usando anticuerpos monoclonales anticitocinas y citocinas recombinantes como tratamiento en las fases tempranas de la infección, probaron que la respuesta tipo Th1 estaba asociada al crecimiento parasitario restringido observado en las primeras semanas de infección. Por el contrario, la respuesta Th2 estaba asociada a la sobrevivencia y crecimiento incontrolado del parásito en el hospedero. Los ratones BALB/c que recibieron anticuerpos monoclonales anti-IL-10 desarrollaron una respuesta inmune tipo Th1 con una carga parasitaria menor que los controles cuando se infectaron con el cisticerco de *T. crassiceps*. Por el contrario los ratones que recibieron anticuerpos anti-IFN-γ mostraron una respuesta tipo Th2 con un aumento significativo en la carga parasitaria. Los tratamientos con las citocinas recombinantes confirmaron estos resultados, los ratones que recibieron IFN-γ mas IL-2 desarrollaron cargas

parasitarias significativamente menores, mientras que los ratones tratados con IL-10 recombinante murina mostraron un aumento significativo en la susceptibilidad al parásito (Terrazas et al., 1999).

No obstante, que se sabe que en las primeras semanas de la infección murina con el cisticerco de *T. crassiceps* se desarrolla una respuesta tipo Th1, y que conforme la infección se vuelve crónica se da una respuesta tipo Th2, nadie hasta ahora ha descrito los mecanismos que podrían estar participando para inducir dichas respuestas. Recientemente, se ha sugerido que la presencia de glicoconjugados y/o proteínas podría ser una vía común de los helmintos para inducir una polarización de la respuesta inmune hacia Th2 (Okano et al, 1999; Hollan et al, 2000) pero las posibles células blanco y los receptores para estas moléculas no están aún definidos.

El modelo murino de infección con el cisticerco de *T. crassiceps* nos brinda la posibilidad de tener *in vivo* los dos tipos de respuestas inmunes claramente polarizadas, lo que nos permite tratar de identificar las células blanco que pudieran estar involucrados en la diferenciación de una respuesta inicial tipo Th1 a una tipo Th2 observadas en esta infección.

Fig.1.- Ciclo de vida del parásito *Taenia crassiceps*



***Leishmania*: Modelos de parásitos intracelulares**

Las especie *Leishmania* son parásitos *trypanosomatidos* intracelulares obligados de las células macrófagos-dendriticas de los hospederos vertebrados. Las formas de la enfermedad producidas por la infección con este parásito en los humanos pueden ser variables, dependiendo principalmente de la especie de *Leishmania*, la lesión cutánea generada puede sanar por si mismas (por ejemp. *Leishmania major*, *Leishmania tropica* y *Leishmania mexicana*) o progresar a una infección sistémica fatal (por ejempl. *Leishmania donovani*). La *Leishmania* tiene un ciclo de vida dimórfico (Fig. 2) consistente de promastigotes extracelulares que se multiplican y desarrollan dentro del intestino medio de mosquito vector, y de amastigotes intracelulares que residen y multiplican dentro de las vacuolas fagolisosomales de los macrófagos (Sacks et al., 2000). La relación entre el parásito y su célula hospedero es particularmente interesante, no solo porque los macrófagos son capaces de tener una actividad microbicida potente, si no también por su capacidad de presentar antígeno desde donde ellos pueden orquestar el desarrollo de la respuesta inmune. Resulta sumamente interesante el hecho de que una infección exitosa por estos parásitos, sea el resultado de una invasión generalizada de los macrófagos, lo que implica que los parásitos son capaces de evadir los efecto nocivos de defensa de estas células. Los mecanismos precisos de cómo esto ocurre no han sido del todo establecidos, sin embargo, en los últimos años se han hecho grandes progresos al respecto con la utilización de modelos murinos de infecciones parasitarias. En particular la infección por *Leishmania* en ratones ha sido un excelente modelo para estudiar los problemas asociados con los parásitos intracelulares (Alexander J et al., 1999).

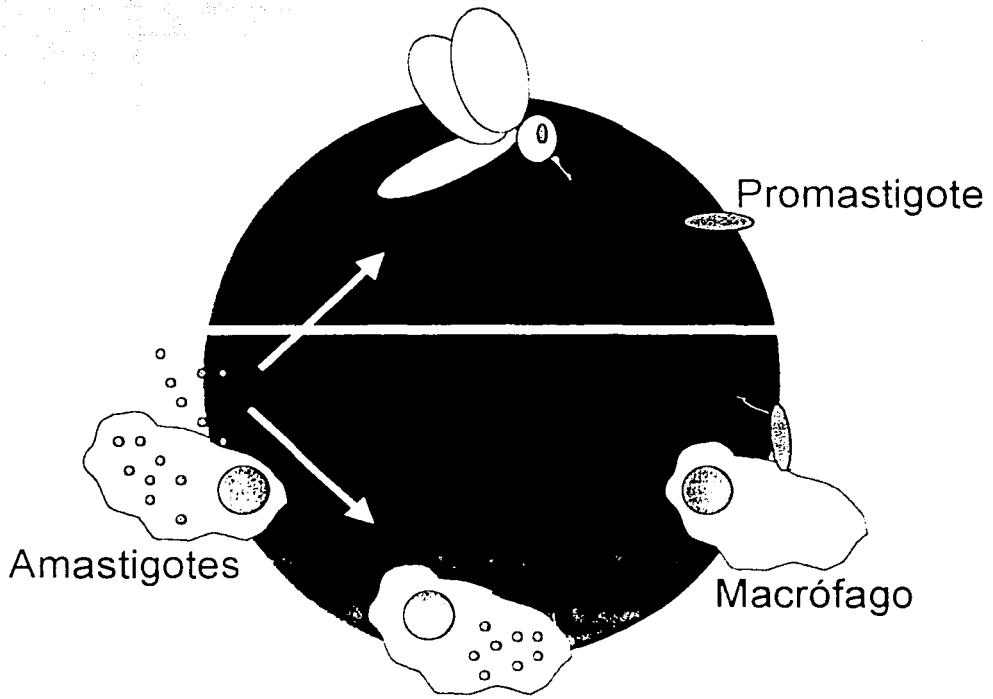
Leishmania major (LV39/ MRHO/SU/59/P-Strain). La infección murina por *Leishmania major*, ha sido un modelo experimental ampliamente usado para establecer la inmunología de esta infección. La infección murina con estos parásitos, en algunos casos se resuelve por si mima y en otros resulta fatal, dependiendo del fondo genético de la cepa del ratón (Behin et al. 1979; Handman et al., 1979). Este fenómeno de resistencia y susceptibilidad por observado en la infección por *L. major*

ha sido explicado basándose en las diferentes propiedades funcionales de las células TCD4$^+$ diferenciadas en las infecciones que se resuelven *versus* las que no se resuelven. Durante los primeros 4-5 días después de la infección con *L. major* hay una respuesta mixta en el tipo de citocinas (IL-2, IL-4 e IL-13) que producen las células TCD4$^+$, sin embargo, las cepas de ratones que resuelvan la infección regulan rápidamente la transcripción de IL-4. Así, la respuesta celular T anti-*L.major* en los ratones resistentes como son CBA o C57BL/6 se caracteriza por la inducción de células TCD4$^+$ Th1, secretoras de IFN-γ e IL-2. En cambio, en los ratones altamente susceptibles como son los BALB/c hay una diferenciación preferencial de células TCD4$^+$ Th2, productoras de IL-4 e IL-10 (Heinzel et al., 1991; Scout ,1989). La infección experimental murina con *L. major* es considerada el ejemplo *in vivo* mas simple de la existencia del paradigma Th1/Th2 (Carrera et al., 1996). Además, en este modelo el papel de las células Th1 y Th2 como mediadoras de resistencia y susceptibilidad, respectivamente, ha sido demostrado ampliamente. Sin embargo hasta ahora no han sido propuestos los posibles mecanismos involucrados en el desarrollo de la respuesta Th1-protectora o Th2-facilitadora en esta infección. EL modelo murino de infección con *L. major* nos brinda la posibilidad de identificar otros factores (como la IL-18 y MIF) que pudieran estar involucrados en el desarrollo de la respuesta tipo Th1-protectora o en la supresión de la misma.

Leishmania mexicana (M379 P-Strain). La leishmaniasis cutánea en América es causada por un complejo de dos parásitos que incluye *L. mexicana* y *L. amazonensis*. Este complejo para fines prácticos se le ha reconocido simplemente como *Leishmania mexicana*. Actualmente está bien documentado que casi todas las cepas de ratón son altamente susceptibles al complejo de *L. mexicana*, ya que una vez que se infectan desarrollan una gran lesión cutánea que no sana. Hasta ahora, se sabe poco a cerca de las bases moleculares involucradas en la susceptibilidad a este complejo. Se sabe que la ausencia de IL-4 así como del factor de transcripción para el receptor de IL-4/IL-13 (STAT-6) en modelos murinos incrementa los niveles de IL-12 que induce el desarrollo de la respuesta tipo Th1, la cual controla la infección. Estos hallazgos indican que la IL-4 y la vía de señalización STAT6 median

la susceptibilidad a *L. mexicana* posiblemente suprimiendo la producción de IL-12 e inhibiendo la respuesta Th1 (Satoskar et al. 1995; Stamm et al. 1998). Sin embargo, hasta ahora no se han identificado las células blanco de la IL-4 y tampoco se ha identificado como esta citocina media la susceptibilidad a la infección por *L. mexicana*. EL modelo murino de infección con el complejo de *L. mexicana* nos brinda la posibilidad de identificar las células blanco y otros factores (como la IL-13, el complejo IL-13/IL-4) que pudieran estar involucrados en la supresión de una respuesta tipo Th1 que es capaz de resolver esta infección.

Fig. 2.- Ciclo de vida del parásito *Leishmania*



OBJETIVO GENERAL

El presente trabajo se desarrolló para determinar la influencia de algunas citocinas, así como la participación de los macrófagos y sus moléculas de membrana como factores inmunológicos involucrados en la polarización de la respuesta inmune en las infecciones parasitarias. Para alcanzar este objetivo general se utilizaron dos modelos murinos de infecciones parasitarias bien establecidos:

Cisticercosis causada por *Taenia crassiceps*, y

Leishmaniosis causada por *Leishmania major* y *Leishmania mexicana*

OBJETIVOS PARTICULARES

1. Establecer si la fuerte inducción de respuestas tipo Th2 generadas por el helminto *T. crassiceps* puede generar un ambiente de citocinas capaz de modular la respuesta inmune a infecciones no relacionadas.

2. Demostrar que en la infección por el helminto *Taenia crassiceps* la respuesta tipo Th1 es primordial para eliminar al parásito, mientras que la respuesta tipo Th2 facilita la instalación de éste parásito.

3. Definir si las células presentadoras de antígeno (macrófagos) participan en la polarización de la respuesta inmune tipo Th1 hacia Th2 durante la infección con cisticercos de *T. crassiceps*.

4. Definir la participación de la IL-18 y MIF en el desarrollo de la respuesta Th1-protectora en la infección murina con *L. major*.

5. Establecer la participación de la IL-12 e IL-13 en la susceptibilidad a la infección murina por *L. mexicana*.

HIPÓTESIS

En las infecciones parasitarias, la producción específica de algunas citocinas son esenciales para dirigir el curso de la difereciación de la respuesta inmune hacia Th1 o Th2, que eventualmente se refleja en protección o susceptibilidad, dependiendo del patógeno que se trate.

Actualmente es conocido que las infecciones por helmintos inducen respuestas inmunes tipo Th2. Dado lo anterior, en los últimos años ha dominado en el área de la inmunoparasitología la idea de que las respuestas inmunes tipo Th2 son cruciales para eliminar las infecciones causadas por los helmintos.

Basados en los antecedentes del modelo experimental de cisticercosis, en este trabajo se propone que:

> La respuesta inmune involucrada en la eliminación del helminto *T. crassiceps* es Th1 dependiente, y que la inmunología de esta infección es similar a aquella observada en infecciones intracelulares como la leishmaniosis. Adicionalmente, se propone que el estado de activacion de los macrófagos participa en la inducción de la respuesta inmune tipo Th2 observada en la cisticercosis.

RESULTADOS

En esta tesis se utiliza un modelo murino de infección por un parásito helminto (*T. crassiceps*) y dos modelos murinos de infección por parásitos intracelulares (*Leishmania major y Leishmania mexicana*) para analizar la diferenciación y función de las subpoblaciones de las células T colaboradoras, en particular para tratar de establecer el papel de las citocinas y de los macrófagos en el desarrollo de las respuestas inmunes generadas por la infección por éstos parásitos. Los resultados de este trabajo de tesis se presentan en forma de 9 artículos agrupados en dos apéndices.

Apéndice I:

ARTÍCULOS RELACIONADOS CON LOS MECANISMOS DE POLARIZACIÓN DE LA RESPUESTA INMUNE POR LA INFECCIÓN DE *T. crassiceps.*

Esta parte de la tesis se enfocó principalmente hacia el estudio de los factores inmunológicos que participan en la polarización hacia la respuesta tipo Th2 observada en las infecciones por helmintos, usando el modelo murino de cisticercosis causada por *Taenia crassiceps.*

Los resultados de esta sección se presentan en forma de 5 artículos. El primero de ellos, se realizó con el fin de establecer si la fuerte inducción de respuestas tipo Th2 (donde predominan IL-4, IL-6 e IL-10) generada por la infección con metacéstodos de *T. crassiceps* puede modular la respuesta inmune a antígenos o infecciones no relacionados con el parásito, ya que existen sólidas evidencias de que esto ocurre en una variedad de helmintos (Actor et al 1993; Kullberg et al 1986; Cox, 2001). El artículo referido se titula **"Susceptibility to *Trypanosoma cruzi* is modified by a previous non-related infection"** por Miriam Rodríguez, Luis I. Terrazas, Ricardo Márquez y Rafael Bojalil que fue publicado en Parasite Immunology en 1999 (21: 177-185). En dicho artículo se demuestra que la infección crónica con *T. crassiceps* induce una fuerte respuesta tipo Th2, que es capaz de incrementar la susceptibilidad a *Trypanosoma cruzi*, cuya eliminación, en muchos casos, se ha reportado Th1-dependiente (Aliberti et al., 1996), aunque en este artículo se muestran

algunas evidencias que apoyan más la hipótesis de una respuesta mixta Th1/Th2 para la restricción de *T. cruzi*.

Posteriormente se hicieron varios trabajos para establecer la participación de la respuesta inmune tipo Th1 y tipo Th2 en la infección por *T. crassiceps*. Los resultados de estos trabajos están presentados en tres artículos. El primer trabajo al respecto se muestra, bajo el titulo: Cutting Edge: **Susceptibility to the larval stage of helminth parasite *Taenia crassiceps* is mediated by Th2 response induced via STAT6 signaling**; por Miriam Rodríguez-Sosa, John R. David, Rafael Bojalil, Abhay R. Satoskar y Luis I. Terrazas, que fue publicado en Journal of Immunology en 2002 (168: 3135-3139). Este trabajo se hizo para establecer más claramente la participación de la respuesta inmune dependiente de Th2 en esta infección. En este artículo se reporta que los ratones genéticamente deficientes (knock-out) en la molécula STAT6 (mediador en la vía de señalización interna para IL-4/IL-13, molécula crítica para la diferenciación a Th2) son capaces de desarrollar una fuerte respuesta tipo Th1 capaz de resolver la infección con cisticercos de *T. crassiceps*, en contraste con los ratones silvestres que desarrollaron una respuesta tipo Th2 con una alta carga parasitaria. Estos hallazgos apoyan la hipótesis de que la vía de señalización mediada por STAT6 es crítica para suprimir la respuesta tipo Th1 requerida para controlar la cisticercosis murina y también sugiere que las citocinas tipo Th2 favorecen el desarrollo de la susceptibilidad a la cisticercosis vía la activación de STAT6.

Un trabajo adicional se requirió para establecer la participación de la respuesta inmune tipo Th1 en esta infección. El segundo artículo que ya ha sido aceptado para su publicación (Apéndice I) bajo el título: **Altered T helper responses in CD40 and IL-12 deficient mice reveal a critical role for Th1 responses in eliminating the helminth parasite *Taenia crassiceps*** por Miriam Rodríguez-Sosa, Abhay R. Satoskar, John R. David and Luis I. Terrazas, demuestra que la ausencia de IL-12 favorece la multiplicación del metacéstodo de *T. crassiceps*, ya que la carga parasitaria en los ratones deficientes para IL-12p35 se incrementó significativamente con respecto a los ratones silvestres debido a una deficiente respuesta tipo Th1 contrastada por una inducción de niveles altos de citocinas y anticuerpos de una respuesta tipo Th2 (IL-4, IL-13, IL-5, IgG1 e IgE). Este hecho confirmó que, la

respuesta tipo Th1, mediada por la IL-12, es necesaria para controlar la infección por el helminto *T. crassiceps*. Por otro lado, los ratones deficientes en CD40 fueron capaces de controlar la infección por cisticercos de *T. crassiceps*, lo que se asoció a niveles disminuidos de IL-4, IL-5 e IL-13. Lo cual sugiere que CD40 participa, de alguna manera, en el desarrollo de la respuesta inmune facilitadora mediada por Th2.

Finalmente, en el artículo titulado **Macrophage migration inhibitory factor (MIF) plays a critical role in mediating protection against the helminth parasite *Taenia crassiceps*** por Miriam Rodríguez-Sosa, Lucia E. Rosas, John R. David, Rafael Bojalil, Abhay R. Satoskar and Luis I. Terrazas; publicado en Infection and immunity en 2003 (71: 1247-1254), se demuestra que los animales deficientes en MIF (una citocina pro-inflamatoria) tuvieron una susceptibilidad 3 veces mayor a la infección por *Taenia crassiceps* con respecto a los ratones silvestres. Siendo significativo el hecho que los ratones deficientes en MIF e infectados con *T. crassiceps* presentaron altos niveles de IL-6 e IL-13 y una marcada disminución en la actividad de los macrófagos.

En conjunto los resultados de los últimos tres trabajos descritos en la parte superior demuestran claramente que una respuesta inmune tipo Th2 favorece el establecimiento de *T. crassiceps*, mientras que una respuesta tipo Th1 está fuertemente asociada a su eliminación. Estos resultados contrastan con el dogma de que una respuesta tipo Th2 es protectora en las infecciones por helmintos.

Por otro lado, no obstante que se demostró que la infección por *T. crassiceps* induce una respuesta altamente polarizada tipo Th2 en su hospedero capaz de modular la respuesta inmune a otros antígenos no relacionados con el parásito, hasta ahora no se había propuesto qué células blanco podrían estar involucradas en dicha respuesta. Los resultados presentados en el artículo: **Chronic helminth infection induces alternatively activated macrophages expressing high levels of CCR5 with low interleukin-12 production and Th2-biasing ability**, por Miriam Rodríguez-Sosa, Abhay R. Satoskar, Rodrigo Calderón, Lorena Gómez-García, Rafael Saavedra, Rafael Bojalil, and Luis I. Terrazas; publicado en Infection and Immunity en 2002 (70: 3656-3664), sugieren una posible vía común que podrían utilizar los helmintos para polarizar la respuesta inmune ante nuevos retos antigénicos. Se

demuestra que hay una activación diferencial de los macrófagos dependiendo del tiempo de infección con el cisticerco. Los macrófagos (F4/80+) de infecciones agudas producen altos niveles de IL-12 y óxido nítrico y niveles disminuidos de IL-6 e PGE2 con la habilidad de inducir una fuerte respuesta proliferativa antígeno específica de células $TCD4^+$. En contraste, los macrófagos de infecciones crónicas producen altos niveles de IL-6 y PGE_2 con niveles bajos de IL-12 y óxido nítrico, asociados a una pobre habilidad de inducir respuesta proliferativa antígeno-especifica de las células $TCD4^+$. La falla para inducir proliferación no se asoció a una deficiente expresión de moléculas accesorias, dado que presentaron una sobreexpresión de MHC-II, CD40, B7-2, CD23 y CCR5 conforme la se hacia crónica. Los macrófagos de la infecciones crónicas, además, fueron capaces de inducir a células $TCD4^+$ a producir IL-4, pero no IFN-γ, un fenómeno contrario al observado con los macrófagos de infecciones agudas. Por otro lado, el bloqueo de B7-2 e IL-6, así como la inhibición de la producción de PGE_2 no restaura la respuesta proliferativa de las células $TCD4^+$. Adicionalmente, con el uso de ratones deficientes de STAT6, se demostró que esta vía es esencial para que se diferencien los macrófagos como alternativamente activados con diferentes capacidades de presentar antígeno y con la propiedad de polarizar la respuesta inmune hacia el tipo Th2.

Apéndice II:

ARTÍCULOS RELACIONADOS CON LOS MECANISMOS DE POLARIZACIÓN DE LA RESPUESTA INMUNE EN LAS INFECCIONES MURINAS POR *Leishmania major y Leishmania mexicana*

En esta sección se trató de establecer la participación de algunas interleucinas (IL), como la IL-18, el factor Inhibidor de la migración de macrófagos (MIF), la IL-12 e IL-13 en la respuesta inmune generada por la infección de dos parásitos intracelulares, *Leishmania major* y *Leishmania mexicana*. Los resultados se presentan en el apéndice II en forma de 4 artículos.

En el primer trabajo titulado: **Genetically resistant mice lacking IL-18 gene develop Th1 response and control cutaneous *Leishmania major* infection** por Monteforte G.M., Takeda K., Rodríguez-Sosa M., Akira S. and David J.R. and A. R. Satoskar; publicado en The Journal of Immunology en 2000 (164:5890-5893), se analizó el curso de la infección cutánea de *L. major* en ratones deficientes en IL-18 ($IL\text{-}18^{-/-}$) comparándolos con ratones silvestres ($IL\text{-}18^{+/+}$). Los ratones $IL\text{-}18^{-/-}$ desarrollaron lesiones cutáneas mas grandes durante la fase temprana de la infección, pero eventualmente resolvieron la infección igual que los ratones silvestres. Sin embargo, las células de los ganglios linfáticos estimuladas con Ag a las dos semanas de infección de los ratones $IL\text{-}18^{-/-}$ como de los $IL\text{-}18^{+/+}$ produjeron niveles similares de IFN-γ. Por otro lado, los ratones $IL\text{-}18^{-/-}$ produjeron significativamente más IL-12 e IL-4. En la semana 10 post-infección tanto los $IL\text{-}18^{-/-}$ como $IL\text{-}18^{+/+}$ resolvieron la infección. En este punto, las células linfáticas de ambos grupos produjeron IL-12 e IFN-γ pero no IL-4. Además, la administración de anticuerpos anti-IFN-γ a los ratones $IL\text{-}18^{-/-}$ los hizo susceptibles a *L. major*. Estos resultados demostraron que la IL-18, una citocina que participa en el desarrollo de una respuesta tipo Th1, puede estar participando en el control temprano del desarrollo de la lesión cutánea causada por la infección con *L. major*, pero que no es crítica para el desarrollo de la respuesta Th1 protectora y la resolución de la infección.

En el segundo trabajo titulado: **Migration-inhibitory factor gene-deficient mice are ausceptible to cutaneous *Leishmania major* infection** por Satoskar A. R., Bozza M., Rodríguez Sosa M., Lin G. and J.R. David; publicado en Infection and

Immunity en 2001 (69: 906-911), se determinó el papel del factor inhibidor de la migración de macrófagos endógeno en el desarrollo de la inmunidad protectora contra la leishmaniosis cutánea. Se analizó el curso de la infección cutánea de *Leishmania major* en ratones deficientes en MIF ($MIF^{-/-}$) y en ratones silvestres ($MIF^{+/+}$). Los ratones $MIF^{-/-}$ fueron altamente susceptibles a la enfermedad y desarrollaron lesiones significativamente más grandes y con mayor número de parásitos que los ratones $MIF^{+/+}$. Los macrófagos peritoneales activados con IFN-γ de los ratones $MIF^{-/-}$ tuvieron una respuesta leishmanicida deficiente y produjeron un nivel significativamente menor de óxido nítrico y superóxido *in vitro*. Sin embargo, los macrófagos de los ratones $MIF^{-/-}$, produjeron mucho más IL-6 que los macrófagos de los ratones silvestres. Estos hallazgos demostraron que MIF endógeno juega un papel importante en el desarrollo de la inmunidad protectora contra *L. major*, *in vivo*. Además, esto demuestra que la susceptibilidad de los ratones $MIF^{-/-}$ a la infección de *L. major* es debido a la capacidad leishmanicida disminuida de los macrófagos más que la desregulación de la respuesta Th1 y Th2.

Por otro lado, en la infección por *L. major* es reconocido que algunas cepas de ratón, que tienden a hacer preferencialmente respuestas tipo Th2, son altamente susceptibles (BALB/c) y por el contrario las cepas de ratones con tendencia a desarrollar respuestas tipo Th1 son resistentes a esta infección (C57BL/6) (Alexander J et al., 1999). Sin embargo en el caso de la infección con el complejo de *Leishmania mexicana* todas las cepas de ratón son altamente susceptibles a esta infección (Alexander et al., 1999). Evidentemente la inmunología de la infección por *Leishmania mexicana* es diferente a la inmunología de la infección por *L. major*. Sin embargo, hasta ahora no se han establecido claramente estas diferencias. Por esta razón se decidió abordar la infección de *L. mexicana* para tratar de establecer la participación de algunas citocinas en esta infección.

Estudios recientes han reportado que las células T de ratones infectados con *L. amazonensis* fallan en responder a la IL-12 debido a una expresión deficiente del receptor para IL-12. En el artículo titulado **Susceptibility to *Leishmania mexicana* infection is due to the inability to produce IL-12 rather than lack of IL-12 responsiveness** por Rodriguez-Sosa M., Monteforte Gina M., and A. R. Satoskar ,

publicado en Immunology and Cell Biology en 2001 (79: 320-322), se demostró que las células de los ganglios linfáticos de ratones C57BL/6 y 129Sv/Ev infectados con *L. mexicana* responden eficientemente a la IL-12 exógena *in vitro* y producen IFN-γ. Además se comprobó que la omisión del gen de la señal transductora y activadora de la transcripción (STAT) 4 y 6 (STAT4/STAT6$^{-/-}$) los hizo susceptibles a *L. mexicana*, lo que demostró que una inhabilidad para producir IL-12 más que una falta de respuesta a esta citocina es la responsable de la susceptibilidad a *L. mexicana*. Además también se estableció que la vía mediada por STAT4 es crítica para el desarrollo de la inmunidad protectora contra la leishmaniasis sin importar la especie de *Leishmania* y/o el fondo genético del ratón.

Por otro lado, estudios recientes han demostrado que la IL-13 es un mediador de la susceptibilidad a la infección cutánea de *L. major* por una vía independiente de IL-4 (Mohrs et al., 1999). Para determinar si la IL-13 también está jugando un papel similar en la patogénesis de la infección cutánea de *L. mexicana* se analizó el curso de la infección por *L. mexicana* en ratones deficientes para IL-13 e IL-4/IL-13 con fondo genético C57BL/6x129sv/Ev y se compararon con los ratones silvestres infectados similarmente. Los resultados pueden verse con mayor detalle en el artículo titulado, **IL-13 gene-deficient mice are susceptible to cutaneous *L. mexicana* infection** por Rodríguez-Sosa M., Rosas L.E., Mackenzie A. N. J. and A. R. Satoskar, publicado en European Journal of Immunology 37: 3255-3260 (2001).

Brevemente, en este trabajo se observó que los ratones IL-13$^{-/-}$ fueron tan susceptibles como los silvestres a la infección por *Leishmania mexicana*, ambos desarrollaron rápidamente una gran lesión. En contraste, los ratones IL-4/IL-13$^{-/-}$, infectados de manera similar, fueron altamente resistentes y la mayoría no desarrollaron lesión, aunque algunos desarrollaron una muy pequeña úlcera con un número muy reducido de parásitos la cual sanó después de 12 semanas. A través del curso de la infección los ratones IL-13$^{-/-}$ y los ratones silvestres produjeron significativamente más anticuerpos IgG1-antígeno específico asociado a una respuesta tipo Th2, comparados con los ratones IL-4/IL-13$^{-/-}$. Todos los grupos produjeron niveles similares del anticuerpo IgG2a-antígeno especifico asociado a una respuesta Th1. A la semana 12 post-infección las células de ganglio estimuladas con

antígeno de *L. mexicana* (LmAg) de los ratones IL-4/IL-13$^{-/-}$ produjeron significativamente más IL-12 e IFN-γ comparados con los IL-13$^{-/-}$ y los silvestres. Sin embargo, las células de ganglio tanto de los ratones IL-13$^{-/-}$ como de los silvestres produjeron IL-4 tras la estimulación *in vitro* con LmAg, aunque los silvestres presentaron niveles significativamente mayores de IL-4. Todos estos hallazgos demuestran que la IL-13 no está involucrada como mediador de la susceptibilidad a *L. mexicana*. Además, estos hallazgos también indican que la IL-4 y no la IL-13 es la citocina preferencialmente involucrada en la patogénesis de la infección cutánea por *L. mexicana*.

DISCUSIÓN GENERAL

Los modelos de infecciones parasitarias han sido muy útiles para caracterizar y demostrar el papel de las subpoblaciones de linfocitos Th en la regulación de la respuesta inmune *in vivo*. Muchos de los parásitos conocidos inducen respuestas inmunes altamente polarizadas, dependiendo de la naturaleza del patógeno y/o del fondo genético del hospedero. En general diversos autores opinan que los parásitos intracelulares inducen respuestas dominadas por Th1. mientras en el otro extremo, los extracelulares generan preferencialmente respuestas dominadas por citocinas tipo Th2 (Jankovic et al, 2001). Esto ha llevado a la generalización y al dogma que respuestas inmunes mediadas por Th1 restringen el desarrollo de enfermedades parasitarias intracelulares (Leishmaniosis, Toxoplasmosis, Trypanosomiasis, Malaria), mientras que las Th2 eliminan las infecciones causadas por helmintos (Jankovic y Sher, 1996). No obstante que existen claras evidencias de que en varias infecciones por helmintos, principalmente aquellos cuyo ciclo de vida está restringido al tracto gastrointestinal, la respuesta tipo Th2 es esencial para eliminar a estos parásitos (*N. brasiliensis, Trichinella spiralis*) donde la respuesta efectora es STAT6 dependiente y puede ser mediada a través de células cebadas y producción de mucus en el intestino (Urban et al, 2000; Finkelman et al, 2000). Sin embargo, existen también otras infecciones por helmintos en las cuales esta respuesta no es efectiva para eliminar al parásito, principalmente en aquellos cuyo ciclo de vida es más complejo y no se limita al tracto gastrointestinal. El mejor ejemplo es el de *S. mansoni*, en el cual la fuerte respuesta tipo Th2 generada, no elimina al parásito sino que favorece el proceso patológico de la infección (Fallon, 2000). El otro ejemplo es el de *Taenia crassiceps*, en el cual se ha demostrado que inicialmente existe una respuesta tipo Th1 que posteriormente es desplazada por una Th2, lo que finalmente favorece la colonización parasitaria (Terrazas et al, 1998).

En este trabajo se abordaron dos aspectos importantes en la inmunoregulación de enfermedades parasitarias por helmintos; el primero es el hecho de que una respuesta tipo Th2 no necesariamente es protectora en helmintiasis, y el segundo es que las células presentadoras de antígenos pueden ser fuertemente afectadas por la

presencia de estos parásitos e influir en el inicio, desarrollo y sostenimiento de respuestas inmunes tipo Th2.

Los hallazgos reportados aquí establecen claramente que una respuesta tipo Th1 es esencial para generar protección en contra de *T. crassiceps*, pues al contrario de lo que sucede en otras infecciones por helmintos, la ausencia de la molécula de señalización de IL-4 e IL-13, STAT6, generó alta resistencia a la infección en nuestro modelo. Estos hallazgos están en claro contraste con aquellos recientemente reportados por Urban et al. (2000), donde ratones $STAT6^{-/-}$ no pudieron eliminar al parásito intestinal *T. spiralis*. Tales diferencias pueden explicarse por el sitio donde se desarrolla la infección (Nabors et al, 1995). Aquí, nosotros encontramos en los animales $STAT6^{-/-}$ una marcada asociación entre altos niveles de IL-12, IFNγ y óxido nítrico con una disminución de más del 90% de la carga parasitaria de *T. crassiceps*. Por el contrario, los animales silvestres desarrollaron una respuesta inmune altamente polarizada hacia Th2, con una elevada producción de IgE e IgG1, IL-4 e IL-13 concomitantemente con altos números de parásitos en la cavidad peritoneal. Lo anterior definitivamente descarta una participación primordial de la IgE en la eliminación de *T. crassiceps*, pues los animales $STAT6^{-/-}$ no produjeron este tipo de anticuerpo. Este hallazgo es contrario también al dogma de que la IgE es esencial para eliminar helmintos (Capron, 1991).

Con el propósito de evaluar directamente el alcance de otras citocinas en los procesos de polarización de los fenotipos Th1 o Th2 así como para tratar de definir más claramente su participación en la resistencia a la cisticercosis por *T. crassiceps*, se estudió la respuesta celular T-antígeno específica contra el parásito en ratones genéticamente deficientes tanto en la citocina pro-inflamatoria conocida como factor inhibidor de la migración de macrófagos (MIF) como en IL-12.

MIF ha sido asociado con fuertes respuestas inflamatorias (Bozza et al, 1999), y tiene un papel fundamental en la susceptibilidad al choque endotóxico, donde coadyuva a mantener altos niveles de TNFα, IL-12 y NO. Dado que los animales $STAT6^{-/-}$ habían presentado elevados los niveles de estos compuestos, sugiriendo una participación importante de los mismos en el control de la cisticercosis, se decidió realizar el experimento con ratones $MIF^{-/-}$ para confirmar la participación de estos

últimos en la resistencia a *T. crassiceps*. Los datos observados demostraron que los niveles de anticuerpos y varias de las citocinas producidas por los linfocitos de bazo en respuesta a los antígenos específicos de *T. crassiceps* fueron ligeramente alterados en los ratones $MIF^{-/-}$, comparando con los animales silvestres. Sin embargo, la carga parasitaria se triplicó en estos ratones $MIF^{-/-}$. Esto sugería la participación de otras células en el control de la infección. De este modo se decidió analizar aquellas células que estaban en más cercano contacto con los parásitos, que eran los macrófagos peritoneales. Al analizar la respuesta de estas células encontramos que la producción de IL-12, TNF-α y NO en los ratones $MIF^{-/-}$ estaba significativamente inhibida, en comparación con los animales silvestres y $STAT6^{-/-}$, pero por el contrario estas mismos macrófagos produjeron altos niveles de IL-6, una citocina que se le ha asociado con una posible actividad de polarizar la respuesta inmune hacia Th2 (Rincón et al., 1997). En conjunto estos datos sugieren que los macrófagos tienen un papel regulador y posiblemente efector en esta infección. Esto fue consistente con los resultados de los ratones deficientes en MIF infectados con *L. major*, los cuales resultaron más susceptibles a esta infección, cuya resolución es Th1-dependiente (Satoskar et al, 2001).

Con estos nuevos datos, la idea de que citocinas relacionadas con una respuesta tipo Th1 eran necesarias para eliminar al cisticerco, estaba más fuertemente fundamentada. Con esto en mente se decidió realizar un último ensayo para eliminar cualquier duda. Así, conociendo que una de las citocinas más claramente relacionadas con el inicio y mantenimiento de una respuesta tipo Th1 es la IL-12 (Afonso et al., 1994; Park y Scott, 2001), y que se había detectado elevada en los animales $STAT6^{-/-}$ y baja en los silvestres con una relación inversa con respecto al número de parásitos encontrados. El experimento crucial para dejar fuera de dudas la participación primordial de esta citocina en la protección contra cisticercosis era o bien neutralizar la IL-12 o utilizar ratones deficientes en IL-12. Como es conocido que la neutralización de citocinas con anticuerpos monoclonales es transitoria y no reflejaría el efecto a más largo plazo de la ausencia de IL-12, se optó por utilizar ratones deficientes en IL-12. Después de 8 semanas de infección observamos un incremento de 100% en la susceptibilidad a *T. crassiceps* en los

ratones genéticamente deficientes en IL 12, lo que confirmaba el papel fundamental de esta citocina para desarrollar protección en contra de esta parasitosis a través del desarrollo de respuestas polarizadas hacia Th1.

De manera interesante, los animales infectados en estos experimentos, no mostraron niveles de IL-18 significativamente alterados. La IL-18 es una citocina producida por macrófagos que se ha propuesto podría influir en el desarrollo de respuestas tipo Th1 durante infecciones parasitarias (Cai et al., 2000), lo que confirmó a la IL-12 como esencial para generar una respuesta tipo Th1, aún en ausencia de IL-18. Esta idea es fuertemente apoyada por los resultados obtenidos en la infección de ratones IL-18$^{-/-}$ con *L. major*, donde se demostró que esta citocina no juega un papel crítico para el desarrollo de la respuesta inmune protectora, dado que los ratones deficientes en IL-18 fueron capaces de resolver la infección a la semana 12 (Monteforte et al, 2000).

En este sentido la generación de la respuesta inmune por el helminto *T. crassiceps* sale del concepto establecido de que los helmintos son eliminados por una respuesta tipo Th2; al contrario, la inmunología de la infección por *T. crassiceps* tiene características muy similares a la de los parásitos intracelulares *L. major* y *L. mexicana*, donde se requiere de una respuesta tipo Th1 para su eliminación.

