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"Caracterización inmunológica de granulomas procedente de bovinos naturalmente infectados por *Mycobacterium bovis*."

TESIS

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PRESENTA

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ABSTRACT

Bovine tuberculosis is a chronic inflammatory disease that causes the formation of granulomas. The study of this structure has improved our understanding of the pathogenesis of tuberculosis. However, the immune response presented by granulomas of young cattle naturally infected with *Mycobacterium bovis* has not been fully studied. In previous work, we observed that naturally infected granulomas of bovines with bovine tuberculosis under four months of age lack a connective tissue capsule, ten times fewer multinucleated giant cells on average per lesion, and a greater number of acid-fast bacilli than granulomas. identified in cattle older than one year. This suggests a deficient immune response against infection by *M. bovis* in granulomas from calves, which will present fewer cells and cytokines related to the control of bovine tuberculosis compared to adult bovines. Therefore, in this study we have used immunohistochemistry and digital pathology analysis to identify cell populations, cytokines, and the presence of mycobacteria in 3,439 granulomas, obtained from 10 Holstein Friesian cattle under four months of age and 15 cattle over one year of age. naturally infected by *M. bovis*. The results show a statistically higher number of mycobacteria, T lymphocytes (CD3+), IFN-γ, TNF-α and inducible nitric oxide synthase (iNOS). Likewise, fewer macrophages (MAC387+), B lymphocytes (CD79+), gamma delta cells (WC1+), vimentin, myofibroblasts (α-SMA) and TGF-β in granulomas of young bovines compared to adult bovines. Our results show a higher production of proinflammatory cytokines in granulomas from calves, the absence of a connective tissue capsule associated with a lower number of fibroblasts and a lower production of anti-inflammatory cytokines compared to granulomas from bovines older than one year. Showing an exacerbated proinflammatory response in calves' granulomas with a greater amount of necrosis and less microbicidal capacity compared to bovine granulomas naturally infected with *M. bovis*. These results suggest that the immune response present in naturally infected bovine granulomas depends on the age at which the animals are infected.

RESUMEN

La tuberculosis bovina es una enfermedad inflamatoria crónica que provoca la formación de granulomas. El estudio de esta estructura ha mejorado nuestra comprensión de la patogenia de la tuberculosis. Sin embargo, la respuesta inmune que se presentan los granulomas de bovinos jóvenes naturalmente infectados con *Mycobacterium bovis* no ha sido completamente estudiada. En un trabajo previo, observamos que granulomas de bovinos con tuberculosis bovina menores de cuatro meses naturalmente infectados, carecen de una cápsula de tejido conectivo, diez veces menos cantidad células gigantes multinucleadas en promedio por lesión y mayor cantidad de bacilos ácido alcohol resistentes que los granulomas identificados en bovinos mayores de un año. Esto sugiere, una respuesta inmune deficiente contra la infección por *M. bovis* en los granulomas de animales jóvenes, los cuales presentaran menor cantidad de células y citocinas relacionadas con el control de la tuberculosis bovina en comparación con bovinos adultos. Por lo tanto, en este estudio hemos utilizado inmunohistoquímica y análisis de patología digital para identificar poblaciones celulares, citocinas y la presencia de micobacterias en 3.439 granulomas, obtenidos de 10 bovinos menores de cuatro meses y 15 bovinos mayores de un año de la raza Holstein Friesian naturalmente infectados por *M. bovis.* Los resultados muestran estadísticamente mayor cantidad de micobacterias, linfocitos T (CD3+), IFN-γ, TNF-α y óxido nítrico sintasa inducible (iNOS). Así mismo, menor cantidad de macrófagos (MAC387+), linfocitos B (CD79+), células gamma delta (WC1+), vimentina, miofibroblastos (α-SMA) y TGF-β en granulomas de bovinos jóvenes comparado con bovinos adultos. Nuestros resultados muestran una mayor producción de citocinas proinflamatorias en los granulomas de bovinos jóvenes, la ausencia de cápsula de tejido conectivo asociada a la menor cantidad de cantidad de fibroblastos y menor producción de citocinas antinflamatorias comparado con los granulomas de bovinos mayores de un año resultados. Mostrando una respuesta proinflamatoria exacerbada en jóvenes con mayor cantidad de necrosis y menor capacidad microbicida en comparación con granulomas de bovinos naturalmente infectados con *M. bovis.* Estos resultados sugieren que la respuesta inmune presente en los granulomas de bovinos naturalmente infectados depende de la edad en la que los animales son infectados.

INTRODUCCIÓN

La tuberculosis bovina causada por *Mycobacterium bovis* (*M. bovis*) afecta a diferentes mamíferos incluyendo los seres humanos, ocasionando problemas de salud pública y pérdidas económicas a la industria ganadera estimadas en 3 mil millones de dólares anuales debido a; bajas en la producción láctea, decomisos parciales o totales de los órganos con lesiones, detectados en la inspección *post mortem* en rastro, así como costos asociados a la operación de programas para su erradicación (Buddle et al., 2011; Majoor et al., 2011; Tsairidou et al., 2014 and Waters et al., 2014). Esta enfermedad se caracteriza por ser un proceso inflamatorio crónico, que afecta principalmente a los nódulos linfáticos craneales y pulmones, provocando linfadenitis y neumonía granulomatosa, respectivamente. Histológicamente se observan granulomas los cuales están formados por agregado de células inflamatorias como: neutrófilos, macrófagos, macrófagos epitelioides, células gigantes multinucleadas y linfocitos los cuales rodean a las micobacterias que frecuentemente se encuentran en el centro de la lesión. Reflejando una interacción entre los mecanismos de defensa innatos y adaptativos del huésped y los factores de virulencia de la micobacteria.

En los estudios con bovinos infectados experimentales con *M. bovis* se han descrito las lesiones granulomatosas, las cuales, se han clasificado en cuatro estadios de acuerdo a criterios morfológicos como son; la presencia o ausencia de necrosis, mineralización y cápsula de tejido conectivo (Wangoo et al. 2005). Esta estructura ha sido objeto de numerosos estudios con la finalidad de entender cómo el huésped controla las micobacterias o cómo estas sobreviven a la respuesta inmune, lo cual ha permitido comprender mejor la patogenia de la enfermedad (Aranday-Cortes et al. 2013). No obstante, poco se sabe de las características macroscópicas y microscópicas del

granuloma producido por *M. bovis* en bovinos naturalmente infectados.

MARCO TEÓRICO
Patogenia de la tuberculosis bovina

M. bovis miembro del complejo *Mycobacterium tuberculosis*, induce lesiones granulomatosas crónicas afectando principalmente a los pulmones y nódulos linfáticos asociados, de bovinos naturalmente infectados, así mismo puede afectar a diferentes órganos relacionado con la ruta de ingreso, donde las lesiones pueden ser localizadas, afectando solo un órgano o generalizadas involucrando varios órganos (Domingo et al. 2014).

La transmisión de *M. bovis* en el ganado bovino, se origina por el contacto con animales domésticos o salvajes infectados, principalmente a través de la vía respiratoria, seguida de la vía digestiva debida al consumo de alimentos contaminados como pastura, agua, leche etc. Lo cual ha sido comprobado, mediante infecciones experimentales, donde se ha inoculado por las mismas vías de infección cepas virulentas de *M. bovis* causando lesiones similares a las encontradas en infecciones naturales (Domingo et al. 2014). Una vez que la micobacteria ingresa al huésped, es reconocida por las células fagocíticas, las cuales inician el reclutamiento de macrófagos, células dendríticas y neutrófilos entre otras células de la respuesta inmune que tratan de eliminar las bacterias y controlar la infección. Los macrófagos son considerados la principal población celular infectada por las micobacterias, estas células fagocíticas interactúan con las células de la respuesta inmune innata y adquirida iniciando la formación del granuloma, la cual puede formarse a partir del día 7-11 después de la infección por *M. bovis* (Pollock et al. 2006).

El granuloma en la tuberculosis bovina

El granuloma es la lesión característica de la tuberculosis, compuesta de células de la respuesta inmune innata y adquirida, tratando de controlar la infección crónica causada por las micobacterias, así mismo puede ser formado por una respuesta inflamatoria crónica causada por diferentes agentes antigénicos o por cuerpos extraños inertes que son difíciles de eliminar (Ndlovu and Marakalala 2016). En el ganado bovino la infección por *M. bovis* causa lesiones granulomatosas que morfológicamente son similares a los ocasionados por *M. tuberculosis* en el humano, con la diferencia de que las lesiones necróticas en humanos pueden convertirse en licuefactivas con abundantes bacilos (Waters et al. 2014b).

Macroscópicamente estas lesiones se presentan como nódulos de color blanco o amarillo, algunas lesiones presentan calcificación, que crepita al corte, estas lesiones pueden ser aisladas y medir unos milímetros u ocupar gran parte del órgano afectado (**Figura 1**).
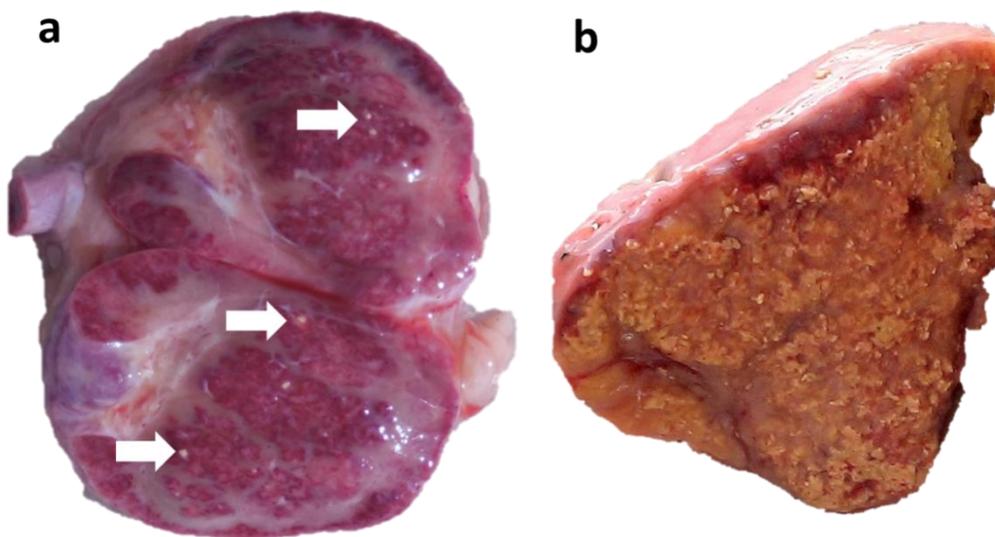
Figura 1: Nódulos linfáticos mediastínicos de bovino con lesiones sugestivas a tuberculosis. (A) Lesiones multifocales, del tamaño de la cabeza de un alfiler señaladas por las flechas **(B)** Lesión que

ocupa la mayor parte del parénquima con abundante necrosis caseosa la cual crepita al corte. Imágenes obtenidas por Jacobo Carrisoza-Urbina.

Microscópicamente los granulomas se caracterizan por formar un agregado de células inflamatorias compuesto principalmente por neutrófilos, macrófagos, macrófagos epitelioides, células gigantes, linfocitos T de tipo CD4 y CD8, así como células plasmáticas y micobacterias las cuales se encuentran en el centro de la lesión infectando los macrófagos y en las zonas con necrosis. En el 2005 Wangoo *et al.* proponen una clasificación de los granulomas, que consiste en cuatro estadios o fases por los que evolucionan las lesiones de bovinos infectados experimentalmente con *M. bovis*, tomando como criterios de clasificación, la presencia o ausencia de: células gigantes, capsula de tejido conectivo, necrosis y calcificación. El Estadio I: representa la etapa inicial de la lesión, donde se han observado grupos de macrófagos epitelioides, células de tipo Langhans con linfocitos intercalados sin presencia de necrosis, algunas veces se observa un infiltrado de neutrófilos. Estadio II: se encuentran macrófagos epitelioides, células de tipo Langhans multinucleadas, mayor cantidad de linfocitos y necrosis caseosa en el centro de la lesión. Los granulomas del estadio III: están completamente encapsulados, con abundantes células gigantes, la necrosis caseosa está bien desarrollada, pero la mineralización es mínima. El Estadio IV: presenta necrosis y mineralización con una gruesa cápsula de tejido conectivo, macrófagos epitelioides y células gigantes multinucleadas que rodean la extensa área de necrosis, también se ha observado acumulo de linfocitos distribuidos cerca de la cápsula fibrótica.

Numerosos estudios se han enfocado en caracterizar las lesiones granulomatosas de bovinos infectados experimentalmente, principalmente en animales mayores a seis

meses de edad, lo cual ha sido de gran utilidad para comprender mejor la patogenia de la enfermedad y permitir evaluar la protección de nuevas vacunas (Palmer et al. 2007; Widdison et al. 2011; Menin et al. 2013a; Palmer et al. 2015). Sin embargo, se desconoce el tipo de respuesta inmune presente en los granulomas de bovinos jóvenes infectados naturalmente por *M. bovis.* A continuación, se describe brevemente lo que se conoce de la función de las células que forman el granuloma de la tuberculosis bovina.

Macrófagos

Los macrófagos forman parte de la respuesta inmune innata contra bacterias patógenas, así mismo tienen un papel fundamental en la patogénesis de la tuberculosis, siendo considerados el principal reservorio durante la infección temprana y crónica de las micobacterias patógenas, las cuales tienen la capacidad de sobrevivir y llevar a cabo su ciclo biológico dentro del macrófago infectado. Esto ha planteado la duda de si estas células son capaces de controlar la infección o solo funcionan como un vehículo que permiten la diseminación de las micobacterias (Russell 2001).

Los macrófagos y las células dendríticas son las principales en iniciar la formación del granuloma, estas células producen citocinas y quimiocinas que provocan el reclutamiento de diferentes tipos celulares de la respuesta inmune innata y adquirida, incluidos neutrófilos, células asesinas naturales, linfocitos T, linfocitos B y fibroblastos (Guirado et al. 2013). En el granuloma se ha observado diferentes fenotipos de macrófagos como son: células epitelioides, células espumosas, células gigantes de tipo Langhans lo que puede deberse a la flexibilidad de estas células en cambiar su fenotipo y fisiología de acuerdo con el microambiente donde se encuentran (Mosser

and Edwards 2008). No obstante, también se ha identificado que la micobacteria puede participar en la diferenciación celular de los macrófagos hacia células gigantes multinucleadas (Herrtwich et al. 2016).