En conjunto todos los resultados obtenidos en este trabajo de tesis muestran que en la infección murina por *T. crassiceps*, de igual manera que en la infección por *L. major* y *L. mexicana*, la respuesta inmune protectora es tipo Th1, dependiente de IL-12 (pero independiente de IL-18), dado que en los tres modelos de infección parasitaria, la ausencia de IL-4 y/o STAT6 induce la producción de niveles muy altos de IL-12, que hace al hospedero resistente a estas infecciones, con una participación importante de MIF como activador de macrófagos capaces de producir óxido nítrico e IL-12 (fig. 3). Este hecho en común apoya la hipótesis de que la inhibición del desarrollo de la respuesta inmune protectora es orquestada por la IL-4 a través de la vía de señalización STAT6. Así, nosotros proponemos que la susceptibilidad se asocia a la producción de las citocinas IL-4 e IL-13, que podrían generar una disminución de los niveles de oxido nítrico e IL-12 producidos por los macrófagos que

podrían ser una de las poblaciones efectoras en la eliminación de *T. crassiceps* (Fig. 4).

Por otro lado, una cuestión crítica y que no se ha resuelto hasta ahora concierne a los eventos tempranos que finalmente dirigen la polarización de la respuesta inmune hacia Th1 versus Th2 *in vivo* (Jankovic et al, 2001). Recientes descubrimientos sugieren que diferentes funciones de las células presentadoras de antígeno (APC), además de su producción de citocinas, podrían ser elementos clave en el proceso de diferenciación Th1/Th2. De los muchos factores que participan en el proceso de diferenciación de las células $TCD4^+$ vírgenes hacia el fenotipo Th1 ó Th2, la presencia de IL-12 o IL-4 respectivamente, al momento de la activación de las células $TCD4^+$ es considerado como el paso primordial para tal diferenciación (Tarleton et al., 2000; Satoskar et al., 2000). Recientemente, se ha descrito que ciertos parásitos intracelulares (*T. cruzi, T. gondii, L. major*) pueden, a través de sus productos, o por sí mismos, disparar la producción de IL-12 por las APC (básicamente células dendríticas), lo cual es congruente con la idea de que IL-12 es un determinante crucial para la polarización de Th1 durante la infección con estos agentes (Aliberti et al 2000, Bliss et al, 1999; Zumbuschenfeldi et al, 1997).

La idea de que los agentes patógenos pueden inducir la producción de citocinas por diversas células a través de sus productos metabólicos está fuertemente sostenida por diversos reportes recientes, que proponen a una familia de receptores trasmembranales, conocidos como Toll-like receptors (TLRs), como los elementos responsables del reconocimiento inmune innato (tanto en mamíferos como en *Drosophilla*) de los productos altamente conservados del metabolismo de los patógenos microbianos (PAMPs), como son el lipopolisacarido (LPS), peptidoglicano (PGN), acidos lipoteicoicos (LTA) y otros componentes de la pared celular microbiana (Medzhitov R et al., 1997). Una característica común de los PAMPs es que son producidos únicamente por microorganismos y no por las células del hospedero, así la detección de los PAMPs por los TLRs puede representar un paso en el reconocimiento inmune de lo propio versus lo no propio.

Sin duda alguna falta mucho para definir claramente la participación de los TLRs en la activación de las respuestas efectoras (Th1 vs Th2) de la inmunidad

adaptativa. Sin embargo existen algunos antecedentes que sugieren que los TLRs expresados sobre las células presentadoras de antígeno podrían regular algunas de las señales accesorias requeridas para la activación de la respuesta inmune adaptativa, como son las moléculas coestimuladoras y las citocinas. Esto podría darse a través del reconocimiento de los PAMPs con la consecuente activación de las respuestas inmunes adaptativas efectoras Th1 vs Th2. Esta idea está fuertemente reforzada por los experimentos donde la ausencia de MyD88 en ratones (una proteína que media la señal de transducción por los TLRs) los hace defectuosos en la activación antígeno-especifica de las células Th1, pero no para desarrollar respuestas inmunes tipo Th2 (Schnare et al.. 2001).

Por otro lado, recientemente se le ha dado gran importancia a la inducción de CCR5 (receptor de las quimiocinas MIP-1 y RANTES) como parte del proceso inicial de polarización hacia Th1 mediado por IL-12 (Sato et al, 1999; Aliberti et al 2000; Iwasaki et al 2001), de modo que aparentemente existe una co-dependencia CCR5/IL-12 para una eficaz polarización hacia Th1. Con respecto a estos procesos en el desarrollo de la respuesta en infecciones por helmintos, poco o nada es conocido. Además de las células dendríticas como las células maestras en la presentación de antígeno, existen otras dos poblaciones de células bien conocidas como APC: estas son las células B y los macrófagos. Aquí nosotros observamos que los marcadores de membrana en macrófagos cambiaban conforme la infección avanzaba, lo que nos pareció interesante, sobre todo en el marco de la regulación de la respuesta inmune por helmintos, donde se ha sugerido una participación fundamental de los macrófagos (Loke et al, 2000; Allen y Loke, 2001).

De acuerdo con recientes nomenclaturas los macrófagos pueden asociarse a dos poblaciones claramente distinguibles. Una es la de macrófagos que son clásicamente activados por respuestas tipo Th1, básicamente por el IFN-γ (Gordon S., 2003). Estos macrófagos producen altos niveles de IL-12 y NO en respuesta a LPS e IFNγ (Goerdt y Orfanos, 1999), y los "macrófagos alternativamente activados", por las citocinas tipo Th2, IL-4 e IL-13 (Gordon S., 2003). Estos macrófagos "alternativamente activados" presentan un patrón de secreción de moléculas totalmente distinto (Goerdt y Orfanos, 1999). El papel que posiblemente tiene este

tipo de macrófagos es el de supresión (Loke et al 2000) y se han reportado recientemente en filariasis (Allen y Loke, 2001).

De acuerdo con nuestros resultados, la población de macrófagos en la cavidad peritoneal de los ratones infectados con *T. crassiceps* sufren una alteración gradual conforme el tiempo de infección avanza y el número de parásitos se incrementa. Esta alteración se dio a nivel de producción de citocinas: en infecciones avanzadas decayó significativamente la producción de IL-12, mientras se incrementó la de IL-6. Sin embargo, la alteración también se observó a nivel de la expresión de moléculas de superficie, así las más sobresalientes fueron la sobreexpresión de CD23 (marcador de "macrófagos alternativamente activados") y la de CCR5. Contrariamente a lo que se observa en infecciones por parásitos intracelulares (Aliberti et al 2000), en nuestro modelo no hay asociación entre la expresión de CCR5 y altos niveles de IL-12, lo cual podría ser una característica intrínseca de las infecciones por helmintos que apoyaría el hecho de la generación de respuestas tipo Th2 en este tipo de infecciones. Debido a las diferencias fenotípicas entre macrófagos durante el transcurso de la infección, se decidió determinar si estas diferencias podrían reflejarse en su capacidad para inducir respuestas inmunes a nuevos estímulos antigénicos no relacionados con el parásito. De este modo, se usaron macrófagos provenientes de diferentes tiempos de infección para conocer su participación en la polarización de la respuesta inmune. Nuestros resultados claramente indican que estos "macrófagos alternativamente activados" tienen diferente capacidad para inducir una respuesta inmune en células $TCD4^+$, ya que macrófagos con alta expresión de CD23 y CCR5, pero con escasa producción de IL-12 indujeron poca respuesta proliferativa en las células $CD4^+$; en cambio, promovieron la secreción de IL-4 e IL-6. Por el contrario, los macrófagos de animales sanos o de infecciones tempranas con una producción normal de IL-12 y baja expresión de CD23 y CCR5 promovieron una mayor repuesta proliferativa asociada con una mayor producción de IFNγ en las células $TCD4^+$, mientras que no se detectaron niveles importantes de IL-4 en esos co-cultivos. En conjunto, esto indica que los macrófagos pueden ser influidos diferencialmente por la presencia de helmintos, y que tienden a desarrollar un fenotipo de "alternativamente activados". Es

posible que este fenómeno influya en la respuesta inmune de individuos presentando infecciones importantes con helmintos.

El papel de CCR5 en macrófagos es poco conocido (a excepción de su papel como co-receptor del virus del SIDA, Bentwich, 1999), contrario de lo que se conoce de la presencia de este marcador en celulas dendríticas, en células CD4 y en células CD8, en donde en todos los casos se le ha asociado con alta producción de IL-12 e IFNγ (Aliberti et al, 2000; Iwasaki et al, 2001). Por otro lado, la expresión de CCR5 es regulada por sus ligandos MIP-1α y RANTES ya que estos generan la internalización de CCR5 (Mack et al, 2001), de este modo la alta expresión de CCR5 observada en los macrófagos de los animales altamente parasitados podría deberse a la ausencia o escasa producción de estas quimiocinas en cisticercosis. El papel de "macrófagos alternativamente activados" ha sido descrito en filariasis, otra infección por helmintos, donde se le ha asignado un papel supresor de la proliferación celular, pero que mantiene la secreción de citocinas en las células TCD4$^+$ (Allen y Loke, 2001), lo cual correlaciona con nuestros hallazgos. Sin embargo, nuestro trabajo va mas allá, pues demostramos que esta activación diferencial se da paulatinamente y que está correlacionada con la expresión de CD23 y CCR5, y que el desarrollo de estos "macrófagos alternativamente activados" es totalmente dependiente de IL-4 e IL-13, ya que los animales STAT6$^{-/-}$ no desarrollaron este fenotipo.

Por otro lado, la baja capacidad para estimular a los linfocitos TCD4$^+$ de los "macrófagos alternativamente activados" no puede atribuirse a la falta de expresión de moléculas coestimuladoras, pues las moléculas más importantes relacionadas con la presentación de antígeno estuvieron altamente expresadas en estos macrófagos, como fue la expresión de MHC-II, B7-2, y CD40. Una posible explicación a la baja inducción de proliferación que presentaron estas células, podría ser que a pesar de expresar altos niveles de MHC-II estas moléculas podrían estar ya completamente cargadas/saturadas con antígenos del parásito, lo cual dificultaría el acceso del nuevo antígeno a las moléculas del MHC-II y no habría una adecuada presentación, de tal modo que los escasos niveles de MHC-II disponibles para el antígeno no relacionado al parásito no serían suficientes para inducir una adecuada proliferación, pero si suficientes para inducir la producción de citocinas antígeno específicas (IL-4 e IL-6).

Esta hipótesis esta apoyada por los resultados de los experimentos hechos con dos microorganismos intracelulares: el primero con la bacteria *Listeria monocytogenes*, en los cuales se demostró que los macrófagos que han ingerido el material bacteriano son deficientes en su capacidad para presentar un Ag proteico (no relacionado) a células T especificas para el mismo (Leyva-Cobian et al. 1988), y el segundo con *Leishmania major*, donde se demostró que los macrófagos parasitados tienen la función presentadora de Ag (no relacionado) alterada vía una interferencia con la carga intracelular de las moléculas del MHC-II con el péptido antigénico (Ulric et al., 1993).

Con estos datos en conjunto nosotros sugerimos que la presencia de "macrófagos alternativamente activados" en helmintiasis tiene un papel crítico en la polarización hacia una respuesta tipo Th2. Probablemente el desarrollo de estos macrófagos esté relacionado con la persistencia del antígeno parasitario o bien por algún antígeno en particular de helmintos. Esto abre la posibilidad de buscar moléculas del parásito que induzcan este tipo de células reguladoras que podrían prevenir una fuerte respuesta tipo Th1 o bien servir como adyuvantes para inducir respuestas tipo Th2. Recientes investigaciones han empezado a considerar que la presencia de helmintos en los países en vías de desarrollo no solo es un problema de salud publica, sino que podrían ser una posible explicación a las menores incidencias de enfermedades autoinmunes mediadas por respuestas tipo Th1 en estos países (Bundy, 1999; Bach 2001). De este modo, en este trabajo de tesis se propone que la regulación de la respuesta inmune por helmintos podría enfocarse, en el futuro, hacia la búsqueda de moléculas de estos parásitos para su posible utilización en la inmunoregulación de otras enfermedades (Black et al, 2001; Elliot et al, 2001). Aquí se muestran evidencias de que esto puede ser posible.

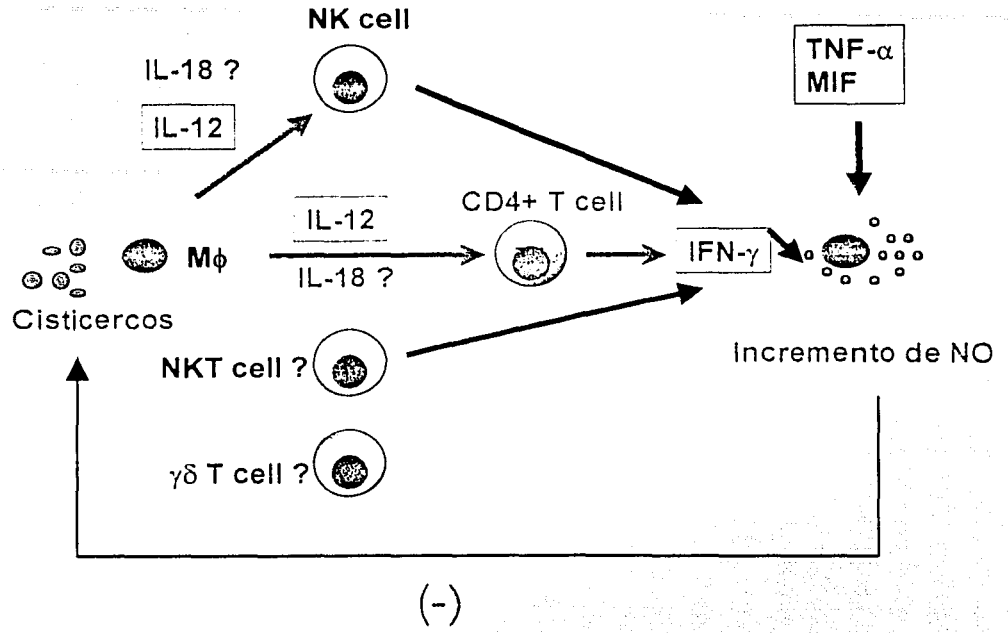


Fig 3. Mecanismo inmunológico de resistencia en la cisticercosis experimental murina. En un primer reconocimiento del parásito, los macrófagos producen niveles altos de IL-12, lo cual favorece la diferenciación de las células TCD4$^+$ hacia el tipo Th1. Las células Th1 producen niveles altos de IFN-γ que favorece la producción de IL-12 y oxido nítrico (NO) por los macrófagos que finalmente restringe el crecimiento del parásito e incluso lo elimina. La presencia de MIF por su parte, parece también favorecer la actividad toxica del macrófago ya que su ausencia lo afecta negativamente.

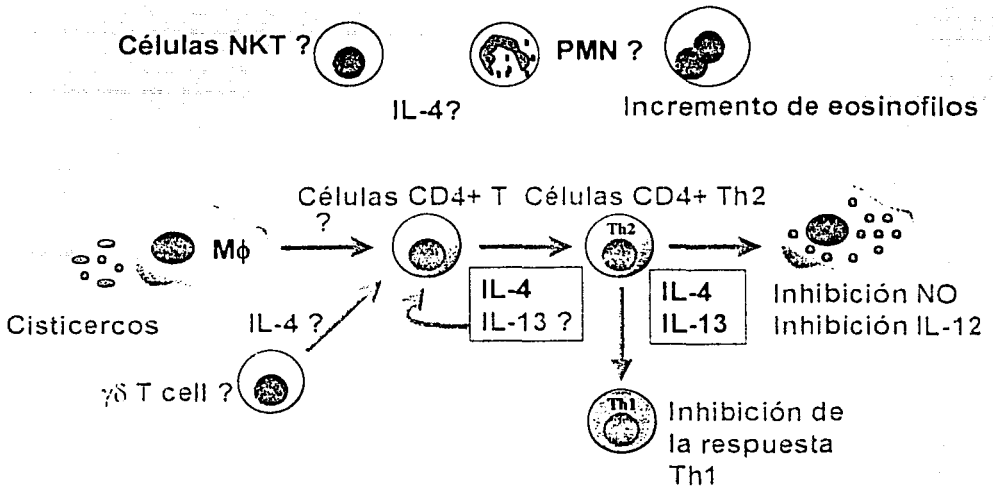


Fig 4. Mecanismo inmunológico de susceptibilidad en la cisticercosis experimental murina. La presencia crónica del cisticerco induce la diferenciación de las células TCD4$^+$ hacia el tipo Th2 a través de STAT-6 probablemente por la presencia de IL-4, aun cuando la fuente inicial no se ha establecido. Las células Th2 producen niveles elevados de IL-4 e IL-13 con un subsiguiente incremento en los eosinófilos e inhibición de la respuesta inmune Th1. La producción de oxido nítrico e IL-12 producidos por los macrófagos se afecta negativamente. Estas condiciones permiten el rápido e incontrolado crecimiento del cisticerco.

CONCLUSIONES

Con los resultados obtenidos de esta tesis se puede concluir lo siguiente:

1) La fuerte inducción de respuestas tipo Th2 generada por la infección con metacéstodos de *T. crassiceps* modula la respuesta inmune y susceptibilidad a infecciones no relacionadas (*T. cruzi*).

2) La respuesta tipo Th1 orquestada por la presencia de IL-12 es esencial para controlar la infección por el helminto *T. crassiceps*.

3) El factor inhibidor de la migración de macrófagos (MIF) juega un papel crítico como mediador de protección contra *T. crassiceps*, su ausencia resulta en una deficiente funcionalidad de los macrófagos.

4) La vía de señalización mediada por STAT6 es crítica para suprimir la respuesta tipo Th1 requerida para controlar la cisticercosis murina.

5) La infección por el helminto *T. crassiceps* puede inducir diferentes poblaciones de macrófagos con diferentes capacidades de presentar antígeno y con la propiedad de polarizar la respuesta inmune hacia el tipo Th2. Esta podría ser una vía común de los helmintos para polarizar la respuesta inmune ante nuevos retos antigénicos.

6) En el modelo murino de infección cutánea por *Leishmania major*, la IL-18 no es crítica para el desarrollo de la respuesta Th1 protectora, sin embargo parece estar involucrada en el control temprano de la lesión generada por la infección.

7) En el modelo murino de infección por *Leishmania major*, MIF juega un papel crítico en el desarrollo de una respuesta inmune protectora, participando en la actividad leishmanicida de los macrófagos.

8) En el modelo murino de infección por *Leishmania mexicana*, la IL-13 no esta involucrada como mediador de la susceptibilidad como es el caso de la IL-4.

9) En el modelo murino de infección por *Leishmania mexicana*, la IL-12 vía STAT4 es esencial para el desarrollo de la respuesta inmune protectora. La susceptibilidad se asocia a la falta de producción de IL-12 mas que a una falta de respuesta a ésta citocina.

10) La respuesta inmune protectora contra la infección por cisticercos de *T. crassiceps* presenta características mas parecidas a la de una infección por un parásito intracelular, como *Leishmania*, que a la clásicamente atribuida a las infecciones por helmintos. En ambos casos, la IL-12 es crucial para el desarrollo de una respuesta inmune protectora mediada por Th1. La IL-4, a través de la vía STAT6, es crucial en el desarrollo de la susceptibilidad mediada por una respuesta tipo Th2. En los dos modelos de infección el mecanismo por el cual el parásito regula negativamente la respuesta Th1, posiblemente sea, a través de inhabilitar a los macrófagos del hospedero para producir IL-12 así como MIF.

Estas observaciones contrastan con el dogma de que organismos extracelulares, como los helmintos, son controlados por respuestas mediadas por células T $CD4^+$ que se diferencian hacia Th2.

APENDICE I

Artículos relacionados con los mecanismos de polarización de la respuesta inmune en la infección murina por *Taenia crassiceps*

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Susceptibility to *Trypanosoma cruzi* is modified by a previous non-related infection

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SUMMARY

Helminth infections are frequently massive, chronic and strong inductors of Th2-type cytokines. This implies that infection by such parasites could alter the susceptibility to subsequent infections by other pathogens, particularly intracellular parasites. We therefore explored whether a persistent infection, caused by Taenia crassiceps *cysticerci, in BALB/c mice could affect susceptibility to a later infection by* Trypanosoma cruzi. *We found that the presence of the cysticerci indeed modified the immune response and the susceptibility to* T. cruzi, *and that these modifications depended on the time-course evolution of the initial infection. Coinfection with the protozoan in the early stages of the helminth infection, induced a delay on the onset of parasitaemia, early specific production of IFN-γ and high specific production of IL-4. A significant increase in susceptibility to* T. cruzi *was observed only when mice were coinfected in late stages when the helminth load is greater and a Th2 type response against it is predominant. The* in vitro *specific response to* T. cruzi *antigens was then characterized by low levels of both IFN-γ and IL-4. These findings suggest that chronic helminth infections could potentially have a significant influence over the immune response and hence susceptibility to other pathogens.*

Keywords *coinfection, susceptibility to* T. cruzi, *helminth infection*

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INTRODUCTION

A large proportion of the worldwide human population lives in developing countries, where helminth infections represent a common cause of disease (Maizels et al. 1993). Individuals who are born and live in populations with inadequate sanitary conditions are exposed to massive helminth infections. In rural populations, these kind of diseases are commonly associated with those caused by intracellular micro-organisms such as *Mycobacterium tuberculosis*, *Plasmodium* sp. and *Trypanosoma cruzi*. The natural coexistence of two or more pathogens infecting an individual, implies that the presence of one of them could (nonspecifically) alter the susceptibility to subsequent infections by other pathogens.

The protective response against some infectious agents, mainly viruses and intracellular parasites is mediated by CD4 + Th1 cells (producers of IFN-γ and IL-2, Mossman *et al.* 1986, Clerici & Shearer 1994, Reiner & Locksley 1995) but not Th2 cells (producers of IL-4, IL-5, IL-6 and IL-10, Hermanek *et al.* 1993, Reiner & Locksley 1995). In contrast, a Th2-dependent response can protect against some extracellular parasites (e.g. *Trichuris muris*, Pearce & Reiner 1995).

Most helminth infections induce a Th2 type response (Svetic *et al.* 1993; Lawrence *et al.* 1995; Pearce & Reiner 1995). This could account for any increase in susceptibility to other pathogenic agents against which a Th1 response is required, or inversely, account for any reduction in susceptibility to those pathogenic agents against which a Th2 response is necessary. Examples of both cases have been found in mice infected with *Schistosoma mansoni*. The polarization of the immune response to a predominant Th2-type can alter the ability to respond to a nonparasitic antigen (sperm whale myoglobin, Kullberg *et al.* 1992), as well as to eliminate vaccinia virus (Actor *et al.* 1993) or, in contrast, increase resistance to another helminth (*T. muris*) against which a Th2-dependent response is

protective (Curry *et al.* 1995). The observed nonspecificity of immune modulation suggests that other helminth infections could also influence the susceptibility to other important pathogens such as intracellular parasites. However, this hypothesis has not been directly explored until now.

As with many other helminths, infection with the cysticerci of *Taenia crassiceps* in BALB/c mice induces a Th2-type immune response, which is not clearly seen until the eighth week after infection. Before that, an initial and gradually declining Th1-type cytokine profile is observed (Terrazas *et al.* 1998).

In order to address the questions of (a) whether *T. crassiceps* infection of BALB/c mice could affect the susceptibility to a latter coinfection with an intracellular parasite such as *Trypanosoma cruzi*, and (b) whether the changing environment regarding number of parasites and cytokine profile induced by *T. crassiceps* infection may differentially influence the outcome of the coinfection with *T. cruzi*, we compared the *T. cruzi* parasitaemia and the *in vitro* cytokine production in BALB/c mice (a) solely infected with *T. cruzi* and (b) coinfected at different times after the initial *T. crassiceps* infection.

We found that the presence of *T. crassiceps* cysticerci modified the susceptibility and the immune response to *T. cruzi*, depending on the time-course evolution of the initial infection. The main implication of these findings is that chronic, massive helminth infections could potentially modify both the immune response and susceptibility to other pathogens.

MATERIALS AND METHODS

Mice

Inbred female BALB/c mice, originally from Jackson Laboratories (Bar Harbor, Maine, USA) were used. They have been maintained in controlled conditions in our animal facilities for more than 20 generations.

Parasites and infections

(A) Metacestodes of *Taenia crassiceps* (ORF) were harvested from the peritoneal cavity of female BALB/c mice after 2–4 months of infection. The cysticerci were washed four times in phosphate-buffered saline (PBS, 0·15 M NaCl, 0·01 M sodium phosphate buffer, pH 7·2) and selected for infection. Experimental infection was achieved by intraperitoneal (ip) injection with 10 small (approximately 2 mm in diameter) nonbudding cysticerci of *T. crassiceps* suspended in 0·3 ml PBS per mouse.

B) *Trypanosoma cruzi* Ninoa strain was obtained from a Mexican patient in 1978 (Monteón *et al.* 1996); these parasites have been propagated in BALB/c mice by subsequent i.p. passages. Single infection and coinfection were performed with this strain of *T. cruzi*, and were achieved by i.p. injection with 10^3 freshly collected blood-stream trypomastigotes in PBS-Alsever's solution per mouse. The coinfection with *T. cruzi* was performed at 2, 4, 8, or 12 weeks after the infection with *T. crassiceps*. Sex-and age-matched uninfected mice, and as well as mice infected only with *T. cruzi* were used as controls. The level of *T. cruzi* parasitaemia was monitored at several days after inoculation by counting the number of parasites in 5 μl of blood drawn from the tail vein; data are reported as the number of parasites per ml (Carlier *et al.* 1987; Cardillo *et al.* 1996).

Soluble antigen from *Trypanosoma cruzi*

T. cruzi extract was obtained as described elsewhere (Castellani *et al.* 1997). Briefly, epimastigotes of *T. cruzi* were maintained by sequential culture in LIT medium (liver infusion tryptose, Difco, Detroit, MI, USA); epimastigotes were obtained, washed three times in PBS and centrifuged at 5000 r.p.m. for 15 min. Protease inhibitors were added (0·1–2 μg/ml Aprotinin; 0·5–2 mM EDTA; 1–5 mM PMSF; 1 μg/ml Pepstatin; 50 μg/ml TLCK; all from Sigma, St. Louis, MO, USA), parasites were sonicated six times for 10 s at 50 watts. Parasite destruction was determined using a microscope. Parasite extracts were then centrifuged at 10 000 r.p.m. for 30 min to separate the soluble fraction which was kept at – 70°C until used. Protein concentration was determined by the Lowry method (Lowry *et al.* 1951).

Cell preparations and culture conditions

After infections and coinfections, mice were bled by cardiac puncture and subsequently killed by cervical dislocation. Spleens were removed in sterile conditions from infected and control mice. Spleen cells were obtained by mincing and filtering, washed and resuspended in culture medium of RPMI 1640 supplemented with 10% foetal bovine serum, 100 units of penicillin/streptomycin, 2 mM glutamine, 25 mM HEPES buffer, and 1% non essential amino acids (all from GIBCO, BRL Grand Island, NY, USA).

Splenocytes were suspended at 5×10^6 cells/ml in the same medium. One hundred μl of the cell suspensions were placed into 96-well flat bottom culture plates (Costar, Cambridge, MA, USA) and stimulated with either 100 μl of concanavalin-A (Con-A; Sigma, St. Louis, MO, USA) mitogen solution (2 μg/ml), or with soluble extract of *Trypanosoma cruzi* (15 μg/ml). Plates were then incubated at 37°C and 5% CO_2 during 72 h or 6 days with Con-A or *T. cruzi* antigen, respectively. Eighteen h prior to culture termination, 0·5 μCi of tritiated thymidine (methyl-3H

TDR, sp. act. 247·9 GBq/mmol, NEN, Boston, MA, USA) were added to each well. After further incubation for 18 h, splenocytes were harvested onto glass filter papers and processed for liquid scintillation counting.

Evaluation of cytokine production *in vitro*

Cell suspensions of lymphoid cells prepared as described above were diluted in 10% FBS-supplemented RPMI-1640 to give 5×10^6 cells/ml. Final 1 ml cell suspensions were placed in each well of a 24-well plate (Costar) and incubated with 2 μg/ml of Con-A for 48 h at 37°C and 5% CO_2, or with 15 μg/ml of soluble antigen from *T. cruzi* for 72 h in similar conditions. After centrifugation, supernatants were collected, aliquoted and stored at −20°C until used. The cytokines IFN-γ, IL-4, and IL-10 were measured using sandwich ELISA methods according to manufacturer's instructions (PharMingen, San Diego, CA, USA). The pairs of cytokine-specific monoclonal antibodies and recombinant cytokines were all obtained from PharMingen. IL-2 was measured by a bioassay using CTLL-2 cells as described elsewhere (Gillis *et al.* 1978), using for blocking monoclonal antibodies anti-IL-2 and anti-IL-4 (PharMingen). Recombinant murine IL-2 (PharMingen) was used as standard curve.

Immunoglobulin determination

T. cruzi-specific IgG1, IgG2a and IgG2b levels were also assessed by ELISA. Briefly, 96 well ELISA plates (Costar) were coated with 100 μl/well (5 μg/ml) soluble extract from *T. cruzi* diluted in Tris pH 7·8 buffer. The plates were left overnight at 4°C. They were then washed thoroughly with phosphate buffer saline (PBS) containing 0·05% Tween 20 (PBS-T; Merck, France) and were blocked with PBS supplemented with 1% bovine serum albumin (PBS-BSA) for one h at room temperature (RT). After that, they were incubated in the presence of 1:100 dilution (in PBS-BSA) of each serum sample from normal or from infected mice for two h at RT. After extensive washing with PBS-T, the plates were incubated for 45 min at RT with peroxidase-labelled goat antimouse isotype (anti IgG2b, anti IgG1 and IgG2a at 1/1000 dilutions) (Zymed, San Francisco, CA, USA). Plates were washed and revealed with ABTS-solution (Zymed). Results were expressed as the maximal OD.

Statistical analysis

The statistical significance of the effects of the experimental variables were determined by nonparametric tests: Mann–Whitney U-Wilcoxon Rank, and a *t*-test in the factorial designs, as considered appropriate.

RESULTS

Immune environment induced by *T. crassiceps*

In order to demonstrate the immune microenvironment present at the moment of the coinfection with *T. cruzi*, different assays were performed. The results obtained were very similar to those recently described (Terrazas *et al.* 1998): as the infection of BALB/c mice with the cysticerci of *T. crassiceps* progressed and the number of parasites increased, the immune response was characterized by decreasing proliferation and secretion of IL-2 and IFN-γ by spleen cells in response to Con-A and also decreasing IgG2a specific antibodies production, associated with increasing levels of IL-4 and IgG1 (Table 1), suggesting a Th1 to Th2 shift during this infection.

Modification of the parasitaemia by *T. cruzi*

To determine if a persistent infection caused by a helminth (*T. crassiceps*) can modify the susceptibility to a second

Table 1 Immune response in female mice early and late infected with *Taenia crassiceps*

		Con-A response	Cytokine production (pg/ml)			Antibody production	
Wk of infection	Parasite Intensity	(cpm)	IL-2	IFN-γ	IL-4	IgG2a	IgG1
0	–	118165±15466	964±243	1430±280	420±24	0.003	0.013
2	4.2±1.8	94522±7442	1047±64	1126±138	421±21	0.435	0.131
4	153.3±53	59015±6358	568±87	464±28	468±18	0.164	0.346
8	440.2±117	10818±1567	200±22	539±49	641±72	0.093	1.500
12	1308±327	4892±1259	75±22	254±38	1104±98	0.028	2.200

Cytokine production is in response to Con-A. Specific antibody production against *T. crassiceps* was detected by ELISA (O.D. 405 nm) in sera diluted 1:100. Values represent the mean±SE; *n*=at least six mice per group. SE in antibody production was less than 10%.

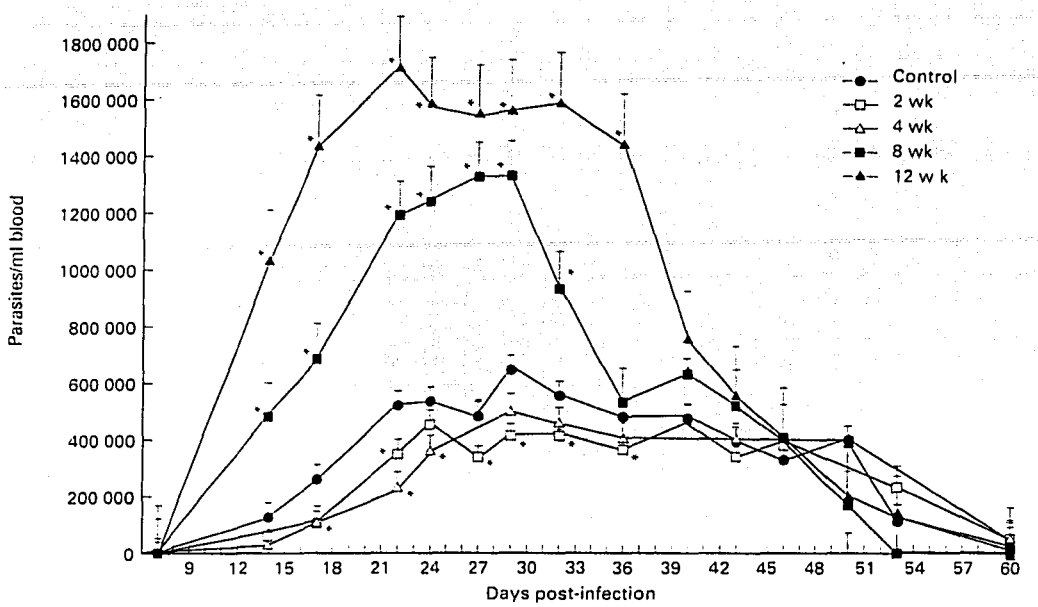

Figure 1 Evolution of parasitaemia after i.p. infection of mice with 1000 trypomastigotes of *T. cruzi*. Control mice were infected only with *T. cruzi*. Coinfected mice were initially infected with ten cysticerci of *T. crassiceps* and after 2, 4, 8 or 12 week were coinfected with *T. cruzi*. 2 wk, 4 wk, 8 wk and 12 wk refer to the week of coinfection after the original *T. crassiceps* infection. Data shown represent mean ± SE of at least nine mice per data point, corresponding to three different experiments. $^*P < 0.05$ with respect to control values obtained the same day.

nonrelated parasite such as *T. cruzi*, we infected BALB/c mice i.p. with ten cysticerci of *T. crassiceps*. These animals were coinfected with 1000 trypomastigotes ip of *T. cruzi* Ninoa strain 2, 4, 8 or 12 weeks after the initial infection. The evolution of the parasitaemia following this second infection was evaluated at different days during two months, and compared to that of mice solely infected with *T. cruzi*.

Figure 1 shows that when coinfection was performed at early times (two and four weeks after the primary infection), the coinfected mice had a significant delay ($P < 0.05$) of one week in the initiation of parasitaemia by *T. cruzi*, but finally presented the same maximal level of parasites as the control mice (approximately 500 000/ml). In contrast, when infection with *T. cruzi* was initiated at latter stages of the infection with *T. crassiceps* (eight and 12 weeks), at the time when a predominant Th2-type cytokine environment is expected (Terrazas *et al.* 1998 and Table 1), the coinfected mice had an early and dramatic increase in susceptibility to *T. cruzi*. The increase in the number of parasites/ml blood was significant from days 14 or 17 to day 29 of the coinfection. Statistical differences with the control at the maximal level of parasitaemia were $P = 0.004$ and $P = 0.005$ when coinfected at eight and 12 weeks, respectively (Figure 1). These results show that the number of blood-circulating trypomastigotes varied as a function of the development of the infection with *T. crassiceps*.

Proliferative response to Con-A of spleen lymphocytes

To examine the cellular proliferative response at different stages of the *T. cruzi* parasitaemia, spleen cells from non-infected (normal control), as well as from *T. cruzi* infected (control) and *T. crassiceps* and *T. cruzi* coinfected mice were obtained at day 9 (the beginning of the parasitaemia), at day 27 (the maximal level of the parasitaemia in the control mice) and at day 60 (when circulating parasites have disappeared). Lymphocytes were then stimulated with Con-A and the proliferative response was measured by tritiated thymidine incorporation (^{3}H-TDR) in a 72-h culture.

Figure 2 shows that lymphocytes from *T. cruzi* infected (control) mice had a significant decrease in the proliferative response to Con-A at the three different time-periods evaluated ($P < 0.05$) with respect to uninfected mice. In early coinfected mice (two and four weeks), the lymphocyte

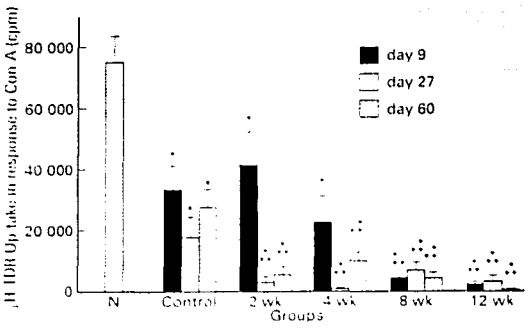

Figure 2 Lymphocyte proliferation in freshly isolated spleen cells upon stimulation with Con-A for 72 h. The bars represent the mean incorporation of ^{3}H-TDR ± SE of triplicate assays of at least nine mice per group, at days 9, 27 and 60 after infection or coinfection with *T. cruzi*. N = normal non infected mice. Control mice for this experiment were only infected with *T. cruzi*. 2 wk, 4 wk, 8 wk and 12 wk refer to the week of coinfection after the original infection with *T. crassiceps*. $^*P < 0.05$ with respect to normal values, $^{**}P < 0.05$ with respect to control values obtained the same day.

proliferative response to Con-A was similar to that of control singly infected mice only at day 9. However, this response decreases significantly ($P < 0.05$) at days 27 and 60 after coinfection. In late coinfections, lymphocyte proliferative response to Con-A was significantly decreased ($P < 0.05$) since day 9 to day 60 of the parasitaemia with respect to control mice. Thus, coinfection with *T. crassiceps* decreases further the inhibited proliferative response to Con-A of mice lymphocytes seen with *T. cruzi* infections.

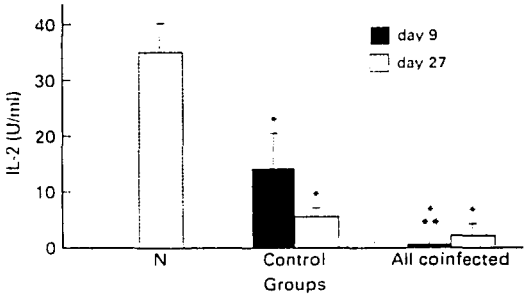

Figure 3 Production of IL-2 by freshly isolated spleen cells upon stimulation with Con-A for 48 h. IL-2 production was estimated by a bioassay using the CTLL-2 cell line. The bars represent the mean cytokine concentration ± SE of triplicate assays of at least nine mice per group, at days 9 and 27 after infection or coinfection with *T. cruzi*. N, normal non infected mice. Control mice for this experiment were only infected with *T. cruzi*. The bar named **all coinfected** represents the mean values of all coinfected mice (coinfected at 2, 4, 8 and 12 weeks after the original infection with *T. crassiceps*). $^*P < 0.05$ with respect to normal values, $^{**}P < 0.05$ with respect to control values obtained the same day.

Cytokine production by spleen cells

Cytokines were measured in the supernatants of cell cultures stimulated with Con-A or with specific *T. cruzi* antigen (total extract).

As previously reported (Kierzenbaum *et al.* 1994), lymphocytes from *T. cruzi* infected mice produced low levels of IL-2 in response to Con-A compared to non infected mice ($P < 0.05$, Figure 3). Minimal IL-2 production was observed at the maximal level of the parasitaemia (day 27, $P < 0.05$, Figure 3). Because there were no significant differences between the different coinfection times, the IL-2 data is presented combined for all coinfected groups. Lymphocytes from all coinfected mice secreted even lower quantities of IL-2 compared to lymphocytes from the singly infected mice ($P < 0.001$, Figure 3). When lymphocytes were stimulated with specific *T. cruzi* antigens, no IL-2 production was observed in any group of mice (data not shown).

For all the other cytokines measured there was no evidence of low production. Lymphocytes of *T. cruzi* infected (control) mice, secreted significantly higher quantities of IFN-γ at day 9 and of IL-4 at day 27, and lower levels of IL-10 at day 27 than noninfected (normal) mice (Figure 4a,b,c). Cells from early coinfected mice (2–4 wk) stimulated with Con-A secreted similar levels of IFN-γ (Figure 4a) and IL-4 (Figure 4b) during all the course of the infection, and higher levels of IL-10 (Figure 4c) at the beginning of the infection than normal and control mice. Similarly, late coinfected mice (8–12 wk) secreted comparable levels of IFN-γ than early coinfected mice during all the infection (Figure 4a), but higher levels of IL-4 at day 9 and lower levels of IL-10 at day 27 than normal and control mice (Figure 4b,c).

When lymphocytes were stimulated with specific *T. cruzi* antigens, *T. cruzi* infected (control) mice secreted significantly lower levels of IFN-γ at the beginning of the infection (Figure 4d, day 9) but higher levels at the peak of the infection (day 27) than normal mice. This control group did not produce specific quantities of IL-4 (Figure 4e), but it did secrete significantly higher levels of IL-10 at day 27 (Figure 4f) than noninfected mice. In contrast, early coinfections induced an early (day 9) specific production of IFN-γ and IL-4, both significantly higher than in the mice solely infected with *T. cruzi* (Figure 4d,e), with no specific production of IL-10 at this day (Figure 4f). This initial cytokine pattern correlated with significantly lower levels of circulating parasites during the third and fourth weeks of the

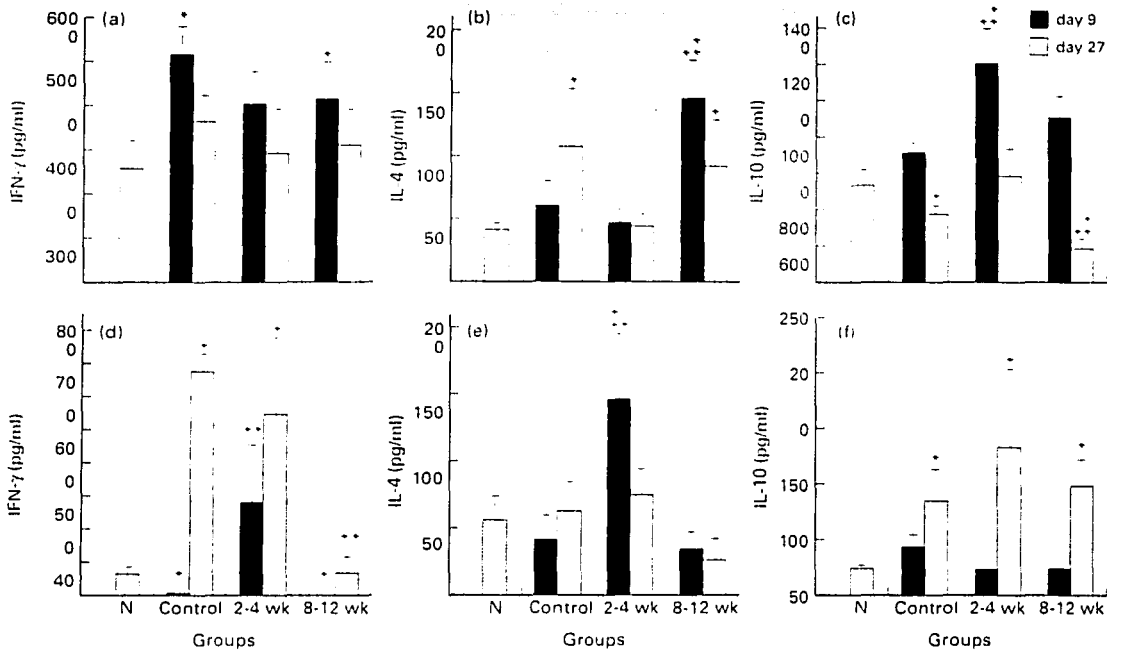

Figure 4 Production of IFN-γ (a, d), IL-4 (b, e) and IL-10 (c, f) by freshly isolated spleen cells upon stimulation with Con-A for 48 h (a, b, c) or specific *T. cruzi* antigens (soluble extract) for 72 h (d, e, f). Cytokine production was estimated by ELISA. The bars represent the mean cytokine concentration ± SE of triplicate assays of at least nine mice per group, at days 9 and 27 after infection or coinfection with *T. cruzi*. N, normal noninfected mice. Control mice were only infected with *T. cruzi*. 2–4 week and 8–12 week refer to early or late coinfection after the original infection with *T. crassiceps* (coinfected at 2 or 4, and 8 or 12 week after infection with *T. crassiceps*). $^{*}P<0.05$ with respect to normal values. $^{**}P<0.05$ with respect to control values obtained on the same day.

coinfection in early coinfected animals compared to the single infected ones (Figure 1). By day 27, the cells of these early coinfected mice still produced high levels of IFN-γ (Figure 4d) in response to *T. cruzi* antigens, but the levels of IL-4 had significantly decreased (Figure 4e), and IL-10 production was increased (Figure 4f), giving rise to a very similar production pattern of these three cytokines (IFN-γ, IL-4 and IL-10) to that of the single infected mice (Figure 4d,e,f). Interestingly, this was associated with statistically comparable numbers of circulating parasites between early coinfected and single infected animals at and beyond this point (Figure 1, days 27–60). In late coinfected mice, we observed a decreased specific production of IFN-γ (Figure 4d) and IL-4 (Figure 4e) at any of the days tested, but IL-10 was produced at similar levels to those detected in singly infected mice (Figure 4f). At day 27 of the parasitaemia, the singly infected mice and the early coinfected mice had a significant production of IFN-γ, not seen in late coinfected mice (Figure 4d), and by this day the late coinfected animals were dramatically more susceptible to *T. cruzi* ($P<0.01$, Figure 1).

Specific antibody production

The presence of three specific antibody isotypes (IgG2a, IgG2b and IgG1) against antigens from *T. cruzi* in the serum of the studied animals were analysed, at three different time points of the parasitaemia: days 9, 27 and 60. At day 9 none of the groups produced any detectable titres of specific anti *T. cruzi* antibodies (data not shown).

As seen in Figure 5a,b, the titres of all isotypes of specific antibodies against *T. cruzi* increased as the infection progresses, IgG2a being the most prominent. With the exception of a lower production of specific antibodies by mice coinfected at the fourth week of the infection with the cysticerci of *T. crassiceps* (Figure 5a), the presence of this

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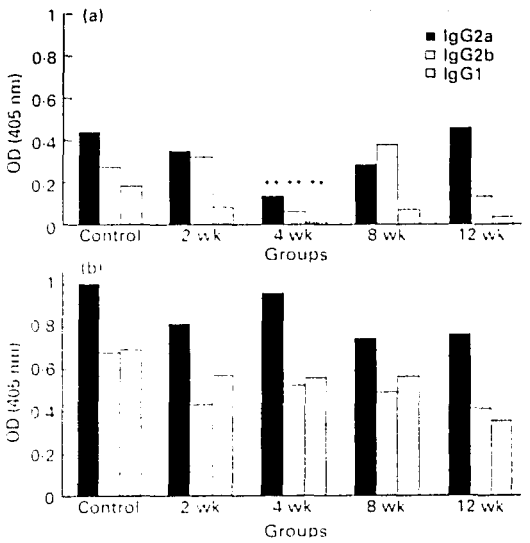

Figure 5 Specific production of IgG2a, IgG2b and IgG1 anti-*T. cruzi* antibodies. All sera were processed by ELISA, using a crude extract of *T. cruzi* as the source of specific antigen. The mean normal value was 0·02. The bars represent the mean antibody concentration of triplicate assays of at least nine mice per group, at day 27 (a) and 60 (b) after infection or coinfection with *T. cruzi*. 2 week, 4 week, 8 week and 12 week refer to the week of coinfection after the original infection with *T. crassiceps*. $^{**}P < 0{\cdot}05$ with respect to control values obtained the same day.

parasite does not seem to affect the antibody response against *T. cruzi*.

DISCUSSION

Our results demonstrate that a primary infection with the cysticerci of *Taenia crassiceps* can affect the susceptibility of BALB/c mice to a subsequent infection (coinfection) with the intracellular parasite *Trypanosoma cruzi*. However, we found that the presence of the cysticerci of *T. crassiceps* by themselves is not enough to render the mice more susceptible to an infection by *T. cruzi*. The modifications induced by the cysticerci of *T. crassiceps* vary as a function of the evolution of this infection (Figure 1). When BALB/c mice were coinfected with *T. cruzi* two or four weeks after the infection with *T. crassiceps* (early coinfection), we observed a transitory but significant delay in the initiation of the parasitaemia with *T. cruzi*. In contrast, when the coinfection was initiated at eight or 12 weeks after the primary infection (late coinfection), the parasitaemia had an early increase, leading to a four times greater parasite load over the single infected mice at the peak of the parasitaemia.

The clear differences in susceptibility to *T. cruzi* as a function of the development of the primary *T. crassiceps* infection, could be related to the variable immune mechanisms sequentially induced by this persistent infection, to a possible immunosuppression generated by it or even to the production of a hypothetical 'growth factor' by the cestode that acts directly on the trypanosome. Arguments against the immunosuppressive explanation are that the infection by *T. crassiceps* cysticerci is not an exhausting one, that is, it does not provoke weight loss, anaemia or lymphopenia (data not shown), and even after very long periods of infection with this helminth, the mice still produce high levels of specific antibodies and of certain cytokines such as IL-10 and IL-6 (Terrazas *et al.* 1998). Furthermore, the spleen cells obtained from coinfected animals produced similar levels of IFN-γ, IL-4 and IL-10 in response to Con-A (Figure 4a,b,c), IL-10 in response to specific *T. cruzi* antigens (Figure 4f), and specific antibodies to *T. cruzi* (Figure 5) compared to singly infected mice. Because there are significant differences in the number of cysticerci already present when early or late coinfections with *T. cruzi* are initiated, we cannot discard the possibility of a 'growth factor' produced by the helminth that would need high concentrations to exert its effects. However, even supposing that such a factor exists its effects would be additive to its effects to those related to the *in vivo* immune environment found at the moment of the coinfection (Terrazas *et al.* 1998 and Table 1). We believe that the variable cytokine environment found after *T. crassiceps* infection exerts an influence over the immune response induced by the second challenge, resembling what happens *in vitro*, where the microenvironment of cytokines present at the time of the antigenic challenge can influence the precursor cells' differentiation towards any of the T CD4 + lymphocyte subpopulations (Maggi *et al.* 1992, Kamogawa *et al.* 1993, Bradley *et al.* 1995, Trinchieri 1995, Wynn *et al.* 1995, Magram *et al.* 1996). The *ex vivo* cytokine responses to *T. cruzi* specific antigens were found to be congruent with this last interpretation, as we found them to be different when the coinfection was initiated at early times compared to when it was initiated at late times after the initial infection with *T. crassiceps*.

In late coinfected animals there was no specific production of IFN-γ which could explain the increase in susceptibility detected in this group, since IFN-γ seems necessary to provide protection against *T. cruzi* (Cardillo *et al.* 1996, Golden & Tarleton 1991, Tarleton 1991, Torrico *et al.* 1991, Silva *et al.* 1992, Hunter *et al.* 1996). However, even a high endogenous production of this cytokine seems insufficient to protect against this parasite (Golden & Tarleton 1991,

Rottenberg *et al.* 1993, Zhang & Tarleton 1996) when it is not accompanied by other cytokines such as IL-4 or even a complex mixture composed of at least IL-2, IL-3, IL-4 and IL-5 (Golden *et al.* 1991). Accordingly, the only stage at which we found no or very low numbers of parasites compared to single infected mice was when IFN-γ and IL-4 were both specifically produced at high levels in early coinfected animals (Figures 1, 4d.e. day 9), whereas a decrease in IL-4 secretion in this same group was related to an increase in the number of parasites (Figures 1 and 4e, day 27). These findings agree with the hypothesis that a mixed response is required to achieve protection against *T. cruzi* infection (Zhang & Tarleton 1996), which could be related to the life cycle of the parasite, where the endogenous production of IFN-γ would be required at least in the intracellular initial phases of *T. cruzi* infection, and IL-4 and other Th2-linked responses (IL-10 and IgG1 antibodies) would be required to control circulating extracellular parasites. The concomitant production of IFN-γ and IL-4 reported in this paper is of interest, because whereas at the clonal level it has been described that IFN-γ and IL-4 synthesis show an inverse correlation (Mossman *et al.* 1986), there are several reports showing this mixed pattern in mice infected with *T. cruzi* (Golden *et al.* 1991, Zhang *et al.* 1996) when measuring secretion of whole cell populations as in this study.

The findings shown in this paper demonstrate that the presence of a parasite that persists for a long period in its host is able to significantly modify the host's susceptibility to other non related pathogen. Although the *T. crassiceps* infection in the mouse could be questioned as a model for a chronic helminth infection in humans, basically because of its location in the peritoneal cavity, the fact is that, as happens with many intraintestinally located helminths (Hermanek *et al.* 1993, Maizels *et al.* 1993, Svetic *et al.* 1993, Pearce *et al.* 1995), this parasite can reach a large biomass and can change the cytokine environment, and hence the possible mechanisms of response. By establishing chronic infections and probably by inducing strong Th2-type responses (Actor *et al.* 1993; Kullberg *et al.* 1992; Svetic *et al.* 1993; Curry *et al.* 1995; Lawrence *et al.* 1995; Kullberg *et al.* 1996), helminths could have a potentially significant influence over the nature of the immune response of infected individuals and hence modify their susceptibility to subsequent infections with other important pathogens, at least those against which a Th1-type response is required, which include *M. tuberculosis* (Marin *et al.* 1995), *L. major* (Reiner *et al.* 1995), *Plasmodium* sp. (Stevenson & Tam 1993; Taylor-Robinson *et al.* 1993) and possibly HIV (Clerici & Shearer 1993; Clerici & Shearer 1994). This is of relevance when it is considered that long persisting parasites such as helminths are widely distributed in many developing countries (Maizels *et al.* 1993), where they coexist with other pathogens that have a greater impact on the mortality rates. The potential risk that whole populations could have an increased susceptibility to dangerous pathogens such as intracellular micro-organisms, should invite study of the possible epidemiological relevance of helminth infections and the impact of controlling them on the incidence or the pathogenesis of other associated infections. The presence of helminth infections could represent a much more important challenge for public health than recognized until now.

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Cutting Edge: Susceptibility to the Larval Stage of the Helminth Parasite *Taenia crassiceps* Is Mediated by Th2 Response Induced Via STAT6 Signaling[1]

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Using STAT6$^{-/-}$ BALB/c mice, we analyzed the role of STAT6-induced Th2 response in determining the outcome of murine cysticercosis caused by the helminth parasite *Taenia crassiceps*. After *T. crassiceps* infection, wild-type BALB/c mice developed a strong Th2-like response; produced high levels of IgG1, IgE, IL-4, as well as IL-13; and remained susceptible to *T. crassiceps*. In contrast, similarly infected STAT6$^{-/-}$ mice mounted a strong Th1-like response; produced high levels of IgG2a, IL-12, IFN-γ, as well as nitric oxide; and efficiently controlled *T. crassiceps* infection. These findings demonstrate that Th2-like response induced via STAT6-mediated signaling pathway mediates susceptibility to *T. crassiceps* and, furthermore, that unlike the case in most helminths, immunity against *T. crassiceps* is mediated by a Th1-like rather than Th2-like response. *The Journal of Immunology*, 2002, 168: 3135–3139.

Cysticercosis is a helminth infection caused by the larvae of the cestode *Taenia solium*, affecting humans and pigs. This disease is considered a public health problem in South America and Asia (1) but has been extended in the past few years to developed countries as shown by case reports published more frequently (2, 3). Cysticercosis in humans results from ingestion of *Taenia* eggs from excreta in the environment. Although cysticerci in muscle may be relatively symptomless, those in brain cause neurocysticercosis, which may clinically manifest as seizures, hydrocephalus, aseptic meningitis, and altered mental status (1, 3).

In the experimental model of murine cysticercosis, infection of inbred mice with *Taenia crassiceps* induces a strong Th2-like response similar to that observed after infection with helminths such as *Nippostrongylus brasiliensis* and *Trichuris muris* (4). Although it is widely accepted that Th2-like response mediates protective immunity against most helminths (5), its role in mediating protection against murine cysticercosis is not clear (6).

Previous studies have found that although *T. crassiceps*-infected mice develop a Th1-like response during the early phase of infection, they eventually develop a Th2 response that is associated with an increase in parasite loads (7). Furthermore, one study found that administration of IFN-γ-neutralizing Abs to *T. crassiceps*-infected mice during the early phase of infection rendered them more susceptible to cysticercosis (8). These findings suggest that whereas Th2-type response may be involved in mediating susceptibility, Th1-type response may play a role in the development of protective immunity against cysticercosis.

Recent studies using STAT6$^{-/-}$ mice have shown that the STAT6-mediated IL-4/IL-13 signaling pathway is critical for Th2 differentiation (9–11). For example, STAT6$^{-/-}$ mice fail to mount a significant Th2 response and cannot control worm burdens after infection with gastrointestinal helminth parasites (12, 13). Conversely, STAT6$^{-/-}$ mice develop a Th1-like response and control infections caused by intracellular protozoan parasites such as *Leishmania mexicana* and *Trypanosoma cruzi* (14, 15), indicating that the STAT6-mediated signaling pathway inhibits development of protective immunity by inhibiting Th1 development.

The purpose of this study was to determine the role of a Th2-type response induced via STAT6-mediated signaling in the outcome of murine cysticercosis caused by the helminth *T. crassiceps*. To approach this question, we compared the course of *T. crassiceps* infection in STAT6$^{-/-}$ BALB/c mice with that in wild-type BALB/c mice. In addition, we analyzed the Ab profiles in sera, cellular responses, and cytokine profile in both spleen cells and peritoneal macrophages. Our data demonstrate that the Th2-type response induced via the STAT6-signaling pathway mediates susceptibility in cysticercosis. They also demonstrate that in the absence of STAT6-mediating signaling, susceptible BALB/c mice develop a Th1 response and control *T. crassiceps* infection.

Materials and Methods

Mice

Six- to 8-wk-old female STAT6$^{-/-}$ and STAT6$^{+/+}$ mice on a genetic BALB/c background were purchased from The Jackson Laboratory Animal Resources Center (Bar Harbor, ME), and maintained in the specific pathogen-free facilities at Harvard School of Public Health animal facilities in accordance with institutional guidelines.

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Parasites and infection

Metacestodes of *T. crassiceps* were harvested under sterile conditions from the peritoneal cavity of female BALB/c mice after 2–4 mo of infection. Mice were infected by i.p. injection with 20 small nonbudding cysticerci and sacrificed at wk 2, 4, 8, and 12 postinfection. Parasites were harvested from the peritoneal cavity and counted.

Cell preparations, culture conditions, and cytokine assays

Spleen cells were obtained and cultured as described previously (7). Briefly, single-cell suspensions were prepared in RPMI 1640 supplemented with 10% FBS, 100 U of penicillin/streptomycin, 2 mM glutamine, 25 mM HEPES buffer, and 1% nonessential amino acids (all from Life Technologies, Gaithersburg, MD). Erythrocytes were lysed, and viable cells were adjusted (3×10^6 cells/ml); 100 μl/well were placed into 96-well flat-bottom culture plates (Costar, Cambridge, MA) and stimulated with *T. crassiceps* Ag (TcAg[1]; 25 μg/ml) at 37°C for 96 h. Eighteen hours before culture termination, 0.5 μCi/well [^{3}H]thymidine (NEN, Boston, MA) were added. Cells were harvested and counted using a beta plate counter. Values are represented as cpm.

Supernatants from these cultures were analyzed for IFN-γ, IL-4 (BD PharMingen, San Diego, CA) and IL-13 (R&D Systems, Minneapolis, MN) production by ELISA.

Cytokine and nitric oxide production by peritoneal macrophages.

Peritoneal exudate cells (PECs) were obtained from mice at 2, 4, 8, and 12 wk after *T. crassiceps* infection. PECs were adjusted to 5×10^6/ml in RPMI supplemented and plated in 6-well plates (Costar). After 2 h at 37°C and 5% CO_2, nonadherent cells were removed, and adherent cells were gently scraped using cold PBS and readjusted to 1×10^6/ml. Viability at this point was >90%. These cells constituted >90% of macrophages according to FACS analysis (F4/80$^+$). One milliliter was plated, and cell activation was performed in 24-well plates (Costar) with LPS (5 μg/ml; *Escherichia coli* 111:B4; Sigma Aldrich, St. Louis, MO) followed by incubation for 48 h. IL-6, IL-12 (BD PharMingen), and nitric oxide (Griess reaction) were examined in supernatants. Total PECs were analyzed by cytospin preparation stained with Wright-Giemsa stain (Sigma Aldrich), and 400 cells were counted by slide.

Ab ELISA

Blood was collected from tails of *T. crassiceps*-infected STAT6$^{+/+}$ and STAT6$^{-/-}$ mice. Ag-specific IgG1 and IgG2a levels were determined by ELISA as previously described (16). Results are expressed as the endpoint titer. Total IgE production was detected by Opt-ELISA (BD PharMingen).

Statistical analysis.

Comparisons between STAT6$^{+/+}$ and STAT6$^{-/-}$ groups considered in this work were made using Student's unpaired *t* test. A value of $p < 0.05$ was considered significant. The statistical significance of the sera titers were determined by nonparametric tests using the Mann-Whitney *U*-Wilcoxon rank test.

Results and Discussion

It is widely accepted that the Th2-like response induced via the STAT6-mediated signaling pathway (through IL-4/IL-13 receptors) plays a critical role in mediating protective immunity against most helminths (17–19). For example, STAT6-mediated signaling promotes protective immunity against *Trichinella spiralis* (13) and *N. brasiliensis* (12). In the present study, both STAT6$^{+/+}$ and STAT6$^{-/-}$ mice showed a progressive increase in the parasite numbers in their peritoneal cavities and displayed comparable parasite burdens at 2 and 4 wk after infection with *T. crassiceps* (Fig. 1). Interestingly, as infection progressed, parasite burdens increased significantly in STAT6$^{-/-}$ mice as compared with STAT6$^{+/+}$ mice that successfully controlled the infection by wk 12 postinfection (Fig. 1). These findings demonstrate that STAT6-mediated signaling pathway is involved in pathogenesis of *T. crassiceps* infection.

[1] Abbreviations used in this paper: TcAg, *Taenia crassiceps* soluble Ag; PEC, peritoneal exudate cell.

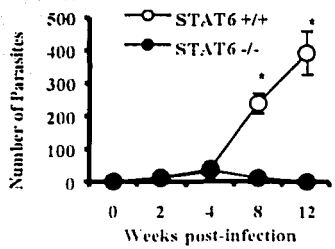

FIGURE 1. STAT6$^{-/-}$ mice efficiently control *T. crassiceps* infection. Course of i.p. *T. crassiceps* infection in STAT6$^{-/-}$ (●) and STAT6$^{+/+}$ (○) mice after infection with 20 cysticerci. Data are expressed as the mean ± SE of 4 mice per group. *, $p < 0.01$ comparing STAT6$^{-/-}$ vs STAT6$^{+/+}$ at the same time point. Similar results were observed in three independent experiments.

Previous studies have demonstrated that the STAT6-mediated signaling pathway prevents development of protective immunity against intracellular parasites such as *L. mexicana* and *Trypanosoma cruzi* by inhibiting Th1 development (14, 15). Furthermore,

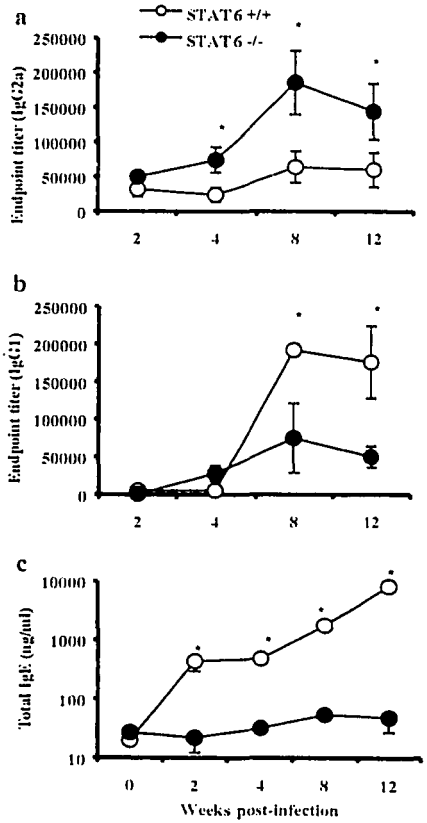

FIGURE 2. Kinetics of Ab production during *T. crassiceps* infection in STAT6$^{-/-}$ (●) and STAT6$^{+/+}$ (○) mice. *a*, Anti-*T. crassiceps*-specific IgG2a; *b*, anti-*T. crassiceps*-specific IgG1; *c*, total IgE. Values are the mean ± SE ($n = 4$ animals) and are representative of three independent experiments. *, $p < 0.05$ comparing STAT6$^{-/-}$ vs STAT6$^{+/+}$ at the same time point.

we previously found that mice treated with IFN-γ and IL-2 during the early course of *T. crassiceps* infection restrict the parasite growth, suggesting that the Th1 response may mediate protective immunity against this parasite (8). Therefore, we measured levels of Th1-associated IgG2a as well as Th2-associated IgG1 and IgE Abs in STAT6$^{-/-}$ and STAT6$^{+/+}$ mice at different time points after infection with *T. crassiceps*. During wk 2 and 4 postinfection, *T. crassiceps*-infected STAT6$^{+/+}$ and STAT6$^{-/-}$ mice displayed comparable titers of TcAg-specific IgG1, but IgG2a Abs were significantly higher in STAT6$^{-/-}$ mice as early as 4 wk after infection (Fig. 2, *a* and *b*). By wk 8 postinfection and thereafter, STAT6$^{+/+}$ mice displayed significantly higher titers of TcAg-specific IgG1 than did STAT6$^{-/-}$ mice, which produced significantly more TcAg-specific IgG2a (Fig. 2, *a* and *b*). Similar differences were observed in the levels of total IgG1 and IgG2a (data not shown). Although Th2-associated IgE has been shown to play a role in mediating immunity against helminths (20, 21), we found that *T. crassiceps*-infected STAT6$^{-/-}$ mice efficiently controlled parasite burdens despite producing significantly lower levels of IgE as compared with similarly infected STAT6$^{+/+}$ mice, suggesting that IgE may have a limited role in mediating protective immunity against *T. crassiceps* (Fig. 2*c*).

The spleen cells from *T. crassiceps*-infected STAT6$^{+/+}$ and STAT6$^{-/-}$ mice displayed different patterns of proliferative responses after in vitro stimulation. Whereas TcAg-stimulated splenocytes from STAT6$^{+/+}$ mice displayed higher proliferative responses during the early phase of infection, those from STAT6$^{-/-}$ mice developed significantly stronger responses in chronic infections as compared with wild-type mice (Fig. 3*a*). At wk 4, 8, and 12 postinfection, TcAg-stimulated spleen cells from STAT6$^{-/-}$ mice produced greater levels of IFN-γ than did those from STAT6$^{+/+}$ mice (Fig. 3*b*). In contrast, as the infection progressed, TcAg-stimulated spleen cells from STAT6$^{+/+}$ mice produced significantly more IL-4 and IL-13 than those from STAT6$^{-/-}$ mice, which produced low levels of these cytokines only during early phase of infection (Fig. 3, *c* and *d*), demonstrating the inability of these mice to maintain a sustained Th2-type response (10, 11). Interestingly, higher levels of Th2-type cytokines in STAT6$^{+/+}$ mice were associated with higher parasite loads. Taken together, these findings suggest that unlike other helminths, the Th2-type response is not essential for mediating protective immunity against *T. crassiceps*. They also indicate that Th2-type response may be detrimental for cysticercosis. Moreover, others using radiation-attenuated vaccine and IL-12 as an adjuvant against *Schistosoma* have shown that a Th1-like response can mediate protective immunity in this model (22, 23).

Several studies have demonstrated that macrophages play a critical role in immunity against many intracellular pathogens by their ability to secrete Th1-inducing cytokines such as IL-12 and IL-18 as well as produce NO that is not only microbicidal (24) but also cytotoxic to larvae of *Schistosoma* (25). Although peritoneal macrophages do not adhere in situ to *T. crassiceps* (C. Larralde, unpublished observations), we hypothesized that these cells may be involved in mediating protective immunity against *T. crassiceps* metacestodes in STAT6$^{-/-}$ mice by secreting Th1-inducing cytokines such as IL-12 and by releasing NO. Hence, we analyzed IL-6, IL-12, and NO production by adherent peritoneal macrophages from *T. crassiceps*-infected mice. Macrophages from STAT6$^{-/-}$ mice obtained during the early phase of infection (2 wk) produced levels of IL-12 similar to those from STAT6$^{+/+}$ mice (Fig. 4*a*). In contrast, IL-6 was detected in lower levels in the same supernatants (Fig. 4*b*). As infection progressed, macrophages from chronically infected STAT6$^{+/+}$ mice produced increased levels of IL-6 (Fig. 4*b*), but low levels of IL-12 (Fig. 4*a*). These patterns of macrophage response were opposed to those observed in STAT6$^{-/-}$ mice, which showed a low production of IL-6 but a sustained and significantly higher production of IL-12 in late infections (Fig. 4, *a* and *b*). NO production was maintained in steady levels until the 4th wk after infection in STAT6$^{+/+}$ mice; however, as the infection became more chronic (8–12 wk), the NO levels dropped significantly (Fig. 4*c*). In contrast, macrophages from *T. crassiceps*-infected STAT6$^{-/-}$ mice produced sustained

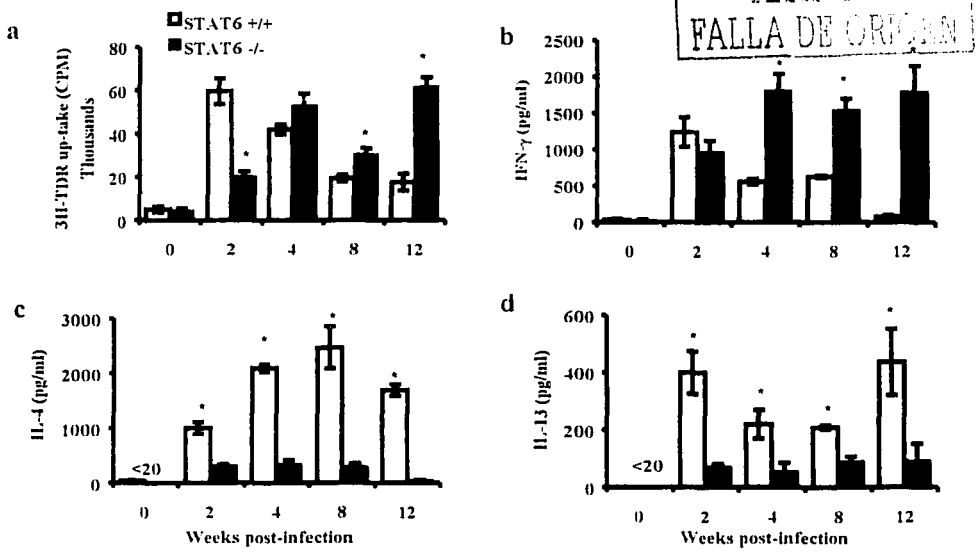

FIGURE 3. Kinetics of in vitro proliferative response and cytokine production by TcAg-stimulated spleen cells from STAT6$^{-/-}$ (■) and STAT6$^{+/+}$ (□) mice. *a*, Ag-specific proliferative response of splenocytes (96 h); *b*, specific IFN-γ; *c*, specific IL-4; *d*, specific IL-13 production by splenocytes after 72 h in vitro stimulation with TcAg (25 μg/ml). Data are expressed as in Fig. 2.

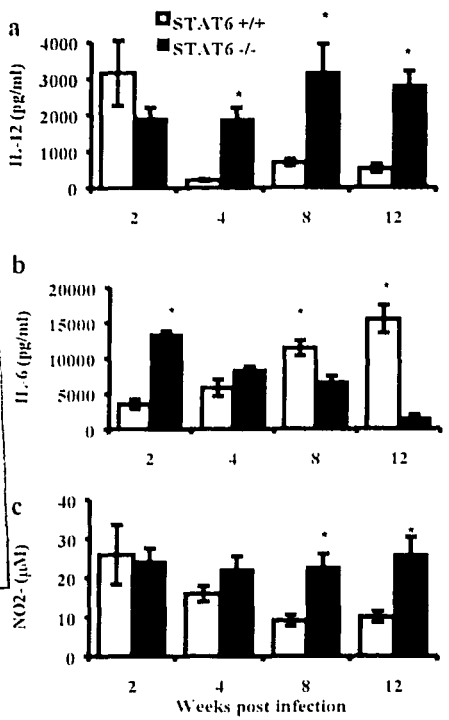

FIGURE 4. Peritoneal macrophages from STAT6$^{-/-}$ and STAT6$^{+/+}$ *T. crassiceps*-infected mice display different responses. Macrophages were obtained at different time points after infection and stimulated with LPS (5 μg/ml) during 48 h; supernatants were analyzed for IL-12p70 (*a*), IL-6 (*b*), and NO (*c*) production. Data are expressed as in Fig. 2.

levels of NO throughout the course of infection (Fig. 4*c*). Taken together, these observations suggest that the STAT6 signaling pathway mediates susceptibility to *T. crassiceps*, at least in part, by inhibiting macrophage IL-12 and NO production. This is supported by previous studies demonstrating that IL-4 and IL-13 inhibit production of IL-12 and NO from macrophages in a STAT6-dependent manner (26, 27). Indeed, reduced IL-6 production in STAT6$^{-/-}$ mice may also contribute to enhancement of Th1 response, given that IL-6 has been shown to play a critical role in differentiation of IL-4 producing CD4^{+} T cells (28).

Eosinophils are considered one of the most efficient effectors cells in several helminth parasitic diseases (21, 29). Therefore, we evaluated the inflammatory infiltrate in the peritoneal cavity of STAT6$^{-/-}$ and STAT6$^{+/+}$ mice in early and late infections. At 2 wk postinfection, STAT6$^{+/+}$ mice recruited up to 11 ± 1.9% of eosinophils, whereas STAT6$^{-/-}$ recruited significantly lower percentages (1 ± 0.2%; $p < 0.05$). At late infection (8 wk), STAT6$^{-/-}$ mice maintained a higher recruitment of eosinophils, rising to 18 ± 1.8%. Conversely, STAT6$^{+/+}$ did not present eosinophils at all (<1%) by this time; instead, they had an increased infiltration of lymphocytes as compared with STAT6$^{-/-}$ mice (25 ± 2.5% vs 7 ± 1.5%, respectively) but similar numbers of macrophages, suggesting a role for these cells in the elimination of the parasite and at the same time ruling out the eosinophils as potential effector cells in murine cysticercosis. These findings oppose the dogma that eosinophils are the key cells that play a critical role eliminating helminths (7, 20, 21, 29). In contrast, our data favor a possible active participation of macrophages in eliminating *T. crassiceps*, perhaps by producing NO. Additionally, we analyzed other cell populations of the peritoneal cavity throughout the infection. Basophils/mast cells were detected in low percentages in STAT6$^{+/+}$ mice at early infections (1.2 ± 0.6%), and lower in late ones (0.2 ± 0.2%). STAT6$^{-/-}$ mice recruited 0.4 ± 0.1% at wk 2 and 0.6 ± 0.4% at late infection. Both STAT6$^{+/+}$ and STAT6$^{-/-}$ mice had comparable numbers of neutrophils (2 ± 0.4% and 3.5 ± 0.9%) throughout the infection. These findings suggest that other effector cells such as basophils/mast cells and neutrophils may not play a significant role in mediating resistance against *T. crassiceps* infection in STAT6$^{-/-}$ mice.