Histológicamente los granulomas iniciales causados por *M. bovis* en el ganado bovino están formados principalmente por macrófagos epteliodes y células gigantes multinucleadas que incrementan en número conforme aumente el avance de la lesión, posteriormente se localizan rodeando el centro de necrosis **Figura 2.**
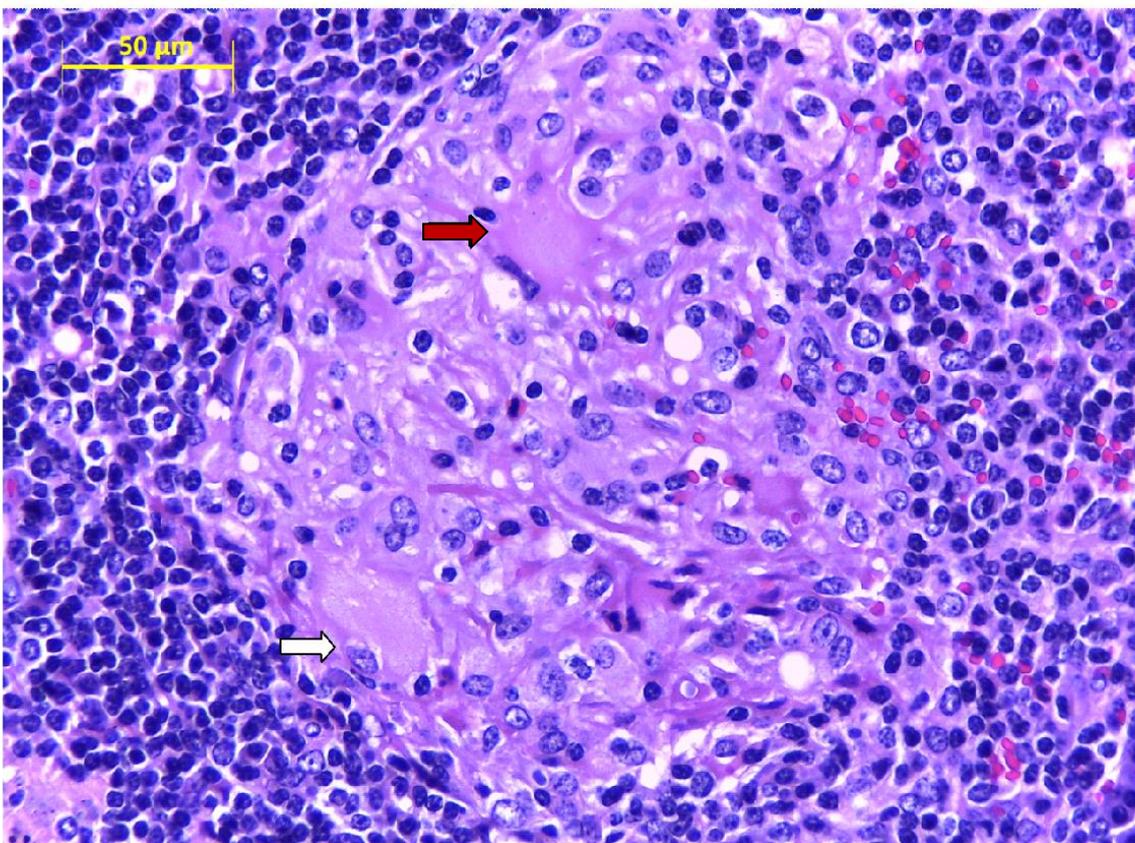
Figura 2: **Granuloma estadio I o inicial en nódulo linfático de bovino naturalmente infectado por *M. bovis*.**
H&E, 400x. Mostrando un acúmulo de macrófagos epitelioides (flecha blanca) y células gigantes (flecha roja). Imágenes obtenidas por Jacobo Carrisoza-Urbina.

Células gigantes

Las células gigantes multinucleadas han sido un marcador histopatológico de lesiones sugestivas de tuberculosis. No obstante, es posible encontrarlas también en enfermedades granulomatosas crónicas como algunas alergias, en enfermedades autoinmunes, en vasculitis, en la enfermedad inflamatoria intestinal, en la sarcoidosis, así como en los granulomas inducidos por cuerpos extraños, entre otras.

Se pensaba que la formación de este tipo de células en la tuberculosis ocurría a través de la fusión de célula-célula como se había demostrado en osteoclastos (Helming and Gordon, 2009). Sin embargo, recientemente se demostró que las células gigantes se forman por modificación en la división celular y un defecto mitótico. Herrtwich *et al.* (2016) demostraron *in vitro* que la estimulación de progenitores de macrófagos y células dendríticas con M-CSF y lipoproteínas bacterianas, da como resultado la formación de células multinucleadas. Lo cual demuestra la participación de la micobacteria en la formación de estas células, las cuales frecuentemente se encuentran infectadas (**Figura 3**) (Herrtwich et al. 2016).

En el granuloma del ganado bovino las células gigantes expresan mediadores químicos como iNOS y diferentes citocinas como TNF-α, IFN-γ, TGF-β, IL-17A e IL-10 en diferentes grados (Pereira-Suárez et al. 2006; Palmer et al. 2016;). Además, estas células parecen tener una correlación con la progresión de lesión ya que se han observado mayoritariamente en lesiones con necrosis y calcificación, así mismo se observa, una menor presencia de estas células en animales vacunados con BCG y posteriormente desafiados con *M. bovis*. Adicionalmente en un estudio donde histológicamente se compararon las lesiones causadas por *M. caprae* y *M. bovis* en jabalís infectados naturalmente, se encontró una mayor cantidad de células gigantes en las lesiones provocadas por *M. caprae* que se correlacionaron con un mayor grado

de patogenicidad (García-Jiménez et al. 2013).
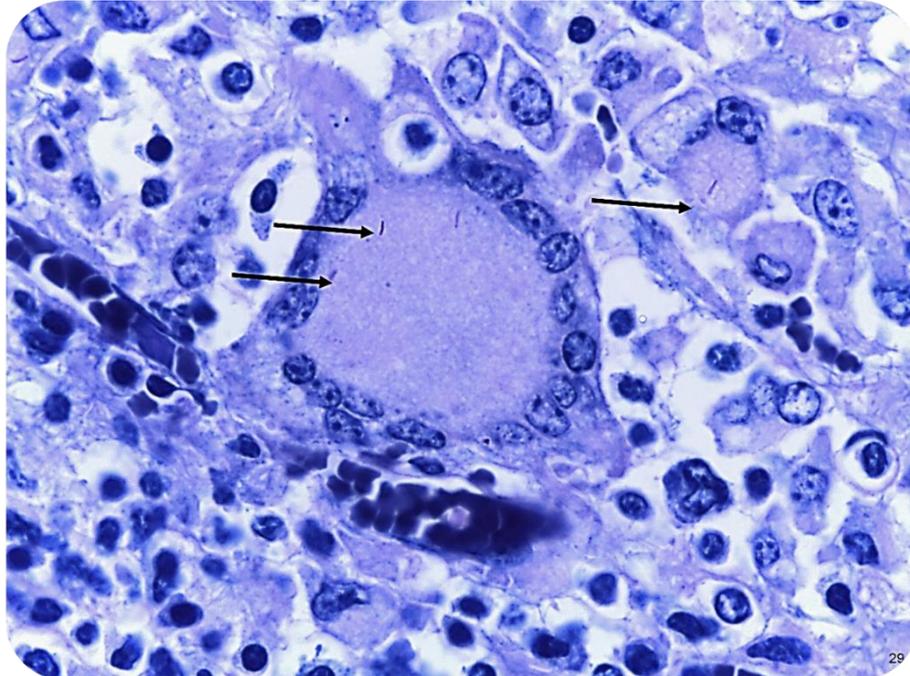
Figura 3: Célula gigante multinucleada en granuloma inicial con BAAR. Se observa una célula gigante multinucleada con bacilos cercanos a los núcleos señalados con flechas. Zielh-Neelsen, 1000x. Imágenes obtenidas por Jacobo Carrisoza-Urbina.

Neutrófilos

Los neutrófilos son fagocitos profesionales de vida corta que son reclutados rápidamente al sitio de infección, donde fagocitan a las bacterias mediante reconocimiento directo y opsonización, promoviendo una rápida fusión del fagosoma con el lisosoma y matando a las bacterias por reacciones de oxidación mediante la producción de especies reactivas de oxígeno (ROS) y nitrógeno, jugando un papel fundamental en el inicio de la respuesta inmune innata (Lowe et al. 2012). Sin embargo, es polémico si los neutrófilos pueden eliminar a las micobacterias que fagocitan, especialmente las cepas virulentas, debido a que se ha reportado que los neutrófilos son las principales células con mayor carga de bacilos encontradas en los lavados

bronqueo-alveolares y en el esputo de pacientes con tuberculosis activa, las cepas virulentas de M. tuberculosis pueden vivir dentro de los neutrófilos a pesar de los componentes microbicidas que estas células poseen y se ha demostrado que los bacilos pueden escapar, induciendo la muerte por necrosis del neutrófilo de una manera independiente de ROS (Corleis et al. 2012). En neutrófilos de bovinos infectados se observa que *M. bovis* también es capaz de sobrevivir y escapar de las células, además de inducir autofagia de una manera aún no determinada (Wang et al. 2013).

La participación de los neutrófilos en el granuloma del bovino causado por *M. bovis* ha sido poco estudiada, algunos estudios han reportado una baja participación de estas células (Wangoo et al. 2005 y Palmer et al. 2007). Aunado a esto, los rumiantes son considerados una especie "linfocitaria", debido a su alto porcentaje de linfocitos en sangre periférica 60-75 % y un bajo porcentaje de neutrófilos del 20-30%, comparado con el ser humano donde los neutrófilos alcanzan valores de 70-80%. No obstante, los neutrófilos se han observado en etapas tempranas del granuloma del ganado bovino, principalmente en lesiones localizadas en el pulmón lo que demuestra su posible participación de estas células en la formación del granuloma (Johnson et al. 2006). Las cabras afectadas naturalmente por *M. bovis*, muestran lesiones cavitarias semejantes a las encontradas en los humanos en las cuales se ha observado un mayor número de neutrófilos comparado con lesiones necróticas (Sanchez et al. 2011). En humanos este tipo de lesiones se asocia con tuberculosis activa, donde se observa una gran cantidad de neutrófilos, siendo estas células las principalmente infectadas por el bacilo (Dallenga and Schaible 2016).

La participación de los neutrófilos en la patogenia de la tuberculosis ha sido controversial, estas células han sido referidas como el "caballo de Troya", debido a

que podrían participar en la diseminación del bacilo a sitios distales de la infección por su posible falla en su capacidad microbicida hacia las micobacterias (Eruslanov et al. 2005). Si los neutrófilos son capaces de eliminar o no al bacilo, puede ser debido a diferentes factores tanto del huésped como del microambiente del tejido infectado, de la etapa de la infección y por otra parte de la virulencia de la micobacteria (Lowe et al. 2012).

Células T γδ

Los linfocitos T γδ constituyen entre el 5 y 10% del porcentaje de las células T en la circulación sanguínea en humanos y en ratones respectivamente, interesantemente en rumiantes jóvenes estas células representan hasta un 50-70%, el cual disminuye a un 12% en animales adultos (Kabelitz 2011). En bovinos se han identificados dos subgrupos de estas células, basándose en la expresión del antígeno WC1 (workshop cluster antigen-1) y sus isoformas de WC1.1 y WC1.2. Donde el primer subgrupo se caracteriza por secretar IFN-γ y el segundo por ser más sensible a la estimulación por mitógenos (Price et al. 2010). Estas células tienen características tanto del sistema inmune innato y adaptativo, por lo que también son consideradas como células T transitorias de la respuesta inmune (Vantourout and Hayday 2013).

Estudios en bovinos infectados experimentalmente con *M. bovis* han reportado una correlación entre la cantidad de células T γδ con el grado de organización de las lesiones, principalmente al inicio de la formación del granuloma (Plattner et al. 2009). También se ha visto que estas células se acumulan rápidamente en nódulos linfáticos de la cabeza y tejido pulmonar después de la vacunación intranasal con la cepa de *M. bovis* BCG en becerros y así mismo aumentan la proporción de linfocitos WC1+

Tγδ productores de INF-y en sangre periférica (Guzman et al. 2012).

A pesar del reciente progreso en él estudió de células T WC1+ γδ, aún se desconoce su función e importancia en la respuesta inmune. Sin embargo, el ganado bovino ofrece una alternativa como modelo de estudio de estas células y en el diseño de vacunas contra la tuberculosis dirigida al reclutamiento de células WC1+γδ que podrían ofrecer buenos resultados (Vantourout and Hayday 2013).

Células B

En la patogenia de la tuberculosis la respuesta inmune celular es considerada la de mayor participación en la defensa contra las micobacterias, en comparación con la respuesta inmune humoral la cual ha sido subestimada en la intervención contra los patógenos intracelulares (Rao et al. 2015). Sin embargo, recientes estudios han evidenciado la participación de linfocitos B y los anticuerpos en mecanismos como: la activación del complemento, opsonización, modulación de la inflamación y actividad antimicrobiana directa contra micobacterias como se muestra en la Figura 4.
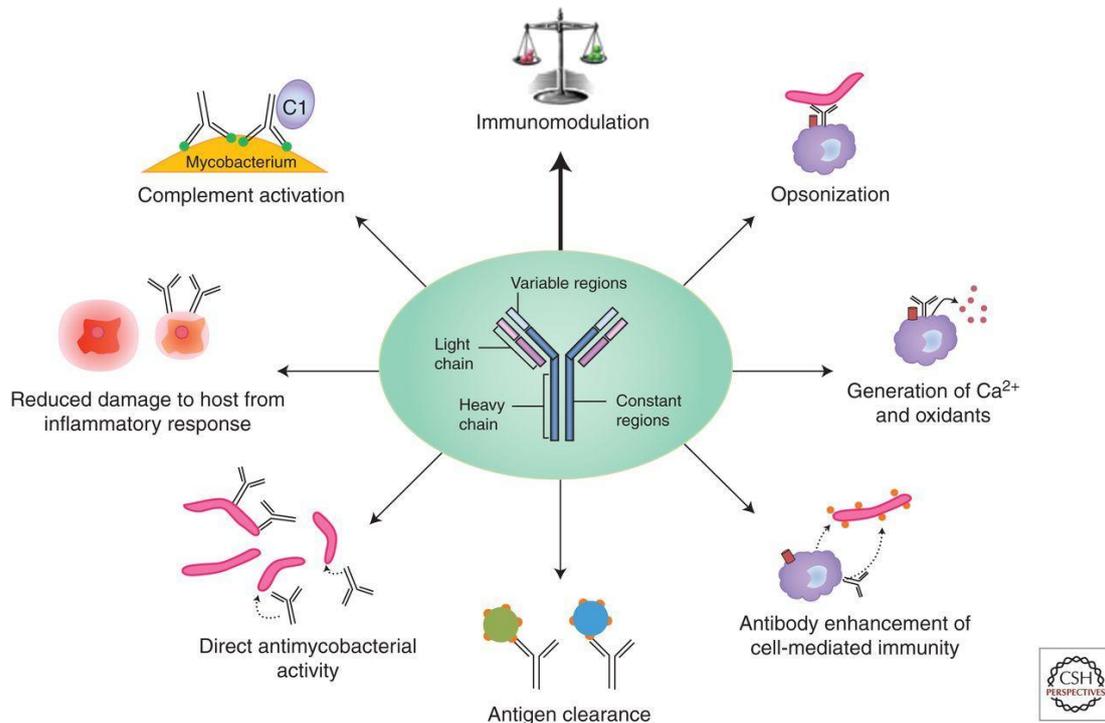
Figura 4: Diferentes funciones de los anticuerpos en la respuesta contra las micobacterias.
Tomado de: **http://perspectivesinmedicine.cshlp.org/content/5/3/a018432/F1.expansion.html**

En algunos reportes las células B también forman parte de las lesiones granulomatosas, constituyendo un 20% del infiltrado (Co et al. 2004) y se ha observado que forman agregados de células parecidos a los centros germinales encontrados en los nódulos linfáticos, que contienen principalmente células vírgenes, células plasmáticas, células B de memoria y un infiltrado de linfocitos T CD4+ y CD8+ (Ulrichs et al. 2004). Estos nódulos de células B, también se han identificado en enfermedades que cursan con procesos inflamatorios crónicos como la esclerosis múltiple y la artritis reumatoide, así como en la infección por el virus de la influenza y *Helicobacter spp* (Kozakiewicz et al. 2013). En las lesiones granulomatosas causados por la infección con miembros del complejo *M. tuberculosis* en ratones, humanos, venados, bovinos entre otras especies también se han observado agregados de células B en la periferia de las lesiones (Ulrichs et al. 2004; Johnson et al. 2006; García-Jiménez et al. 2012; Aranday-Cortes et

al. 2013). En un estudio realizado en cabras infectadas naturalmente con *M. caprae*, en donde se compararon las poblaciones celulares de granulomas y lesiones cavitarias, se observó una mayor cantidad de linfocitos B y células plasmáticas en los granulomas comparado con las lesiones cavitarias (Sanchez et al. 2011).