In conclusion, STAT6$^{-/-}$ BALB/c mice mount a strong Th1-like response; produce high levels of IL-12, IFN-γ, and NO; and efficiently control *T. crassiceps* infection. In contrast, STAT6$^{+/+}$ BALB/c mice develop a predominant Th2-like response associated with high levels of IL-4, IL-13, IgG1, IgE, and eosinophilia and display significantly higher parasite loads. Our findings support the hypothesis that STAT6-mediated signaling is critical for the suppression of the Th1 responses required for controlling murine cysticercosis and also suggest that Th2 cytokines favor the development of susceptibility during cysticercosis infection via STAT6 activation.

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Altered T helper responses in CD40 and interleukin-12 deficient mice reveal a critical role for Th1 responses in eliminating the helminth parasite *Taenia crassiceps*

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Abstract

A key feature of helminth infections is the induction of strong Th2-biased immune responses in their hosts. We have previously found that Th2-like responses mediate susceptibility to the helminth parasite *Taenia crassiceps*, probably by inhibiting Th1 responses required for the development of protective immunity against this parasite. Here we show that mice lacking interleukin-12p35 (IL-12p35 −/−) following *T. crassiceps* infection, failed to mount a Th1 response, but developed a strong Th2-type response, produced higher levels of IgG1, IgE, interleukin-4, interleukin-5 as well as interleukin-13 than wild-type mice, and became highly susceptible to the larval stage of this cestode. In contrast, similarly-infected CD40 deficient BALB/c mice (CD40 −/−) displayed impairment of both Th1 and Th2-type responses associated with low levels of interferon-γ as well as IgE, interleukin-4, interleukin-5 and interleukin-13, but efficiently controlled *T. crassiceps* infection. Together, these findings suggest a detrimental role for Th2-biased responses during the larval stage of *T. crassiceps* infection. Furthermore, they also suggest a pivotal role for CD40 in developing Th2-type responses.

Keywords: Parasite-helminth; CD40; Interleukin-12; Th1/Th2; *Taenia crassiceps*

1. Introduction

Infectious agents often bias the host immune response, leading to polarised cytokine production. In contrast to pro-inflammatory and Th1-biased responses seen with most intracellular pathogens (Alberti et al., 2000; Bliss et al., 2000), infection with the majority of helminth parasites leads to an immune response which is Th2-type biased. Cysticercosis is a helminth infection caused by the larvae of the cestode *Taenia solium* affecting humans and pigs. This disease is considered a Public Health problem in South America and Asia, but has been extended in the last few years to developed countries (Sciutto et al., 2000).

In an experimental model of cysticercosis, infection of inbred mice with *Taenia crassiceps* induces a strong Th2-type response, similar to that observed after infection with other helminthes such as *Nippostrongylus brasiliensis* and *Trichuris muris* (Finkelman et al., 1997). Although it is widely accepted that Th2-like responses mediate protective immunity against most helminths (Bancroft et al., 1997; Urban et al., 1995), their role in mediating protection against murine cysticercosis is in doubt (Terrazas et al., 1998; Toenjes et al., 1999).

A series of studies found that although *T. crassiceps*-infected mice develop a Th1-like responses during the early phase of infection, they eventually develop a Th2 response that is associated with an increase in parasite load (Terrazas et al., 1998; Villa and Kuhn, 1996), whereas the administration of interferon (IFN)-γ neutralising antibodies to *T. crassiceps*-infected mice during the early phase of infection rendered them more susceptible to cysticercosis (Terrazas et al., 1999). Furthermore, using STAT6 −/− mice we have demonstrated that the STAT6-mediated interleukin (IL)-4/IL-13 signalling pathway is critical for development of protective immunity by inhibiting Th1 development

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(Rodriguez-Sosa et al., 2002a). Thus, a Th1-type response may play a critical role in protective immunity against experimental cysticercosis.

IL-12 is known to induce IFN-γ production and promote Th1-type responses that are essential for controlling infections with intracellular parasites such as *Leishmania major* and *Toxoplasma gondii* (Cella et al., 1996; Gazzinelli et al., 1996; Manetti et al., 1993; Mattner et al., 1996). On the other hand, IL-12 has also been shown to mediate pathological damage during helminth infections (Bancroft et al., 1997; Finkelman et al., 1994; Rottman et al., 1997), even favouring intestinal installation of the nematode *Trichuris muris* (Bancroft et al., 2001). CD40 is a membrane molecule expressed in a variety of cell types, such as B cells, as well as antigen presenting cells (APC) that include macrophages and dendritic cells (DC) (Cella et al., 1996; Kawabe et al., 1994). CD40 plays a pivotal role in the interaction APC-T cells (Cella et al., 1996; Grewal et al., 1997; Kawabe et al., 1994) where its interaction with CD154 has been shown to play an essential role in cell-mediated immune responses, especially Th1 development during many intracellular parasitic diseases and fungal infections (Cosyns et al., 1998; Grewal et al., 1997). Moreover, a recent study also reported that CD40/CD154 interaction is essential for Th2 development and reducing mortality during infection with the helminth parasite *Schistosoma mansoni* (MacDonald et al., 2002a).

In this study we investigated the roles of IL-12 and CD40 in the regulation of T helper responses during murine cysticercosis, caused by the helminth *T. crassiceps*, and in determining its outcome, by analysing the course of *T. crassiceps* infection in both IL-12p35 − /− and CD40 − /− BALB/c mice and comparing this with similarly infected wild-type BALB/c mice. Additionally, we also analysed antibody profiles in sera, cellular responses and cytokines profiles in both spleen cells and macrophages.

2. Materials and methods

2.1. Mice

Six- to 8-week-old female BALB/c mice, IL-12p35 − /− and CD40 − /− mice in a BALB/c background were purchased from Jackson Laboratories and maintained in a pathogen-free environment at the Harvard Medical School animal facility in accordance with Institutional guidelines. In some experiments STAT6 − /− mice in a BALB/c background (Jackson Laboratories) were used as resistant controls.

2.2. Parasites and infection

Metacestodes of *T. crassiceps* were harvested from the peritoneal cavity of female BALB/c mice 2–4 months after infection. The cysticerci were washed four times in sterile PBS (0.15 M, pH 7.2). Experimental infection was achieved by i.p. injection with 20 small (ca. 2 mm diameter) non-budding cysticerci of *T. crassiceps* suspended in 0.3 ml PBS per mouse. Infections were done at the same time in order to perform all assays on the same day, using age-matched uninfected mice as controls. Parasite load was measured at 8 weeks p.i. by counting all parasites found in the peritoneal cavity after extensive washes with PBS.

2.3. Cell preparations, culture conditions and cytokine assays

Spleen cells were obtained and cultured as previously described (Terrazas et al., 1998). Briefly, single cell suspensions were prepared in RPMI 1640 supplemented with 10% FBS, 100 units of penicillin/streptomycin, 2 mM glutamine, 25 mM HEPES buffer, and 1% non-essential amino acids (all from Gibco BRL). Erythrocytes were lysed and viable cells adjusted to 3×10^6 cells/ml and 100 µl/well, placed into 96-well flat-bottomed culture plates (Costar) and stimulated with *T. crassiceps* soluble antigen (25 µg/ml) at 37 °C for 96 h. Eighteen hours prior to culture termination, 0.5 µCi/well of [^{3}H]thymidine (NEN) was added. Cells were harvested, and counted using a β-plate counter. Values are represented as c.p.m. Supernatants from these cultures were harvested, centrifuged and analysed by ELISA for IFN-γ, IL-4, IL-5 (PharMingen) and IL-13 (R&D Systems) production.

2.4. Cytokine and nitric oxide production by peritoneal macrophages

Peritoneal exudate cells were obtained from mice at 8 weeks after *T. crassiceps* infection. Peritoneal cells were adjusted to 5×10^6/ml in RPMI supplemented and plated in six-well plates (Costar). After 2 h at 37 °C and 5% CO_2, non-adherent cells were removed and adherent cells were gently scrapped using cold PBS. These cells were centrifuged and re-adjusted to 1×10^6/ml. Viability at this point was > 90%. These cells constituted > 90% macrophages according to fluorescence activated cell sorting analysis carried out in a FACS-calibur with Cell Quest software by using fluorescein isothiocyanate monoclonal antibody F4/80$^+$ (Serotec). One millilitre was then plated and cell activation performed in 24-well plates (Costar) with lipopolysaccharide (LPS) (5 µg/ml, *Escherichia coli* 111:B4; Sigma), followed by incubation for 48 h. IL-18, IL-12 (Pharmingen) and nitric oxide (Griess reaction) were then examined in supernatants.

2.5. Antibody ELISAs

Blood was collected from the tails of *T. crassiceps*-infected IL-12p35 − /−, CD40 − /−, STAT6 − /− and BALB/c wild-type mice. Antigen-specific IgG1 and IgG2a levels were determined by ELISA as previously described

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(Rodriguez et al., 1999). Results are expressed as the endpoint titres. Total IgE production was determined by Opt-ELISA (Pharmingen).

2.6. Statistical analysis

Comparisons among wild-type, IL-12p35 −/−, STAT6 −/− and CD40 −/− groups considered in this work were made using Student's unpaired *t*-test. $P < 0.05$ was considered significant. The statistical significance of the sera titres were determined by non-parametric tests using Mann–Whitney *U*/Wilcoxon rank.

3. Results

3.1. Absence of IL-12 favours T. crassiceps infection,whereas absence of CD40 favours resistance

In order to clarify the role of one of the most important Th1-inducer cytokine such as IL-12, in cysticercosis, we performed experiments in IL-12p35 −/− mice. In the present study, we infected age-matched wild-type (BALB/c) and IL-12p35 −/− mice with 20 cysticerci and followed the infection for 8 weeks. Significantly increased parasite burdens were observed in IL-12p35 −/− mice compared with BALB/c mice (Fig. 1). These findings suggest that IL-12 is involved in resistance to *T. crassiceps* infection.

Next, we developed experiments with CD40 −/− mice to analyse the role of this molecule in both T helper responses and susceptibility to murine cysticercosis. Surprisingly, CD40 −/− mice had increased resistance to *T. crassiceps* infection (Fig. 1), suggesting a negative role for CD40 in resistance in this helminth infection. In these experiments we used STAT6 −/− mice as resistant controls to *T. crassiceps* infection. Further, we confirmed here that STAT6 −/− mice were highly resistant to larval

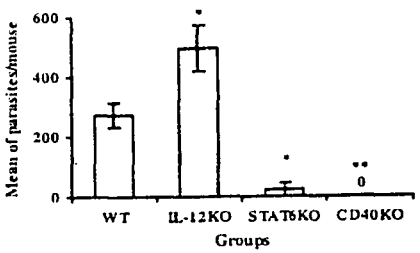

Fig. 1. IL-12p35 −/− mice are highly susceptible, whereas CD40 −/− mice are highly resistant to *Taenia crassiceps* infection. Mice were infected i.p. with 20 cysticerci of *T. crassiceps* and parasite loads were measured at 8 weeks post-infection. Data are expressed as the mean ± S.E. of four mice per group. * $P < 0.01$ comparing IL-12p35 −/−, CD40 −/− and STAT6 −/− versus BALB/c wild-type mice. ** $P < 0.05$ comparing CD40 −/− versus STAT6 −/− mice. Similar results were observed in two independent experiments.

infection with *T. crassiceps* (Fig. 1) (Rodriguez-Sosa et al., 2002a).

3.1.1. Antigen-specific IgG1 and total IgE production are enhanced and IgG2a production is reduced in IL-12p35 −/− T. crassiceps-infected mice, whereas CD40 −/− infected mice did not switched to neither IgG nor IgE

Next, we measured levels of Th1-associated IgG2a as well as Th2-associated IgG1 and IgE antibodies in IL-12p35 −/−, CD40 −/− and STAT6 −/− and wild-type mice after 8 weeks of infection. *Taenia crassiceps*-infected IL-12p35 −/− mice displayed significantly ($P < 0.05$) higher titres of antigen-specific IgG1, but significantly lower titres of IgG2a antibodies, compared with wild-type mice (Fig. 2a,b, $P < 0.05$). On the other hand, levels of total IgE were significantly higher ($P < 0.05$) in these IL-12p35 −/− mice (Fig. 2c). As expected, CD40 −/− infected mice did not switch to IgG1, IgG2a or IgE (Fig. 2a–c), whereas STAT6 −/− infected mice developed lower titres of IgG1, significantly higher titres of IgG2a ($P < 0.01$) and basal levels of IgE (Fig. 2a–c).

3.1.2. Specific splenocyte proliferation and cytokine profile are altered in both IL-12p35 −/− and CD40 −/− T. crassiceps-infected mice

In order to determine whether the cellular response was altered in the different groups of infected mice, we analysed the proliferation of splenocytes and their cytokine profile in response to specific antigen after 8 weeks of infection. Fig. 3a shows that IL-12p35 −/− infected mice had a reduced proliferative response to these antigens. Likewise, splenocytes from CD40 −/− infected mice presented a low proliferation rate compared with wild-type mice. In contrast, STAT6 −/− infected mice showed a strong antigen-specific proliferative response. Regarding the cytokine profiles, IL-12p35 −/− mice made significantly higher levels of IL-4, IL-5 and IL-13 but produced markedly less IFN-γ in response to *T. crassiceps* antigens, compared with wild-type mice (Fig. 3b–e, $P < 0.05$). Similarly stimulated spleen cells from *T. crassiceps*-infected CD40 −/− mice displayed basal levels of all these cytokines. As reported previously, concomitantly infected, STAT6 −/− mice developed strong Th1-biased responses (Rodriguez-Sosa et al., 2002a) (Fig. 3b–e).

Because the reduced proliferation and cytokine production seen in splenocytes from CD40 −/− infected mice might be due to a reduced functionality or defective cellular proliferation to stimulation, we decided to examine the ability of splenocytes from wild-type and CD40 −/− *T. crassiceps*-infected mice to proliferate and to produce cytokines in response to plate-bound anti-CD3. Following in vitro stimulation with anti-CD3, spleen cell from CD40 −/− infected mice displayed a strong proliferative response while those from the wild-type infected mice showed a reduced response, to the same stimuli (Fig. 4a).

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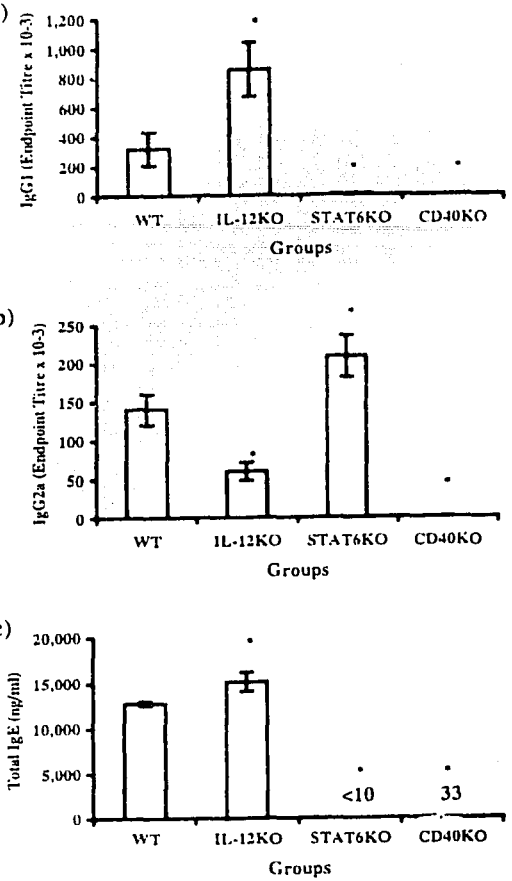

Fig. 2. Comparison of antigen-specific IgG1, IgG2a and total IgE production during *Taenia crassiceps* infection in IL-12p35 − /−, CD40 − /−, STAT6 − /− and wild-type mice. (a) Antigen-specific IgG1, (b) Antigen-specific IgG2a and (c) Total IgE. The graphs show the mean ± S.D. ($n = 4$ animals) and are representative of two independent experiments. * $P < 0.05$ comparing IL-12p35 − /−, CD40 − /− and STAT6 − /− versus wild-type mice.

Moreover, as seen in Fig. 4b, anti-CD3 stimulated splenocytes from CD40 − /− *T. crassiceps*-infected mice produced lower levels of IL-4, IL-5 and IL-13 but more IFN-γ than wild-type infected mice (Fig. 4b).

3.2. Macrophage response is altered during *T. crassiceps* infection

The larval stage of *T. crassiceps* typically resides in the peritoneal cavity of the murine host. To determine the role of IL-12 and CD40 in the development of the macrophage response during this helminth infection, we performed experiments with macrophages isolated from the peritoneal cavities of IL-12, CD40, and STAT6 deficient mice, as well as wild-type infected mice at 8 weeks following *T. crassiceps* infection, and tested their ability to respond to a conventional Th1-type stimuli such as LPS (MacDonald et al., 2002b). As expected, IL-12p70 was not detected in IL-12p35 − /− mice and IL-12p70 production was highly reduced in wild-type infected mice compared with similarly infected STAT6 − /− and CD40 − /− *T. crassiceps*-infected mice (Fig. 5a). Furthermore, LPS-stimulated macrophages from *T. crassiceps*-infected IL-12p35 − /− mice produced significantly less nitric oxide (NO) compared with wild-type mice, but produced similar levels of IL-18 (Fig. 5b,c). On the other hand, macrophages from CD40 − /− and STAT6 − /− *T. crassiceps*-infected mice displayed significantly higher levels of NO ($P < 0.05$), while IL-18 levels remained similar to those observed in wild-type mice (Fig. 5b,c).

4. Discussion

Taken together, our results provide new and detailed evidence that IL-12-induced Th1-type response is necessary for the control of larval stages of the helminth parasite *T. crassiceps*, whereas a Th2-type response appears to be required for parasite establishment. These requirements are more evident in *T. crassiceps*-infected IL-12p35 − /− mice that fail to develop an efficient Th1-type response and display greater parasite burdens than the controls, despite their stronger Th2-biased antigen-specific response. In contrast, similarly infected CD40 − /− mice that fail to mount a Th2-type response efficiently clear the infection with this parasite.

Given that infection by helminths is universally associated with high levels of IgE, eosinophilia, IL-4, IL-5 and IL-13, and all of them are associated with Th2-type responses, the dogma that Th2-type responses are responsible for elimination of helminths emerged several years ago (Allen and Maizels, 1997). The data presented here oppose this dogma, as wild-type mice, even though they developed the classical response to helminthes, with elevated levels of Th2-associated cytokines and antibodies, harboured the heaviest infections. Our results differ from those reported in gastrointestinal-helminth infections (Bancroft et al., 1997, 2001; Blackwell and Else, 2001; Finkelman et al., 1997; Urban et al., 1998), where this kind of response efficiently eliminated infection. However, our data on the possible involvement of IFN-γ and NO production as being crucial in eliminating a tissue-dwelling helminth, such as *T. crassiceps* cysticerci, support the findings reported in two previous studies, showing that IL-12 dependent Th1 responses mediate protection against the nematode *Brugia malayi* (Lawrence et al., 1995) and the trematode *S. mansoni* (Anderson et al., 1998; Mountford et al., 1996; Smythies et al., 1992). Moreover, Loke et al. (2000) also showed that cytokine production skewed towards Th2-type following *B.*

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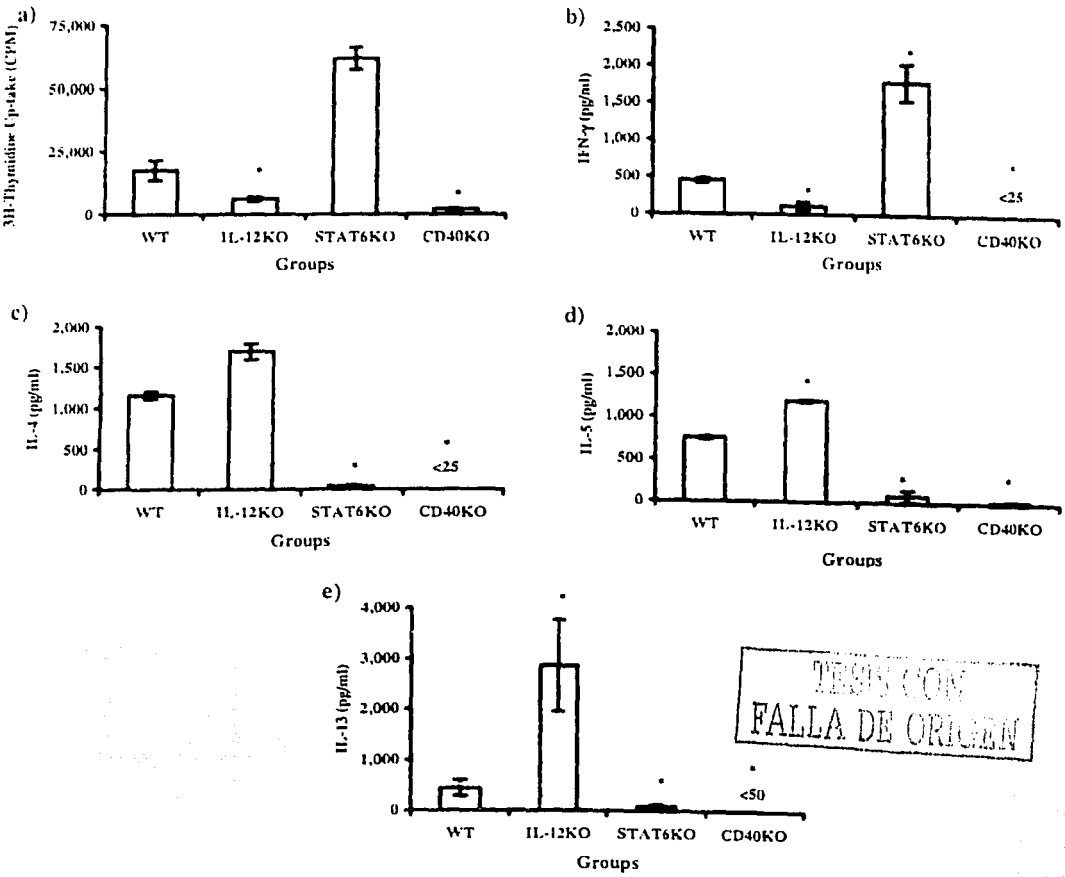

Fig. 3. Comparison of antigen-specific-induced splenocyte responses of IL-12p35 −/−, CD40 −/−, STAT6 −/− and wild-type mice following 8 weeks of *Taenia crassiceps* infection. Splenocytes were obtained and stimulated with 25 μg/ml of *T. crassiceps* soluble antigen. (a) Antigen-specific proliferative response of splenocytes (96 h); (b) specific interferon-γ; (c) specific interleukin-4; (d) specific interleukin-5; (e) specific interleukin-13 production by splenocytes following 72 h in vitro stimulation. Data are expressed as in Fig. 2.

mulayi infection, paralleled heavy parasite loads. Together, these studies suggest that the commitment towards a Th2 pattern of cytokine production during an extra-intestinal helminth infection favours or enhances parasite establishment and survival. In support of this view, we showed here that although IL-12p35 −/− mice that fail to mount an optimal Th1 response and develop a greater Th2 response than wild-type mice, fail to control *T. crassiceps* infection. These findings support our recent report where we found that mice lacking STAT-6 are highly resistant to the larval stage of *T. crassiceps*, despite developing a poor Th2-type response during *T. crassiceps* infection (Rodriguez-Sosa et al., 2002a). Notwithstanding that most gastrointestinal helminth parasites are expelled by a STAT-6 dependent Th2 type response (Bancroft et al., 2000; Urban et al., 2000), previous studies, with this and other helminth-parasite models (Bungiro et al., 1999; Emery et al., 1998; Lawrence et al., 1995; Manoutcharian et al., 1999; Oswald et al., 1998; Terrazas et al., 1999), as well as the present study, suggest that Th2-type responses efficiently eliminate gastrointestinal worms, but not necessarily the extra-intestinal life phases of helminths.

Although Th2-associated IgE has been shown to play a role in mediating immunity against helminths (Gounni et al., 1994; Khalife et al., 2000), we found that *T. crassiceps*-infected CD40 −/− mice efficiently controlled parasite burden despite their defect in IgE switching. In contrast, IL-12 p35 −/− mice developed higher levels of IgE but remained highly susceptible to the infection, suggesting that IgE may have a limited role in mediating protective

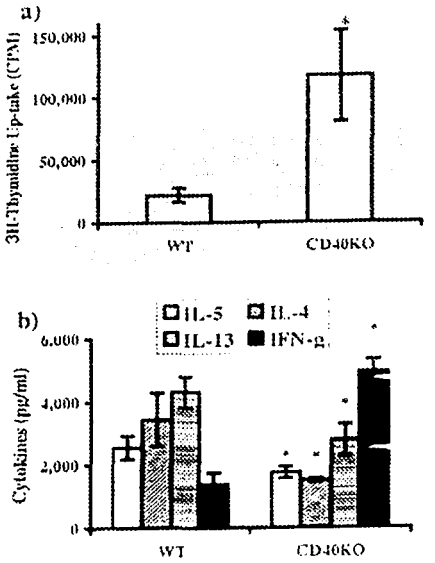

Fig 4. *Taenia crassiceps*-infected CD40 − / − mice show effective in vitro proliferative response to anti-CD3 stimulation. (a) Proliferation of splenocytes from wild-type or CD40 − / − infected mice in response to plate-bound anti-CD3. (b) Cytokine production in response to anti-CD3. Spleen cells were processed as mentioned in Fig. 3 and stimulated with 1 μg/ml of plate-bound anti-CD3. Cytokines were measured in supernatants after 72 h in culture. Wild-type and CD40 − / − uninfected mice developed similar responses to that observed on CD40 − / − *T. crassiceps*-infected mice (data not shown). Data are expressed as in Fig. 2.

immunity against *T. crassiceps*. These data are consistent with our previous report where mice lacking STAT6 did not produce IgE, but were resistant to *T. crassiceps* infection (Rodriguez-Sosa et al., 2002a). Nevertheless, our observations do not exclude the role of parasite-specific IgE in the elimination of *T. crassiceps*.

Previous studies have demonstrated that the absence of CD40-mediated signalling in CD40 − / − or CD154 − / − mice prevents development of protective immunity against intracellular parasites such as *Cryptosporidium parvum* (Cosyns et al., 1998), *Leishmania major* (Kamanaka et al., 1996), *Toxoplasma gondii* (Reichmann et al., 2000) and *Trypanosoma cruzi* (Chaussabel et al., 1999) by inhibiting Th1 development. Moreover, an early study (Lu et al., 1996) demonstrated that blockade of CD40L in vitro altered Th 2 responses against the helminth *Heligmosomoides polygyrus*. A more recent study found that CD154 − / − BALB/c mice show a significant increase in morbidity and mortality during *S. mansoni* infection, suggesting that CD40/CD154 interaction is essential for the development of appropriate immune response and preventing potentially life threatening complications during infection (MacDonald et al., 2002a). Interestingly, in the present study we found that CD40 − / − mice were highly resistant to *T. crassiceps* infection compared with wild-type BALB/c mice.

It is likely that the inability of spleen cells from CD40 − / − mice to mount an antigen specific response may be due to the absence of CD40/CD154 co-stimulatory pathway in these mice. In fact, early studies have shown that CD40/CD154 interaction is essential for mounting antigen-specific T-cell response during infection with pathogens such as *L. major* (Kamanaka et al., 1996) and *S. mansoni* (MacDonald et al., 2002a). Hence, we decided to examine the ability of splenocytes from *T. crassiceps*-infected CD40 − / − and wild-type BALB/c mice to proliferate

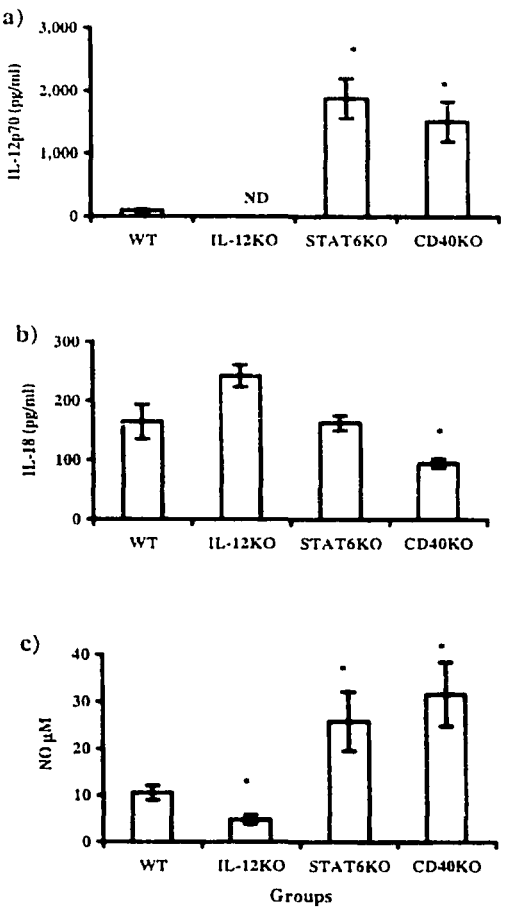

Fig. 5. Peritoneal macrophages from IL-12p35 − / −, CD40 − / −, STAT6 − / − and wild-type *Taenia crassiceps*-infected mice display different responses. Macrophages were obtained at 8 weeks after infection and stimulated with lipopolysaccharide (5 μg/ml) during 48 h. supernatants were analysed for (a) interleukin-12p70, (b) interleukin-18 and (c) nitric oxide production. Values from uninfected mice for each strain of mice were subtracted to its respective group. Data are expressed as in Fig. 2.

and produce cytokines in response to plate-bound anti-CD3 stimulation. Following in vitro stimulation with anti-CD3 antibody, spleen cells from *T. crassiceps*-infected CD40 −/− mice mounted a significantly stronger proliferative response than wild-type mice. Interestingly, despite their ability to mount a stronger proliferative response after stimulation with anti-CD3, spleen cells from *T. crassiceps*-infected CD40 −/− mice produced significantly lower levels of Th2-associated IL-4, IL-5 and IL-13 but more Th1-associated IFN-γ than wild-type mice. The data presented here confirm some of the previous findings by MacDonald et al. (2002a) in an experimental model of murine schistosomiasis, which showed that spleen cells from *S. mansoni*-infected CD154 −/− mice proliferate poorly in response to soluble egg antigen, but mount a significant proliferative response following anti-CD3 stimulation.

Several studies have demonstrated that macrophages play a critical role in immunity against many intracellular pathogens by their ability to secrete Th1-inducing cytokines such as IL-12 and IL-18, and produce NO, that is microbicidal (Bogdan et al., 2000). Furthermore, NO produced by macrophages has also been shown to be cytotoxic to *Schistosoma* larvae (Ahmed et al., 1997). Previous studies have shown that chronic helminth infection induces alternatively activated macrophages that produce low levels of IL-12, as well as NO, and have Th2-biasing ability (Allen et al., 1996, MacDonald et al., 1998; Rodriguez-Sosa et al., 2002b). Therefore, we compared the ability of macrophages isolated from the peritoneal cavity from *T. crassiceps*-infected IL-12p35 −/−, CD40 −/−, STAT6 −/−, and wild-type BALB/c mice to secrete Th1-inducing cytokines (IL-12 and IL-18), as well as to produce NO following in vitro stimulation with LPS. Peritoneal macrophages from *T. crassiceps*-infected wild-type mice produced significantly lower levels of IL-12p70 and NO than cells from similarly infected STAT6 −/− and CD40 −/− mice. However, macrophages isolated from all three groups produced comparable levels of IL-18. These findings support observations in a recent study, which found that CD40 −/− DC also produce high levels of IL-12 to similar stimuli (MacDonald et al., 2002b). Interestingly, CD40 −/− macrophages did produce NO following LPS stimulation, despite the lack of a robust IFN-γ production in these mice in response to parasite antigen. It is possible that this may be due to a compensatory increase in other cytokines, such as tumour necrosis factor-α and macrophage migration inhibitory factor (MIF), or chemokines such as RANTES and MIP-1α, that have been shown to induce NO production from macrophages (Aliberti et al., 1999; Juttner et al., 1998). In fact, in a recent study we found that MIF −/− mice that are highly susceptible to *T. crassiceps* infection produce IFN-γ, but their macrophages display a marked impairment of NO production after LPS stimulation (Rodriguez-Sosa et al., in press). These findings, taken together with our previous observation that macrophages from chronically *T. crassiceps*-infected mice highly express CD40 and induce a Th2 biased response, but cannot induce Th1 development in CD4 cells (Rodriguez-Sosa et al., 2002b), suggest that CD40 is involved in developing Th2 responses in helminth infections. A similar phenomenon has been recently observed using DC CD40 −/− and DC CD154 −/− stimulated with helminth antigens (MacDonald et al., 2002b; Straw et al., 2003).

Acknowledgements

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Macrophage Migration Inhibitory Factor Plays a Critical Role in Mediating Protection against the Helminth Parasite *Taenia crassiceps*

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To determine the role of endogenous migration inhibitory factor (MIF) in regulation of immune response during murine cysticercosis caused by the helminth parasite *Taenia crassiceps*, we analyzed the course of *T. crassiceps* infection in $MIF^{-/-}$ BALB/c mice. $MIF^{-/-}$ mice were highly susceptible to *T. crassiceps* and developed significantly higher parasite loads compared to similarly infected $MIF^{+/+}$ mice. Throughout the course of infection, *Taenia crassiceps* soluble antigen-stimulated spleen cells from both $MIF^{+/+}$ and $MIF^{-/-}$ mice produced significant and comparable levels of interleukin-4 (IL-4), but those from $MIF^{-/-}$ mice produced significantly more IL-13, as well as gamma interferon (IFN-γ), suggesting that the susceptibility of $MIF^{-/-}$ mice to *T. crassiceps* was not due to the lack of IFN-γ production. Interestingly, low levels of both total and specific immunoglobulin G2a were observed in $MIF^{-/-}$ cysticercotic mice despite the high IFN-γ levels; in addition, peritoneal macrophages obtained from *T. crassiceps*-infected $MIF^{-/-}$ mice at different time points failed to respond efficiently to stimulation in vitro with lipopolysaccharide plus IFN-γ and produced significantly lower levels of IL-12, tumor necrosis factor alpha, and NO compared to those from $MIF^{+/+}$ mice. These findings demonstrate that MIF plays a critical role in mediating protection against *T. crassiceps* in vivo. Moreover, these findings also suggest that impaired macrophage function rather than the lack of Th1 development may be responsible for mediating susceptibility to *T. crassiceps*.

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that is produced by different types of cells, such as activated macrophages, T cells, and the pituitary gland (3). MIF has been associated with proinflammatory functions (11, 23) and has been detected elevated in several inflammatory diseases (37, 40). For example, it is known that MIF-deficient mice are highly resistant to lipopolysaccharide (LPS)-induced septic shock because their inability to produce tumor necrosis factor alpha (TNF-α) (6). MIF is also involved in the pathogenesis of arthritis and chronic colitis (11, 37) and has been shown to play a role in regulation of innate immune response (29). Although few studies have shown that MIF is an important factor playing a critical role in determining the outcome of infections caused by intracellular pathogens such as *Plasmodium chabaudi* and *Leishmania major* (27, 38), its role in the regulation of immunity against extracellular pathogens has not been evaluated.

Cysticercosis is a helminth infection caused by the larvae of the cestode *Taenia solium* affecting humans and pigs. This disease is not only endemic in South America and Asia but in the last few years has also become a public health problem in developed countries, as indicated by recent case reports (39). Although cysticerci in muscle may be relatively symptomless, those in the brain cause neurocysticercosis that may clinically manifest as seizures, hydrocephalus, aseptic meningitis, and altered mental status (39). Cysticercosis caused by *Taenia crassiceps* naturally affects rodents, and the final hosts are canines; this parasite has the advantage of an asexual budding reproduction (10). Notwithstanding, there are reports demonstrating that immunocompromised humans can develop *T. crassiceps* cysticercosis (16, 25). Experimental murine cysticercosis caused by *T. crassiceps* has been a useful model for understanding and defining the biological factors affecting susceptibility and resistance to this disease. Thus, genetic, immunological, and hormonal factors have been related to resistance in this model (14, 15, 20, 22, 31, 41). Likewise, some of these observations have been confirmed in the natural hosts for *T. solium* (30, 33, 34).

Infection of inbred mice with *T. crassiceps* induces a strong Th2-like response (42, 44) similar to that observed after infection with helminths such as *Nippostrongylus brasiliensis* and *Trichuris muris* (13). Although Th2-like response developed via STAT6-mediated pathway plays a critical role in protective immunity against most helminths (45), we have recently demonstrated that $STAT6^{-/-}$ mice are highly resistant to *T. crassiceps*, indicating that a STAT6-dependent Th2 response is involved in mediating susceptibility to cysticercosis (35). Moreover, these findings also suggest that STAT6-dependent path-

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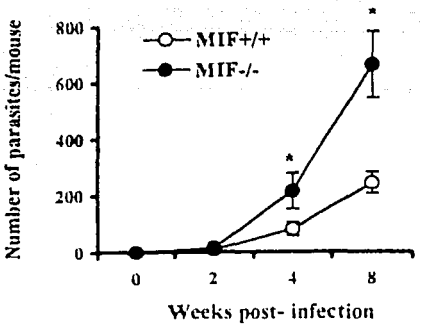

FIG. 1. MIF$^{-/-}$ mice are highly susceptible to *T. crassiceps* infection. The course of intraperitoneal *T. crassiceps* infection in MIF$^{-/-}$ (●) and MIF$^{+/+}$ (○) mice after infection with 20 cysticerci was determined. The data are expressed as the means ± the standard errors of six mice per group. *, $P < 0.01$ (MIF$^{-/-}$ versus MIF$^{+/+}$ mice at the same time point). Similar results were observed in two other independent experiments.

way mediates susceptibility to *T. crassiceps* at least in part by inhibiting macrophage NO production.

The purpose of the present study was to analyze the role of endogenous MIF in the regulation of immune response and in determining the outcome of murine cysticercosis caused by *T. crassiceps*. To approach this question, we compared the course of *T. crassiceps* infection in MIF$^{-/-}$ BALB/c mice with that in the wild-type BALB/c mice. In addition, we analyzed the antibody profiles in the sera, cellular responses, and cytokine profile in both spleen cells and peritoneal macrophages. Our data demonstrate that proinflammatory cytokine MIF plays a critical role in mediating protection against cysticercosis. Furthermore, they also suggest that enhanced susceptibility of MIF$^{-/-}$ mice to *T. crassiceps* could be associated with the dysregulation of macrophage function and their inability to produce TNF-α and NO rather than lack of Th1 development.

MATERIALS AND METHODS

Mice. Six- to eight-week-old female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were maintained in a pathogen-free environment at the Harvard Medical School animal facility in accordance with Institutional guidelines. MIF$^{-/-}$ mice were developed as described previously (6) and backcrossed for more than 10 generations to a BALB/c genetic background.

Parasites and infections. Metacestodes of *T. crassiceps* (ORF strain) were harvested under sterile conditions from the peritoneal cavity of female BALB/c mice after 2 to 4 months of infection. The cysticerci were washed four times in sterile phosphate-buffered saline (PBS; 0.15 M, pH 7.2). Experimental infection was achieved by intraperitoneal injection with 20 small (~2 mm in diameter) nonbudding cysticerci of *T. crassiceps* suspended in 0.3 ml of PBS per mouse, and infections were carried out at different times in order to perform all of the assays on the same day with age-matched uninfected mice as controls at each time point. At weeks 2, 4, and 8 mice were killed, and their peritoneal cavities were exhaustively washed to recover and count all of the parasites.

Cell preparations, culture conditions, and cytokine assays. Spleen cells were obtained and cultured as described previously (42). Briefly, single cell suspensions were prepared in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U of penicillin-streptomycin, 2 mM glutamine, 25 mM HEPES buffer, and 1% nonessential amino acids (all from Gibco-BRL, Grand Island, N.Y.). Erythrocytes were lysed, and viable cells were adjusted to 3×10^6 cells/ml. Then, 100-μl portions were placed into each well in 96-well flat-bottom culture plates (Costar, Cambridge, Mass.), followed by stimulation with *T. crassiceps* soluble antigen (TcAg; 25 μg/ml) at 37°C for 96 h. At 18 h prior to culture termination, 0.5 μCi of [^{3}H]thymidine (NEN, Boston, Mass.) was added per well. Cells were harvested in a 96-well harvester (Tomtec, Turku, Finland) and counted by using a β-plate counter. Values are represented as counts per minute. Supernatants from these cultures were analyzed for gamma interferon (IFN-γ), interleukin-4 (IL-4; BD Pharmingen, San Diego, Calif.), and IL-13 (R&D Systems, Minneapolis, Minn.) production by enzyme-linked immunosorbent assay (ELISA).

TcAg was obtained from freshly and sterile isolated cysticerci from BALB/c female mice within 2 to 4 months of infection, and parasites were extensively washed with PBS and homogenized by using a Tissue Tearor (Dremel, Racine, Wis.) with cycles of 2 to 3 min on ice. Homogenized cysticerci were centrifuged to 10,000 rpm for 1 h at 4°C. The supernatant was collected, and protein levels were determinate by using the Bradford method. TcAg for cell cultures was sterilized by filtration and reevaluated for its protein content.

Peritoneal macrophage extraction and pattern of response to LPS plus IFN-γ. Peritoneal exudate cells (PECs) were obtained from the peritoneal cavities of mice infected with *T. crassiceps* for 2, 4, and 8 weeks or from uninfected mice. The cells were washed twice with Hanks balanced salt solution, and erythrocytes were lysed by resuspending the cells in Boyle's solution (0.17 M Tris and 0.16 M ammonium chloride). After two more washes, viable cells were counted by trypan blue exclusion. PECs were adjusted to 5×10^6/ml in RPMI medium and then cultured in six-well plates (Costar, Cambridge, Mass.). After 2 h at 37°C and in 5% CO_2, nonadherent cells were removed by washing them with warm supplemented RPMI medium. Cold Ca^{2+} Mg^{2+}-free PBS was added to adherent cells for 5 min, and immediately the adherent cells were gently detached by using a sterile rubber policeman. The plates were rinsed twice with Ca^{2+} Mg^{2+}-free PBS to collect residual cells. These cells were centrifuged and readjusted to 10^6/ml. Viability was determined at this step by trypan blue exclusion; the viability was usually ≥95%. These cells constituted ≥80% of the macrophages according to fluorescence-activated cell sorting analysis carried out in a FACSCalibur with CellQuest software (BD Bioscience, Mountain View, Calif.), by using the fluorescein isothiocyanate-conjugated monoclonal antibody F4/80 (Serotec, Oxford, United Kingdom). Then, 1 ml was plated, and cell activation was performed in 24-well plates (Costar) with LPS (1 μg/ml, *E. coli* 113:B4; Sigma, St. Louis, Mo.) plus 2 ng of recombinant murine IFN-γ (BD Pharmingen)/ml, followed by incubation for 48 h at 37°C and 5% CO_2. Supernatants were harvested, centrifuged, and examined by ELISA for IL-6, IL-12, and TNF-α production (the antibodies and cytokines were from Pharmingen) and for nitric oxide (Griess reaction) production. Total PECs were analyzed by using a Cytospin preparation stained with Wright-Giemsa stain (Sigma); 400 cells were counted per slide.

Nitric oxide production by macrophages was assayed by determining the increase in nitrite concentration (28) by the Griess reaction adapted to Microwell plates.

Antibody ELISA and cytokines in sera. Blood was collected from tails of individual (no pools were made) *T. crassiceps*-infected MIF$^{+/+}$ and MIF$^{-/-}$ mice. Total levels of immunoglobulin G1 (IgG1) and IgG2a were determined by using for IgG2a a capture antibody (clone R11-89), a detection antibody (clone R19-15), and a standard antibody (purified mouse IgG2a, clone G155-178), all of them obtained from BD Pharmingen. For total IgG1 detection, we used the clones A85-3 (capture), A85-1 (detection), and MOPC-31C (standard), also all from BD Pharmingen. The serum dilutions used to estimate the total levels of IgG2a and IgG1 were 1:2,000. The specific endpoint titers of IgG1 and IgG2a were determined by ELISA as previously described (42). Total IgE production (serum dilution, 1:20) was detected by Opt-ELISA (BD Pharmingen).

Additionally, sera from these mice were used to look for circulating cytokines, such as IL-4, IL-12, and IFN-γ, by using the antibodies and methods described above.

Statistical analysis. Comparisons between the MIF$^{+/+}$ and MIF$^{-/-}$ groups considered in this study were made by using the Student unpaired *t* test. A *P* value of ≤0.05 was considered significant. The statistical significance of the serum titers was determined by using Mann-Whitney U Wilcoxon rank nonparametric tests.

RESULTS AND DISCUSSION

MIF has been shown as a pivotal cytokine that mediates host inflammatory and immune responses (17). Previous studies have demonstrated that the absence of MIF prevents the development of protective immunity against intracellular para-

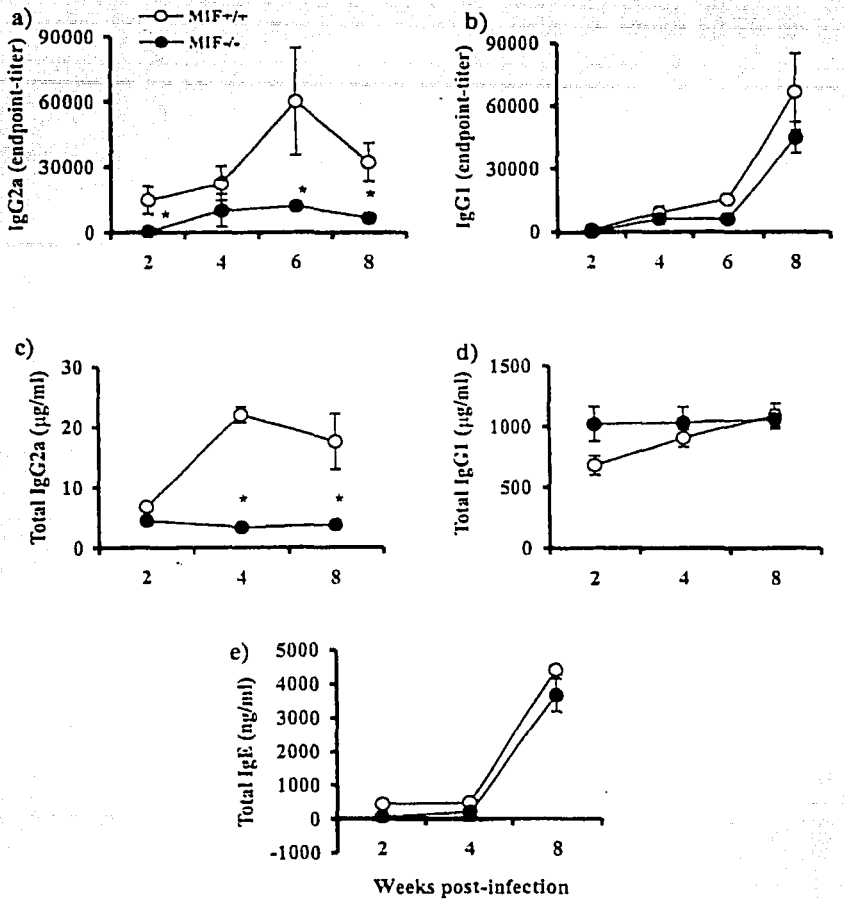

FIG. 2. Kinetics of total and specific antibody production during *T. crassiceps* infection in $MIF^{-/-}$ (●) and $MIF^{+/+}$ (○) mice. (a) Specific IgG2a; (b) specific IgG1; (c) total IgG2a; (d) total IgG1; (e) total IgE. The graphs show the means ± the standard errors (n = six animals), and the results are representative of three independent experiments. *, $P < 0.05$ ($MIF^{-/-}$ versus $MIF^{+/+}$ mice at the same time point).

sites such as *Leishmania* by inhibiting macrophage microbicidal activity rather than by preventing the development of a Th1-like response (38). In experimental cysticercosis, an extraintestinal helminthic infection, several immunological factors have been associated with susceptibility and resistance (14, 43). For example, we have previously demonstrated that blocking IFN-γ results in a higher susceptibility to the parasite (43). Moreover, we recently found that mice lacking the STAT6 signaling pathway develop a Th1-type response associated with increased macrophage-derived proinflammatory cytokines after *T. crassiceps* infection which restricted parasite growth, suggesting that Th1 response and macrophage activation are required for the development of protective immunity against this parasite (35, 43).

In the present study, both $MIF^{+/+}$ and $MIF^{-/-}$ mice showed a progressive increase in the parasite numbers in their peritoneal cavities and displayed comparable parasite burdens at 2 weeks after infection with *T. crassiceps* (Fig. 1). Interestingly, as infection progressed, the parasite burdens increased significantly in $MIF^{-/-}$ mice compared to $MIF^{+/+}$ mice that showed ~3-fold-lower parasite loads by week 8 postinfection (Fig. 1). These findings demonstrate that endogenous MIF is involved in mediating resistance to *T. crassiceps* infection.

Next, we measured levels of Th1-associated IgG2a, as well as Th2-associated IgG1and IgE antibodies, in $MIF^{+/+}$ and $MIF^{-/-}$ mice at different time points after infection with *T. crassiceps*. Infected $MIF^{+/+}$ mice displayed higher titers of specific IgG2a antibodies than $MIF^{-/-}$ mice throughout the course of the infection, reaching the most significant difference at week 6 postinfection. In contrast, $MIF^{-/-}$ mice displayed a

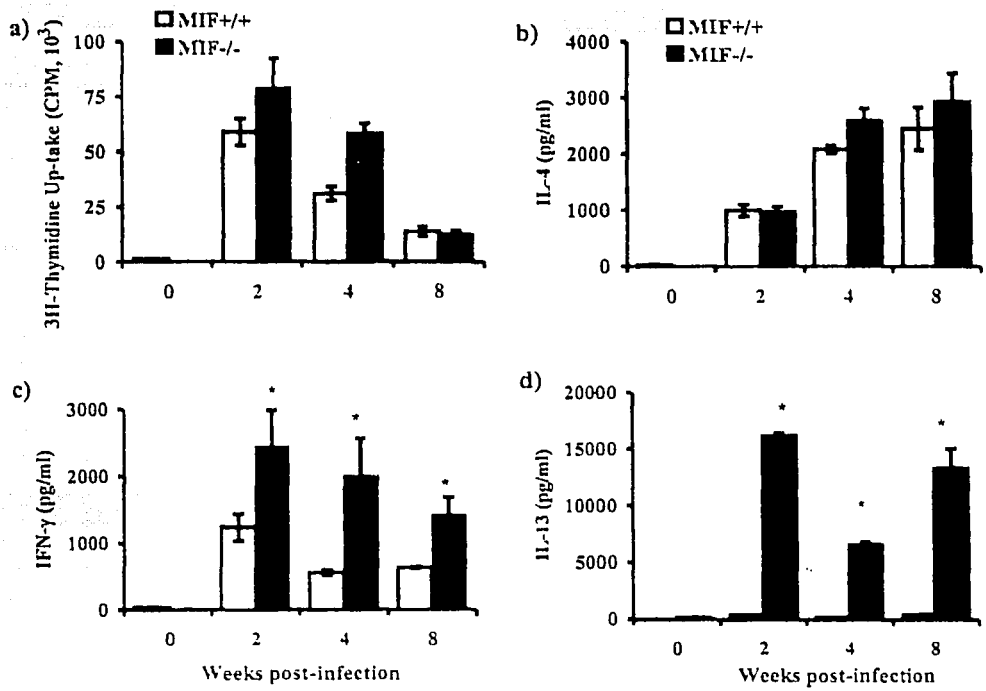

FIG. 3. Kinetics of in vitro proliferative response and cytokine production by TcAg-stimulated spleen cells from MIF$^{+/+}$ (■) and MIF$^{-/-}$ (□) mice. (a) Antigen-specific proliferative response of splenocytes (96 h). (b to d) Specific IL-4 (b), specific IFN-γ (c), and specific IL-13 (d) production by splenocytes after 72 h of in vitro stimulation with TcAg (25 μg/ml). The data are expressed as in Fig. 2.

similar production of specific IgG1 compared to MIF$^{+/+}$ mice that was maintained until the eighth week after infection (Fig. 2b).

In order to test whether the lower specific titers of IgG2a detected in MIF$^{-/-}$ mice reflected an overall lack of this isotype, we decided to measure the total levels of this antibody. As shown in Fig. 2c, the levels of total IgG2a from MIF$^{-/-}$ infected mice were significantly lower than MIF$^{+/+}$ infected mice, confirming that there is a low production of IgG2a. To exclude the possibility that the low IgG2a antibody production in MIF$^{-/-}$ mice was a general tendency of the antibody response, we measured the total levels of IgG1. Figure 2d shows that this kind of antibody was not affected in the absence of MIF, suggesting a specific downregulation in IgG2a production. However, these data contrast with the previous finding of Bacher et al. (2), who observed an inhibitory effect of anti-MIF treatment versus antigen-specific levels of total IgG. Differences in the times of antibody measurement (days versus weeks in our study) and the absence of subclass determination in their work could explain the lack of consensus.

Although Th2-associated IgE has been shown to play a role in mediating immunity against helminths (18, 21), we found that *T. crassiceps*-infected MIF$^{-/-}$ mice did not control parasite burdens despite producing similar levels of IgE compared to infected MIF$^{+/+}$ mice, suggesting that IgE may have a limited role, if any, in mediating protective immunity against *T. crassiceps* (Fig. 2e). These observations also support those of our recent study, which demonstrated that *T. crassiceps*-infected STAT6$^{-/-}$ mice are able to control infection despite producing only basal levels of IgE (35).

Previous studies have shown that cytokines produced by Th1 and Th2 subsets of CD4$^+$ cells play an important role in determining the outcome of cysticercosis (24, 26, 43). Moreover, we have shown that the resistance of STAT6$^{-/-}$ mice to *T. crassiceps* is associated with an enhanced development of Th1 response with increased IFN-γ production (35). Hence, to determine whether the susceptibility of MIF$^{-/-}$ mice to *T. crassiceps* was due to the dysregulation in Th1/Th2 cytokine profile, we measured cytokine production by TcAg-stimulated spleen cells from *T. crassiceps*-infected MIF$^{+/+}$ and MIF$^{-/-}$ mice. After in vitro stimulation with TcAg, spleen cells from both groups displayed a very similar pattern of proliferative responses, indicating that the higher susceptibility of MIF$^{-/-}$ mice is not due to a defect in the ability of spleen cells to respond to parasite antigens (Fig. 3a). At weeks 4 and 8 postinfection, TcAg-stimulated spleen cells from MIF$^{+/+}$ and MIF$^{-/-}$ mice produced comparable levels of IL-4, but those from the latter produced significantly more IL-13 (Fig. 3b and

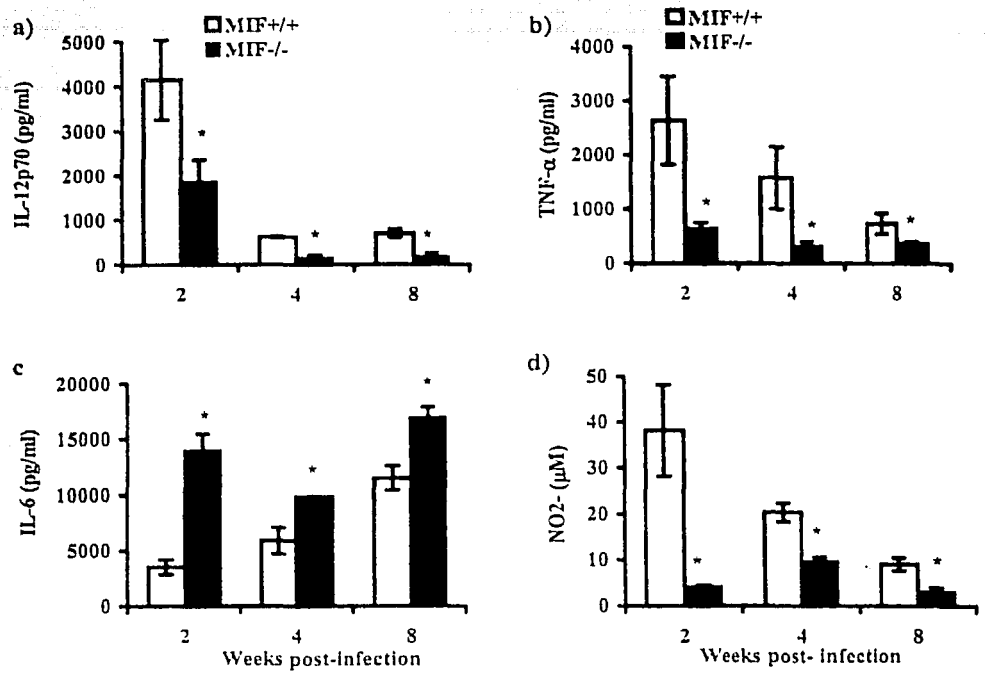

FIG. 4. Peritoneal macrophages from MIF$^{+/+}$ and MIF$^{-/-}$ *T. crassiceps*-infected mice display different responses. Macrophages were obtained at different time points after infection and stimulated with LPS (1 μg/ml) + IFN-γ (2 ng/ml) for 48 h; supernatants were then analyzed for IL-12p70 (a), TNF-α (b), IL-6 (c), and NO (d) production. The data are expressed as in Fig. 2.

d). Interestingly, at these time points spleen cell culture supernatants from MIF$^{-/-}$ mice also contained significantly more IFN-γ compared to those from MIF$^{+/+}$ mice (Fig. 3c), demonstrating the ability of these mice to mount a sustained mixed Th1/Th2-type response. Taken together, these findings support our previous observation and suggest that unlike other helminths (46), the Th2-type response is not essential for mediating protective immunity against *T. crassiceps* cysticercosis. Furthermore, they also show that the lack of MIF does not lead to impairment of IFN-γ production during *T. crassiceps* infection; this latter observation is similar to findings with MIF$^{-/-}$ mice during infection with the intracellular protozoan parasite *L. major* (38).

Several studies have demonstrated that macrophages play a critical role in immunity against many intracellular pathogens by their ability to secrete Th1-inducing cytokines such as IL-12 and IL-18 and to produce NO that is not only microbicidal (4) but has also been shown to be cytotoxic to the larvae of some helminths (1). We recently hypothesized that these cells may be involved in mediating protective immunity to *T. crassiceps* metacestodes in STAT6$^{-/-}$ mice by secreting Th1-inducing cytokines such as IL-12 and by releasing NO. Hence, we analyzed IL-12, IL-6, TNF-α, and NO production by adherent peritoneal macrophages obtained from MIF$^{+/+}$ and MIF$^{-/-}$ mice that were infected with *T. crassiceps*. Peritoneal macrophages from MIF$^{-/-}$ mice obtained during the early phase of infection (2 weeks) produced lower levels of IL-12 compared to those from MIF$^{+/+}$ mice in response to LPS+IFN-γ stimulation (Fig. 4a). As infection progressed, both MIF$^{+/-}$ and MIF$^{-/-}$ macrophages showed an impairment of their ability to secrete IL-12 at weeks 4 and 8 postinfection (Fig. 4a). At these time points, however, the levels of IL-12 in culture supernatants from MIF$^{-/-}$ macrophages were still significantly lower compared to those from MIF$^{+/+}$ macrophages (Fig. 4a). Similar differences were also observed in TNF-α production between LPS+IFN-γ-stimulated peritoneal macrophages from *T. crassiceps*-infected MIF$^{+/+}$ and MIF$^{-/-}$ mice (Fig. 4b). Interestingly, at all three time points examined, macrophages from MIF$^{-/-}$ mice produced significantly more IL-6 (Fig. 4c). Throughout the course of infection, macrophages from *T. crassiceps*-infected MIF$^{-/-}$ mice produced significantly lower levels of NO compared to those from similarly infected MIF$^{+/+}$ mice, although levels of NO in both groups dropped significantly as the infection became more chronic (Fig. 4d). These observations also support findings in previous studies demonstrating that blockade of MIF in vivo inhibits TNF-α production (7) and, conversely, macrophages exposed to MIF produce more TNF-α, IL-12, and NO (8, 11). Taken together, our observations suggest that MIF may mediate host resistance against *T. crassiceps*, at least in part, by increasing IL-12,

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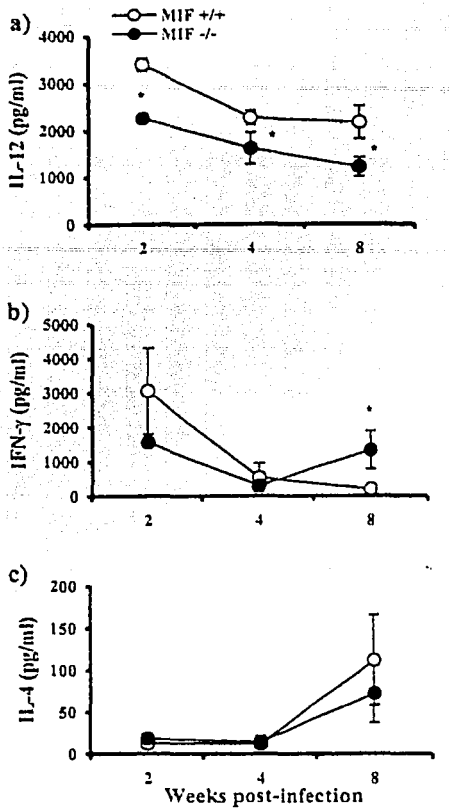

FIG. 5. Circulating cytokine profile during *T. crassiceps* infection. Sera from MIF$^{+/+}$ and MIF$^{-/-}$ infected mice were collected at different times postinfection and analyzed for IL-12 (a), IFN-γ (b), and IL-4 production by ELISA. The data are the means ± the standard deviations from four to five animals per group and time. *, $P < 0.05$.

TNF-α, and NO production from macrophages. Since IL-6 has been shown to inhibit macrophage superoxide and NO production (19), it is likely that increased IL-6 production by MIF$^{-/-}$ macrophages may contribute at least partially to the enhanced susceptibility to cysticercosis. Our recent findings that macrophages from highly resistant STAT6$^{-/-}$ mice produce low levels of IL-6 but higher levels of NO would also support this hypothesis (35).

Additionally, we analyzed the levels of circulating cytokines as indicative of its in vivo production. Figure 5a shows that IL-12 levels in sera were significantly lower at weeks 2, 4, and 8 in MIF$^{-/-}$ mice compared to MIF$^{+/+}$ infected mice at the same points, even though in both groups a tendency to decrease was observed. In contrast, IFN-γ levels (Fig. 5b) were similar at weeks 2 and 4 postinfection, but at week 8 the MIF$^{-/-}$ mice produced significantly higher levels of this cytokine. As we observed during in vitro stimulations, IL-4 levels

TABLE 1. Peritoneal cell infiltration during *T. crassiceps* infection[a]

Cell type	Mean ± SEM in:			
	MIF$^{+/+}$ mice at:		MIF$^{-/-}$ mice at:	
	4 wk	8 wk	4 wk	8 wk
Macrophages	58.9 ± 3.6	74.3 ± 1.3	37.7 ± 2.4[b]	59.0 ± 0.6[b]
Lymphocytes	25.3 ± 2.3	7.3 ± 1.5	30.4 ± 2.5	10.5 ± 4.4
Eosinophils	16.7 ± 1.9	13.8 ± 1.8	23.1 ± 0.2	21.0 ± 3.6[b]
Basophils	3.0 ± 0.6	0.25 ± 0.2	0.8 ± 0.1	2.6 ± 1.4
Neutrophils	5.0 ± 0.9	3.5 ± 0.7	7.7 ± 0.4	5.4 ± 1.0

[a] PECs were recovered after *T. crassiceps* infection (at 4 and 8 weeks), and cytospin preparations were stained with Wright-Giemsa staining solution to identify the different populations recruited. The data represent the means of four mice per group.

[b] $P < 0.05$ (comparison of the same populations at the same time points of MIF$^{+/+}$ versus MIF$^{-/-}$ mice).

were similar in both groups of mice and higher levels were detected in more advanced infections (Fig. 5c). Together, these data reflected our in vitro findings.

An interesting observation in this study is that *T. crassiceps*-infected MIF$^{-/-}$ mice failed to control infection despite producing high levels of IFN-γ. Furthermore, *T. crassiceps*-infected MIF$^{-/-}$ mice produced low levels of Th1-associated total IgG2a, as well as of TcAg-specific IgG2a. Additionally, peritoneal macrophages obtained from *T. crassiceps*-infected MIF$^{-/-}$ mice were poor responders to in vitro LPS+IFN-γ stimulation and produced low levels of TNF-α, IL-12, and NO, suggesting that macrophages in MIF$^{-/-}$ mice may fail to respond to endogenous IFN-γ produced in MIF$^{+/+}$ mice and get activated to produce NO possibly required for elimination of the parasite. Although the mechanisms that are responsible for the lack of IFN-γ responsiveness in MIF$^{-/-}$ macrophages are not clear, it is likely that alterations in the IFN-γR levels or STAT1 signaling pathway could be involved. We are currently investigating these possibilities in our laboratory. Interestingly, a recent study has found that MIF$^{-/-}$ macrophages are hyporesponsive to LPS due to the reduced expression of the pathogen recognition receptor TLR-4 and the decreased activity of PU.1 that is required for optimal TLR-4 expression (36). Hence, it is likely that this mechanism may be at least in part responsible for impaired macrophage function. Finally, an alternate explanation could be related to the extraordinary high levels of IL-13 detected in MIF$^{-/-}$ *T. crassiceps*-infected mice, which could be associated to the deactivation of macrophages and the low response to IFN-γ, as has been suggested (5).

Classically, eosinophils have been considered as one of the most efficient effector cells in several helminth parasitic diseases (9, 18). Therefore, to determine whether susceptibility of MIF$^{-/-}$ mice to *T. crassiceps* was related to a deficient eosinophil recruitment, we compared the relative proportions of eosinophils in total PECs recruited in MIF$^{+/+}$ and MIF$^{-/-}$ mice at 4 and 8 weeks postinfection. The data obtained at these points in wild-type mice are very close to those recently reported (32). Interestingly, we found that MIF$^{-/-}$ infected mice recruited higher percentages of eosinophils compared to MIF$^{+/+}$ mice, whereas the percentage of peritoneal macrophages recruited in MIF$^{-/-}$ mice was significantly lower (Table 1). Taken together, these findings suggest that, in contrast to types of other helminthic infection, eosinophils do not ap-

pear to play a destructive role against this parasite, since MIF$^{+/-}$ mice harbored higher parasite loads. These data are also opposed to those recently reported that speculated about the possible role of MIF as a molecule involved in eosinophil recruitment (12), since MIF$^{-/-}$ infected mice maintained the ability to recruit high numbers of eosinophils (Table 1). This increase in eosinophil recruitment observed in MIF$^{+/-}$ at 8 weeks postinfection appears to be IL-5 independent because levels of IL-5 secreted by spleen cells at this point were similar between MIF$^{+/-}$ and MIF$^{-/-}$ (750 ± 49 versus 775 ± 145 pg/ml, respectively), suggesting that other factors, such as eotaxin, could be involved in this phenomenon. Finally, the analysis of PECs recruited during the infection showed differences in the macrophage populations between MIF$^{-/-}$ and MIF$^{+/-}$ mice; these differences are probably due to a defect in macrophage recruitment and activation as possible mechanisms involved in parasite installation.

In conclusion, the data presented here show that MIF plays an essential role in the resistance to murine cysticercosis caused by *T. crassiceps*. However, we cannot yet specifically establish the mechanisms involved in contributing to this resistance. Nevertheless, a defective response to IFN-γ, both in vivo and in vitro, was consistently observed, which probably favored parasite growth. Thus, the higher susceptibility of MIF$^{+/-}$ mice to *T. crassiceps* infection was associated with low levels of macrophage-derived factors such as IL-12, TNF-α, and NO. This work expands upon a previous study in which we demonstrated that macrophage activation appears essential to control cysticercosis (35). Our findings suggest that MIF is required for an optimal activation of macrophages to induce inflammatory responses for controlling murine cysticercosis. However, further investigation focused in MIF$^{-/-}$ macrophage impaired function is still needed to clarify these findings.

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Chronic Helminth Infection Induces Alternatively Activated Macrophages Expressing High Levels of CCR5 with Low Interleukin-12 Production and Th2-Biasing Ability

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Helminth infections induce Th2-type biased immune responses. Although the mechanisms involved in this phenomenon are not yet clearly defined, antigen-presenting cells (APC) could play an important role in this process. Here, we have used peritoneal macrophages (F4/80+) recruited at different times after challenge with *Taenia crassiceps* as APC and tested their ability to regulate Th1/Th2 differentiation. Macrophages from acute infections produced high levels of interleukin-12 (IL-12) and nitric oxide (NO), paralleled with low levels of IL-6 and prostaglandin E_2 (PGE_2) and with the ability to induce strong antigen-specific $CD4^+$ T-cell proliferation in response to nonrelated antigens. In contrast, macrophages from chronic infections produced higher levels of IL-6 and PGE_2 and had suppressed production of IL-12 and NO, associated with a poor ability to induce antigen-specific proliferation in $CD4^+$ T cells. Failure to induce proliferation was not due to a deficient expression of accessory molecules, since major histocompatibility complex class II, CD40, and B7-2 were up-regulated, together with CD23 and CCR5 as infection progressed. These macrophages from chronic infections were able to bias $CD4^+$ T cells to produce IL-4 but not gamma interferon (IFN-γ), contrary to macrophages from acute infections. Blockade of B7-2 and IL-6 and inhibition of PGE_2 failed to restore the proliferative response in $CD4^+$ T cells. Furthermore, studies using $STAT6^{-/-}$ mice revealed that STAT6-mediated signaling was essential for the expansion of these alternatively activated macrophages. These data demonstrate that helminth infections can induce different macrophage populations that have Th2-biasing properties.

After stimulation, $CD4^+$ T cells differentiate into distinct subsets characterized by their functions and their cytokine profiles (Th1 or Th2 cells). Th1 cells produce high levels of interleukin 2 (IL-2) and gamma interferon (IFN-γ), which are strong inducers of cell-mediated immunity, whereas Th2 cells produce cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13, which provide signals for B-cell activation and antibody production (34). Although the mechanisms that generate both subsets have been studied in detail, there is not enough convincing evidence of one isolated process inducing polarization of CD4 T cells towards a Th1 or Th2 outcome. Nevertheless, several factors, such as the major histocompatibility complex (MHC) haplotype, dose and nature of the antigen, route of antigen administration, costimulatory molecules, cytokine microenvironment, and type of antigen-producing cells (APC) (2, 6, 7, 24), have been shown to play a role in the polarization of the immune response. Since it is possible that most in vivo responses do not take place in a milieu with adequate levels of cytokines to stimulate T cells, professional APC may play an important role in Th differentiation by secreting the essential cytokines locally and providing the ligands for T-cell-receptor and costimulatory signals. Thus, the state of activation of APC could be a decisive factor inducing the polarization of the immune response.

A key feature of helminth infections is the induction of strong Th2-biased immune responses in their hosts. Why and how helminths induce Th2-type biased responses are still unresolved questions. It has been suggested that excreted/secreted products from helminth parasites play an important role in these Th2 responses (16, 36). However, the precise mechanism of action or the target cell for these products is still uncertain. Indeed, parasitic helminth infections can modify the normal host immune response against another nonrelated antigenic stimulus (8, 21) and/or also modify the susceptibility to others parasites (15, 38) or viruses (1), suggesting an important influence of helminth infections in the immunological microenvironment.

Also, experimental murine cysticercosis caused by the helminth *Taenia crassiceps* induces polarized type 2 cytokine profiles; another key feature is a gradual shifting from an initial restrictive Th1-type response to a late permissive Th2-type response in the infected host (41, 45). We also have previously reported that infection with *T. crassiceps* cysticerci renders their host more resistant or more susceptible to a secondary

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nonrelated infection depending on the time course of *T. crassiceps* implantation (38). A series of recent studies suggest that a subset of macrophages, called alternatively activated, plays a role in the Th2 biased response induced in nematode infections (4, 25, 26, 28). Therefore, we hypothesized that these alternatively activated macrophages can be a more general mechanism to down-modulate T-cell proliferation and favor Th2 development in helminth infections. To test this hypothesis, we isolated peritoneal macrophages from *T. crassiceps*-infected mice during acute as well as chronic infection and used them as APC to determine whether they induce preferential Th2 differentiation.

We found that macrophages obtained during chronic infections showed a poor ability to induce proliferative responses in $CD4^+$ T cells when used as APC but favored IL-4 production. Furthermore, these cells expressed high levels of both CD23 and CCR5, which were associated with low IL-12 and nitric oxide (NO) production, but secreted high levels of IL-6 and prostaglandin E_2 (PGE_2). This contrasted with macrophages recruited early during infections, which induced stronger $CD4^+$-T-cell proliferation and high levels of IFN-γ. Our data indicate that the state of activation of APC induced by a chronic helminth infection modulates the final pattern of response of $CD4^+$ T cells previously sensitized with a nonrelated parasite antigen. This is a possible mechanism for Th2 biased responses in helminth infections.

MATERIALS AND METHODS

Mice. Six- to eight-week-old female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were maintained in a pathogen-free environment at the Harvard Medical School animal facility in accordance with institutional guidelines. In some experiments STAT6-KO mice in a BALB/c background (Jackson Labs) were used.

Parasites and infections. Metacestodes of *T. crassiceps* (ORF strain) were harvested from the peritoneal cavity of female BALB/c mice after 2 to 4 months of infection. The cysticerci were washed four times in sterile phosphate-buffered saline (PBS) (0.15 M, pH 7.2). Experimental infection was achieved by intraperitoneal (i.p.) injection with 20 small (diameter, ca. 2 mm) nonbudding cysticerci of *T. crassiceps* suspended in 0.3 ml of PBS per mouse, and infections were done at different times in order to perform all assays at the same day, using age-matched uninfected mice as controls for each time point.

Peritoneal macrophage extraction and pattern of response to LPS. Peritoneal exudate cells (PECs) were obtained from the peritoneal cavities of mice infected with *T. crassiceps* for 2, 4, 8, and 12 weeks or from uninfected mice injected with thioglycolate (3%) 4 days earlier. The cells were washed twice with Hanks' solution, and erythrocytes were lysed by resuspending cells in Boyle's solution (0.17 M Tris and 0.16 M ammonium chloride). Following two washes, viable cells were counted by trypan blue exclusion. PECs were adjusted to 5×10^6 cells/ml in RPMI supplemented and cultured in six-well plates (Costar, Cambridge, Mass.). After 3 h at 37°C and 5% CO_2, nonadherent cells were removed by washing with warm supplemented RPMI medium. Adherent cells were removed with cold PBS and a scraper and readjusted to 10^6 cells/ml. Viability was determined at this step by trypan blue exclusion; viability was usually >95%. One milliliter was plated, and cell activation was performed in 24-well plates (Costar) by addition of lipopolysaccharide (LPS) (5 μg/ml, *E. coli* 111:B4; Sigma, St. Louis, Mo.) followed by incubation for 48 h at 37°C and 5% CO_2. Supernatants were harvested, centrifuged, and examined by enzyme-linked immunosorbent assay (ELISA) for production of IL-6, IL-12, IL-10 (antibodies and cytokines were obtained from Pharmingen [San Diego, Calif.]), IL-18, and transforming growth factor beta (TGF-β) (obtained from R&D Systems). Peritoneal adherent cells constituted >90% of macrophages according to fluorescence-activated cell sorter (FACS) analysis (F4/80$^+$).