En las lesiones causadas por *M. bovis* en bovinos infectados experimentalmente, se han observado agregados de células B principalmente en lesiones avanzadas, estadios III y IV rodeando la cápsula de fibrosis (Aranday-Cortes et al. 2013). Como dato interesante, en animales vacunados con *M. bovis BCG* y desafiados con *M. bovis,* se ha observado un aumento de estas células en estadios II al IV (Johnson et al. 2006; Salguero et al. 2016). Por lo que se ha considerado que estas células participan en el control de *M. bovis* tanto directa como indirectamente, controlando la respuesta inmune local en la lesión.

ANTECEDENTES.

En un estudio previo se evaluaron granulomas procedentes de 32 bovinos de la raza Holstein Friesian infectados naturalmente por *M. bovis*, provenientes de una cuenca lechera de la región central de México. En el muestreo 46.8 % (15/32) de los animales fueron menores de 4 meses de edad y 53.2% (17/32) fueron mayores de un año. Macroscópicamente el 100% (32/32) de los animales incluidos, presentaron lesiones sugestivas a tuberculosis en la cadena de nódulos linfáticos mediastínicos y el 50% (16/32) de las lesiones se identificaron en pulmones; de estos animales se analizaron microscópicamente un total de 1143 lesiones granulomatosas, de las cuales el 34.6% (396/1143) fueron de bovinos mayores de un año que se clasificaron de acuerdo a la nomenclatura de Wangoo *et al*. 2005 (**Figura 5**), Los tipos de granulomas con mayor frecuencia encontrados fueron estadio IV con un 34.3% (136/396), y estadio I en un 29.0% (115/396) que frecuentemente fueron satélites de estadios III y IV. Sorpresivamente, las lesiones en los bovinos menores de 4 meses mostraron un patrón atípico que no permitió identificar su clasificación. Estos granulomas presentaron grandes áreas de necrosis que se extendían en la mayor parte del órgano afectado, acompañadas de calcificación central, ausencia de cápsula de tejido conectivo, un promedio de 1.4 células gigantes por lesión comparado con 14.5 observadas en bovinos mayores de un año (**Figura 6 y 7**). Finalmente, en el 84.3% (27/32) de los casos estudiados se identificó DNA de *M. bovis* mediante PCR y en el resto de los casos sólo se observó la presencia de bacilos acido alcohol resistente en el tejido. Los resultados sugieren que los bovinos menores de 4 meses naturalmente infectados por *M. bovis* forman granulomas atípicos incapaces de contener la

infección. Este conocimiento puede ser útil en un mejor entendimiento de la resistencia natural del huésped a la infección por micobacterias.
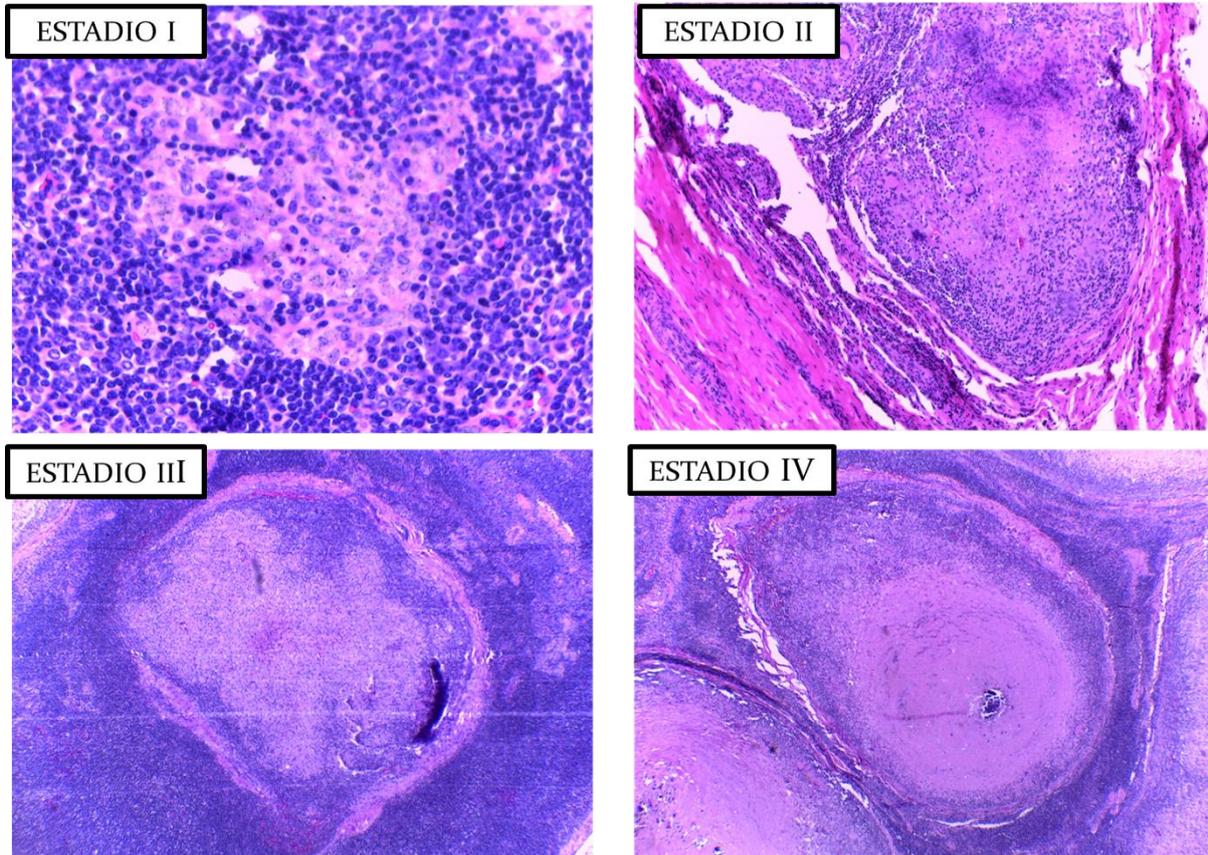
Figura 5: Clasificación histopatológica de granulomas en nódulos linfáticos de bovinos. H&E (a.-400x, b-d.-200x) Estadio I: pequeños focos de células inflamatorias infiltradas, principalmente macrófagos, macrófagos epitelioides y linfocitos intercalados. Estadio II: Estructura de mayor tamaño donde se encuentran macrófagos epitelioides, células de tipo Langhans multinucleadas, mayor cantidad de linfocitos y el inicio de necrosis caseosa en el centro de la lesión. Estadio III: lesión bien organizada característica del granuloma el cual está completamente encapsulado con una delgada capa de tejido conectivo, la necrosis caseosa es evidente, pero la mineralización es mínima. Estadio IV: Se observa mineralización y abundante necrosis, rodeada por numerosos linfocitos, macrófagos epitelioides, células gigantes y una cápsula gruesa de tejido conjuntivo. Imágenes obtenidas por Jacobo Carrisoza-Urbina.
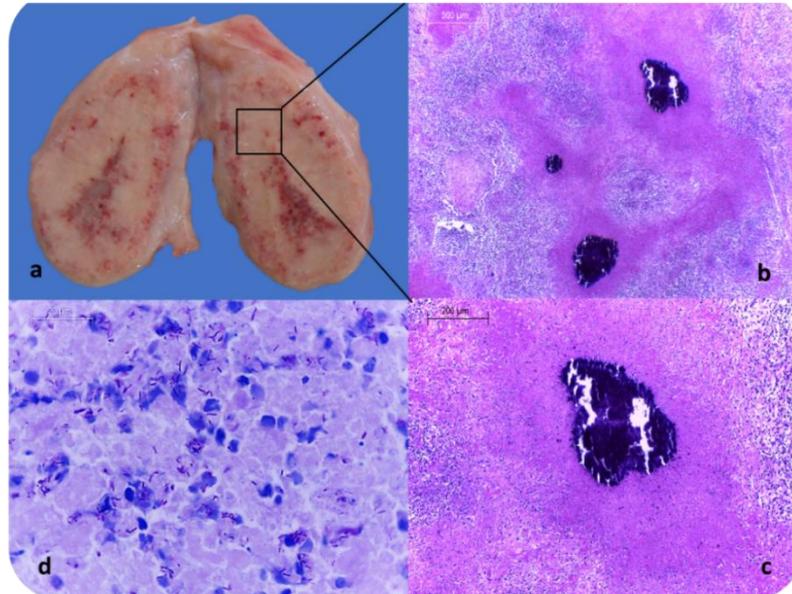
Figura 6: Linfadenitis granulomatosa en bovinos menores de cuatro meses de edad. (**A**) Nódulos linfáticos que al corte presenta extensas áreas de necrosis caseosa con petequias. (**B**) H&E, 40x de nódulo linfático que muestra extensas áreas de necrosis sin bordes definidos con calcificación, (**C**) [Aumento de sección c, H&E, 200x (**D**) Nódulo linfático con abundantes bacilos acido alcohol resistente extracelulares, Zielh Neelsen, 1000x. Imágenes obtenidas por Jacobo Carrisoza-Urbina.
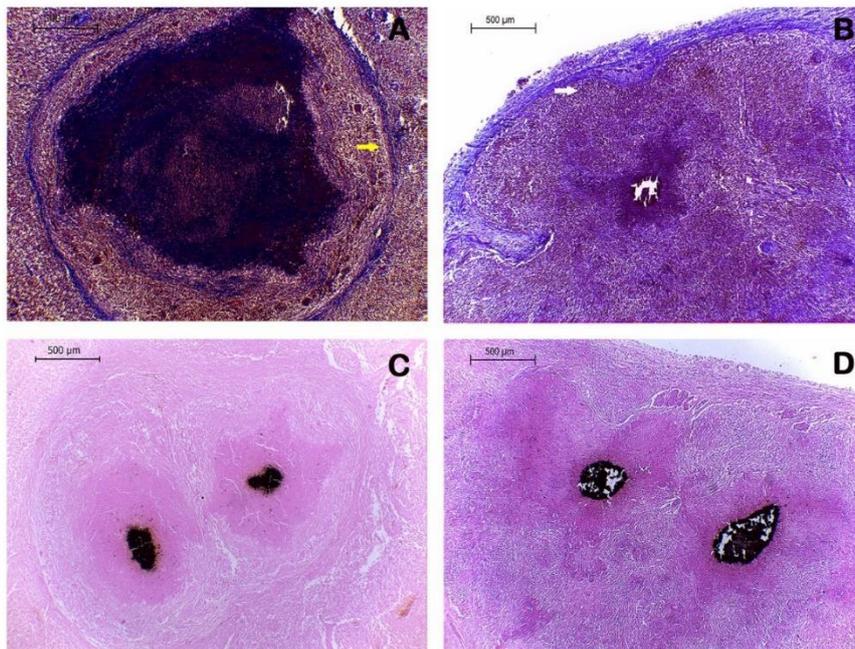
Figura 7: Granulomas en becerros infectados naturalmente por *M. bovis* sin cápsula de tejido conectivo. (A) Tricromica de Masson 40x granulomas estadio IV donde se observa la presencia de tejido conectivo señalado con flechas blancas. **(B)** Tricromica de Masson 40x Estadio III-IV en becerro sin presencia de cápsula de tejido conectivo **(C)** Tinción de Von Kossa 40x. Granuloma estadio IV con precipitados de calcio de color negro al centro de la lesión **(D)** Tinción de Von Kossa 40x de granuloma estadio III-IV en becerro con precipitados de calcio. Imágenes obtenidas por Jacobo Carrisoza-Urbina.

PLANTEAMIENTO DEL PROBLEMA

El granuloma es considerado la lesión característica de la tuberculosis que tiene como función aislar y controlar las micobacterias, así como restringir el daño tisular previniendo la inflamación crónica del tejido circundante (Domingo et al. 2014b). La estructura del granuloma ha sido asociada con la progresión o control de la enfermedad, los granulomas con poca necrosis bien delimitados por una cápsula de tejido conectivo se asocian con una menor cantidad de bacilos comparado con granulomas inadecuadamente formados como es el caso de personas coinfectadas con *M. tuberculosis* y VIH en los cuales las lesiones que presentan, muestran extensas áreas de necrosis, limitada encapsulación de tejido conectivo y una alta cantidad de bacilos (Diedrich et al. 2016). Por otra parte, en el modelo de ratón C3HeB/FeJ, una cepa susceptible a tuberculosis los animales infectados con *M. bovis* presentan lesiones con extensas áreas de necrosis, neutrófilos, una ineficiente cápsula de tejido conectivo y abundantes bacilos que llevan a la muerte de los animales a las 5 semanas post infección (Bouté et al. 2017).

Previamente hemos caracterizado macroscópica y microscópicamente granulomas de bovinos mayores de un año infectados naturalmente por *M. bovis* observando lesiones similares a las reportadas por Wangoo *et al* 2005. Por otra parte, se hemos observado que en animales menores de cuatro meses se presentaban granulomas "atípicos" los cuales presentan gran cantidad de bacilos extracelulares, necrosis y ausencia de una cápsula de tejido conectivo, lo cual sugiere que este grupo de animales es incapaz de controlar la infección por *M. bovis*. Sin embargo, se desconoce la respuesta inmune presente en las lesiones granulomatosas de estos animales. Por tal motivo, en el presente trabajo se pretende caracterizar

inmunológicamente los granulomas de bovinos naturalmente infectados por *M. bovis.* Este estudio ayudará a comprender mejor la patogénesis de la enfermedad. Además, el conocimiento generado, será útil para un mejor entendimiento de la resistencia natural del huésped a la infección por micobacterias.

HIPÓTESIS

Los granulomas procedentes de bovinos menores de cuatro meses naturalmente infectados por *M. bovis* que presentan mayor cantidad de; necrosis, bacilos ácido alcohol resistente y ausencia de cápsula de tejido conectivo tendrán una menor cantidad de células y citocinas de la respuesta inmune asociadas al control de la tuberculosis, en comparación con granulomas de bovinos mayores de un año.

OBJETIVO GENERAL

Caracterización *in situ* de la respuesta inmune en granulomas de bovinos mayores de un año y menores de cuatro meses, naturalmente infectados por *M. bovis.*

Objetivos específicos

- Realizar las necropsias y colectar muestras de tejidos de bovinos con lesiones sugestivas a tuberculosis.

- Identificar la presencia de *M bovis* mediante análisis bacteriológico y de biología molecular.

- Caracterizar histológicamente granulomas procedentes de bovinos naturalmente infectados con *M. bovis*, de acuerdo con Wangoo et al. (2005).

- Cuantificar el porcentaje de macrófagos, linfocitos T, linfocitos B y linfocitos gamma delta en granulomas de bovinos infectados naturalmente por *M. bovis* menores de cuatro meses y mayores de un año.

- Cuantificar el porcentaje de iNOS, TNF-α, IFN-γ y TGF- B en granulomas de bovinos infectados naturalmente por *M. bovis* menores de cuatro meses y mayores de un año.

- Cuantificar el número de bacilos presentes en los granulomas de bovinos naturalmente infectados por *M. bovis in situ* en bovinos menores de cuatro meses de edad.

- Analizar la posible asociación entre la población celular y concentración de mediadores inmunológicos con la estructura morfométrica de los granulomas y la cantidad de bacilos ácido alcohol resistentes.

MATERIAL Y MÉTODOS

Colección de muestras.

Se colectaron 36 muestras de tejido de una explotación de ganado bovino lechero de la raza Holstein Friesian la cual cuenta con aproximadamente 28,000 animales y con una prevalencia de la enfermedad mayor a 16% (Situación actual de Tuberculosis Bovina, Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria). Se obtuvieron muestras de tejido con lesiones sugestivas a tuberculosis al examen *post mortem*, en un muestreo por oportunidad de bovinos que fueron sacrificados. Posteriormente se seleccionaron bloques de parafina con diferentes secciones de tejidos de nódulos linfáticos mediastínicos de 15 bovinos mayores de un año (adultos) y 10 bovinos menores de cuatro meses de edad (jóvenes) de la raza Holstein Friesian. La **Tabla 1** muestra la edad y las causas de muerte de los animales incluidos en el estudio. Previamente, en estos tejidos se identificó a *M. bovis* mediante el aislamiento bacteriológico y por PCR en bloques de tejidos con parafina finalmente los granulomas identificados fueron caracterizados de acuerdo con wangoo et al. 2005 y se realizó una nueva clasificación de las lesiones identificada en el grupo de jóvenes (Carrisoza-Urbina et al. 2019).

Tabla 1.- Edad y causas de muerte en bovinos estudiados.

Groupo	Número de caso	Edad	Sexo	Causas de muerte
Adultos	1	4 Años	H	Infertilidad
	2	3 Años	H	Timpanismos gaseoso
	3	3 Años	H	Pericardits traumática
	4	3 Años	H	Fracura de tibia
	5	5 Años	H	Timpanismos gaseoso
	6	5 Años	H	Infertilidad
	7	3 Años	H	Desconocido
	8	5 Años	H	Timpanismos gaseoso
	9	3 Años	H	Infertilidad
	10	2 Años	H	Timpanismos gaseoso
	11	5 Años	H	Desconocido
	12	5 Años	H	Timpanismos gaseoso
	13	3 Años	H	Pericardits traumática
	14	5 Años	H	Desconocido
	15	1 Año	H	Timpanismos gaseoso crónico
Jóvenes	16	4 Meses	M	Neumonía
	17	8 Días	H	Insuficiencia cardiorrespiratoria.
	18	1 Mes	H	Diarrea y deshidratación
	19	1.5 Meses	H	Neumonía
	20	3 Meses	H	Neumonía
	21	3 Meses	H	Timpanismos gaseoso
	22	2 Meses	H	Peritonitis
	23	1 Mes	H	Insuficiencia respiratoria aguda
	24	2.5 Meses	H	Neumonía
	25	3.5 Meses	H	Insuficiencia respiratoria aguda

H: Hembra y M: Macho

Declaración de ética

Todos los procedimientos fueron revisados y aprobados por el Comité de Ética y Bienestar Animal de la Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México (CICUA, FMVZ-UNAM), y cumplieron con los lineamientos mexicanos para la investigación animal (JAGP-074).

Patología macroscópica

La técnica de necropsia consistió en inspeccionar el cadáver externa e internamente, disecando los órganos por sistemas. Se colectaron muestras de tejidos de nódulos linfáticos, tejido pulmonar y órganos que presentaron lesiones sugestivas a tuberculosis. Los tejidos con lesiones se seccionarán en cortes de 0.5-1 cm, los cuales fueron colocados en formol al 10%, posteriormente fueron embebidos en parafina para su procesamiento y análisis histopatológico. Para el aislamiento bacteriológico fueron colectados tejidos con lesiones sugestivas a tuberculosis y colocadas en borato de sodio sin cortar para disminuir la contaminación.

A los casos colectados, se les tomaron los siguientes datos: la ubicación de la explotación de origen, la reseña del animal (especie, raza, sexo, edad, etc.), identificación precisa del animal (arete, marcas u otros) y tipo de órganos colectados.

Análisis histopatológico

Las secciones de tejido con lesiones sugestivas a tuberculosis se fijaron en formol al 10% y posteriormente se incluyeron en parafina. Se realizaron cortes seriados de aproximadamente cuatro micras, posteriormente fueron teñidas con hematoxilina y eosina (H&E), tricrómica de Masson, Zhiel Neelsen y Von Kossa estas se examinaron con un microscopio óptico. Mediante la tinción de H&E se identificaron lesiones granulomatosas, las cuales se clasificaron de acuerdo con Wangoo et al (2005), en cuatro estadios. Estadio I (inicial), se observa agregados de macrófagos epitelioides, células gigantes de tipo Langhans con linfocitos intercalados sin presencia de necrosis, algunas veces presentan un infiltrado de neutrófilos. Estadio II (Solido), principalmente compuestas por macrófagos epitelioides, mayor cantidad de células

gigantes multinucleadas, linfocitos y mínima necrosis caseosa al centro de la lesión.

Estadio III (Necrosis mínima): Granuloma completamente encapsulados, con necrosis caseosa bien desarrollada y mínima mineralización. El Estadio IV: presenta necrosis y mineralización con una gruesa capsula de tejido conectivo, macrófagos epitelioides y células gigantes multinucleadas rodean la extensa área de necrosis; también se han observado linfocitos rodeando la lesión.

Las tinciones de tricromía se Masson y Von Kosaa se utilizaron para observar la presencia de tejido conectivo y calcificación respectivamente.

Inmunohistoquímica (IHQ).

El procedimiento de la técnica se resume en la tabla la **Tabla 2**. Brevemente, tejidos fijados en formalina y embebidos en parafina fueron cortados en secciones de 4-5 µm y colocados en laminillas electro cargadas. Se desparafinaron a 60°C por 30 min, se rehidrataron y colocaron en peróxido de hidrógeno al 3% por 15 min para eliminar la actividad de la peroxidasa endógena, posteriormente se sometieron a un proceso de desenmascaramiento de proteínas utilizando tanto métodos físicos como químicos de acuerdo con el anticuerpo primario a utilizar. Los tejidos fueron lavados con agua destilada y colocados en el sistema de inmunotinción Shandon Sequenza™, los lavados entre reactivos se realizaron con solución salina tamponada con fosfato 1x. (PBS: NaCl 138 mM, KCl 3 mM, Na2HPO4 8.1mM, KH2PO4 1.5mM ajustando el pH a 7.4) y solución salina tamponada con tris más tween (TBST: solución salina tamponada con tris de 0,005 mM, pH 7,6 con Tween 20 al 0,05 %). Posteriormente, se agregó el bloqueador de proteínas inespecíficas y se incubaron las muestras con el anticuerpo primario (la concentración y tiempo de incubación fue estandarizada para cada anticuerpo), después de lavar las muestras se colocó el anticuerpo

secundario biotinilado siguiendo las instrucciones del fabricante (ABC Peroxidase Standard Staining Kit), el revelado se realizó con 3,3-Diaminobencidina tetrahidrocloruro (DAB). La contratación se efectuó con hematoxilina de Mayer, finalmente las secciones fueron deshidratadas y montadas con resina.

Tabla 2. Anticuerpos para inmunohistoquímica en tejido de bovino.

Anticuerpo y dilución	Tipo de anticuerpo	Provedor	Tiempo de incubación de anticuerpo	Reactivo de recuperación de antígenos	Solución de lavado
MAC387 1/500	Monoclonal de ratónIgG1	Bio-Rad MAC387	12 h 4°C	Proteinasa K	PBS
CD79 1/50	Monoclonal de ratónIgG1	Dako HM57	12 h 4°C	Proteinasa K	PBS
CD3 1/50	Policlonal de conejoIgG	Biocare Medical SP7	12 h 4°C	Solución amortiguadora de citratos , pH 6.0,	TBST
Vimentina1/100	Policlonal de conejoIgG	Biocare Medical CRM 31:	45 min	Solución amortiguadora de citratos , pH 6.0,	TBST
Actina de musculo liso 1/100	Monoclonal de ratónIgG1	Biocare Medical SP9	45 min	Solución amortiguadora de citratos , pH 6.0,	TBST
Mycobacterium 1/100	Policlonal de conejo	Biocare Medical CP 140	12 h 4°C	Solución amortiguadora de citratos , pH 6.0,	TBST
TGF-β 1/100	Monoclonal de ratónIgG1	Gene Tex TB21	12 h 4°C	Solución amortiguadora de citratos , pH 6.0,	TBST
TNF-α 1/100	Monoclonal de ratónIgG1	Gene Tex CC327	12 h 4°C	Solución amortiguadora de citratos , pH 6.0,	TBST
iNOS 1/500	Policlonal de conejo	Millepore 06-573	45 min	Solución amortiguadora de citratos , pH 6.0,	TBST
INF-γ 1/100	Monoclonal de ratónIgG1	Gene Tex CC330	12 h 4°C	Solución amortiguadora de citratos , pH 6.0,	TBST
WC1 1/500	Monoclonal de ratónIgG1	Invitrogen, CC15	12 h 4°C	Solución amortiguadora de citratos , pH 6.0,	TBST

TBST = Solución salina tamponada con Tris. 0,005 mM, pH 7,6 con Tween 20 al 0,05 %.
PBS: Solución salina Amortiguada por Fosfatos. NaCl 138 mM, KCl 3 mM, Na2HPO4 8,1 mM, KH2PO4 1,5 mM, pH a 7,4.

Procesamiento de tejidos colectados y análisis de imagen digital en granulomas

Los portaobjetos inmunomarcados con diferentes anticuerpos se procesaron digitalmente con un microscopio de barrido (Aperio Scanscope CS, Aperio, CA, EE. UU.), generando imágenes de 40× con una resolución espacial de 0,45 µm/píxel. Las imágenes se analizaron con el software ImageScope (Aperio, CA, EE. UU.), los granulomas se delimitaron eliminando las áreas de necrosis compuestas por restos celulares y calcificación. Se utilizaron algoritmos que detectan y cuantifican la tinción marrón obtenida por IHQ. Esta metodología permitió la cuantificación de las proteínas marcadas en las diferentes pruebas de IHQ. La **Figura 8** resume los procedimientos experimentales utilizados en este estudio y la **Tabla 3** muestra los anticuerpos utilizados y la cantidad de granulomas analizados en cada grupo.

Tabla 3: Anticuerpos utilizados y número de granulomas analizados

Anticuerpo	Granulomas analizados	
	Adultos	Becerros
MAC387	72	146
CD3	105	155
CD79	103	215
WC1	78	202
SMA	88	229
Vimentina	106	226
TNF-α	96	210
INF-γ	108	224
TGF-β	114	201
iNOS	116	254
Anti-mycobacterium	91	300
Total	**1077**	**2362**
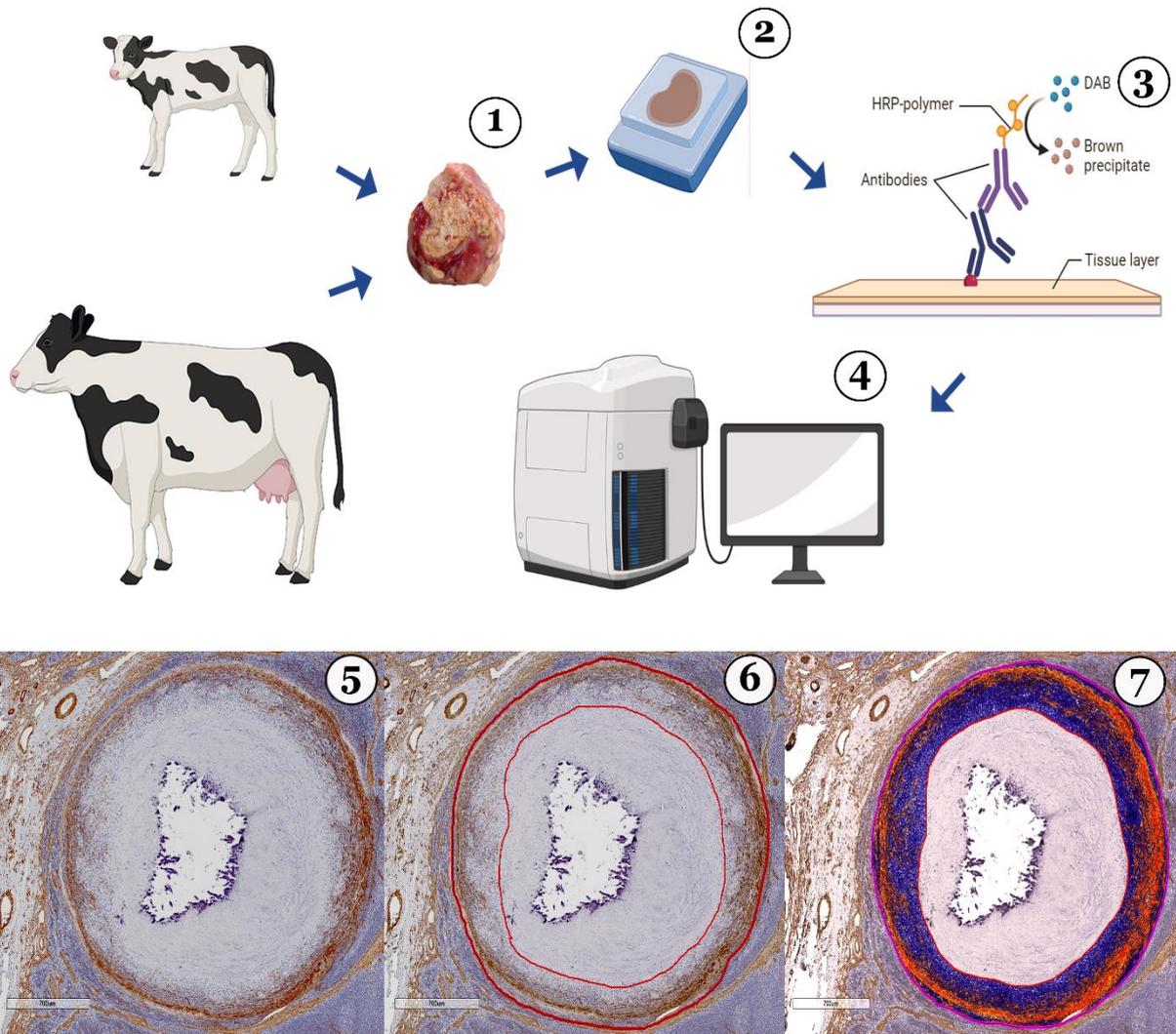
Figura 8: Diagrama de flujo de los procedimientos experimentales: Se incluyeron nódulos linfatico de bovinos de la raza Holstein-Friesian, naturalmente infectados por *M. bovis* [**1**], los tejidos se incluyeron en bloques de parafina [**2**], posteriormente se cortaron secciones seriadas de 4-5 μm e inmunomarcaron [**3**], a continuación se escanearon las laminillas con los tejido marcados, [**4**] se identificaron los granulomas en las imágenes digitalizadas utilizando el sistema de software ImageScope. Finalmente se cuantificó el número de píxeles positivos de cada inmunotinción [**5-7**]. (Creado con BioRender.com, consultado el 4 de septiembre de 2022)

Análisis estadístico

El análisis estadístico se realizó con el programa PASW Statistics 18 y GraphPad Prism 7.0. Los datos fueron analizados mediante la prueba de Shapiro-Wilk y gráficos de Q-Q Plot para verificar la normalidad de los datos. Las comparaciones de el marcaje obtenido en las IHQs de los granulomas de bovinos adultos y jóvenes se realizaron mediante la prueba no paramétrica de Mann-Whitney. Se consideraron diferencias significativas cuando fue menor o igual a $P< 0,05$.

RESULTADOS

Los granulomas de becerros infectados naturalmente por *M. bovis* presentan mayor cantidad de bacterias

Mediante la tinción de Ziehl-Neelsen observamos un mayor número de BAAR en granulomas identificados en nódulos linfáticos mediastínicos de becerros en comparación con bovinos adultos infectados de forma natural por *M. bovis*. Con la finalidad de validar este resultado y cuantificar las poblaciones celulares y citocinas. Utilizamos secciones de nódulos linfáticos colectados de 25 bovinos Holstein Friesian naturalmente infectados con *M. bovis*. En los cuales se analizaron un total de 3.439 granulomas, de los cuales el 31,3% (1077) correspondieron a bovinos adultos y el 68,6% (2362) a becerros. La identificación de micobacterias presentes en los granulomas fue realizada utilizando un anticuerpo policlonal contra *Mycobacterium tuberculosis*.