Flow-cytometric analysis. Expression of costimulatory and accessory molecules was analyzed by flow cytometry of peritoneal cells from uninfected mice and on weeks 2, 4, and 8 following *T. crassiceps* infection. PECs were obtained as mentioned above and cultured in 24-well plates (Costar) overnight at 37°C with 5% CO_2. Nonadherent cells were discarded, and adherent cells were recovered with a plastic scraper and processed for analysis. Adherent PECs were blocked with anti-mouse FcγR antibody (CD16/CD32) and stained with fluorescein isothiocyanate-conjugated monoclonal antibodies against F4/80, MHC-II, B7-2, or B7-1 or phycoerythrin-conjugated antibodies against CD23 or CCR5. All antibodies were purchased from Pharmingen except F4/80, which was obtained from Serotec (Oxford, England). Stained cells were analyzed on a FACSCalibur analyzer using Cell Quest software (Becton Dickinson). Live cells were electronically gated using forward- and side-scatter parameters.

Detection of NO production. NO production by macrophages was assayed by determining the increase in nitrite concentration (33) by the Griess reaction adapted to microwell plates (Costar). Briefly, 50 μl of culture supernatant was mixed with an equal volume of Griess reagent (1.5% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid) and incubated for 10 min at room temperature in the dark, and the absorbance was measured at 570 nm. Values were quantified using serial dilutions of sodium nitrite.

PGE_2 measurement. PGE_2 concentrations in supernatants from macrophages stimulated with 5 μg of LPS/ml were measured by an enzymatic immunoassay kit (Cayman Chemical) following the producers' specifications.

Cell culture and coculture of macrophages-$CD4^+$ cells: antigen presentation assay. All cultures and cocultures were maintained in RPMI 1640 (Gibco BRL) supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin (Sigma)/ml, 5×10^{-5} M 2-β-mercaptoethanol (GIBCO BRL), and 10% fetal bovine serum (HyClone).

Spleen cells from *T. crassiceps*-infected mice were processed as described previously (38) and stimulated with 25 μg of soluble antigen of *T. crassiceps*/ml; supernatants were harvested 72 h after antigen stimulation, and IL-4, IL-13, and IFN-γ were evaluated by ELISA. Coculture of macrophages with primed $CD4^+$ T cells was performed as follows: macrophages were obtained as described, adjusted to 10^6/ml, plated in 96-well plates (Costar), and maintained at 37°C with 5% CO_2 for 1 h in the presence of keyhole limpets hemocyanin (KLH) (Sigma) or chicken egg albumin (OVA) (Sigma), 50 μg/ml. Splenocytes were prepared from mice previously i.p. injected (7 days earlier) with KLH (100 μg/mouse) or OVA (100 μg/mouse) without adjuvant and enriched for $CD4^+$ T cells (>95% by FACS analysis) using CD4 magnetic cell sorter beads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells were plated in 96-well flat-bottom plates (Costar) which were precoated with macrophages from uninfected or infected mice at a 1:2 ratio (macrophages/$CD4^+$) and loaded with OVA or KLH as mentioned above. Cultures were maintained at 37°C with 5% CO_2 for 4 days, and then [^{3}H]thymidine (activity, 185 GBq/mmol; Amersham, Aylesbury, England) (1 μCi/well) was added and incubated for a further 18 h. Cells were harvested on a 96-well harvester (Tomtec, Toku, Finland) and counted using a β-plate counter. Values are expressed as counts per minute from triplicate wells and are the result after subtracting counts per minute from cocultures in the absence of antigen. Supernatants from cocultures were harvested at 72 h and analyzed for IL-4, IL-6, IL-12, and IFN-γ production. In some cocultures (at 8 weeks after infection) PECs, previously loaded with OVA, were fixed with 0.5% paraformaldehyde for 5 to 10 min, washed extensively with RPMI, adjusted, and added to 96-well plates in the presence of $CD4^+$ cells previously sensitized with OVA. These cocultures were processed as described above.

Statistical analysis. Comparisons between control and experimental groups in this work were made using Student's unpaired *t* test. *P* values of ≤ 0.05 were considered significant.

RESULTS

Parasite growth and shifting from a Th1-type response towards a Th2-type response. Studies in murine cysticercosis have shown that the immune response is altered by the chronicity of the infection, going from an early Th1-type to a late Th2-type response as infection progresses (41). Since we were interested in determining the mechanisms that induce highly polarized immune responses in helminth infections, we used this model to study how APCs derived from different immunological environments can alter or modify an expected immune response. In order to show that APCs used in our study were under the influence of a different immunological environment, we assayed the kinetics of parasite growth in the peritoneal cavity, the specific antibody response, and total im-

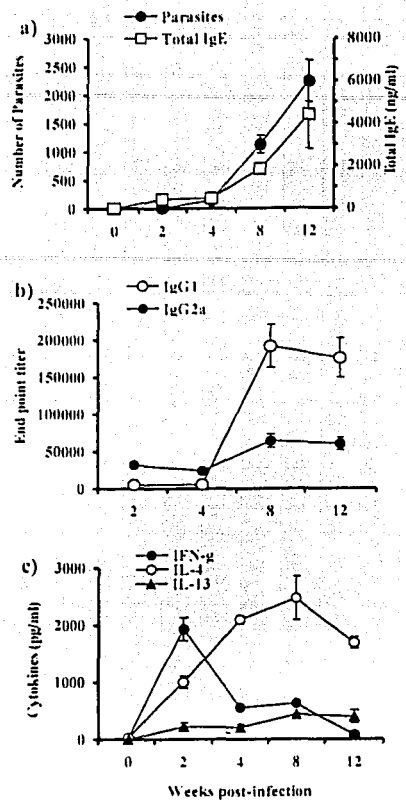

FIG. 1. Course of *T. crassiceps* infection in BALB/c mice and Th1/Th2 markers. (a) Female BALB/c mice were inoculated i.p. with 20 cysticerci of *T. crassiceps*, and parasite loads and total IgE levels were monitored at times indicated. (b) Endpoint titers for antigen-specific antibody production of IgG1 and IgG2a. (c) Antigen-specific production of IFN-γ, IL-4, and IL-13 by splenocytes from *T. crassiceps*-infected mice as markers of Th1/Th2 environment. Data are representative of three independent experiments. Error bars represent ± standard deviations.

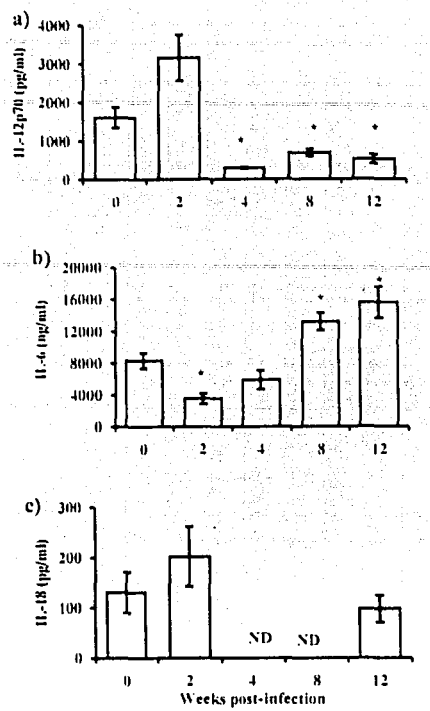

FIG. 2. Cytokine production by macrophages in response to Th1-polarizing antigen (LPS). IL-12 (a), IL-6 (b), and IL-18 (c) production by macrophages from different weeks after *T. crassiceps* infection was determined by ELISA. Adherent PECs were obtained from infected mice at the times indicated, as well as from age-matched uninfected mice. Macrophages (10^6) were stimulated in vitro with 5 μg of LPS/ml for 48 h. Data are the mean ± standard deviation for six animals at each time point. Asterisk, $P < 0.05$ with respect to uninfected mice and mice at 2 weeks postinfection; ND, not determined.

munoglobulin E (IgE) levels after an initial i.p. infection with 20 cysticerci. Furthermore, we analyzed the profile of cytokines released by splenocytes from infected mice after antigen-specific stimulation. The results obtained were similar to those previously described (38, 41), showing that at early infection IgG2a antigen-specific levels were higher than those detected for IgG1, but as *T. crassiceps* infection progressed, increased levels of both total IgE and antigen-specific IgG1 antibodies were detected while the IgG2a levels showed only a slight increase (Fig. 1a and b). On the other hand, antigen-specific IFN-γ was detected at higher levels early in the infection, but as infection turned chronic these levels dropped drastically. In contrast, IL-4 and IL-13 were detected in low levels early in the infection but were increased during chronic infection (Fig. 1c). These results show that although *T. crassiceps*-infected mice initially develop a Th1-type immune response concomitant with a limited parasite growth, later this immune response is progressively polarized towards a Th2-type response.

Helminth infection differentially alters macrophages' cytokine response in a time-dependent manner. The mechanisms involved in the induction of polarized Th2-type responses in cysticercosis and in other helminth infections are still undefined. To determine the effect of *T. crassiceps* infection on macrophage function, we analyzed the cytokine production by adherent peritoneal exudate cells (PECs, mostly macrophages, >90% according to the expression of F4/80) from *T. crassiceps*-infected mice at weeks 2, 4, 8, and 12. Adherent macrophages were stimulated with LPS (5 μg/ml) for 48 h, and supernatants were analyzed for cytokine production (IL-6, IL-10, IL-12, IL-18, and TGF-β). Macrophages obtained in early periods after infection (2 weeks), when the parasite burden was low (Fig. 1a), produced levels of IL-12 similar to or higher than those for macrophages from uninfected mice (Fig. 2a). In contrast, IL-6 was detected in significantly lower levels in the same supernatants (Fig. 2b). Macrophages from late infections produced significantly higher levels of IL-6 (Fig. 2b, $P < 0.05$), but

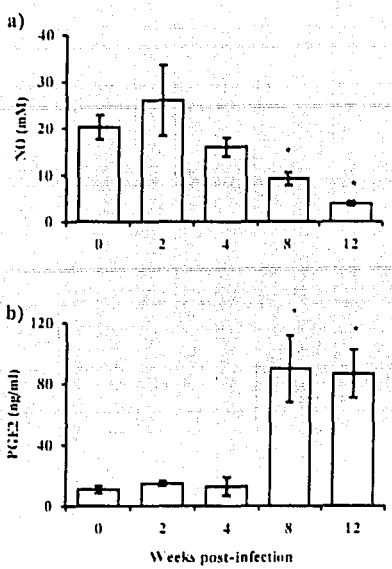

FIG. 3. Other products of macrophages' activity are altered by *T. crassiceps* infection. Production of PGE_2 (a) and NO (b) as measured by ELISA or Griess reaction were assayed from the same culture supernatants from Fig. 2. Asterisk, $P < 0.05$ with respect to uninfected mice and mice at 2 weeks postinfection.

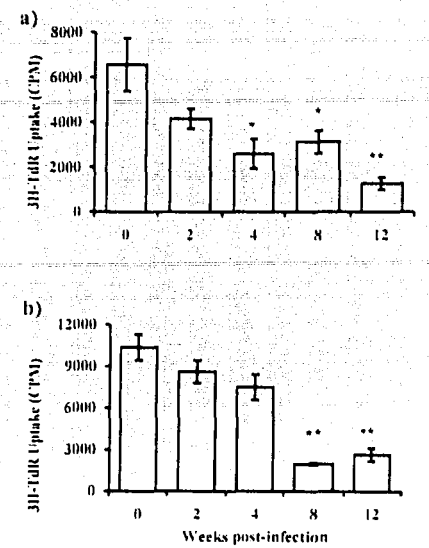

FIG. 4. *T. crassiceps* infection alters the capacity of macrophages as antigen-presenting cells. Macrophages from different weeks after infection were loaded with KLH (a) or OVA (b) and cocultured with $CD4^+$ cells (previously sensitized) for 5 days; proliferation was assayed by uptake of [^{3}H]thymidine (3H-TdR). Data are representative of three different experiments. ± standard errors. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$ (compared to uninfected mice or mice infected for 2 weeks).

IL-12 was significantly suppressed between weeks 8 and 12 postinfection when the numbers of parasites were higher. At both time points there were no significant alterations in the levels of IL-18 (Fig. 2b). IL-10 and TGF-β levels were minimally detected in these same supernatants, and no statistically significant differences were observed in the infection (data not shown).

NO and PGE_2 are two compounds associated with the state of macrophage activation (46). As shown in Fig. 3a, NO production was maintained in steady levels until the fourth week after infection; however, as the infection progressed (8 to 12 weeks), the NO levels dropped significantly ($P < 0.05$). In contrast to IL-12 and NO, PGE_2 production presented a strikingly reverse pattern; that is, only low levels were released by adherent macrophages obtained early after infection, but as infection advanced, these cells produced significantly higher quantities of PGE_2, reaching the maximum level at 8 weeks postinfection, which was sustained at least until week 12 postinfection (Fig. 3b, $P < 0.01$).

Chronic helminth infection alters the APC function of adherent macrophages. It is known that helminth infections can significantly bias immune responses to unrelated soluble antigens introduced in the host towards a Th2 type (8), but the mechanisms related to this phenomenon are not clear. Since adherent PECs are mostly macrophages and they can function as APC, we decided to investigate whether this property in our cell population could be affected by the course of the infection. To examine the APC ability of adherent PECs to prime Th responses in vitro, macrophages obtained at different times after infection or from healthy mice were cocultured with purified $CD4^+$ T cells isolated from mice previously injected with KLH or OVA. Adherent macrophages were obtained and pulsed with antigen (KLH or OVA at 50 μg/ml), and after 2 h $CD4^+$ T cells were plated in the same cultures. Adherent macrophages obtained from healthy or early-infected mice induced strong proliferative responses in $CD4^+$ T cells to either KLH or OVA antigens (Fig. 4). In contrast, cocultures where the APC were obtained from chronically infected mice presented a significantly weaker ability to induce proliferative responses in $CD4^+$ T cells (Fig. 4).

The state of activation of APC alters the pattern of cytokines secreted by $CD4^+$ T cells. Parallel to the proliferative response, supernatants in the cocultures were harvested and analyzed for IL-4, IL-6, IL-12, and IFN-γ production. In supernatants from $CD4^+$ T cells cocultured with APCs obtained in early infections, significantly higher levels of IFN-γ and IL-12 were detected than were detected with those stimulated with APCs from later infections (Fig. 5a and b). In addition, the same supernatants showed low levels of IL-4 and IL-6 in the $CD4^+$ cells cocultured with adherent macrophages from uninfected or early-infected mice. However, as infection progressed, adherent macrophages drove $CD4^+$ cells to produce significantly more IL-4 but lower levels of IFN-γ (Fig. 5c and a), whereas the levels of IL-6 in these supernatants were higher than in early infections (Fig. 5d).

Differential expression of surface molecules is detected on

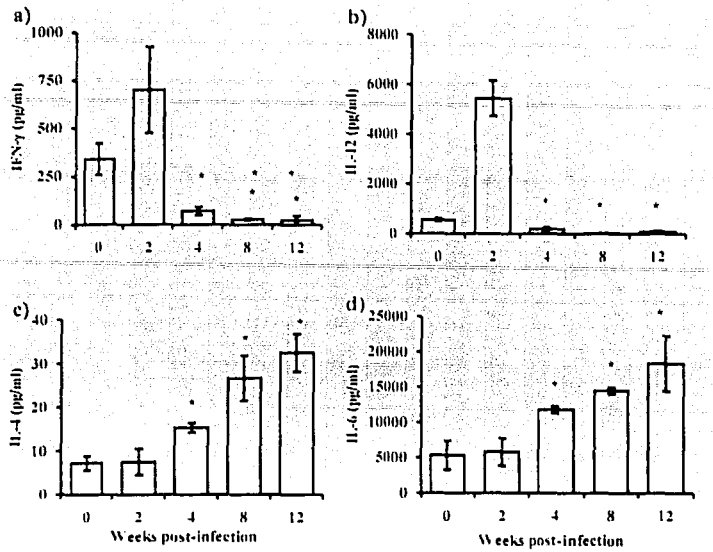

FIG. 5. T-cell-polarizing ability of alternatively activated macrophages in vivo during *T. crassiceps* infection. Adherent peritoneal macrophages were obtained from infected mice at the times indicated, as well as from age-matched uninfected mice. Cells (10^6) were loaded in vitro with 50 μg of OVA for 2 h. $CD4^+$ T cells (2×10^6) from healthy mice previously immunized with OVA were cocultured, and 72 h later supernatants were analyzed for IFN-γ (a), IL-12 (b), IL-4 (c), and IL-6 (d). Data are representative of three independent experiments with at least four animals individually assayed. Error bars indicate ± standard deviations. Asterisk, $P < 0.05$ comparing with macrophages from healthy mice or early (2 weeks)-infected mice.

macrophages, as infection turns chronic. It has been shown that several molecules on the surface of the APC are important for adequate interaction with the responder T cells to induce them to proliferate and secrete cytokines. To determine whether infection with cysticerci could alter the antigen-presenting function in macrophages, the expression of costimulatory molecules and molecules related to antigen presentation were evaluated. We measured the expression of B7-1, B7-2, MHC-II, and CD40 on macrophages extracted from uninfected mice and from *T. crassiceps*-infected mice after 2, 4, and 8 weeks of infection. As infection progressed, macrophages showed an increase of the costimulatory molecules MHC-II and CD40. By 8 weeks postinfection, more of the 90% of macrophages expressed MHC-II and CD40 molecules with high density per cell (Fig. 6), suggesting that macrophages from chronic infection have increasing APC capacity. However, compared with uninfected mice there was not significant change in the surface expression of B7-1 at any point of the infection. In contrast, B7-2 expression was markedly up-regulated in macrophages from late-infected mice (8 weeks) with respect to its expression in uninfected mice and mice infected for 2 weeks (Fig. 6). The higher intensities of relative fluorescence observed in B7-2 expression indicate that chronic infection with *T. crassiceps* cysticerci can dramatically and differentially increase the expression of important molecules involved in antigen presentation.

Because macrophages obtained from different times of helminth infection displayed both different patterns of cytokine production and distinct abilities to induce antigen-specific proliferation, we next looked for additional markers that identified these apparently different populations of macrophages. Double immunofluorescence analysis showed an interesting up-regulation of CD23 and CCR5 in $F4/80^+$ cells (macrophages) as infection advanced (Fig. 7). Expression of CD23 has been associated with a state of activation of macrophages known as alternatively activated macrophages (11), whereas CCR5 expression has been basically associated with inflammatory processes (47). Interestingly, the up-regulation of these markers was accompanied by an increase in parasite load, poor IL-12 production, and a weak ability to stimulate $CD4^+$ T cells.

In an attempt to elucidate other factors that could influence the recruitment or activation of these alternatively activated macrophages, we analyzed the peritoneal population that was expanded in response to *T. crassiceps* infection in STAT6-KO mice. As shown in Fig. 7 (bottom panel), macrophages recruited in STAT6-KO mice after 8 weeks of infection did not up-regulate CD23 and/or CCR5, which were detected at levels very similar to those observed for uninfected mice. It is important to note that STAT6-KO mice are highly resistant to *T. crassiceps* infection and their macrophages produce higher levels of IL-12 (39).

Blockade of IL-6 and B7-2 and inhibition of PGE_2 did not restore CD4 proliferation, but absence of STAT6 did. To determine factors contributing to the low level of proliferation observed in $CD4^+$ T cells when macrophages from chronic infections were used as APC, we performed a series of experiments blocking IL-6, B7-2, and PGE_2. As shown in Fig. 8, blockade of IL-6 and B7-2 with specific monoclonal antibodies

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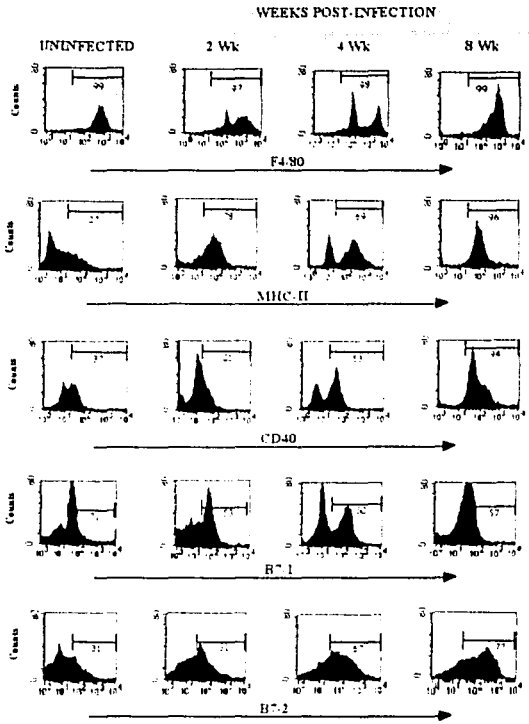

FIG. 6. Phenotypes of peritoneal macrophages isolated from *T. crassiceps* infection. Cell surface expression of F4/80, MHC-II, CD40, B7-1, and B7-2 at 0, 2, 4, and 8 weeks after *T. crassiceps* infection are shown. Data are representative histograms of three separate experiments.

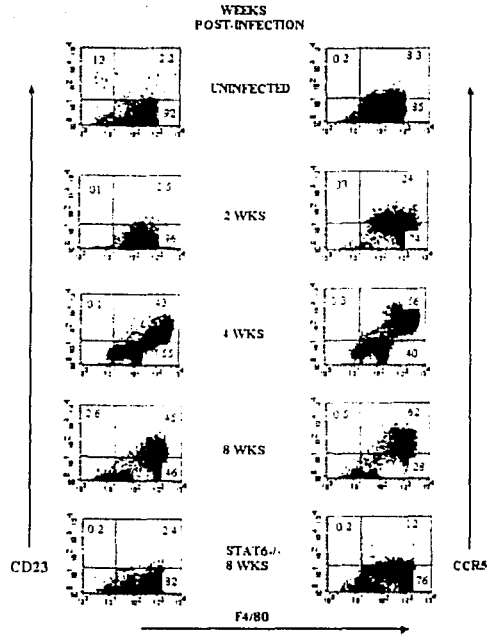

FIG. 7. *T. crassiceps* infection up-regulates the expression of CD23/CCR5 in macrophages. Cell surface expression of F4/80-CD23 and F4/80-CCR5 on peritoneal macrophages obtained at different weeks after infection. Bottom, macrophages obtained from STAT6-KO mice 8 weeks after *T. crassiceps* infection.

and inhibition of PGE_2 with indomethacin did not modify the poor ability to induce proliferation observed previously in macrophages from late infections (Fig. 8a). Interestingly, when macrophages obtained from STAT6-KO mice after 8 weeks of infection with *T. crassiceps* were used as APC, a significantly higher-level proliferative response of $CD4^+$ T cells was observed (Fig. 8a). However, when the cytokine production was measured in the same cocultures, blockade of IL-6 significantly reduced the expected levels of IL-4, whereas IFN-γ was detected in significantly higher concentrations (Fig. 8c and d). Furthermore, we observed that blockade of B7-2 and PGE2 did not alter the cytokine profile previously detected (Fig. 8 b and d). In contrast, macrophages deficient in STAT6 signaling induced significantly lower levels of IL-4 but higher production of IFN-γ compared with wild-type macrophages (Fig. 8 c and d). Finally, in order to know the source of IL-6 detected in the cocultures, macrophages from chronic infections were loaded with OVA, fixed, and used as APC. Data from these cocultures showed that IL-6 is largely produced by macrophages, since these cocultures showed a very low level of IL-6 (Fig. 8b), and again levels of IFN-γ were recovered (IL-4 levels were <5 pg/ml) (Fig. 8 c and d). Thus, macrophages obtained from chronically *T. crassiceps*-infected mice potently drive Th2 development, apparently in an IL-6- and STAT6-dependent manner.

DISCUSSION

Early and sustained interactions between the pathogen and the host participate in the establishment of an adaptive immune response against the pathogen. The majority of intracellular pathogens are potent stimulators of Th1 responses. For example, *Mycobacterium*, *Listeria*, *Trypanosoma*, and *Toxoplasma* all drive Th1-type responses, in part, due to the rapid induction of IL-12 and IFN-γ by innate immune cells responding through expressed molecules, termed pattern recognition receptors, which bind conserved structures shared by large groups of pathogens (3, 5). In contrast, helminth infections generally bias the immune response towards a Th2-type profile that may also be associated with induction of antigen-specific or nonspecific anergy (8, 38). The mechanisms involved in this phenomenon are not yet completely defined.

To date, cytokines and APC cells represent two of the major determinant factors in the differentiation of naive and uncommitted $CD4^+$ T cells into effectors Th1 and Th2 cells (17). In addition, the activation status of APC has been gaining importance as an explanation of how the final commitment of Th1/Th2 cells is achieved, since they are both a source of cytokines

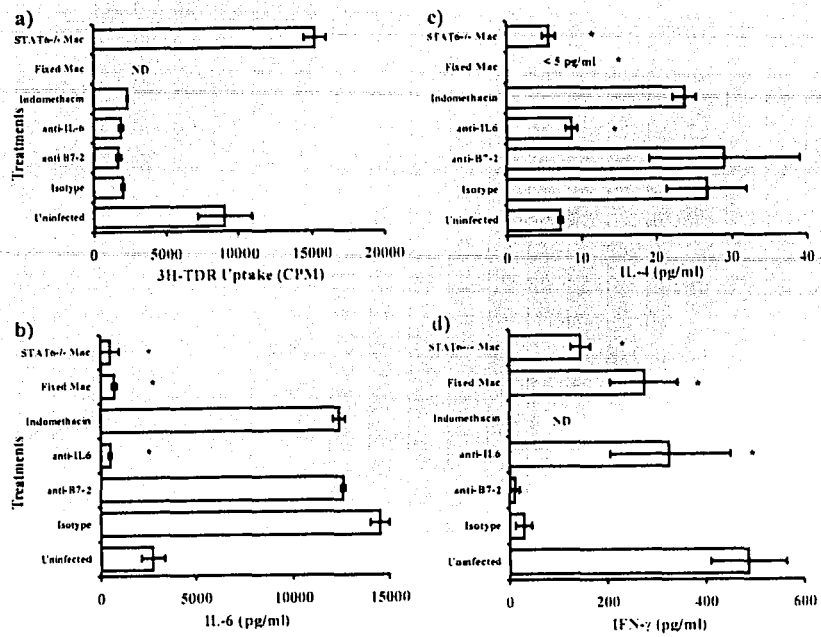

FIG. 8. Effect of fixation, blockade of IL-6/B7-2, inhibition of PGE_2, and absence of STAT6 signaling of macrophages in $CD4^+$ T-cell proliferation and cytokine production. (a) Macrophages from mice at 8 weeks postinfection were used as APC and cocultured as for Fig. 4. In addition, blocking antibodies for IL-6 and B7-2, indomethacin, fixed macrophages, and STAT6-KO macrophages were used in these cocultures. (a) Proliferation was assayed by [^{3}H]thymidine (3H-TDR) incorporation. (b to d) Cytokine levels detected in the presence and absence of blocking antibodies, indomethacin, fixed macrophages, or STAT6-KO macrophages. Asterisk, $P < 0.05$ compared to isotype control. ND, not determined.

and a source of antigen (17). In the present study our data demonstrate that following chronic exposure to the helminth *T. crassiceps*, a population of macrophages with an increased expression of MHC-II, CD40, B7-2, CD23, and CCR5 is expanded. This population produces low levels of IL-12 both in response to LPS and in coculture with $CD4^+$ T cells plus antigen and also exhibited a very limited ability to induce antigen-specific $CD4^+$ T-cell proliferation. Moreover, $CD4^+$ T cells stimulated with these APCs produced more IL-4 instead of IFN-γ. In contrast, macrophages obtained after a short period following the helminth infection (2 weeks) expressed low levels of B7-2, CD23, and CCR5, produced more IL-12, and induced high levels of proliferation in $CD4^+$ T cells, which preferentially produced IFN-γ.

Recently it has been shown that exposure of immature APCs to helminth antigens in vitro can influence the Th1/Th2 commitment (18, 25, 26, 28, 30). However, this study is the first demonstration in vivo that over the course of a helminth infection APCs can undergo such a dramatic change in phenotype and be able to produce a differential pattern of cytokines in such a manner that on encountering a new antigen the immune response is biased towards Th2 differentiation.

IL-12 plays a critical role in the development of Th1 responses, up-regulating the production of IFN-γ by NK and $CD4^+$ T cells (32). Our data showed that IL-12 production was down-regulated as infection progressed. In chronic infections, macrophages switch to a phenotype that produces more IL-6 and PGE_2 but less IL-12 and NO. The absence of APC-derived IL-12 could be a default pathway in helminth infections to rapidly alter the outcome of immunity to new antigenic challenges or infections. In addition, the presence of IL-6 could facilitate this transition, since IL-6 has been shown to induce differentiation of IL-4-producing $CD4^+$ T cells (37). Thus, IL-6 produced by APCs could play an important role in the initiation of Th2 differentiation in helminth infections, as has been recently suggested (23). In our study, this idea is supported by the findings that blockade of IL-6 in cocultured macrophages from late infections with $CD4^+$ T cells suppressed IL-4 production. Furthermore, when macrophages were fixed and used as APC, levels of IL-6 were at minimum, and in both cases IFN-γ reached levels similar to those detected in cocultures with macrophages from healthy mice, suggesting that IL-6 may be an important factor favoring Th2 commitment in cysticercosis. Also, the higher level of production of PGE_2 by macrophages from chronic infections could be associated with the low production of IL-12 and NO whereas favoring IL-6 production (12, 19, 22, 44). Thus, several APC-dependent factors are involved in the Th2 differentiation during a helminth infection.

Besides the cytokines produced by antigen-presenting cells, there are other factors, such as altered peptide ligands and costimulatory molecules (B7-1, B7-2), that can influence the

differentiation of T cells (7, 24). We found that B7-2 expression was up-regulated as infection progressed, whereas B7-1 was not. Despite the fact that there is strong evidence of the involvement of B7-2 in Th2-biased responses in different systems (43), in helminth infections it has been shown that neither B7-1 nor B7-2 blockage affects the development of the immune response (9, 13). Similarly, in the present study blockage of B7-2 in vitro did not modify the outcome of IL-4/IFN-γ production or proliferation in cocultured $CD4^+$ T cells/macrophages. These data suggest that costimulation through B7-2 is not involved in the induction of Th2-type responses by macrophages from late *T. crassiceps* infections.

Peritoneal macrophages taken from chronic infections displayed features shared with alternatively activated macrophages, which can be induced by IL-4, IL-13, IL-10, and TGF-β, and they express innate immune receptors and CD23 (10, 11). These alternatively activated macrophages have been reported in filariasis, where they appear to play a role in inducing Th2 differentiation (25, 29). Moreover, these macrophages also produce IL-10 and TGF-β and apparently play an important role in down-modulating inflammation and immunity. Though our results differed in IL-10 and TGF-β involvement and in the different surface markers, our data on IL-4 dependence, low-level induction of proliferative ability, time required to appear, and Th2 biasing activity of alternatively activated macrophages in murine cysticercosis are in accordance with the findings of MacDonald et al. (28) and Loke et al. (26), who implanted the nematode *Brugia malayi* in the peritoneal cavity of mice to recruit suppressor macrophages. Loke et al. also showed that cytokine production in their system was skewed towards the Th2 type, paralleled with suppression in the proliferative response (25). Together, these studies suggest that the commitment towards a Th2 pattern of cytokine production by $CD4^+$ cells following interaction with alternatively activated macrophages may explain the reduced levels of IFN-γ and IL-12 observed with certain helminth infections and the biased Th2-type response to new antigenic challenges consistently reported in helminth coinfections (1, 21, 22).

In our model, macrophages isolated from a chronic infection shared some of the features observed in alternatively activated macrophages, as mentioned before, and in addition they expressed high levels of CCR5/CD23 as well as molecules associated with antigen presentation. Interestingly, peritoneal macrophages taken from STAT6$^{-/-}$ *T. crassiceps*-infected mice (8 weeks) did not show these features. They had low CD23 and CCR5 expression and successfully induced antigen-specific proliferation as well as IFN-γ production in $CD4^+$ T cells when they were used as APC, revealing that the development of these alternatively activated macrophages is critically dependent on the STAT6-mediated signaling pathway and also implying a role for IL-4 and/or IL-13 in the expansion and activation of these macrophages during this helminth infection.

An exciting question regards the early source of IL-4 in response to helminth antigens; accessory cells and naïve Th cells have been suggested as sources of this key cytokine (14, 27), but conclusive studies are still missing. In our hands, ex vivo analyses of macrophages from *T. crassiceps*-infected mice (with no further in vitro stimulation) by reverse transcription-PCR suggest that these cells are not a source of IL-4 (data not shown). However, further studies are necessary to know whether or not helminth antigen does stimulate macrophages directly to produce IL-4; this is an area of growing interest (27).

The role of high-level expression of CCR5 in macrophages is not well understood (20); however, in T cells it has been associated with inflammation and is IL-12 dependent (35). The high-level expression of CCR5 detected in macrophages from heavily parasitized mice could be related to the absence of CCR5 ligands, RANTES/MIP-1α (31). On the other hand, the relationship between CCR5 in macrophages and their poor ability to induce proliferation in $CD4^+$ T cells is an interesting finding that needs more detailed study. Indeed, it is noteworthy that this population of macrophages produces low levels of IL-12 even in response to a strong Th1-type stimuli, such as LPS (30), and also in the cocultures with antigen-specific $CD4^+$ T cells where the CD40-CD40L system is participating. These observations are in sharp contrast with dendritic cells expressing high levels of CCR5 in response to acute exposure to protozoan antigens, which were high producers of IL-12 and play a significant role in inducing Th1 responses (3, 32). This discrepancy between failure to produce IL-12 and high CCR5 expression in macrophages from chronic *T. crassiceps*-infected mice could be an intrinsic feature for helminth infections and possibly plays a role in the bias towards Th2 immune responses to both the parasite's antigen and nonrelated antigens in helminthic diseases (1, 15, 38).

In conclusion, we found that chronic infection with the helminth parasite *T. crassiceps* can differentially regulate the T-cell-stimulatory ability of APC and favor a Th2-type response to new antigenic encounters. This mechanism appears to be STAT6 dependent and possibly involve IL-4 and IL-13. However, a recent body of evidence suggests an alternative route to Th2 biasing in helminth infections, which implicates APCs as a possible direct target of helminth products to finally deliver Th2 signals (30, 40, 42).

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APENDICE I I

Artículos relacionados con los mecanismos de polarización de la respuesta inmune en las infecciones murinas por *Leishmania major* y *Leishmania mexicana*

Genetically Resistant Mice Lacking IL-18 Gene Develop Th1 Response and Control Cutaneous *Leishmania major* Infection[1]

Gina M. Monteforte,* Kiyoshi Takeda,† Miriam Rodriguez-Sosa,* Shizuo Akira,†
John R. David,* and Abhay R. Satoskar[2]*

IL-18 has been shown to play a critical role in the development of a Th1 response and immunity against intracellular pathogens. To determine the role of IL-18 in the development of protective immunity against *Leishmania major*, we have analyzed the course of cutaneous *L. major* in IL-18-deficient C57BL/6 mice (IL-18$^{-/-}$) compared with similarly infected wild-type mice (IL-18$^{+/+}$). After *L. major* infection, IL-18$^{-/-}$ mice may develop larger lesions during early phase of infection but eventually will resolve them as efficiently as IL-18$^{+/+}$ mice. By 2 wk after infection, although Ag-stimulated lymph node cells from *L. major*-infected IL-18$^{+/+}$ and IL-18$^{-/-}$ mice produced similar levels of IFN-γ, those from IL-18$^{-/-}$ mice produced significantly more IL-12 and IL-4. By 10 wk after infection, both IL-18$^{+/+}$ and IL-18$^{-/-}$ mice had resolved *L. major* infection. At this time, lymph node cells from both IL-18$^{+/+}$ and IL-18$^{-/-}$ mice produced IL-12 and IFN-γ but no IL-4. Furthermore, administration of anti-IFN-γ Abs to IL-18$^{-/-}$ mice rendered them susceptible to *L. major*. These results indicate that despite the role IL-18 may play in early control of cutaneous *L. major* lesion growth, this cytokine is not critical for development of protective Th1 response and resolution of *L. major* infection. *The Journal of Immunology*, 2000, 164: 5890–5893.

Leishmania are obligate intracellular parasites that cause a wide range of diseases such as cutaneous, mucocutaneous, and visceral leishmaniasis (1). The murine model of cutaneous *Leishmania major* infection has been well characterized and frequently has been used as a functional model of Th1 and Th2 cell responses (2). Control of cutaneous *L. major* infection in resistant mice such as C3H and C57BL/6 is associated with the development of IL-12-induced Th1-type response and the production of IFN-γ (2–4). In contrast, susceptible BALB/c mice develop large nonhealing lesions after *L. major* infection and mount a Th2-type response that is associated with the production of the cytokines IL-4 and IL-10 (2, 5).