La IHQ nos ayudó a confirmar significativamente una mayor cantidad de bacteria en granulomas de becerros (**Figura 9A**). La mayor sensibilidad de esta técnica permitió

identificar la presencia de células con morfología bacilar, así como, restos celulares, los cuales se observaron en forma de vacuolas y gránulos citoplasmático, posiblemente asociados con el procesamiento y fagocitosis de las micobacterias por parte de los macrófagos. La mayoría de los granulomas de ambos grupos presentaron diferentes grados de tinción positiva a micobacterias en el citoplasma de: macrófagos, MΦ epitelioides y células gigantes multinucleadas (CGM) (**Figura 9B**). Los granulomas de bovinos adultos que presentaban centros necróticos con mineralización y rodeados por una cápsula de tejido conectivo frecuentemente no presentaban tinción positiva, en este tipo de lesiones la tinción se observó únicamente en las CGM (**Figura 9C**). En granulomas de becerros, la marca fue extracelular y predominantemente en áreas necróticas **(Figura 9D)**. El citoplasma de diferentes tipos de células que conforman el granuloma y las células que rodean la lesión presentaron diferentes grados de tinción en ambos grupos. El mayor número de bacterias observado en los granulomas de los becerros en comparación con los bovinos adultos puede estar relacionado con el tipo de respuesta inmune. Por lo tanto, el siguiente paso fue identificar las principales poblaciones celulares y citoquinas asociadas con la inmunopatología de la tuberculosis y que participan en la formación del granuloma.

Mycobacterium
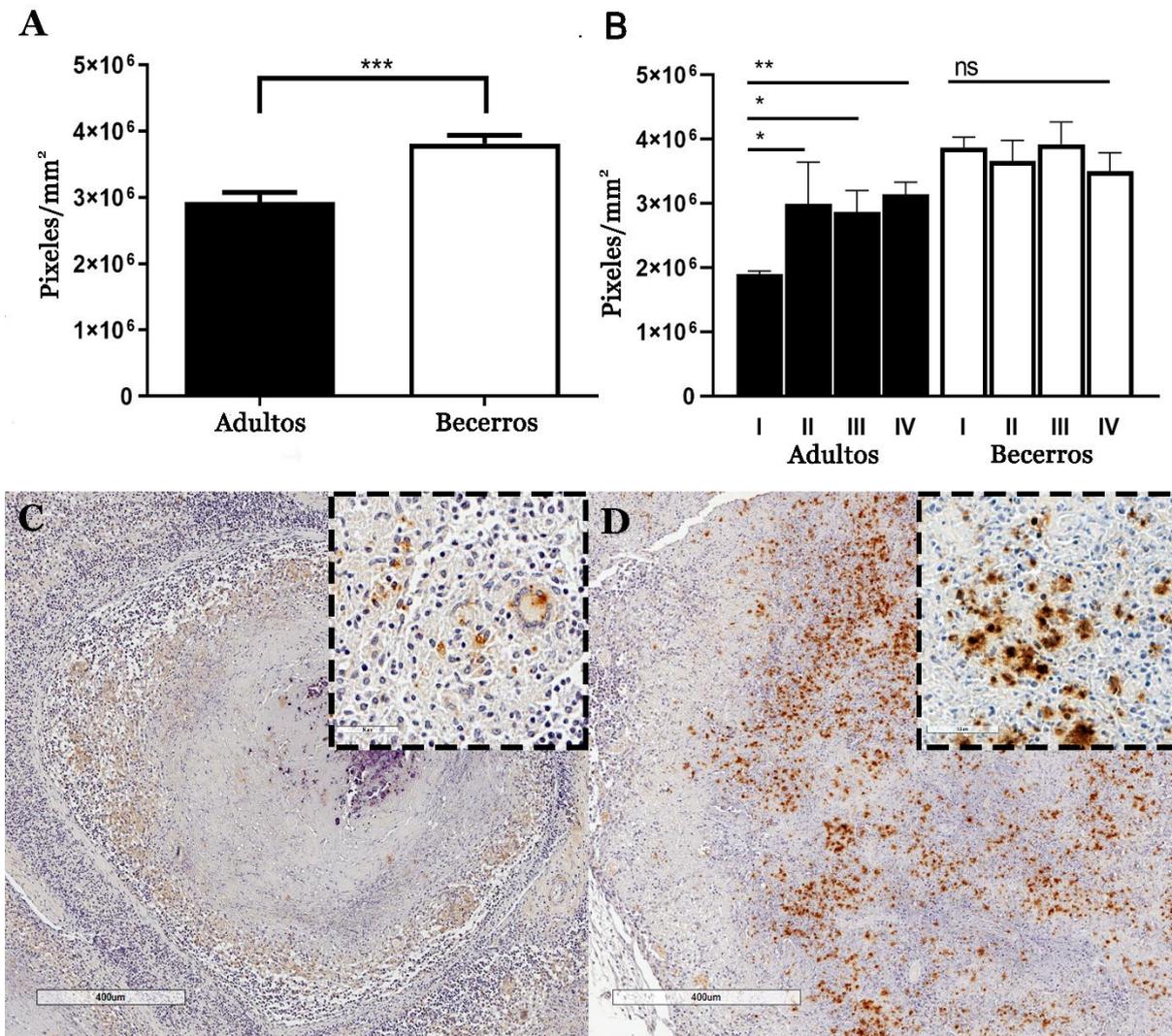
Figura 9: Los granulomas de becerros infectados naturalmente con *M. bovis* muestran un mayor número de micobacterias en comparación con bovinos adultos. A) Número promedio de píxeles positivos para la tinción IHQ de *M*ycobacterium en granulomas y estadios de bovinos adultos y jóvenes, respectivamente. **B**) Prueba de Mann-Whitney * *P* < 0.05, * * *P* < 0.01, y * * * *P* < 0.001. **C)** y **D**) Microfotografía de IHQ de *M*ycobacterium, 40×. **C)** Granuloma bovino adulto estadio IV con débiles áreas marrones positivas alrededor de la necrosis, primer plano que muestra células con morfología macrofágica y una célula gigante con marca citoplasmática. **D)** Granuloma de ternero que muestra una gran cantidad de tinción positiva en el área necrótica: el recinto muestra inmunomarcaje citoplasmático y extracelular.

Los granulomas de bovinos jóvenes carecen de una cápsula de tejido conectivo.

Uno de los principales hallazgos del análisis histopatológico fue la ausencia de cápsula de tejido conectivo en los granulomas de becerros naturalmente infectados por *M. bovis.* Se ha demostrado que los fibroblastos y miofibroblastos son las principales poblaciones celulares que forman las cápsulas de tejido conectivo en los granulomas causados por *M. bovis* (14). Con la finalidad de evidenciar la presencia de estas células en los granulomas, realizamos el marcaje de estas células, observando significativamente mayor cantidad de fibroblastos marcados con vimentina ($P < 0,001$) y miofibroblastos marcados con α-SMA ($P < 0,001$) en los granulomas de bovinos adultos en comparación con los becerros (**Figura 10**). La tinción de vimentina fue mayor en todos los estadios, observando principalmente en los fibroblastos que forman la cápsula de tejido conectivo alrededor de los granulomas de bovinos adultos, en comparación con los becerros, también se observó en MΦs epitelioides y CGMs, que se intercalaron en el área celular de los granulomas. La distribución de los fibroblastos positivos a vimentina en granulomas de becerros fue dispersa sin llegar a formar la cápsula de tejido conectivo (**Figuras 10A-10D**). La IHQ de α-SMA permitió identificar la presencia de miofibroblastos, los cuales se observan formaron un anillo alrededor de los estadios III y IV en los granulomas de bovinos adultos. Los estadios iniciales de ambos grupos mostraron α-SMA positivo intercalado con MΦ epitelioide y linfocitos, mostrando más células positivas en los granulomas de becerros en etapa I (**Figuras 10E-10F**). En los granulomas estadios III y IV de bovinos, se observan células positivas α-SMA tanto en la cápsula de tejido conectivo como intercaladas con el resto de las células que forman la lesión. En los granulomas

de becerros los miofibroblastos se distribuyeron irregularmente, sin formar una cápsula alrededor de la lesión (**Figuras 10G y 10H**).
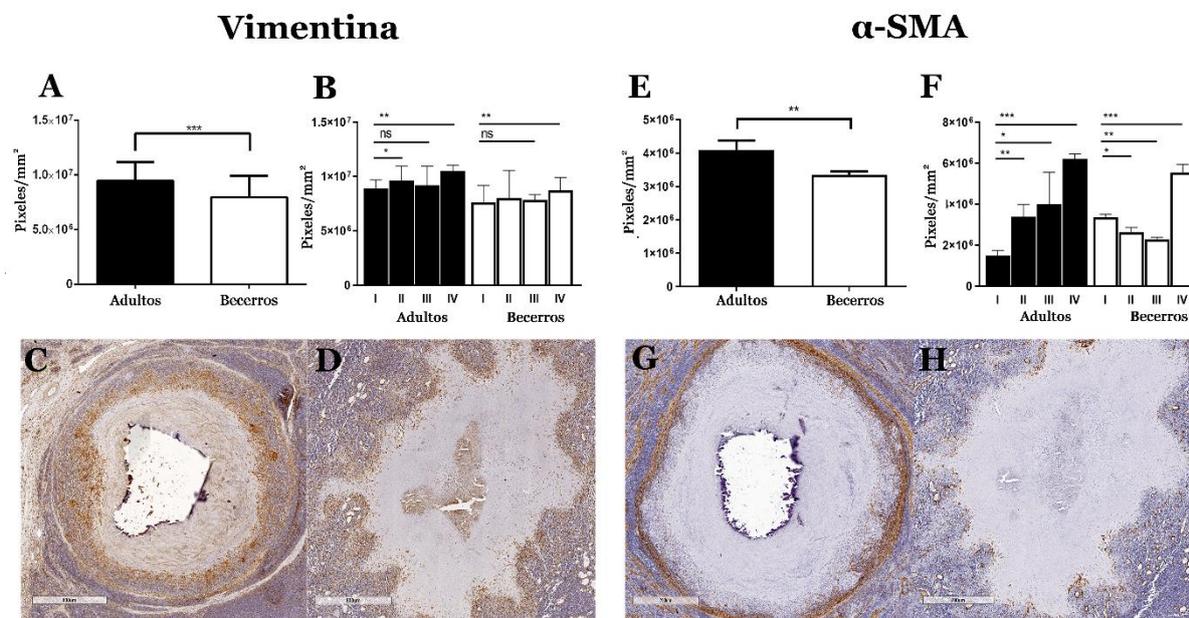
Figura 10: La ausencia de cápsula de tejido conectivo en granulomas de becerros se asocia con un menor número de fibroblastos y miofibroblastos. A-B y **E-F**) Se cuantificó la inmunotinción de vimentina y α-SMA en los granulomas y estadios en bovinos de adultos y becerros, prueba de Mann-Whitney *$P < 0.05$, **$P < 0.01$, y ***$P < 0.001$ respectivamente. **C** y **D**) IHQ de vimentina en granulomas estadios IV muestran tinción abundante, principalmente en la cápsula de tejido conectivo del granuloma de bovinos adulto en comparación con el granuloma de becerros respectivamente. **G** y **H**) IHQ de α-SMA en granulomas estadio IV muestran miofibroblastos formando la cápsula de tejido conectivo en granuloma de bovino adulto. No así, en el granuloma de becerro en el cual se muestra células positivas alrededor de la necrosis sin formar una cápsula de tejido conectivo.

Los granulomas de bovinos adultos y becerros naturalmente infectados por *M. bovis*, tienen una proporción celular diferente.

Los granulomas de los becerros presentaron mayor cantidad de bacterias y una ausencia de cápsula de tejido conectivo, con menor cantidad de fibroblastos y miofibroblastos. En conjunto, estos resultados sugieren una diferencia funcional en la respuesta inmune, la cual puede estar relacionada con el tipo de células que

constituyen los granulomas. Por lo que, mediante IHQs identificamos las principales poblaciones celulares que conforman el granuloma. Observando significativamente mayor cantidad de tinción de MAC387 (macrófagos/monocitos), WC1 (células T γδ), células CD79+ (linfocitos B) y menos células CD3+ (linfocitos T) en granulomas de bovinos adultos en comparación con los de becerros (**Figura 11**). Los granulomas de bovinos adultos mostraron mayor cantidad de MΦs, MΦs epitelioide y CGM, los cuales son más abundantes en los estadios I y II y disminuyendo en los estadios III y IV en ambos grupos, lo cual puede ser asociado con el cambio de poblaciones celulares que tienen los granulomas conforme su maduración (**Figuras 11A-11D**). En el caso de las células T γδ se observó que, en granulomas de bovinos adultos, el número de células positivas fue aumentando conforme aumenta el estadio, con gran cantidad de células positivas en los estadios III/IV. Mientras que en los becerros encontramos un pequeño número de estas células positivas en los diferentes estadios (**Figuras 11E-11H**). Los linfocitos B identificados mediante CD79+ se ubicaron entre el área celular de los estadios iniciales y alrededor de las lesiones que presentan calcificación y necrosis. También se observaron agregados multifocales o nichos de células CD79+ en algunos estadios avanzados de ambos grupos (**Figuras 11I-11L**). Finalmente, los linfocitos T identificados mediante CD3+ se intercalaron con los MΦs epitelioides en los estadios iniciales y posteriormente se observaron rodeando la necrosis en ambos grupos (**Figuras 11M-11P**).
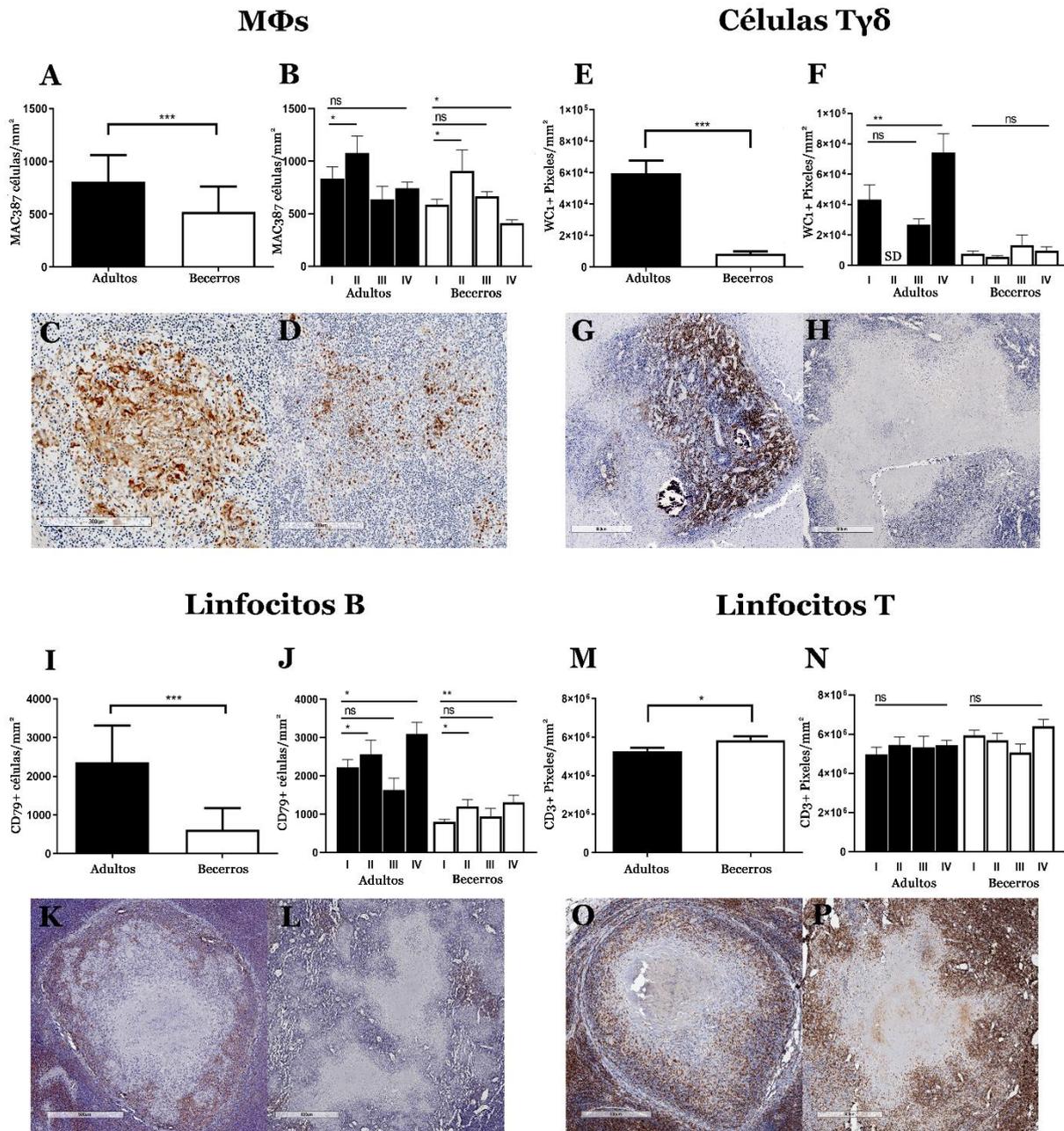
Figura 11: La proporción celular en granulomas de bovinos adultos y jóvenes infectados naturalmente con _M. bovis_ es diferente A-P) Expresión promedio e imágenes representativas de la inmunotinción de MAC387 (macrófagos), WC1 (células T γδ), CD79 (células B) y CD3 (células T) respectivamente, en los estadios y en el total de granulomas de bovinos adultos y becerros, prueba de Mann-Whitney *$P < 0,05$, ** $P < 0,01$ y *** $P < 0,001$. **C)**, 100x) Granuloma estadio II de adulto, muestra abundantes células MAC387+, incluidas algunas CGM. **D)**, 100x) Granuloma de becerro con células positivas alrededor de un área necrótica. **G)**, 40x) Granuloma estadio IV de adulto con abundante tinción de células WC1+ (células T γδ) en el centro de la lesión. **H)**, 40x) Granuloma de un becerro con escasas células WC1+. **K)**, 40x) Granuloma de adulto con células CD79+ rodeando la lesión. **L)**, 40x) Granuloma de becerro, con algunos agregados de células CD79+ alrededor del área necrótica. **O)** y **P)**, 40x) Células CD3+ rodeando la necrosis e intercaladas con el resto de las células que forman el granuloma en adultos y becerros, respectivamente. **ND,** no determinado (por falta de granulomas del adulto estadio II).

Los granulomas de becerros presentan mayor respuesta proinflamatoria en comparación con los adultos infectados naturalmente por *M. bovis.*

Las diferencias observadas en las poblaciones celulares de los granulomas de becerros y bovinos adultos naturalmente infectados sugieren una respuesta inmune diferente en ambos grupos. Para explorar más a fondo esta hipótesis, cuantificamos las citocinas y los mediadores inflamatorios asociados con la inmunopatología de la tuberculosis en humanos y bovina, como el interferón gamma (IFN-γ), la forma inducible del óxido nítrico sintasa (iNOS), el factor de crecimiento transformante-beta (TGF- β) y factor de necrosis tumoral α (TNF-α) (Flynn and Chan 2001; Pollock and Neill 2002; Palmer et al. 2022). Aunque estas citocinas proinflamatorias se han asociado con el control de la infección por micobacterias, observamos significativamente mayor cantidad de IFN-γ, iNOS y TNF-α y menor TGF-β en los granulomas de becerros en comparación con los granulomas de adultos. El mismo patrón en los diferentes estadios de los granulomas fue similar (**Figura 12**). La marca de interferón se observó principalmente de forma intracelular en los linfocitos que rodean las áreas de necrosis y alrededor de la cápsula de tejido conectivo. En ambos grupos, los estadios iniciales presentaron células IFN-γ+ mezcladas con MΦ epitelioides, mientras que los granulomas de estadio II y IV muestran tinción de IFN-γ en las áreas circundantes a la necrosis. Curiosamente, hubo una mayor cantidad de células IFN-γ+ en el granuloma estadio III de ganado adulto en comparación con los terneros (**Figuras 12A-12D**). Se detectó una mayor expresión de iNOS en granulomas y estadios de becerros en comparación con los adultos. La tinción se observó generalmente en el citoplasma de MΦs, CGMs epitelioides y las células

gigantes multinucleadas (**Figuras 12E-12H**). El marcaje de TNF-α se observó en el citoplasma de MΦs epitelioides, CGMs y en algunos fibroblastos que rodean los granulomas. También se identificó la marca extracelularmente, con mayor intensidad alrededor de secciones necróticas y menor intensidad en la periferia de la lesión (**Figuras 12I -12L**). Finalmente, TGF-β, una citocina antiinflamatoria se observó extracelularmente y en el citoplásmica de MΦ epitelioides y CGM de ambos grupos. La tinción de TGF-β fue muy intensa en fibroblastos que forman la cápsula de tejido conectivo en granulomas en etapa III-IV de bovinos adultos en comparación con becerros (**Figuras 12M-12P**). Estos resultados sugieren la existencia de un microambiente proinflamatorio exacerbado que provoca una incapacidad para controlar la infección por *M. bovis* en bovinos naturalmente infectados.
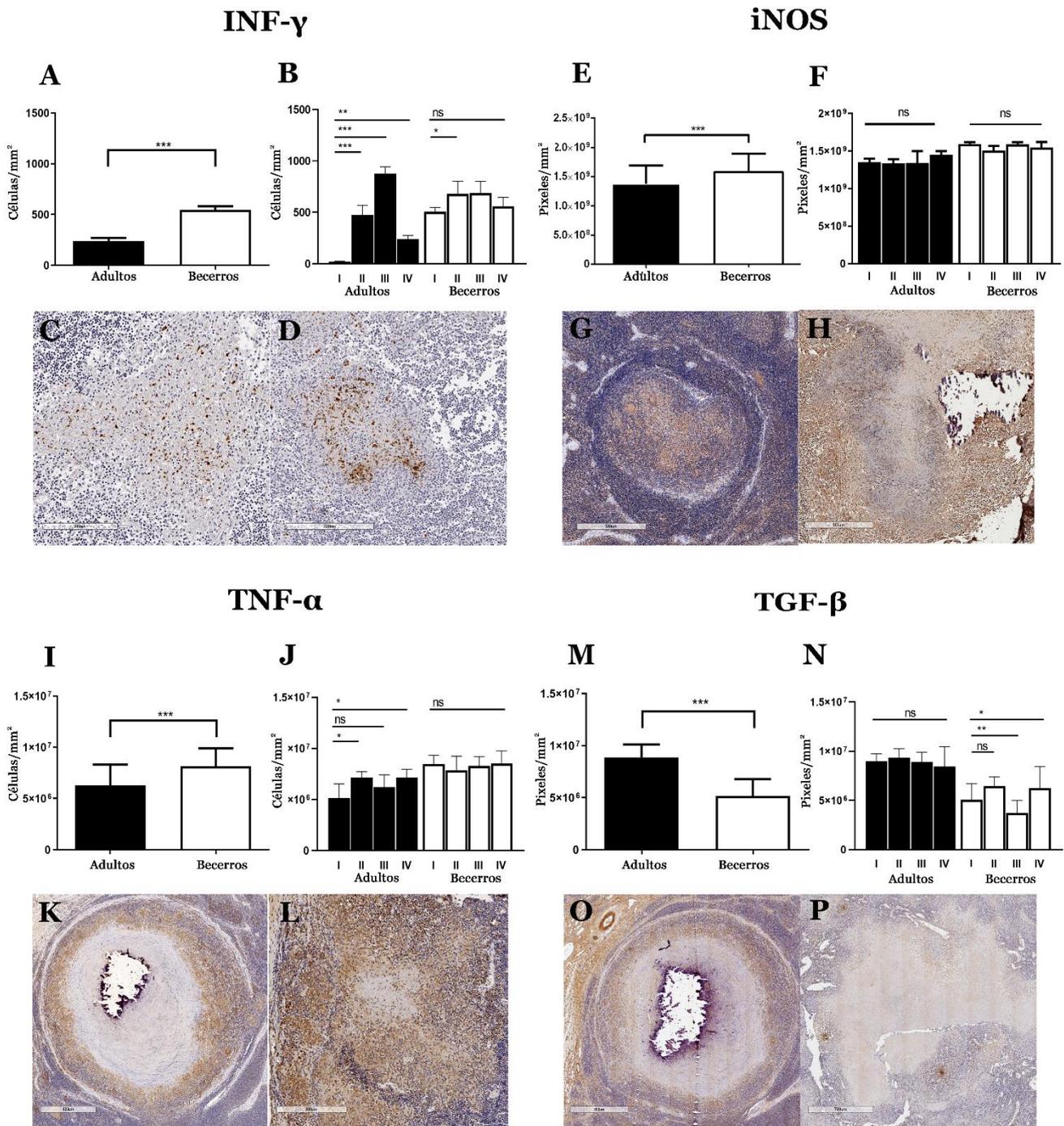
Figura 12: Los granulomas de becerros muestran una mayor respuesta proinflamatoria en comparación con adultos. **A-P**) Promedio de expresión e imágenes representativas de la inmunotinción para IFN-γ, la forma inducible del óxido nítrico sintasa (iNOS), factor de necrosis tumoral alfa (TNF-α) y factor de crecimiento transformante beta (TGF-β), respectivamente, en granulomas de adultos y becerros; Prueba de Mann-Whitney *$P < 0,05$, **$P < 0,01$ y ***$P < 0,001$. **C-D**, 100x) Granulomas iniciales que muestran células IFN-γ+ con morfología linfocitaria en el centro de la lesión. (**G-H**, 40x) Tinción de iNOS alrededor de necrosis, células positivas con morfología de macrófagos y CGM con diferentes intensidades de tinción en adultos y becerros, respectivamente. (**K**, 40×; **L**, 100×) Las células TNF-α+ y la tinción extracelular alrededor de la necrosis, las células positivas tienen una morfología MΦs epitelioide y CGM muestran una intensidad de tinción diferente en adultos y becerros, respectivamente. (**O-P**, 40x) Granulomas marcados con TGF-β, se aprecia la marca

extracelularmente y en el citoplasma de MΦ epitelioides, CGM y en fibroblastos (**D1-D2**, 40x) La tinción de TGF-β se observa extracelularmente en el citoplasma; de MΦ epitelioides, algunas CGM y principalmente en células de fibroblastos.

Presencia de fibrina en granulomas de bovinos naturalmente infectados por *M. bovis.*

La gran cantidad de TNF-α identificado en los granulomas de becerros y la presencia material homogéneo con características de fibrina nos sugirió la presencia de fibrina en estas lesiones. Por lo que, mediante la tinción de hematoxilina ácida fosfotúngstica se identificó la presencia de fibras de fibrina con un tono morado intenso en los granulomas de bovinos adultos y jóvenes. Apreciando gran cantidad de esta marca e las lesiones de bovinos jóvenes en el centro y la periferia de las lesiones (**Figura 14 y 15**). Este resultado puede ser asociado con la mayor cantidad de TNF-α observado en los granulomas de becerros.
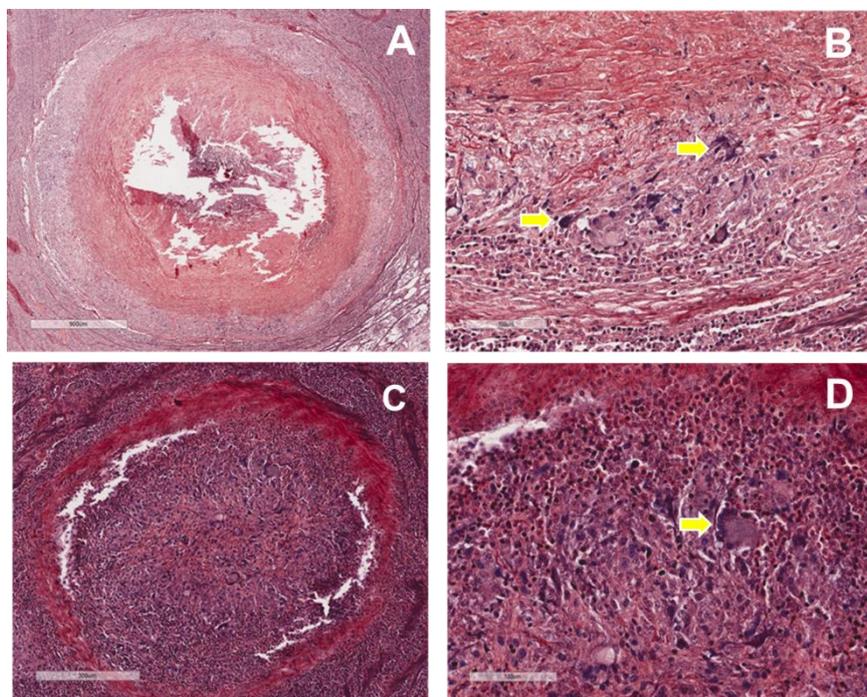
Figura 14: Tinción de hematoxilina ácida fosfotúngstica en granulomas de bovinos mayores de un año. (**A.**- 500x, **B**.-100x, **C**.-200x y **D**.-100x) **A**) Estadio IV y **B**) aumento de la lesión en **A, C**) Estadio II y

D) aumento de la lesión en **C**. Las flechas señalan la presencia de fibrina de color azul obscuro en granulomas de bovinos adultos.
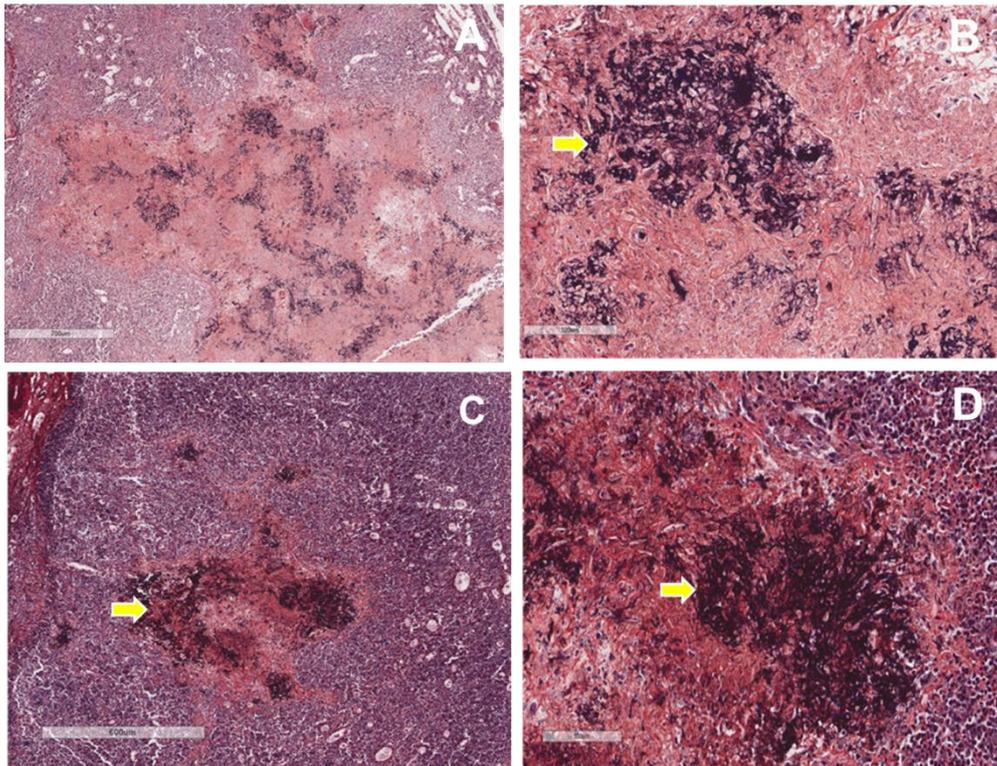
Figura 15: Tinción de Hematoxilina ácida fosfotúngstica en granulomas de bovinos menores de cuatro meses. (**A**.- 700x, **B**.-100x, **C.-**600x y **D**.-100x) A) Estadio IV y **B**) aumento de la lesión en **A, C**) Estadio II y **D**) aumento de la lesión en **C.** Las flechas señalan la presencia de una gran cantidad de fibrina de color azul obscuro en granulomas de bovinos jóvenes.