IL-18 is a recently discovered cytokine that is produced by activated macrophages (6). This cytokine has been shown to play a critical role in the development of protective immunity against intracellular pathogens including *Mycobacterium tuberculosis*, *Cryptococcus neoformans*, *Yersinia enterocolitica*, and acute HSV type 1 (7–10). The protective role of IL-18 in these infections has been attributed to its ability to activate NK cells, enhance proliferation of activated T cells, and induce IFN-γ production. Moreover, one study using IL-12/IL-18$^{-/-}$ (double mutant) mice has demonstrated that both IL-12 and IL-18 act in synergy to activate NK cells and induce Th1 development in vivo (11).

Several studies have demonstrated that IL-12 is indispensable for the development of protective immunity against *L. major* (12, 13). A recent study also found that susceptible BALB/c mice treated with recombinant IL-18 required only small quantities of IL-12 to control cutaneous *L. major* infection (14). Although these results demonstrate that exogenously administered IL-18 may act synergistically with IL-12 to induce protection against *L. major* infection in susceptible BALB/c mice, it is not clear whether endogenous IL-18 plays a similar role in the development of protective immunity against *L. major* in resistant mice. Therefore, we examined the development of Th1 response and cutaneous growth of *L. major* in resistant C57BL/6 mice lacking the IL-18 gene. Our results suggest that IL-18 may be involved in controlling early lesion growth but that it is not required for the development of a Th1 response and the resolution of *L. major* infection.

Materials and Methods

Animals

IL-18 gene-deficient C57BL/6 mice were generated as described previously (11) and were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). The mice were bred and maintained in the facility at the Harvard School of Public Health (Boston, MA) according to the guidelines for animal research. Wild-type C57BL/6 and IL-12$^{-/-}$ C57BL/6 mice of the same sex and age were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used as controls in all experiments.

Parasites and infection protocols

L. major LV39 was maintained by serial passage of amastigotes inoculated s.c. into the shaven rumps of BALB/c mice as described previously (15). Groups of C57BL/6 IL-18 gene-deficient and wild-type C57BL/6 mice were infected in the right hind footpad with 2×10^6 stationary-phase promastigotes of *L. major* (LV39). Lesion development was measured using a dial-gauge micrometer (Mitutoyo, Kanagawa, Japan) at weekly intervals up to 10 wk after infection. The increase in the thickness of the right hind footpad was compared with the uninfected left hind footpad.

T cell proliferation assay and cytokine analysis

The draining popliteal lymph nodes were removed from *L. major*-infected mice at 2 and 10 wk after infection. T cell proliferation assays were performed as previously described (15). To the wells of a 96-well flat-bottom

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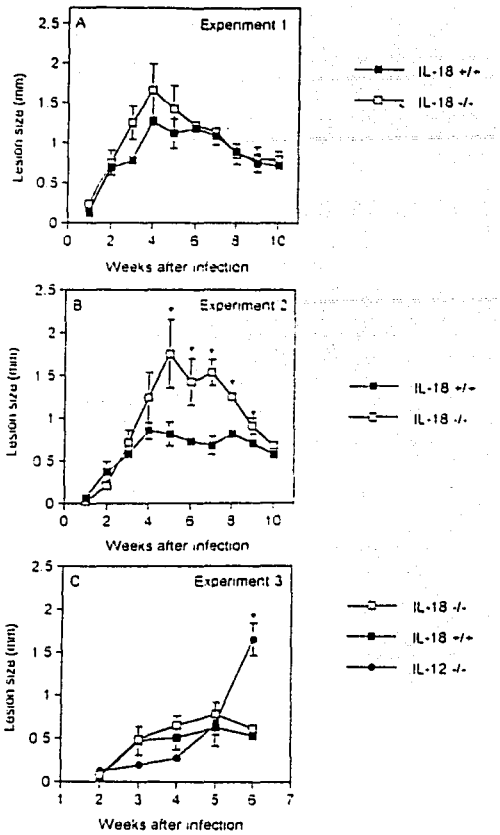

FIGURE 1. Course of *L. major* infection in IL-18$^{+/+}$ and IL-18$^{-/-}$ mice. *A* and *B*, In two independent experiments IL-18$^{+/+}$ and IL-18$^{-/-}$ mice were inoculated with 2×10^6 *L. major* stationary-phase promastigotes into the right hind footpad, and lesion growth was monitored by measuring the increase in lesion size of the infected footpad and comparing it to the thickness of the uninfected left footpad. *C*, In the third independent experiment we compared lesion development in IL-12$^{-/-}$ mice to that of IL-18$^{+/+}$ and IL-18$^{-/-}$ mice. In all three experiments, IL-18$^{-/-}$ mice developed larger lesions in early course of infection than IL-18$^{+/+}$ mice did; however, the differences in lesion sizes were significant in experiment 2 only (*B*). Data are presented as mean lesion size ± SE. *, Statistically significant differences between groups ($p < 0.05$).

tissue culture plate (Costar, Cambridge, MA), 3×10^6 lymph node cells were added. Cells were stimulated with 20 μg/ml of freeze-thawed *L. major* Ag (LmAg)[3] or supplemented medium as a negative control. After incubation at 37°C for 72 h in 5% CO_2, supernatants were collected from parallel cultures for ELISA quantification of cytokine production as described previously (15). Cultures were analyzed for production of IFN-γ (reagents purchased from PharMingen, San Diego, CA; detection limit, 20 pg/ml), IL-12 (PharMingen; detection limit, 20 pg/ml), and IL-4 (PharMingen; detection limit, 3 pg/ml).

Leishmania-*specific ELISA*

Peripheral blood was collected at 3-wk intervals from *L. major*-infected IL-18$^{+/+}$ and IL-18$^{-/-}$ mice. Serum was analyzed for Th2-associated Abs, IgG1, and for Th1-associated Abs, IgG2a. Specific levels of these Abs were measured using ELISA as described previously (16).

[3] Abbreviation used in this paper: LmAg, freeze-thawed *L. major* Ag.

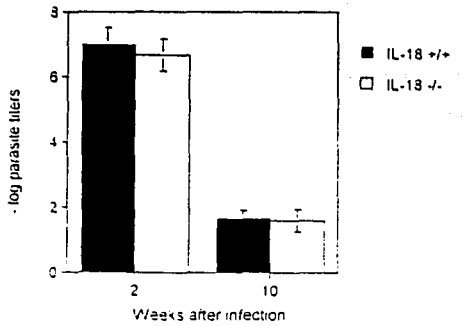

FIGURE 2. Parasite burdens in infected footpads from *L. major*-infected IL-18$^{+/+}$ and IL-18$^{-/-}$ mice determined by limiting dilution analysis. Data are expressed as mean log titer ± SE. Similar results were observed in two independent experiments.

Histopathology

Infected footpads from IL-18$^{+/+}$ and IL-18$^{-/-}$ mice were removed and fixed in decalcifying solution F (Stephens Lab, Riverdale, NJ) for 7 days. The tissues were processed and embedded in paraffin, and 4- to 8-μm sections were cut. The sections were hydrated and stained by routine hematoxylin and eosin staining.

Anti-IFN-γ neutralizing Ab treatment

Rat anti-mouse IFN-γ (clone, XMG 1.2) neutralizing mAb was kindly provided by Dr. Mary Russell. IL-18$^{-/-}$ mice were treated by i.p. administration of 1 mg anti-IFN-γ neutralizing Ab or control Ab 1 day before *L. major* infection and a weekly dose of 1 mg/mouse thereafter for 7 wk.

Statistical significance

Student's unpaired *t* test was used to determine statistical significance of values obtained. Differences in Ab endpoint titers were determined using the Mann-Whitney *U* prime test.

Results and Discussion

The results presented in this study suggest that IL-18 may be involved in controlling early *L. major* lesion growth in resistant C57BL/6 mice but that it is not essential for the development of acquired protective immunity and resolution of *L. major* infection. In addition, these findings also demonstrate that the development of larger lesions in IL-18$^{-/-}$ mice in early course of infection is associated with a significant increase in IL-4 production rather than a decrease in IFN-γ.

Previous studies have demonstrated that IL-12 plays a critical role in mediating protective immunity against *L. major* (14, 17, 18). The protective role of IL-12 in murine *L. major* infection has been attributed to its ability to activate NK cells and induce IFN-γ production required to develop a Th1 cell response (19). However, we have recently demonstrated that endogenous IL-12 can directly induce protective Th1-like response in the absence of NK cells and can control *L. major* infection (15). IL-18 is a recently discovered cytokine that is produced by activated macrophages (6, 20). This cytokine shares immunoregulatory functions with IL-12 and plays a critical role in the host defense against several pathogens (8, 9, 19, 21–23). A recent study has demonstrated that the treatment of susceptible BALB/c mice with exogenous IL-18 together with small quantities of IL-12 significantly enhanced their resistance to cutaneous *L. major* infection, indicating that IL-18 may play a role in the development of protective immunity against *L. major* (14). In the present study, we found that IL-18$^{-/-}$ C57BL/6 mice, although they occasionally developed significantly larger lesions

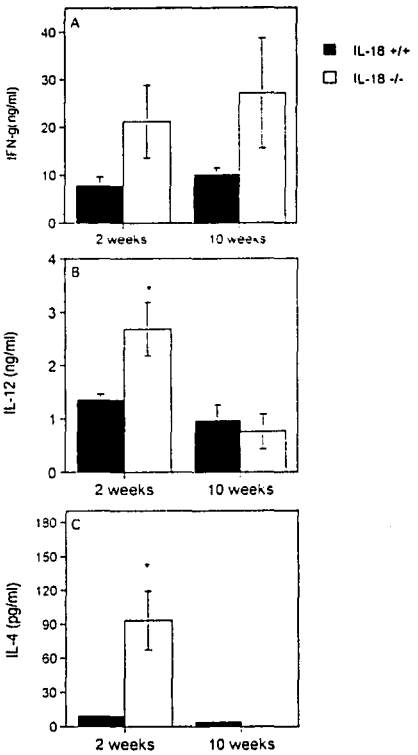

FIGURE 3. Kinetics of in vitro LmAg (20 μg/ml)-induced IFN-γ (*A*), IL-12 (*B*), and IL-4 (C) production by popliteal lymph node cells from *L. major*-infected IL-18$^{-/-}$ and IL-18$^{-/-}$ mice. The data are the mean of six to seven animals at each time point for two of the three experiments. *, Statistically significant differences between each group ($p < 0.05$).

during the early course of infection (Fig. 1*B*), eventually resolved their lesions (Fig. 1, *A–C*) and controlled parasite replication (Fig. 2). Furthermore, infected footpads from both IL-18$^{+/+}$ and IL-18$^{-/-}$ mice displayed preserved skin and an inflammatory infiltrate comprised of lymphocytes and macrophages with few or no parasites. In contrast, in an another replicate experiment, concomitantly infected IL-12$^{-/-}$ C57BL/6 mice developed large nonhealing lesions by week 6 after infection (Fig. 1*C*). The lesions from IL-12$^{-/-}$ mice showed significant ulceration of skin with necrosis and inflammatory infiltrate comprised primarily of heavily parasitized macrophages, neutrophils, and eosinophils. Our findings contradict a recent study that demonstrated that IL-18$^{-/-}$ mice on CD1 genetic background are highly susceptible to *L. major* and develop large nonhealing lesions by day 40 after infection (24). Although different results observed in our study and that by Wei et al. (24) can be attributed to the differences in genetic backgrounds of the strains used, we also monitored disease progression for a longer duration. As reported by Wei et al., *L. major*-infected IL-18$^{-/-}$ mice did develop larger lesions than infected IL-18$^{-/-}$ mice did during early course of infection but eventually resolved them (Fig. 1, *A–C*). Moreover, the difference in early lesion sizes between IL-18$^{-/-}$ and IL-18$^{-/-}$ mice was statistically significant in only one of the three experiments (Fig. 1*B*).

Previous studies indicate that IL-12-activated NK cells are the primary source of IFN-γ early in *L. major* infection that is required

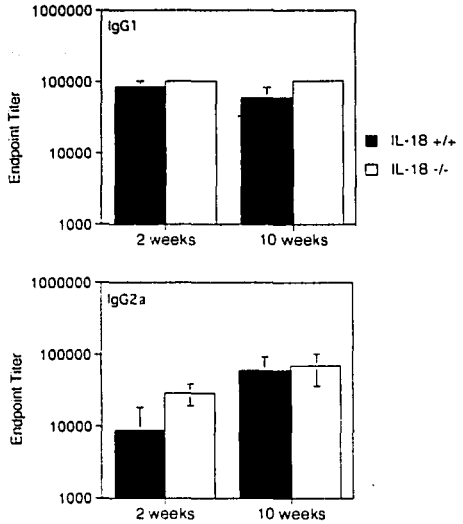

FIGURE 4. Ab responses in *L. major*-infected IL-18$^{-/-}$ and IL-18$^{-/-}$ mice at 2 wk and 10 wk after infection. *A*, LmAg-specific IgG1; and *B*, Lm-Ag-specific IgG2a. Data for IgG1 and IgG2a are presented as mean reciprocal endpoint titer on log scale. Three to five mice were analyzed in each group for two of the three experiments.

for the development of a Th1 response in resistant mice (19). Recent studies have demonstrated that NK cells and the Th1 subset of CD4$^+$ T cells express IL-18R (25). Furthermore, NK cell activity is also significantly impaired in IL-18$^{-/-}$ mice (11). Therefore, we measured IL-12 and IFN-γ production by LmAg-stimulated lymph node cells and also determined the serum levels of Th1-associated LmAg-specific IgG2a Abs in IL-18$^{+/+}$ and IL-18$^{-/-}$ mice at 2 and 10 wk after *L. major* infection. At both of these time points, LmAg-stimulated lymph node cells from both IL-18$^{-/+}$ and IL-18$^{-/-}$ mice produced comparable levels of IFN-γ (Fig. 3*A*), and both groups displayed significant titers of Ag-specific IgG2a Abs at these time points (Fig. 4). Furthermore, *L. major*-infected IL-18$^{-/-}$ mice treated with anti-IFN-γ neutralizing Ab developed significantly larger lesions compared with those on similarly infected IL-18$^{-/-}$ mice treated with control Ab (Fig. 5). These results in-

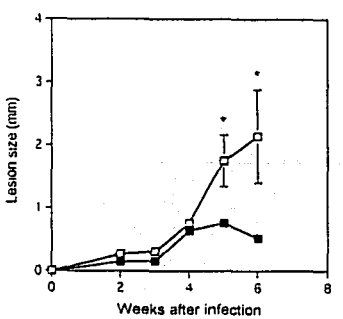

FIGURE 5. Administration of IFN-γ neutralizing Ab to IL-18$^{-/-}$ mice exacerbates cutaneous *L. major* infection. Data are expressed as mean lesion size + SE. *, Statistically significant differences between each group ($p < 0.05$).

dicate that IL-12 alone in the absence of endogenous IL-18 induces protective Th1 response and controls *L. major* infection. This is perhaps not surprising because we recently demonstrated that IL-12 can directly induce Th1 development in mice lacking NK cells and can control *L. major* infection (15). Interestingly, at 2 wk after infection, lymph node cells from IL-18$^{-/-}$ mice produced significantly more IL-12 than those from IL-18$^{+/+}$ mice (Fig. 3*B*). These findings indicate that IL-18 may be involved in down-regulation of IL-12 production as suggested previously (24).

IL-4 has been shown to play a role in mediating susceptibility to cutaneous *L. major* infection. The disease-exacerbating role of IL-4 in cutaneous *L. major* infection has been attributed to its ability to induce Th2 development, inhibit Th1 development, and directly inhibit macrophage leishmanicidal activity (1). In this study, LmAg-stimulated lymph node cells from IL-18$^{-/-}$ mice produced significantly greater amounts of IL-4 at 2 wk after infection than those from IL-18$^{+/+}$ mice (Fig. 3*C*). However, IL-4 was undetectable in lymph node cell culture supernatants from either group at 10 wk after infection. These results indicate that although increased IL-4 production during early phase of *L. major* infection may contribute to the development of large lesions in IL-18$^{-/-}$ mice during early course of infection, it is not sufficient to induce Th2 differentiation and to render IL-18$^{-/-}$ highly susceptible to *L. major* infection.

In conclusion, IL-18$^{-/-}$ mice from a resistant C57BL/6 genetic background may develop larger lesions during early course of *L. major* infection but eventually will resolve them efficiently. Moreover, the resolution of *L. major* infection by IL-18$^{-/-}$ mice is associated with the development of an efficient Th1 response.

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Migration-Inhibitory Factor Gene-Deficient Mice Are Susceptible to Cutaneous *Leishmania major* Infection

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To determine the role of endogenous migration-inhibitory factor (MIF) in the development of protective immunity against cutaneous leishmaniasis, we analyzed the course of cutaneous *Leishmania major* infection in MIF gene-deficient mice ($MIF^{-/-}$) and wild-type ($MIF^{+/+}$) mice. Following cutaneous *L. major* infection, $MIF^{-/-}$ mice were susceptible to disease and developed significantly larger lesions and greater parasite burdens than $MIF^{+/+}$ mice. Interestingly, antigen-stimulated lymph node cells from $MIF^{-/-}$ mice produced more interleukin-4 (IL-4) and gamma interferon (IFN-γ) than those from $MIF^{+/+}$ mice, although the differences were statistically not significant. IFN-γ-activated resting peritoneal macrophages from $MIF^{-/-}$ mice showed impaired macrophage leishmanicidal activity and produced significantly lower levels of nitric oxide and superoxide in vitro. The macrophages from $MIF^{-/-}$ mice, however, produced much more IL-6 than macrophages from wild-type mice. These findings demonstrate that endogenous MIF plays an important role in the development of protective immunity against *L. major* in vivo. Furthermore, they indicate that the susceptibility of $MIF^{-/-}$ mice to *L. major* infection is due to impaired macrophage leishmanicidal activity rather than dysregulation of Th1 and Th2 responses.

Most inbred mice are resistant to cutaneous *Leishmania major* infection and develop small self-resolving lesions (1, 16). It is widely accepted that the ability of genetically resistant mice to resolve cutaneous *L. major* infection is associated with the development of interleukin-12 (IL-12)-induced Th1 response and gamma interferon (IFN-γ) production. The protective role of IFN-γ has been attributed to its ability to induce the Th1 response, inhibit Th2 differentiation, and enhance macrophage leishmanicidal activity (16).

Migration-inhibitory factor (MIF) is a pleiotropic cytokine that is produced by many cells, including macrophages, T cells, and the pituitary gland, during inflammatory responses. MIF inhibits anti-inflammatory effects of corticosteroids and plays a critical role in pathogenesis of sepsis (3, 4). Additionally, MIF also acts as an enzyme and catalyzes the tautomerization of several substrates (21). Experimental studies using MIF-neutralizing antibodies indicate that this cytokine is involved in pathogenesis of autoimmune diseases such as collagen type II-induced arthritis and immunologically induced kidney disease (11, 14). Recent studies show that MIF counteracts the antitumor activity of p53 and appears to link chronic inflammation and tumor formation (9).

Studies from our laboratory and others indicate that MIF may play a critical role in regulation of host immunity or susceptibility to pathogens. For example, we found that $MIF^{-/-}$ mice cleared gram-negative *Pseudomonas aeruginosa* bacteria more efficiently than $MIF^{+/+}$ mice, indicating that MIF is involved in pathogenesis of pulmonary *P. aeruginosa* infection (4). In contrast, oral administration of MIF alone or together with tumor necrosis factor alpha (TNF-α) via transfected attenuated *Salmonella enterica* serovar Typhimurium enhanced resistance of BALB/c mice to *L. major* (23). Furthermore, others demonstrated that MIF with endogenous TNF-α enhances macrophage NO production and induces in vitro killing of *L. major* by macrophages (10). Interestingly, the same study found that both susceptible BALB/c and resistant C57BL/6 mice express high levels of MIF mRNA in their draining lymph nodes following *L. major* infection (10). Therefore, we examined the cutaneous growth of *L. major* infection in MIF gene-deficient C57BL/6 × 129/Sv ($MIF^{-/-}$) mice and compared it with growth in similarly infected age and sex-matched wild-type ($MIF^{+/+}$) mice. In addition, we analyzed the leishmanicidal activity of resident macrophages and measured cytokine production by the draining lymph nodes from *L. major*-infected $MIF^{+/+}$ and $MIF^{-/-}$ mice. Our results indicate that MIF plays a critical role in the development of protective immunity and control of cutaneous *L. major* infection.

MATERIALS AND METHODS

Mice. MIF gene-deficient C57BL/6 × 129/Sv mice were generated by gene targeting as described previously (4). $MIF^{-/-}$ mice were maintained by mating between homozygous mice on the C57BL/6 × 129Sv background. The wild-type littermates were used to generate wild-type $MIF^{+/+}$ mice of the same age and sex that were used as controls in all experiments. Mice were maintained in the specific-pathogen-free facility at the Harvard School of Public Health according to the guidelines for animal research.

Parasites. *L. major* LV39 was maintained by serial passage of parasites in BALB/c mice. Amastigotes isolated from lesions of infected BALB/c mice were grown to stationary phase as described previously (18).

Infection protocol and quantitation of parasite burdens. Mice 8 to 12 weeks old were injected in the hind footpad with 2×10^6 stationary-phase promastigotes enumerated using a Nuebauer hemacytometer. Lesion growth was monitored by measuring the increase in thickness of the infected footpad using a dial-gauge micrometer (18) at weekly intervals up to 12 weeks after infection and comparing this to the thickness of the contralateral uninfected footpad.

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Parasite burdens in the infected footpads were determined by limiting-dilution analysis, as described previously (18).

IL-12 treatment. Recombinant murine IL-12 (R & D Systems Inc.) was used to treat $MIF^{+/+}$ mice in doses described previously (22). Briefly, 1 μg of IL-12 in 100 μl was administered intraperitoneally to each $MIF^{+/+}$ mouse 1 day prior to *L. major* infection, followed by a daily dose of 1 μg per mouse for 5 days thereafter. Control $MIF^{+/+}$ mice received injections of 100 μl of sterile phosphate-buffered saline (PBS).

T-cell proliferation and cytokine assays. T-cell proliferation was determined as previously described (20). $MIF^{+/+}$ and $MIF^{-/-}$ mice were sacrificed on weeks 2, 6, and 12 following *L. major* infection. The inguinal lymph nodes were removed and teased gently to prepare a single-cell suspension. Briefly, 3×10^5 cells were added in triplicate to the wells of sterile 96-well flat-bottomed tissue culture plates (Costar, Cambridge, Mass.) and stimulated with *Leishmania* antigen (LmAg; 20 μg/ml). Supernatants were collected after 72 h of incubation and analyzed for the production of IL-4 (reagent purchased from Endogen, Cambridge, Mass.; detection limit, 10 pg/ml) and IFN-γ (both reagents purchased from PharMingen, San Diego, Calif.; detection limit, 20 pg/ml) by capture enzyme-linked immunosorbent assay (ELISA) (20). Similarly, supernatants from macrophage cultures were also analyzed for IL-6, IL-10, IL-12, and TNF-α production by ELISA.

Assessment of macrophage leishmanicidal activity. Resting peritoneal macrophages were added to the wells of 24-well flat-bottomed plates containing 12-mm-diameter glass coverslips. Cells were incubated at 37°C for 4 h to allow macrophages to adhere to the coverslips. Nonadherent cells were removed by gentle washing, and adherent macrophages were infected with *L. major* promastigotes (parasite-macrophage ratio, 5:1) for 4 h, washed, and cultured in the presence of 200 U of IFN-γ (Genzyme, Cambridge, Mass.) per ml for 72 h. At this time, macrophages were stained by Giemsa stain, and coverslips were mounted upside down on a glass slide; intracellular amastigotes were counted by microscopy.

Assessment of NO_2^- and O_2^- production. The supernatants from the above assays were analyzed for nitrite concentration using Griess reagent as described previously (6). For determining levels of O_2^-, IFN-γ-treated macrophages were stimulated with phorbol myristate acetate, and superoxide dismutase-inhibitable reduction of Fe_3^+ cytochrome *c* to the ferrous form (Fe_2^+) was measured. The amount of O_2^- released was calculated as described previously (15).

Statistical significance. Student's unpaired *t* test was used to determine the statistical significance of values.

RESULTS AND DISCUSSION

We have previously demonstrated that oral administration of MIF alone or together with IFN-γ and TNF-α via transfected attenuated *Salmonella* cells conferred significant protection against *L. major* infection in susceptible BALB/c mice (23). Moreover, others found that MIF enhanced in vitro macrophage leishmanicidal activity (10). In the present study, following infection with 2×10^6 *L. major* stationary-phase promastigotes, $MIF^{+/+}$ mice developed lesions that resolved spontaneously by week 12 postinfection (Fig. 1A). In contrast, similarly infected $MIF^{-/-}$ mice displayed smaller lesions than $MIF^{+/+}$ mice in the early phase of infection but developed large lesions which failed to resolve as infection progressed (Fig. 1A). Concomitantly infected BALB/c mice developed large, rapidly progressive ulcerating lesions by week 6 postinfection (data not shown). At week 12 postinfection, infected footpads from $MIF^{-/-}$ mice also contained significantly more parasites (>100-fold) than those from $MIF^{+/+}$ mice (Fig. 1B). Taken together, these findings indicate that endogenous MIF plays an important role in the development of protective immunity against *L. major* in resistant C57BL/6 × 129Sv/Ev mice.

Several studies have demonstrated that control of *L. major* infection in resistant mice is associated with the development of the IL-12-mediated Th1-like response and the production of IFN-γ, whereas susceptibility to *L. major* infection is associated with development of a Th2-like response and the production of IL-4 (8, 13, 19). A previous study had demonstrated that MIF plays a critical regulatory role in the activation of T cells (2). Furthermore, MIF was produced by the *Leishmania*-specific Th2 clone (D10G4.1, L1/1), but not the Th1 (LNC2, B10/B1) clone following in vitro stimulation with mitogen (2). Therefore, we compared IL-4 and IFN-γ production by LmAg-stimulated lymph node cells from *L. major*-infected $MIF^{+/+}$ and $MIF^{-/-}$ mice at weeks 2, 6, and 12 postinfection to determine whether susceptibility of $MIF^{-/-}$ mice to *L. major* is due to an enhanced Th2-like response and/or an impaired Th1-like response. We also measured serum levels of LmAg-specific Th1-associated immunoglobulin G2a (IgG2a) and Th2-associated IgG1 antibodies. Following in vitro stimulation with LmAg, lymph node cells from both *L. major*-infected $MIF^{+/+}$ and $MIF^{-/-}$ mice displayed significant but similar levels of proliferative responses (data not shown) and produced signif-

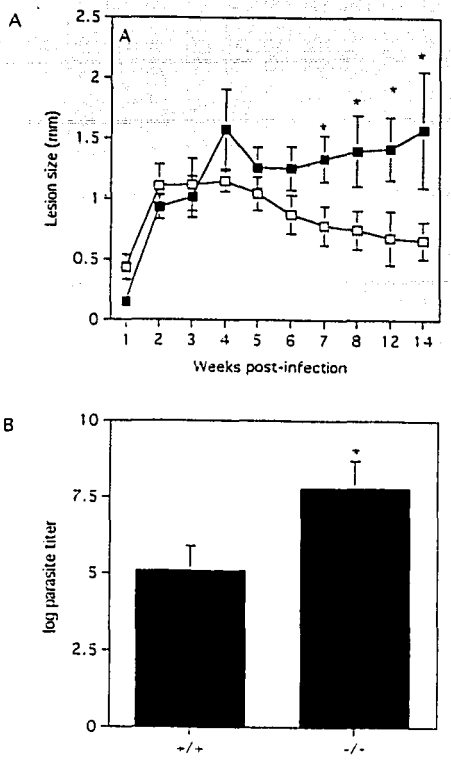

FIG. 1. $MIF^{-/-}$ mice are relatively more susceptible to cutaneous *L. major* infection than $MIF^{+/+}$ mice and develop large lesions. (A) Course of cutaneous *L. major* infection in $MIF^{-/-}$ (solid squares) and $MIF^{+/+}$ (open squares) mice following infection with 2×10^6 stationary-phase promastigotes. Disease progression was monitored by measuring the increase in thickness of the infected footpad and comparing this to the thickness of the contralateral uninfected footpad. (B) Footpad parasite burdens in $MIF^{-/-}$ and $MIF^{+/+}$ mice were determined at week 12 postinfection by limiting-dilution analysis. Data are expressed as the mean titer ± the standard error (SE). Similar results were observed in three and two independent experiments (A and B, respectively).

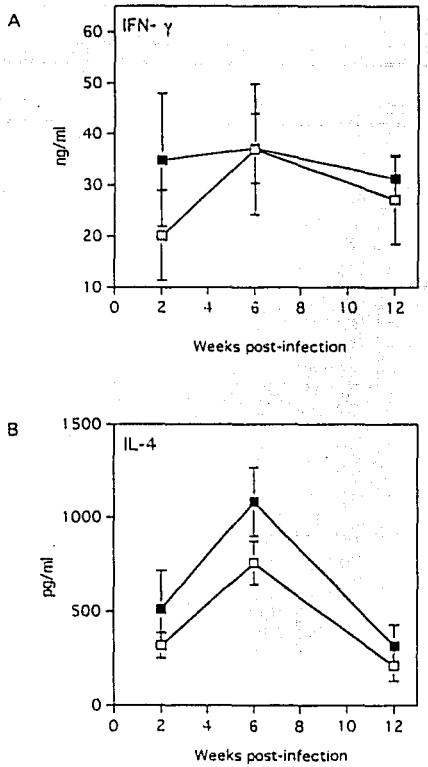

FIG. 2. Kinetics of in vitro cytokine production by LmAg-stimulated lymph node cells from MIF$^{-/-}$ (solid squares) and MIF$^{+/+}$ (open squares) mice. (A) IFN-γ and (B) IL-4 production by draining lymph node cells following in vitro stimulation with LmAg (20 μg/ml) was measured at weeks 2, 6, and 12 postinfection by ELISA. The graph shows the mean (n = 6 to 9 animals) of two separate experiment. Data are expressed as the mean ± SE.

icant amounts of IFN-γ and IL-4. Levels of IFN-γ and IL-4 in lymph node cell culture supernatants from MIF$^{-/-}$ mice were either higher or similar to those from MIF$^{+/+}$ mice, although the differences were not statistically significant (Fig. 2A and B). At week 12 postinfection, MIF$^{+/+}$ and MIF$^{-/-}$ mice displayed comparable titers of IgG1 (34,400 ± 23,200 and 46,050 ± 14,927 in MIF$^{+/+}$ and MIF$^{-/-}$ mice, respectively; $P < 0.375$) and IgG2a (19,200 ± 12,255 and 43,328 ± 17,952 in MIF$^{+/+}$ and MIF$^{-/-}$ mice, respectively; $P < 0.1$). Moreover, in one experiment, although administration of recombinant murine IL-12 to MIF$^{-/-}$ mice significantly inhibited lesion growth in the early course of *L. major* infection between 3 and 6 weeks, these mice eventually developed large lesions comparable to those of control MIF$^{-/-}$ mice treated with PBS (Fig. 3). Although lesion sizes in IL-12-treated MIF$^{-/-}$ mice were smaller than in PBS-treated MIF$^{-/-}$ mice at weeks 7 and 8 postinfection these differences were statistically not significant. As previous studies have clearly demonstrated that IL-12 induces production of IFN-γ from NK cells and T cells, it is most likely that enhanced resistance of IL-12-treated MIF$^{-/-}$ mice against *L. major* in the early course of infection is due to an increase in IFN-γ production. Nevertheless, taken together, these observations suggest that endogenous MIF may not be involved in the regulation of T-cell activation and production of cytokines IL-4 and IFN-γ following *L. major* infection.

Macrophages activated by the cytokines IFN-γ and TNF-α are major effector cells involved in killing of *Leishmania* (12). The leishmanicidal activity of cytokine-activated macrophages has been attributed to their ability to produce microbicidal effector molecules such as nitric oxide and superoxide (12). MIF has been shown to induce TNF-α and NO production in human monocytes (5) and activate murine macrophages to kill *Leishmania major* in vitro (10). Furthermore, we previously demonstrated that susceptible BALB/c mice that were orally administered MIF together with IFN-γ and TNF-α via transfected attenuated *Salmonella* express markedly higher levels of nitric oxide synthase 2 in their lymph nodes and developed significantly smaller lesions than control animals (23). Together, these findings indicate that the protective role of MIF in murine leishmaniasis can be attributed to its ability to induce macrophage leishmanicidal activity by increasing NO production.

To determine whether the susceptibility of MIF$^{-/-}$ mice to *L. major* can be attributed to macrophage dysfunction, we compared the ability of resting peritoneal macrophages from MIF$^{-/-}$ and MIF$^{+/+}$ mice to produce proinflammatory cytokines and kill *L. major* promastigotes following in vitro stimulation with IFN-γ. Four hours after infection with *L. major* promastigotes, macrophages from MIF$^{-/-}$ and MIF$^{+/+}$ mice displayed similar parasite loads (116 ± 13 and 110 ± 18 parasites/200 macrophages in MIF$^{-/-}$ and MIF$^{+/+}$ macrophages, respectively; $P < 0.4$), indicating that initial uptake of parasites is not increased in MIF$^{-/-}$ macrophages. After 72 h, IFN-γ

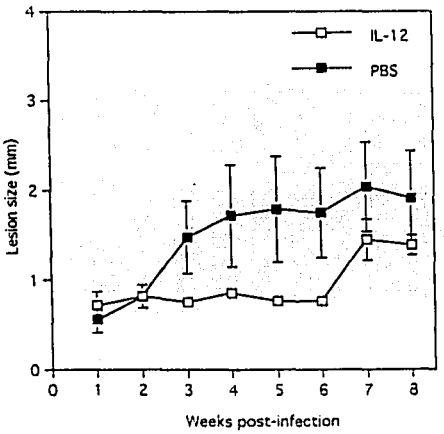

FIG. 3. Effect of recombinant IL-12 treatment on course of *L. major* infection in MIF$^{-/-}$ mice. Disease progression was monitored by measuring the increase in the thickness of infected footpad and comparing this to the thickness of the contralateral uninfected footpad. Data are expressed as mean increase in footpad thickness ± SE. Four mice were used in each group.

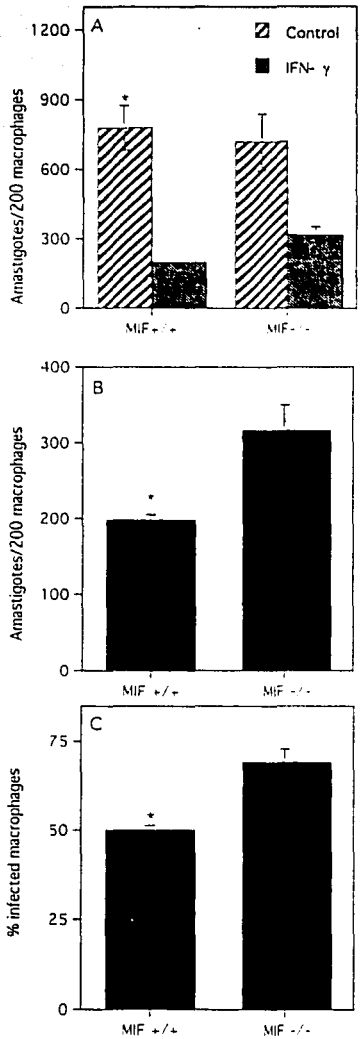

FIG. 4. Resting peritoneal macrophages from MIF$^{-/-}$ mice display impairment of IFN-γ-induced leishmanicidal activity. Macrophages were infected with *L. major* stationary-phase promastigotes as described in Materials and Methods and stimulated with 200 U of IFN-γ per ml for 72 h. The number of amastigotes per 200 infected macrophages (A and B) and the percentage of infected macrophages (C) were determined microscopically by Giemsa staining. This experiment is representative of two performed. Four to five mice were used in each group, and cells were plated in triplicate.

induced significant parasite killing in macrophages from both MIF$^{-/-}$ and MIF$^{+/+}$ mice compared to control macrophages that were not stimulated with IFN-γ (Fig. 4A). At this time point, however, IFN-γ-stimulated macrophages from MIF$^{-/-}$ mice displayed significant impairment of leishmanicidal activity compared to MIF$^{-/-}$ mice (Fig. 4B and C). Furthermore, impairment of the in vitro leishmanicidal activity of MIF$^{-/-}$ macrophages was associated with significantly lower production of superoxide (two- to threefold) and nitric oxide (fivefold) than MIF$^{+/+}$ macrophages (Fig. 5A and B). There were no significant differences in the levels of IL-10, transforming growth factor beta (TGF-β), and IL-12 in culture supernatants from MIF$^{+/+}$ and MIF$^{-/-}$ mice (data not shown). Interestingly, macrophages from MIF$^{-/-}$ mice produced significantly more IL-6 than those from MIF$^{+/+}$ mice (Fig. 6). In contrast, culture supernatants from LmAg-stimulated lymph node cells from MIF$^{+/+}$ and MIF$^{-/-}$ mice contained only basal levels of IL-6, suggesting that MIF$^{-/-}$ T cells do not overproduce IL-6. Several studies indicate that IL-6 is a proinflammatory cytokine; others, however, have reported that it can also exhibit immunosuppressive activity and induce differentiation of IL-4-producing CD4^{+} T cells (17). In fact, IL-6 has been shown to suppress superoxide production in human monocytes and to inhibit their leishmanicidal activity in vitro (7).

In our previous paper (4), we reported that thioglycolate-induced macrophages from MIF$^{-/-}$ mice produced the same

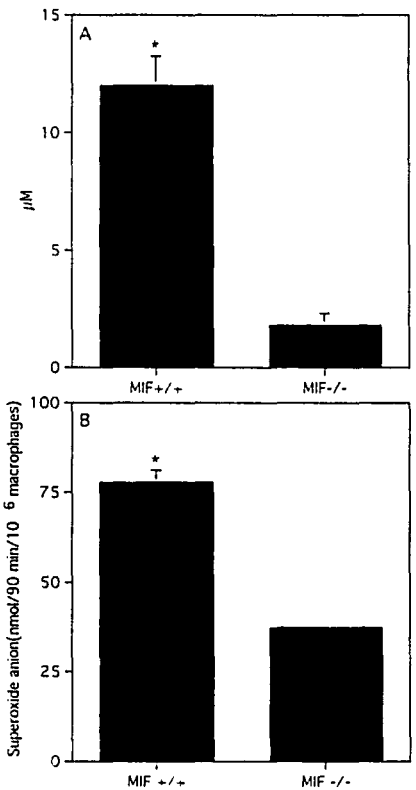

FIG. 5. Production of nitric oxide (NO) and superoxide (O_2^-) is impaired in macrophages from MIF$^{-/-}$ mice. (A) Nitric oxide production and (B) superoxide (O_2^-) production were measured as described in Materials and Methods. Similar results were observed in two independent experiments. Data are expressed as mean ± SE.

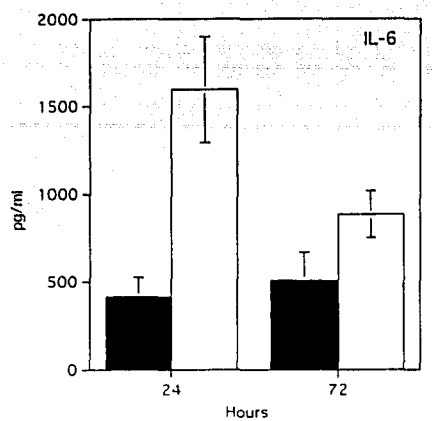

FIG. 6. Macrophages from MIF mice (open squares) produce significantly more IL-6 than those from MIF (squares). Resting peritoneal macrophages were infected with *L. major* promastigotes in vitro and stimulated with IFN-γ (200 U ml). IL-6 production was measured at 12, 24, and 48 h postinfection by ELISA. Data are expressed as the mean of triplicates ± SE. Similar results were observed in three independent experiments.

amount of IL-6 as and a little more NO than wild-type mice. These differences from the present findings are probably due to the much greater stimulus of macrophages used in the earlier study; macrophages induced by thioglycolate may be more activated than resident macrophages. These cells were further stimulated with both lipopolysaccharide (LPS) and IFN-γ rather than with IFN-γ alone, as in the present studies. Therefore, it is perhaps not surprising that thioglycolate-elicited and IFN-γ/LPS-activated peritoneal macrophages from MIF mice killed *Leishmania* as efficiently as MIF macrophages (26% ± 10% and 33% ± 11% infected macrophages in MIF and MIF mice, respectively; $P < 0.4$). The present protocol using resident macrophages and stimulation with IFN-γ alone is probably more closely related to the physiological reality.

There may be several explanations why IFN-γ-activated MIF macrophage have impaired leishmanicidal activity. First, increased IL-6 production may contribute at least partly to increased susceptibility of MIF mice to *L. major* by inhibiting macrophage superoxide production, as reported previously (7). Hence, we are currently generating IL-6/MIF double-knockout mice to investigate this further. Second, as MIF has been shown to induce TNF-α, impaired leishmanicidal activity of resident peritoneal macrophages from MIF mice may be due to reduced TNF-α production. This, however, is unlikely in the present study, as TNF-α was either absent or detectable only at basal levels in culture supernatants from both groups (data not shown). Lastly, it is possible that lack of MIF suppresses expression of IFN-γ and/or TNF receptors on macrophages that are essential for mediating the biological functions of these cytokines. We are presently investigating this hypothesis in studies in our laboratory. Nevertheless, together these results indicate that the protective role of endogenous MIF against *L. major* in resistant mice is mediated by its ability to regulate superoxide and NO production and induce macrophage leishmanicidal activity.

In conclusion, our findings in the present study demonstrate that MIF gene-deficient C57BL/6 × 129Sv/Ev mice are susceptible to cutaneous *L. major* infection and develop larger lesions and greater parasite burdens than their wild-type counterparts. The susceptibility of MIF mice to *L. major* is due to impaired IFN-γ-induced macrophage leishmanicidal activity rather than to the lack of Th1 development or enhanced Th2 development. These observations indicate that endogenous MIF is required for optimal activation of macrophages and control of *L. major* infection in resistant mice. Additionally, the data demonstrate that MIF is not required for activation of T cells and production of Th1-associated IFN-γ and Th2-associated IL-4.

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Research Article

Susceptibility to *Leishmania mexicana* infection is due to the inability to produce IL-12 rather than lack of IL-12 responsiveness

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Summary Almost all inbred mice are highly susceptible to parasites of the *Leishmania mexicana* complex that includes *L. amazonensis* and *L. mexicana*. Recent studies have reported that T cells from *L. amazonensis*-infected mice fail to respond to IL-12 due to impaired IL-12R expression. Here, we demonstrate that lymph node cells from *L. mexicana*-infected C57BL/6 and 129Sv/Ev mice respond efficiently to exogenous IL-12 *in vitro* and produce IFN-γ. Moreover, we also show that deletion of signal transducer and activator of transcription (STAT)4 gene in resistant STAT6$^{-/-}$ mice renders them susceptible to *L. mexicana*. These findings indicate that an inability to produce IL-12 rather than unresponsiveness to this cytokine is responsible for susceptibility to *L. mexicana*. Moreover, the data also demonstrate that the STAT4-mediated pathway is critical for the development of protective immunity against cutaneous leishmaniasis, regardless of the species of *Leishmania* and/or genetic background of the mice.

Key words: *Leishmania mexicana*, STAT4, STAT6.

Introduction

American cutaneous leishmaniasis is caused by parasites of the *Leishmania mexicana* complex that includes *L. amazonensis* and *L. mexicana*.[1] It is well documented that almost all inbred mice are susceptible to *L. mexicana* complex and develop large non-healing lesions following infection with these parasites.[2,3] We have previously demonstrated that IL-4$^{-/-}$ as well as signal transducer and activator of transcription (STAT)6$^{-/-}$ mice produce high levels of IL-12, develop a Th1 response and control *L. mexicana* infection.[4,5] These findings indicate that IL-4 and the STAT6-mediated signalling pathway mediate susceptibility to *L. mexicana* by suppressing IL-12 production and Th1 development. In contrast, other studies indicate that the inability to generate a Th1 response rather than the presence of IL-4 or a Th2 response may be responsible for susceptibility to *L. amazonensis*.[6,7] Moreover, lack of Th1 in *L. amazonensis*-infected mice has been attributed to the inability of CD4$^+$ T cells to respond to IL-12 due to the suppression of IL-12Rβ expression.[7]

Therefore, in this study, we determined whether lack of IL-12 production or inability to respond to this cytokine was responsible for impaired Th1 development and susceptibility to *L. mexicana* infection.

Materials and Methods

Animals

Six to eight-week-old female C57BL/6 and 129Sv/Ev mice were purchased from Taconic Laboratories (Germantown, NY, USA). STAT4/STAT6 gene-deficient C57BL/6 × 129Sv/Ev mice were generated by intercrossing STAT4$^{-/-}$ and STAT6$^{-/-}$ C57BL/6 × 129Sv/Ev mice that were kindly provided by Dr James Ihle (St. Jude's Hospital, Memphis, TN, USA). These mice were bred and maintained in a facility at the Harvard School of Public Health according to the guidelines for animal research.

Parasite and infection protocols

For all experiments, mice were infected by injecting 5 × 10^5 *L. mexicana* (M379) amastigotes into shaven back rump. The disease progression was monitored by measuring lesion diameters weekly until week 10 postinfection.

Antibody ELISA

Mice were bled at different time points from tail snips following *L. mexicana* infection. Blood was centrifuged at 200 × *g*, and serum was collected and tested for *L. mexicana* antigen (LmAg)-specific Th1-associated IgG2a and Th2-associated IgG1 antibodies by ELISA as described previously.[5]

T-cell proliferation and cytokine assays

At week 10 postinfection the mice were killed, their lymph nodes excised and T-cell proliferation assays were performed as described previously.[5] Briefly, 3 × 10^5 lymph node cells were added to the wells of 96-well flat-bottomed tissue culture plates and stimulated with 20 μg/mL of LmAg prepared by repeated freezing and thawing of stationary phase promastigotes. Supernatants from these cultures were analysed for IL-4, IL-12 and IFN-γ production by ELISA.[5]

Results and Discussion

It is widely accepted that IL-12 plays a critical in the development of Th1 response and protective immunity during

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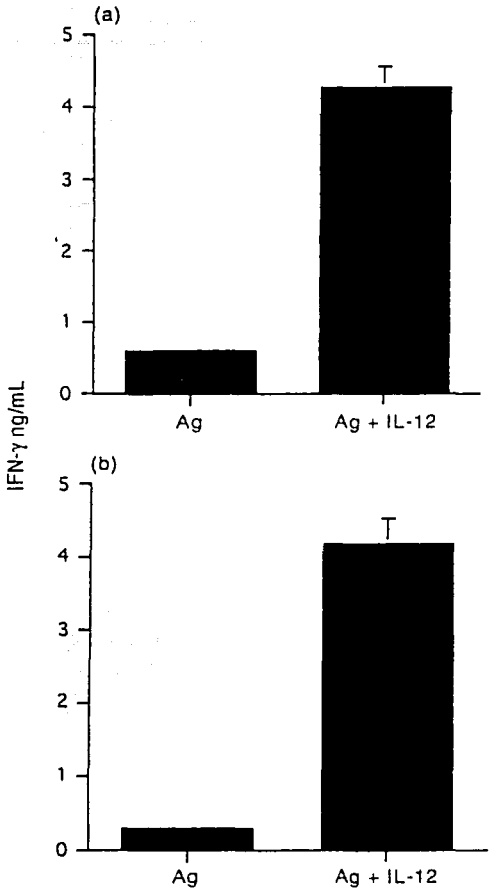

Figure 1 *In vitro* production of IFN-γ by *L. mexicana* antigen (LmAg)-stimulated lymph node cells from (a) C57BL/6 and (b) 129Sv/Ev mice following addition of recombinant murine IL-12. Five animals were used in each group. Data are expressed as means ± SEM and are representative of three experiments.

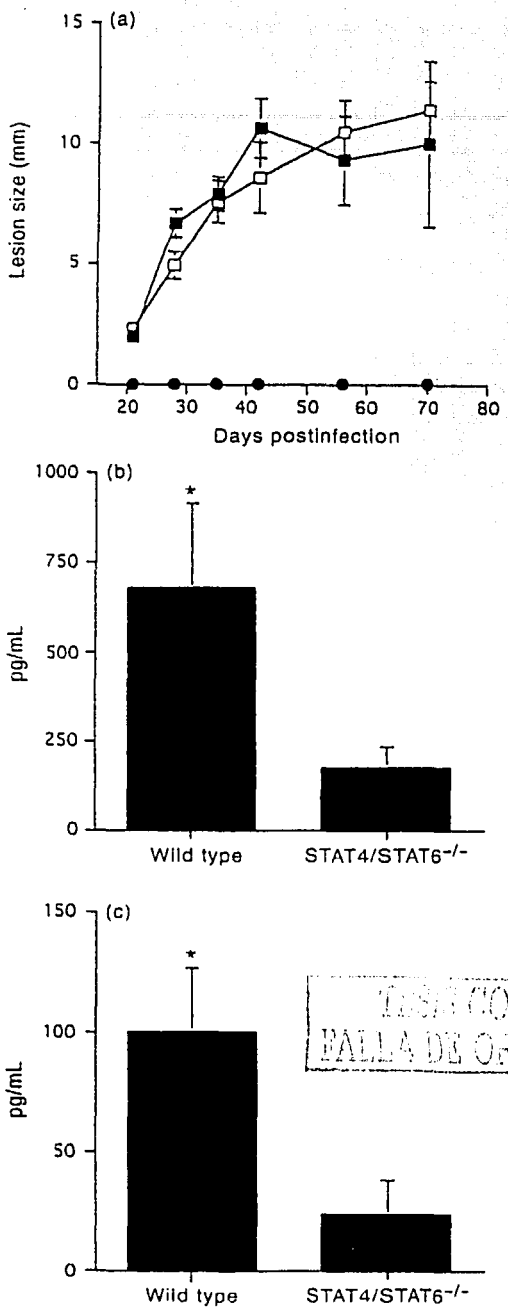

Figure 2 Course of cutaneous *L. mexicana* infection in STAT4/STAT6$^{+/+}$ (■), STAT4/STAT6$^{-/-}$ (□) and STAT6$^{-/-}$ mice (●). (a) Lesion growth was monitored by measuring lesion diameters following *L. mexicana* infection. Data are expressed as mean lesion diameter ± SE. At week 10 postinfection *in vitro* *L. mexicana* antigen (LmAg)-induced (b) IFN-γ and (c) IL-4 by the lymph node cells from STAT4/STAT6$^{+/+}$ (wild type) and STAT4/STAT6$^{-/-}$ mice were measured by ELISA. Four to five animals were used in each group.

cutaneous as well as visceral leishmaniasis.[8,9] As demonstrated previously, C57BL/6 and 129Sv/Ev mice were highly susceptible to *L. mexicana* and developed large non-healing lesions following *L. mexicana* infection (data not shown). At week 10 postinfection, LmAg-stimulated lymph node cells from these mice failed to produce significant levels of IFN-γ, which was associated with basal levels of IL-12 (Fig. 1). However, addition of exogenous recombinant murine IL-12 to these cultures induced significant IFN-γ production by LmAg-stimulated lymph node cells from these inbred strains (Fig. 1). These results demonstrate that T cells, regardless of the genetic background of the mouse, can respond efficiently to IL-12 and produce IFN-γ during *L. mexicana* infection. Moreover, these results also suggest that lack of IL-12 production rather than responsiveness to this cytokine may be responsible for impaired IFN-γ production following *L. mexicana* infection. It is possible that IL-12-induced IFN-γ may be derived partly from other non-$CD4^+$ T cells, such as NK cells. Nevertheless, these findings differ from those observed in previous studies demonstrating that the lack of IL-12 responsiveness resulting from impaired IL-12Rβ expression inhibits and mediates susceptibility to *L. amazonensis*.[7]

We previously found that genetically susceptible C57BL/6 × 129Sv/Ev mice lacking STAT6 gene produce significantly higher levels of IL-12, develop a Th1 response and control *L. mexicana* infection, suggesting that *L. mexicana* infection does not alter *in vivo* responsiveness of T cells to IL-12.[5] If susceptibility to *L. mexicana* was due to the lack of IL-12 responsiveness, $STAT6^{-/-}$ mice would have failed to mount a Th1 response and remained susceptible to *L. mexicana* despite production of high levels of IL-12. Recent studies have demonstrated that $CD4^+$ and $CD8^+$ T cells differ in their regulation of IFN-γ.[10] While STAT4-mediated IL-12R signalling is critical for IFN-γ production in $CD4^+$ T cells via the TCR pathway and Th1 development, $CD8^+$ T cells can produce IFN-γ independently of IL-12.[10] Therefore, we hypothesized that deletion of STAT4 gene in $STAT6^{-/-}$ mice will impair Th1 development and render them susceptible to *L. mexicana*. As anticipated, in the present study we found that $STAT4/STAT6^{-/-}$ C57BL/6 × 129Sv/Ev mice developed rapidly progressive large non-healing lesions similar to $STAT4/STAT6^{-/-}$ mice (Fig. 2a). In contrast, concomitantly infected $STAT6^{-/-}$ mice failed to develop lesions (Fig. 2a). At week 10 postinfection, LmAg-stimulated lymph node cells from STAT4/STAT6 mice produced significantly lower levels of IFN-γ and IL-4 than wild-type mice did (Fig. 2b,c). These observations demonstrate that in the absence of STAT4-mediated IL-12 signalling pathway $STAT6^{-/-}$ mice become susceptible to *L. mexicana*. These findings demonstrate that the IL-12R/STAT4-mediated pathway is functional and indispensable for Th1 development in $STAT6^{-/-}$ mice during *L. mexicana* infection. These findings support our previous observations in a *Leishmania major* model,[11] and indicate that the STAT4-mediated pathway is critical for the development of protective immunity against cutaneous leishmaniasis regardless of the species of *Leishmania* and/or genetic background of the mice.

In conclusion, our data indicate that unlike *L. amazonensis*, failure to develop an efficient Th1 response during *L. mexicana* infection is due to the lack of IL-12 production rather than the inability of T cells to respond to this cytokine. Moreover, the data also demonstrate that in the absence of STAT6, the STAT4-mediated IL-12 signalling pathway is indispensable for the development of protective immunity against *L. mexicana*.

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IL-13 gene-deficient mice are susceptible to cutaneous *L. mexicana* infection

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Recent studies have demonstrated that IL-13 mediates susceptibility to cutaneous *L. major* infection via IL-4-independent pathway. To determine whether IL-13 also plays a similar role in pathogenesis of cutaneous *L. mexicana* infection, we analyzed the course of *L. mexicana* infection in IL-13$^{-/-}$ and IL-4/IL-13$^{-/-}$ C57BL/6×129sv/Ev mice and compared with that in similarly infected wild-type mice. IL-13$^{-/-}$ mice were as susceptible as the wild-type mice to *L. mexicana* and developed rapidly progressing, large non-healing lesions following cutaneous *L. mexicana* infection. In contrast, similarly infected IL-4/IL-13$^{-/-}$ mice were highly resistant and developed either no lesions or small lesions containing few parasites that totally resolved by 12 weeks following infection. Throughout the course of infection IL-13$^{-/-}$ and the wild-type mice produced significantly more Th2-associated *L. mexicana* antigen (LmAg)-specific IgG1 than IL-4/IL-13$^{-/-}$ mice. All three groups produced comparable levels of Th1-associated IgG2a. At week 12 post infection, LmAg-stimulated spleen cells from *L. mexicana*-infected IL-4/IL-13$^{-/-}$ produced significantly higher levels of IL-12 and IFN-γ as compared to those from similarly infected wild-type and IL-13$^{-/-}$ mice. Although both IL-13$^{-/-}$ and the wild-type spleen cells produced IL-4 following *in vitro* antigenic stimulation, the wild-type mice produced significantly more. These findings demonstrate that IL-13 is not involved in mediating susceptibility to *L. mexicana*. Moreover, they also indicate that IL-4 not IL-13 is a dominant cytokine involved in pathogenesis of cutaneous *L. mexicana* infection.

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1 Introduction

American cutaneous leishmaniasis caused by *Leishmania mexicana* clinically manifests as chronic infection that is associated with mutilation of the ear pinna [1]. While most inbred mice develop self-healing lesions when infected subcutaneously with *L. major*, almost all develop large non-healing lesions full of parasites following *L. mexicana* infection [2]. Although several studies have reported that *L. major*-infected susceptible BALB/c mice produce of high levels of IL-4 and mount a Th2 response [3, 4], it is not clear whether IL-4 plays a role in mediating susceptibility to *L. major*. For example, some investigators have shown that IL-4-deficient BALB/c mice are resistant to *L. major* [5], while others found them to be as susceptible as the wild-type BALB/c mice [6]. Nevertheless, we have found that IL-4$^{-/-}$ and STAT6$^{-/-}$ mice, regardless of their genetic background, are resistant to cutaneous *L. mexicana* infection, indicating that IL-4- and STAT6-mediated IL-4 signaling pathways inhibit the development of protective immunity against *L. mexicana* [7–9].

IL-13 is a cytokine that is mainly produced by activated T cells and shares a common receptor component and many biological activities of IL-4 [10, 11]. Thus, similar to IL-4, IL-13 inhibits production of NO and TNF-α from macrophages [10, 12]. Moreover, findings in IL-13$^{-/-}$ mice suggest that IL-13 acts upstream of IL-4 and regulates Th2 differentiation [13]. Recent studies have shown that, while IL-13 is critical for the development of protective immunity against some helminthes [14], it is also involved in granuloma formation and fibrosis during schistosomiasis [15, 16]. Furthermore, two independent studies demonstrated that IL-13 is involved in the pathogenesis of murine asthma [17, 18].

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The first two authors contributed equally to this work.

Abbreviation: LmAg: *L. mexicana* antigen

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Despite these observations, the role of IL-13 in regulation of immunity in cutaneous leishmaniasis is not clear. For example, one study found that IL-4Rα$^{-/-}$ BALB/c mice develop significantly smaller lesions than IL-4$^{-/-}$ BALB/c mice following *L. major* infection, indicating that IL-13 mediates susceptibility to *L. major* [19]. In contrast, others also using IL-4Rα$^{-/-}$ BALB/c mice reported that these mice develop significantly larger lesions than similarly infected IL-4$^{-/-}$ BALB/c mice during the late phase of *L. major* infection [20]. Based on their observations, these authors suggested that IL-13 may play a protective role in chronic leishmaniasis caused by *L. major* [20]. A more recent study, however, has found that IL-13$^{-/-}$ BALB/c mice are highly resistant to *L. major*, whereas expression of IL-13 transgene in resistant C57BL/6 mice rendered them susceptible to *L. major* even in the absence of IL-4 [21]. These results indicate that IL-13 mediates susceptibility to *L. major* via IL-4-independent pathway [21].

Therefore, we have analyzed the course of cutaneous *L. mexicana* infection in IL-13$^{-/-}$ and IL-4/IL-13$^{-/-}$ C57BL/6×129/Sv mice and compared with that in similarly infected wild-type counterparts of matched age and sex. Our findings show that IL-13 does not play a role in pathogenesis of cutaneous leishmaniasis caused by *L. mexicana*. Moreover, they demonstrate that IL-4 alone is sufficient to mediate susceptibility to cutaneous *L. mexicana* infection in the absence of IL-13.

2 Results and discussion

In this study, both IL-13$^{-/-}$ and the wild-type mice developed rapidly growing large non-healing lesions containing large number of parasites following subcutaneous inoculation with *L. mexicana*. In contrast, similarly infected IL-4/IL-13$^{-/-}$ mice were highly resistant and developed either no lesions or small lesions that totally resolved by 12 weeks following infection and contained only a few parasites. These findings demonstrate that IL-13 is not critical for pathogenesis of cutaneous leishmaniasis caused by *L. mexicana* and that IL-4 alone can mediate susceptibility to *L. mexicana* in the absence of IL-13.

IL-13 is a T cell-derived cytokine [10, 12] that is distantly related to IL-4 at the amino acid sequence level. Furthermore, IL-13 also binds to the IL-4Rα chain and induces phosphorylation of STAT6 [10, 12, 22]. Therefore, it is perhaps not surprising that IL-13 shares several biological activities of IL-4 [10, 12, 22, 23]. A recent study found that IL-13$^{-/-}$ mice displayed significantly reduced levels of IgE and CD23 on B cells [13]. Furthermore, CD4$^+$ T cells from these mice produced significantly lower levels of IL-4, IL-5 and IL-10 following *in vitro* stimulation with Con A and inclusion of IL-4 and IL-13 failed to restore IL-5 and IL-10 production in these assays [13]. Interestingly, the same study found that immunization of IL-13$^{-/-}$ mice with a schistosome egg antigen or infection with *N. brasiliensis* induced a significant Th2 response *in vivo* that was associated with the development of cells capable of producing IL-4 and IL-5 [13].

Recent studies have reported that IL-13 also plays an important role in regulation of immune responses during several parasitic diseases. For example, IL-13 is critical for the development of protective immunity against certain gut helminthes [14, 24, 25]. In contrast, IL-13 also mediates immunopathological damage during *Schistosoma mansoni* infection by inducing granuloma formation and fibrosis [15, 16]. Nevertheless, the role of IL-13 in cutaneous leishmaniasis caused by *L. major* is controversial. While two studies using IL-13$^{-/-}$ and IL-4Rα BALB/c mice as well as IL-13 transgenic C57BL/6 mice found that IL-13 mediates susceptibility to *L. major* [19, 21], one study using IL-4Rα$^{-/-}$ and IL-4$^{-/-}$ mice indicated that IL-13 may be protective during chronic *L. major* infection [20]. The reasons for these conflicting observations in the *L. major* model remain to be determined and may be attributed to differences in *L. major* strains used by different groups. However, we have previously demonstrated that both IL-4$^{-/-}$ and STAT6$^{-/-}$ mice, regardless of their genetic background, are highly resistant to *L. mexicana* [7–9]. Although these observations suggest that IL-13 alone is not sufficient to mediate protection or susceptibility to *L. mexicana*, it is not clear whether IL-4 alone can mediate susceptibility to *L. mexicana* in the absence of IL-13. In the present study, we found that IL-13$^{-/-}$ mice were highly susceptible to *L. mexicana* and developed large non-healing lesions similar to the wild-type mice following subcutaneous inoculation with *L. mexicana* (Fig. 1). On the other hand, similarly infected IL-4/IL-13$^{-/-}$ mice were highly resistant and failed to develop detectable lesions during most of the course of study (Fig. 1). Moreover, skin lesions from both the wild-type and IL-13$^{-/-}$ mice showed extensive subcutaneous tissue destruction with ulceration and diffuse inflammatory infiltrate primarily comprised of parasite filled macrophages, neutrophils and few lymphocytes. In contrast, inoculation sites from *L. mexicana*-infected IL-4/IL-13$^{-/-}$ mice displayed well-preserved skin with inflammatory foci comprised of lymphocytes and macrophages with only a few parasites. Furthermore, at 12 weeks post infection, lesions from the wild-type and IL-13$^{-/-}$ mice contained significantly higher (approximately 5 log-folds higher) number of parasites as compared to inoculation sites from IL-4/IL-13$^{-/-}$ mice (Fig. 2). Together, these results demonstrate that IL-13 is not involved in pathogenesis of *L. mexicana* infection and IL-4 in the absence

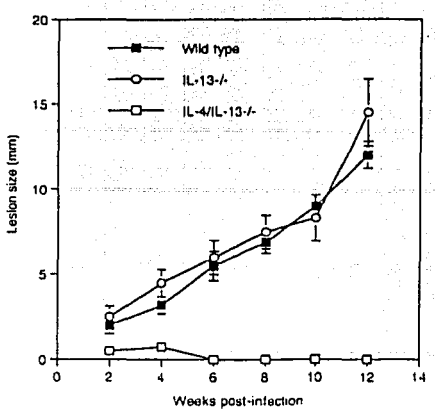

Fig. 1. Course of cutaneous *L. mexicana* infection in wild-type, Il-13$^{-/-}$ and IL-4/IL-13$^{-/-}$ C57BL/6×129Sv/Ev mice. (A) The wild-type, Il-13$^{-/-}$ and IL-4/IL-13$^{-/-}$ mice were inoculated with 5×10^5 *L. mexicana* amastigotes into the shaven back rump and lesion growth was monitored by measuring lesion diameters every week until 12 weeks post infection. Data presented as mean lesion diameter + SE. Similar results were observed in five independent experiments. Data expressed as mean titer + SE. Asterisks indicate statistically significant differences between each group ($p < 0.05$).

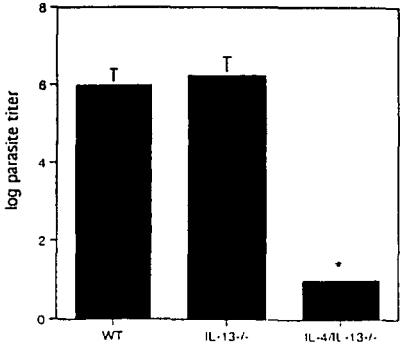

Fig. 2. Assessment of parasite loads in the lesions from *L. mexicana*-infected wild-type, IL-13$^{-/-}$ and IL-4/IL-13$^{-/-}$ mice. At week 12 post infection, the lesions or inoculation sites were excised and parasite burdens were determined by limiting dilution analysis. Data are expressed as mean log titer + SE. Similar results were observed in two independent experiments.

of IL-13 can mediate susceptibility to *L. mexicana*. These findings differ from a recent study, which indicated that IL-13 mediates susceptibility to *L. major* by inhibiting Th1 development independently of IL-4 [21]. This is perhaps not surprising as it is becoming increasingly evident that cutaneous growth of *L. major* is under genetic and immunoregulatory controls different from those associated with the cutaneous growth of *L. mexicana* [26–28].

In previous studies, we had found that ability of IL-4$^{-/-}$ and STAT6$^{-/-}$ mice to control cutaneous *L. mexicana* infection is associated with a preferential development of protective Th1 response due to their inability to mount a Th2 response [7–9]. Similarly, in the present study, *L. mexicana*-infected IL-4/IL-13$^{-/-}$ mice displayed significantly lower levels of Th2-associated *L. mexicana* antigen (LmAg)-specific IgG1 and total IgE as compared to similarly infected wild-type and IL-13$^{-/-}$ mice (Fig. 3A, C). Interestingly, throughout the course of infection, all three groups displayed comparable titers Th1-associated LmAg-specific IgG2a antibody (Fig. 3B) that were barely detectable in *L. mexicana*-infected IL-4$^{-/-}$ mice in our previous study [7]. Nevertheless, similar to IL-4$^{-/-}$ and STAT6$^{-/-}$ mice, LmAg-stimulated spleen cells from *L. mexicana*-infected IL-4/IL-13$^{-/-}$ mice produced significantly more IL-12 and IFN-γ than those from the wild-type and IL-13$^{-/-}$ mice, indicating a Th1-influenced response in IL-4/IL-13$^{-/-}$ mice (Fig. 4A, B). Although both wild-type and IL-13$^{-/-}$ spleen cells produced comparable levels of IFN-γ following antigenic stimulation, the latter produced significantly more IL-12 (Fig. 4A). LmAg-stimulated spleen cells from the wild-type and IL-13$^{-/-}$ mice produced detectable amounts of IL-4 (Fig. 4C). These results extend the findings of our previous studies [7, 8], demonstrating that IL-4 inhibits development of Th1 response during *L. mexicana* infection. Additionally they also indicate that both IL-4 and IL-13 inhibit IL-12 production during *L. mexicana* infection, supporting our recent findings that the susceptibility to *L. mexicana* infection is due to the inability to produce IL-12 rather than lack of IL-12 responsiveness [29].

IL-13 is believed to play an important role in regulation of Th2 cell-dependent immune responses. For example, a recent study found that IL-13$^{-/-}$ mice produce significantly reduced levels of IL-4 and have lower levels of IgE [13]. Furthermore, exogenous IL-13 or IL-4 added to *in vitro* cultured CD4$^+$ T cells from IL-13$^{-/-}$ mice failed to restore production of Th2 type cytokines, indicating that IL-13 is an important regulator of Th2 differentiation [13]. In the present study, we also found that LmAg-stimulated spleen cells from IL-13$^{-/-}$ mice produced significantly lower levels of IL-4 than the wild-type mice (Fig. 4C). However, there were no significant differences in levels of Th2-associated IgG1 and IgE antibodies between the wild-type and IL-13$^{-/-}$ mice, indicating that IgG1 and IgE production during *L. mexicana* is IL-13 independent (Fig. 3C).

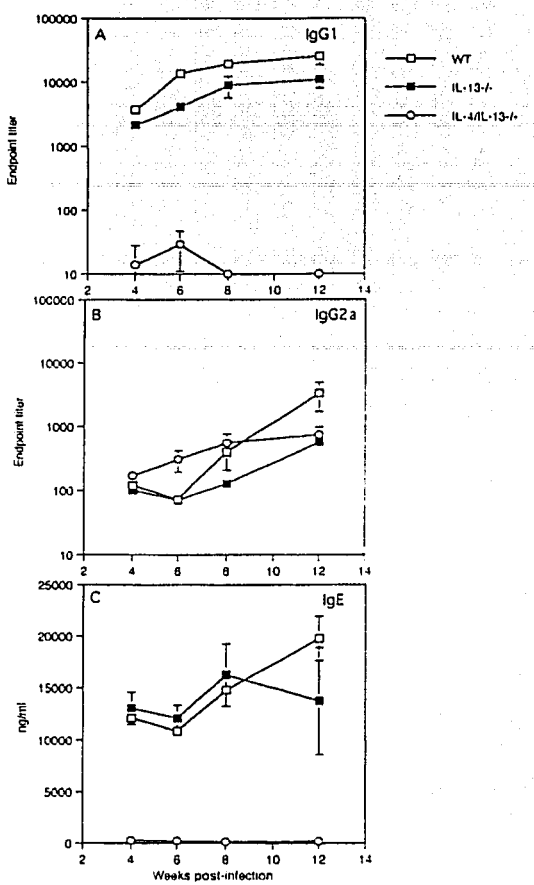

Fig. 3. Kinetics of antibody responsese in *L. mexicana*-infected in wild-type, Il-13$^{-/-}$ and IL-4/IL-13$^{-/-}$ mice at weeks 2, 4, 6, 8, and 12 after injection. (A) LmAg-specific IgG1, (B) Lm-Ag specific IgG2a and (C) total IgE. Data for IgG1 and IgG2a is presented as mean reciprocal endpoint titer on log scale. Data for total IgE is presented as mean IgE level (ng/ml) + SE. Five to six animals were analyzed in each group. Data representative of one out of threee identical experiments with similar results. Asterisks indicate statistically significant differences between each group ($p < 0.05$).

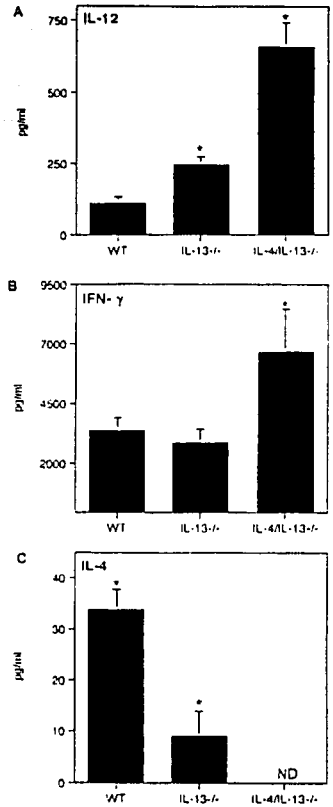

Fig. 4. Cytokine production by LmAg-stimulated spleen cells from *L. mexicana*-infected wild-type, IL-13$^{-/-}$ and IL-4/IL-13$^{-/-}$ mice. (A) IL-12, (B) IFN-γ, and (C) IL-4 production by splenocytes from *L. mexicana*-infected mice were measured at 12 weeks post infection. Four to five animals were analyzed in each group. Similar results were observed in two independent experiments. Asterisks indicate statistically significant differences between each group ($p<0.05$). ND: non-detectable.

In conclusion, genetically susceptible C57BL/6×129Sv/Ev mice lacking IL-13 gene are highly susceptible to *L. mexicana* and develop large lesions containing large number of parasites similar to *L. mexicana*-infected wild-type mice. In contrast, IL-4/IL-13$^{-/-}$ C57BL/6×129Sv/Ev mice produce significantly higher levels of Th1-type cytokines associated with impaired Th2 development and efficiently control cutaneous *L. mexicana* infection. These findings not only indicate that IL-13 is not involved in pathogenesis of *L. mexicana* infection but also demonstrate that IL-4 mediates susceptibility to *L. mexicana* in the absence of IL-13.

3 Materials and methods

3.1 Mice

IL-13$^{-/-}$ and IL-4/IL-13$^{-/-}$ mice on C57BL/6×129Sv genetic background were generated as described previously [13, 21]. The mice were bred and maintained in the facility at the

Harvard School of Public Health according to the guidelines for animal research. Wild-type mice of the same strain combination, age and sex were used as controls in all experiments.

3.2 Parasites and infection

L. mexicana (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated subcutaneously into shaven rumps of 129Sv/Ev mice. For all the experiments, 8–12-week-old, sex-matched IL-13$^{-/-}$, IL-4/IL-13$^{-/-}$ and IL-4/IL-13$^{+/+}$ mice were infected by injecting 5×10^6 *L. mexicana* amastigotes into shaven back rump. The disease progression was monitored by measuring lesion diameters every 2 weeks until week 12 post infection. Lesions from IL-13$^{-/-}$ and IL-4/IL-13$^{+/+}$ mice and inoculation sites from IL-4/IL-13$^{-/-}$ mice were excised and fixed in 10% buffered formalin.

3.3 Antibody ELISA

Blood was collected from tail snips from *L. mexicana*-infected mice at different time points after infection and their sera were analyzed for the levels of *Leishmania*-specific Th1-associated IgG2a and Th2-associated IgG1 as well as total IgE antibodies by ELISA as described previously [9].

3.4 T cell proliferation and cytokine assays

Spleens from *L. mexicana*-infected IL-13$^{-/-}$, IL-4/IL-13$^{-/-}$ and IL-4/IL-13$^{+/+}$ mice were removed at week 12 post infection, and T cell proliferation assays were performed as previously described [9]. Briefly, cell suspensions were prepared by gentle teasing in RPMI 1640 supplemented 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 U penicillin/ml, 100 mg streptomycin/ml and 0.05 mM 2-mercaptoethanol. Erythrocytes were lysed by re-suspending cells in Boyle's solution (0.17 M Tris and 0.16 M ammonium chloride). Following two washes, live cells were enumerated by trypan blue exclusion using Nuebaer hemocytometer and adjusted to 5×10^6/ml. Aliquots containing 5×10^5 cells were plated in triplicate to the wells of 96-well flat-bottom tissue culture plates containing 20 μg/ml LmAg prepared by six cycles of freezing at −70°C and thawing at 37°C. Culture supernatants from these assays were analyzed for production of IL-4, IL-12 and IFN-γ (reagents purchased from BD PharMingen; detection limit for IL-4 is 3 pg/ml and for all others is 20 pg/ml) by capture ELISA as described previously [9].

3.5 Statistical analysis

Student's unpaired *t*-test was used to determine the statistical significance of values obtained. Differences in Ab titers were determined using Mann-Whitney *U* prime test.

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