DISCUSIÓN

El granuloma es la lesión característica de la tuberculosis bovina que afecta principalmente a los ganglios linfáticos y al tejido pulmonar de los bovinos. El desarrollo de esta estructura depende de la respuesta inmune de la huésped asociada, al tipo de población celular, citocinas, quimiocinas y activación celular, así como la estimulación crónica provocada por la virulenta micobacteria. Estos y otros factores determinan el desarrollo, la morfología y el destino de cada lesión. La respuesta inmune y las características morfológicas de los granulomas de *M. bovis* han sido estudiadas principalmente en bovinos mayores de 6 meses de edad (Palmer et al. 2022). Sin embargo, al igual que en los humanos, existe muy poca información sobre la inmunología de la tuberculosis en animales jóvenes, la cual es fundamental comprender para mejorar el diseño de vacunas y pruebas diagnósticas, ya que se ha demostrado que bovinos menores de seis meses de edad responden diferente a la prueba de tuberculina, aumentando la cantidad de falsos negativos, lo cual puede estar asociada al tipo de respuesta inmune en estos animales (Ramos et al. 2020).

Debido a las características observadas en los granulomas de bovinos menores de cuatro meses, naturalmente infectados por *M. bovis*, este proyecto se enfocó en realizar una comparación histopatológica y bacteriológica entre el grupo de bovinos adultos y jóvenes. Identificando un mayor número de lesiones macroscópicas y microscópicas en los nódulos linfáticos mediastínicos y pulmón de bovinos infectados por *M. bovis*, lo que sugiere a la vía aérea como ruta de ingreso de las micobacterias en la tuberculosis bovina. Microscópicamente los estadios tipo IV, se observaron principalmente en animales mayores de un año infectados naturalmente por *M. bovis,*

lo que ilustra la cronicidad de la enfermedad. Así mismo, se observó que bovinos menores de cuatro meses infectados naturalmente por *M. bovis* presentan mayor cantidad de bacilos acido alcoholes resistentes comparado con los bovinos mayores de un año. Lo cual sugiere que el tipo de respuesta inmune en la infección por *M. bovis* será diferente en bovinos menores de cuatro meses y mayores de un año (Carrisoza-Urbina et al. 2019).

Con la finalidad de identificar las principales poblaciones celulares, citocinas, quimiocinas de la respuesta inmune asociadas a la patogenia de la tuberculosis bovina y la cantidad de bacterias que se encuentran en estas lesiones, utilizamos IHQ y análisis de patología digital. Confirmando significativamente ($P < 0.001$) mayor cantidad de micobacterias en granulomas de bovinos jóvenes naturalmente infectados, las cuales se encuentran extracelularmente y en áreas necróticas, así como en el citoplasma de MΦs epitelioides y CGM. Este resultado difiere con lo reportado en bovinos experimentalmente infectados con cepas virulentas de *M. bovis*, donde el número de BAARs es bajo y se encuentran principalmente en el citoplasma de células gigantes multinucleadas (Palmer et al. 2007; Palmer et al. 2022). Sin embargo, nosotros obtuvimos resultados similares en granulomas de bovinos adultos, en los cuales identificamos las bacterias principalmente en el citoplasma de MΦ epitelioides y CGM. En algunas lesiones de bovino adulto no se detectó tinción positiva, especialmente en granulomas estadio III y IV. Ha sido demostrado que este tipo de lesiones crónicas son capaces de matar las micobacterias en infecciones latentes con *M. tuberculosis* en monos (Lin et al. 2013a). Una limitación de la IHQ utilizada en este estudio fue el uso de anticuerpos policlonales porque la tinción no solo detectó la morfología de los bacilos sino también los restos celulares teñidos que se observaron

en forma de vacuolas y polvo citoplasmático, posiblemente asociados con restos celulares debido al procesamiento y fagocitosis de micobacterias. Curiosamente, también se observó inmunomarcaje en células fuera del granuloma en ambos grupos. Este hallazgo se ha señalado en estudios previos, sugiriendo la presencia de micobacterias fuera de la lesión sugiriendo que pueden ser restos de bacterias fagocitadas (Gutiérrez Cancela and García Marín 1993; Mustafa et al. 2006; Ashouri and Nourani 2022).

La presencia de una cápsula fibrótica alrededor de granulomas crónicos es característico de la tuberculosis bovina. Esta cápsula está compuesta principalmente por colágeno tipo I, producido por fibroblastos y miofibroblastos. Mediante la tinción tricrómica de Masson confirmamos la presencia de fibras de colágeno formando la cápsula de tejido conectivo en los granulomas de bovinos adultos (Wangoo et al. 2005; Johnson et al. 2006a). Sin embargo, no fue posible su detección en los granulomas de los becerros. Para confirmar si las lesiones de los bovinos jóvenes carecían de fibroblastos y miofibroblastos, identificamos estas poblaciones celulares mediante IHQ de vimentina y α-SMA respectivamente. Detectando significativamente mayor marca en los granulomas de bovinos adultos comparados con becerros. Sin embargo, el granuloma estadio I de becerros presenta mayor cantidad de miofibroblastos, lo cual sugiere una mayor quimiotaxis de estas células al inicio de la infección. No obstante, en los granulomas estadios III y IV de becerros los fibroblastos y miofibroblastos aparecían desorganizados sin formar una cápsula alrededor de la lesión. La capsula de tejido conectivo alrededor de los granulomas se ha correlacionado con la presencia de TGF-β y la diferenciación de fibroblastos en miofibroblastos productores de colágeno (Ameglio et al. 2005; Menin et al. 2013). Por lo

que, mediante IHC identificamos TGF-β en los granulomas de bovino, observamos significativamente ($p < 0.001$), menor cantidad de esta citocina en granulomas de becerros en comparación con bovinos adultos. Esta citocina se ha asociado con el desarrollo de fibrosis en granulomas de bovinos infectados por *M. bovis* (Wangoo et al. 2005). La función y como se lleva a cabo la formación de la cápsula de tejido conectivo que rodea los granulomas causados por *M. bovis* es poco conocido. Algunos estudios han asociado su presencia en los granulomas con; la cronicidad de la lesión, mejor control bacteriano, limitar el daño tisular del tejido cercano a la lesión y crear un ambiente de hipoxia que puede llegar a inducir la latencia de la infección (Gil et al. 2010; Lin et al. 2013b; Menin et al. 2013; Shkurupiy et al. 2014). En la tuberculosis bovina, la cápsula de tejido conectivo es característica de los granulomas estadio III y IV compuestos principalmente por colágeno tipo I producido por fibroblastos. En bovinos naturalmente infectados, el grosor de la cápsula de tejido conjuntivo ha sido asociada con un menor número de bacterias en los granulomas. La ausencia de cápsulas en los granulomas de terneros que tienen un alto número de bacterias sugiere el papel protector de la cápsula en el ganado naturalmente infectado con *M. bovis.* Por otra parte, no está claro qué factores determinan la formación y el depósito de tejido fibroso en la parte externa de las lesiones. Estudios recientes sugieren que los fibroblastos que forman la cápsula de tejido conectivo pueden ser macrófagos que atraviesan por el fenómeno de transición macrófagos-miofibroblastos (Evans et al. 2020). En este estudio se observó tinción en el citoplásmico y en la membrana de los fibroblastos que formaron el tejido conectivo de la cápsula con el anticuerpo de MAC387, que identifica la proteína MRP14 expresada en monocitos y macrófagos recientemente infiltrados en inflamaciones agudas (Odink et al. 1987; Soulas et al. 2011). Lo cual sugiere

la posibilidad de una transición de macrófagos-miofibroblastos en los granulomas de los bovinos naturalmente infectados.

Se han observado granulomas con mayor carga bacteriana y ausencia de fibroblastos rodeando las lesiones, en monos y humanos que presentan una tuberculosis activa. Este tipo de tuberculosis presenta mayor producción de citoquinas proinflamatorias (Lin et al. 2009; Lin et al. 2013a). Nosotros, observamos significativamente mayor cantidad de INF-γ, TNF-α e iNOS y menos TGF-β en granulomas de becerros en comparación con bovinos adultos, lo que sugiere una mayor respuesta proinflamatoria en los granulomas de becerros. La mayor cantidad de INF-γ puede estar correlacionada con la cantidad de células T CD3+ en respuesta a antígenos micobacterianos observados en granulomas de bovinos jóvenes en comparación con adultos. Se han demostrado una alta respuesta de IFN-γ en sangre periférica en becerros, después de un mes de la infección por *M. bovis,* así como la producción de TNF-α en becerros vacunados con BCG (Nonnecke et al. 2005; Stabel et al. 2021). Estas dos citoquinas son esenciales para activar los mecanismos anti micobacterianos e inducir intermediarios reactivos de nitrógeno por macrófagos activados que juegan un papel crucial en la muerte intracelular de micobacterias. Sin embargo, a pesar de observar una mayor producción de iNOS en los granulomas de los becerros, estos presentaron mayor cantidad de micobacterias en comparación con el ganado adulto. La actividad citotóxica inducida por altas concentraciones de iNOS podría explicar también la menor cantidad de MΦ identificados en los granulomas de becerros (Vanini et al. 2015). Por otra parte, la mayor cantidad de citocinas proinflamatorias, no se relaciona con la cantidad de MΦ presentes en las lesiones de bovinos jóvenes. Una posible explicación es que, los MΦ identificados en los granulomas, provienen principalmente

de la circulación sanguínea. Se ha demostrado que, este tipo de células identificadas con MAC387 son más proinflamatorias, de vida corta y metabólicamente obtienen energía de la glucólisis en comparación con las MΦ del tejido residente (Verrecchia and Mauviel 2007). Este hallazgo se relaciona con la mayor presencia de células MAC387+ identificadas en el tejido sin lesión que rodea los granulomas de becerros en comparación con los bovinos adultos, sugiriendo que los macrófagos y monocitos detectados provienen principalmente del torrente sanguíneo (Odink et al. 1987; Soulas et al. 2011). Sugiriendo que los granulomas de becerros tienen una respuesta más proinflamatoria, este tipo de microambiente se asocia con menor producción de TGF-β, y una disminución en la cantidad de tejido conectivo que rodea los granulomas (Wangoo et al. 2005). Los granulomas son estructuras dinámicas, con una organización espacial que muestra un centro proinflamatorio que puede presentar necrosis y una periferia de células que muestran un perfil proinflamatorio (Mattila et al. 2013; Marakalala et al. 2016). En este estudio podemos inferir que los granulomas de bovinos jóvenes infectados con *M. bovis* tienen una respuesta antiinflamatoria disminuida, por lo que carecen de una adecuada encapsulación de tejido fibroso.

El papel de las células B y las células T γδ en la patogenia de la tuberculosis bovina no se comprende bien. Sin embargo, la presencia de células B en los granulomas se ha asociado con un mejor control de la infección, observándose un mayor número en los granulomas que presentan menos bacterias (Johnson et al. 2006a). En este estudio se observaron linfocitos CD79+ en los diferentes estadios de los granulomas intercalados con el resto de las células y alrededor de la lesión. También formando agregados multifocales de células en algunos estadios avanzados conocidos como "nichos de células". Observando significativamente mayor cantidad de células CD79+

en granulomas de bovino adulto en comparación con jóvenes, que presentaban un mayor número de bacterias. Este resultado respalda el hallazgo de que los granulomas con un mayor número de linfocitos B presentan menor número de micobacterias. Por el contrario, las células T γδ han desempeñado un papel fundamental en la conexión de la inmunidad innata y adaptativa en la respuesta a *M. bovis*. En la circulación sanguínea periférica, las células T γδ representan hasta el 70% de los linfocitos en animales jóvenes y disminuyen a un promedio de 10% a 20% en los bovinos adultos (McGill et al. 2014). El mayor porcentaje de células T, en becerros, sugiere una importante participación en el sistema inmunológico. En los granulomas, las células T γδ se identifican como las primeras en llegar a los sitios de infección; las cuales se han observado entre 7 y 15 días después de la infección experimental con *M. bovis,* sugiriendo su participación en la formación de los granulomas. Asimismo, se ha reportado una correlación de número de células con el estadio del granuloma, lo que se pudo observar en este estudio, donde el número de células T γδ fue aumentando en los granulomas de bovinos adultos en estadios III/IV (Cassidy et al. 2001; Palmer et al. 2019). Sorprendentemente, aunque estas células se encuentran en mayor cantidad en la circulación sanguínea de los becerros, encontramos un pequeño número en los granulomas. Una posible explicación de este resultado es que las células T γδ en bovinos jóvenes permanecen principalmente en el torrente sanguíneo y están presentes en menor medida en el intersticio de los tejidos. Estas observaciones destacan la participación de las células T γδ en la patogenia de la tuberculosis en animales jóvenes.

Las limitantes de este estudio es que desconocemos el grado de virulencia bacteriana, la vía de infección, la dosis bacteriana y el tiempo de infección, lo que

puede correlacionarse con el tipo de lesión observada. Se reconoce que la patología de la tuberculosis bovina es multifactorial. Este estudio se enfatiza la edad de los animales, como un factor importante en el tipo de respuesta inmune y formación de granulomas en bovinos naturalmente infectados con *M. bovis*. En resumen, se observó que los granulomas de los becerros presentan una mayor cantidad de bacterias, ausencia de cápsula de tejido conectivo asociado con a una menor cantidad de fibroblastos y miofibroblastos desorganizados, un predominio de citoquinas proinflamatorias (INF-γ, TNF-α e iNOS), y menor cantidad de MΦs epitelioides, CGM, células T γδ, linfocitos B y TGF-β, en comparación con granulomas de bovino adulto (**Figura 16**). Nuestros resultados sugieren que los becerros presentan una tuberculosis de tipo activo con una respuesta proinflamatoria exacerbada asociada con la mayor cantidad de necrosis observada y una menor capacidad microbicida, provocando que sean más permisivos a la infección y diseminación de micobacterias. La información de este estudio destaca la importancia de una mejor comprensión de la respuesta inmune y la patogénesis de la tuberculosis bovina en animales jóvenes.
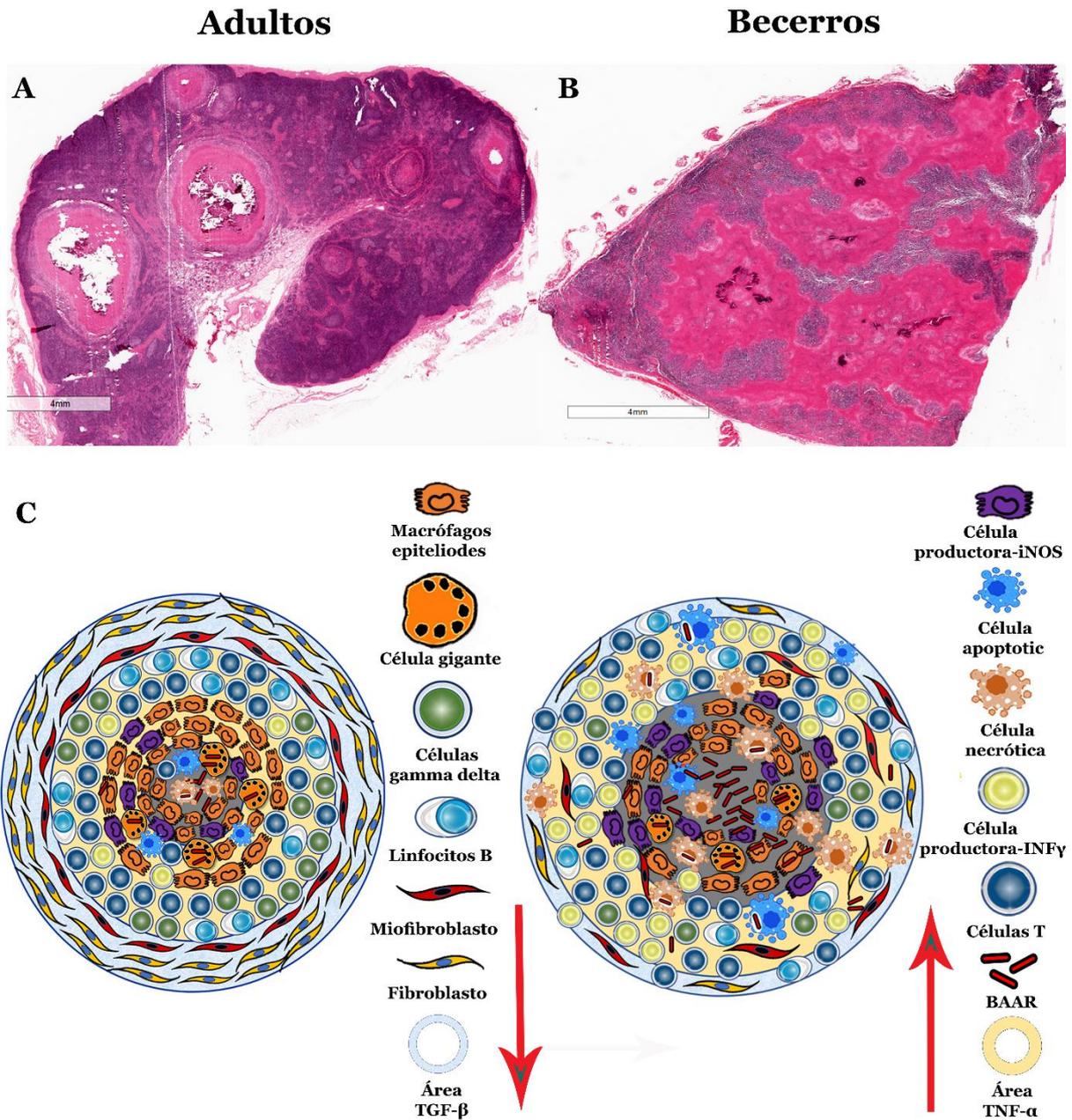
Figura 16: Esquema de las diferencias observadas en granulomas de bovinos naturalmente infectados por *M. bovis* en adultos y jóvenes.

(**A-B,** 5x) Secciones de nódulos linfáticos con granulomas causados por *M. bovis* **A**) Nódulo linfático de bovino adulto con granulomas estadios II y IV. **B**) Nódulo linfático de becerro, mostrando lesiones granulomatosas con gran cantidad de necrosis y ausencia de cápsula de tejido conjuntivo. **C**) Esquema que resume las diferencias identificadas en los granulomas, mostrando menor cantidad de CGM, MΦ epitelioides, células T γδ, células B, fibroblastos, TGF-β y mayor cantidad de iNOS, necrosis, células T, células IFN-α+, TNF-α y micobacterias en granulomas de becerros en comparación con bovinos adultos.

CONCLUSIONES

- Se demostró que los granulomas de becerros naturalmente infectados por *M. bovis*, presentan mayor cantidad de bacterias en comparación con los granulomas de bovino adulto.

- Los granulomas de becerros naturalmente infectados carecen de una cápsula de tejido conectivo, la cual se asocia con una menor cantidad de fibroblastos y miofibroblastos en comparación con bovinos adultos

- Granulomas de becerros, muestran significativamente mayor cantidad de citoquinas INF-γ, TNF-α e iNOS y menor cantidad de TGF- β

- Granulomas de becerros naturalmente infectados por *M. bovis* significativamente tienen menor cantidad de macrófagos, CGM, células T γδ, linfocitos B en comparación con granulomas de bovino adulto

PERSPECTIVAS

- Realizar el análisis morfológico e inmunológico en un mayor número de bovinos adultos y jóvenes naturalmente infectados por *M. bovis.*

- Analizar la virulencia de las cepas aisladas en este estudio, con el objetivo de saber si existe una relación con las características de las lesiones identificadas.

- Realizar ensayos de infección *in vitro* con células de bovinos adultos y jóvenes infectados, cuantificar la producción de citocinas proinflamatorias. Con el objetivo de verificar si los resultados obtenidos son similares a los obtenidos en este estudio.

- Identificar la producción de inmunoglobulinas asociadas a los nichos celulares de linfocitos B en los granulomas de bovinos.

- Cuantificar la cantidad de células gamma delta en nódulos linfáticos de becerros y bovinos adultos sanos. Con el objetivo de verificar si existe una relación con la edad de los animales.
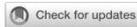
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Mycobacterium bovis naturally infected calves present a higher bacterial load and proinflammatory response than adult cattle

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Granulomas are characteristic bovine tuberculosis lesions; studying this structure
has improved our understanding of tuberculosis pathogenesis. However, the
immune response that develops in granulomas of young cattle naturally infected
with *Mycobacterium bovis* (*M. bovis*) has not been fully studied. Our previous work
described an atypical pattern in granulomatous lesions of cattle younger than 4
months (calves) naturally infected previously *M. bovis* that did not correspond
to the histological classification previously proposed. Histologically, granulomas
from calves lack a connective tissue capsule and have fewer multinucleated giant
cells (MGCs) and more acid-fast bacilli (AFB) than the classic tuberculosis lesions
found in cattle older than 1 year (adults); this suggests a deficient immune response
against *M. bovis* infection in young animals. Therefore, we used IHC and digital
pathology analysis to characterize the *in situ* immune response of granulomas
from young and adult cattle. The immunolabeling quantification showed that
granulomas from calves had more mycobacteria, CD3+ cells, IFN-γ, TNF-α, and
inducible nitric oxide synthase (iNOS) than those of adult cattle. Furthermore,
calf granulomas showed lower immunolabeling of MAC387+, CD79+, and WC1+
cells without connective tissue surrounding the lesion and were associated with
less vimentin, Alpha Smooth Muscle Actin (α-SMA), and TGF-β compared with
granulomas from adult cattle. Our results suggest that the immune responses in
granulomas of cattle naturally infected with *M. bovis* may be age dependent. This
implies that an exacerbated proinflammatory response may be associated with
active tuberculosis, producing more necrosis and a lower microbicidal capacity
in the granulomas of calves naturally infected with *M. bovis*.

KEYWORDS

atypical granulomas, *Mycobacterium bovis*, natural-infection, calcification, bovine
tuberculosis, calves immune-response, proinflammatory response

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1. Introduction

Bovine tuberculosis caused by *Mycobacterium bovis* affects different mammals, including humans. In the livestock industry, *M. bovis* causes losses of approximately 3 billion dollars per year (1, 2). This disease mainly affects cattle's lymph nodes and lungs, where granulomas are formed. These structures isolate and control mycobacteria and restrict tissue damage by preventing chronic inflammation of the surrounding tissue (3). Granuloma formation depends on the recruitment and activation of the cellular immune response and the persistence of the mycobacterial antigenic stimulus. These factors will determine the histology and development of the lesion (4). The structure of the granuloma has been associated with the progression or control of the disease; granulomas with little necrosis and well delimited by cellular and connective tissue capsules are associated with a lower bacillus number than inadequately formed granulomas with extensive necrosis and limited encapsulation. The latter type of lesion has been reported in humans and experimental monkeys with active tuberculosis as well as in individuals co-infected by *Mycobacterium tuberculosis*/HIV (5–7).

We have previously characterized granulomas of cattle naturally infected with *M. bovis* and older than 1 year, observing lesions comparable to those reported by Wangoo et al. (8). In contrast, bovines younger than 4 months presented "atypical" granulomas with many bacilli, necrosis, and absence of a connective tissue capsule, suggesting that this group of calves developed a response that was unable to form a granuloma to control the infection (9). Nonetheless, the immune response present in the granulomas of these animals is unknown. To better understand the immune response of granulomas induced by *M. bovis* at the cellular and molecular levels, we used immunohistochemistry (IHC) and digital pathology analysis to characterize granulomas from cattle older than 1 year and calves younger than 4 months. Our results suggest that calf granulomas present more mycobacteria, a greater proinflammatory response, and a lack of connective tissue capsule compared to the granulomas from adults.

2. Materials and methods

2.1. Sample collection

Mediastinal lymph node samples were collected from 25 naturally infected cattle, 15 were adult Holstein-Friesian dairy cows between one to 5 years of age, and 10 corresponded to calves between 1 week to 4 months of age. These tissues were collected with owner consent, from cattle that exhibited lesions suggestive of tuberculosis in the post-mortem examination. All cattle died from conditions that did not include tuberculosis, the main circumstances of death (euthanasia, emergency slaughter or unassisted death), were metabolic/digestive disorders, pneumonias, traumatism, and mastitis/udder problems, in the Supplementary Table 1 we described the causes of death of each animal. The samples were collected in a dairy basin from the central region of Mexico with a prevalence of bovine tuberculosis higher than 16% (10).

2.2. Histopathological analyses of paraffin-embedded tissues

Samples of lymph nodes, lung tissue, and individual organs that exhibit tuberculosis-suggestive lesions were collected during the necropsy. Tissues were divided for histopathology and bacteriological cultures.

For histopathological analysis, the tissue was fixed in 10% formaldehyde and embedded in paraffin. From the formalin-fixed paraffin-embedded tissues (FFPE) were obtained 4-μm width serial sections and stained with Hematoxylin and Eosin (H&E), Masson's trichrome, Ziehl Neelsen (ZN), and Von Kossa. In these sections were identified granulomas with fibrous tissue capsules, acid-fast bacilli, and calcification. Granulomas were identified and staged according to Wangoo et al. (8) and using a new classification of granulomas in the group of young bovines (9).

2.3. *Mycobacterium bovis* identification

Mycobacterium bovis was identified by bacteriological isolation and by PCR of FFPE tissues. Briefly, part of the collected tissue was used for bacteriological isolation after Petroff's decontamination method under biosecurity conditions (11). To confirm the presence of *M. bovis,* we extracted genomic DNA from the bacteriological isolation and FFPE tissues that had granulomas. In the case of paraffin blocks, 10 to 12 micron sections were obtained by use of a microtome and added to a 1.5 ml centrifuge tube, microtome blades were cleaned with 70% alcohol between slices to avoid cross-contamination of samples. After, 1 ml of xylol was added to each tissue section which was then vortexed and incubated for 5 min. Xylol was subsequently decanted and tissue section was washed twice with absolute ethyl alcohol, allowed to dry, and resuspended in 400 μl of TE with 50 μl of lysozyme (10 mg/ml) and were incubated over-night at 37°C. Then, we used the CTAB (N-cetyl-N, N, N-trimetyl ammonium bromide)/chloroform-isoamyl alcohol protocol, described by Van Helden et al. (12). Next, a nested PCR was performed to amplify the mpb70/m22 genes and identify members of the Mycobacterium tuberculosis complex. We used a commercial kit (TopTaq Master Mix Kit) followed the manufacture instructions. Primers for the mpb70 gene that amplify a product of 372 bp were: mpb70 F (5′-GAACAATCCGGAGTTGACAA-3′) and mpb70 R (5′-AGCACGCTGTCAATCATGTA-3′). For a second reaction a 208 bp product from the same gene was obtained, the M22 F (5′-GCTGACGGCTGCACTGTCGGGC-3′) and M22 R (5′-CGTTGGCCGGGCTGG TTTGGCC-3′) primers were used. Finally, PCR of the RD9 and RD4 genes was used to identify specifically *M. bovis.* For the RD9 gene, selected primers were RD9 F (GTGTAGGTCAGCCCCATCC), RD9 I (CAATGTTT GTTGCGCTGC) and RD9 R (GCTACCCTCGACCAAGTGTT), with a product of 333 bp for *M. tuberculosis* and 206 bp for *M. bovis* and for RD4 gene the primers were RDF (ATGTGCGAGCTGAGCGATG), RD4 I (TGTACTATGCT GACCCATGCG) and RD4 R (AAAGGAGCACCATCGTCCAC), with a product of 268 bp for *M. bovis* and *M. bovis* BCG, for the rest of the members of the *M. tuberculosis* complex a product of 172 bp is amplified (12–14).

2.4. Immunohistochemistry

IHC procedures are summarized in Table 1. Briefly, FFPE tissues were cut into 4–5 μm sections and placed on electrocharged slides (Kling-On Slides-Biocare Medical). The sections were deparaffinized at 60°C for 30 min, rehydrated, and placed in 3% hydrogen peroxide for 15 min to eliminate endogenous peroxidase activity; then, epitope demasking was performed using both physical and chemical methods according to the primary antibody (Table 1). The tissues were washed with distilled water and placed in Sequenza cover plates (Shandon Scientific Loughborough, UK) for immunolabeling. The sections were washed after each step of the staining procedure with phosphate-buffered saline (PBS: 138 mM NaCl, 3 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2HPO4 adjusting the pH to 7.4) and Tris-buffered saline with Tween (TBST: 0.005 mM Tris-buffered saline, pH 7.6 with 0.05% Tween 20). A universal blocking reagent (Background Sniper BS966L10) for reducing nonspecific background staining was added, then samples were incubated with the primary antibody. Antibody concentration and incubation time were standardized for each antibody. After washing twice, MACH 1 Universal HRP-Polymer Detection (Micro-polymer detection) was used following the manufacturer's instructions. A probe (mouse antibodies only) was added to the sections and incubated for 15 min at room temperature. Then, the Polymer was added and incubated for 30 min at room temperature, followed by 3,3′-diaminobenzidine tetrahydrochloride (DAB) for visualization. The slides were rinsed in purified water, counterstained in Mayer's hematoxylin, dehydrated, and mounted with resin.

2.5. Digital image analysis in granulomas

The slides immunolabeled with different antibodies were processed digitally with a scanning microscope (Aperio Scanscope CS, Aperio, CA, USA), generating 40× images with a spatial resolution of 0.45 μm/pixel. Images were analyzed with the ImageScope software (Aperio, CA, USA), and granulomas were delimited by removing the areas of necrosis composed of cell debris and calcification. Various algorithms were used to standardize the adequate detection level for the quantification of the brown staining obtained from IHC. This methodology enabled the quantification of the proteins marked in the different IHC tests. Supplementary Figure 1 summarizes the experimental procedures used in this study, and Supplementary Table 2 shows the antibodies used and the number of granulomas analyzed in each group.

2.6. Statistical analyses

Statistical analysis was performed using the PASW Statistics 18 program and GraphPad Prism 7.0. Shapiro–Wilk test and normal Q-Q plots were tested for normality. Comparisons of immunostaining in granuloma between adult and young cattle were performed by the nonparametric Mann–Whitney test. Significant differences were considered when $p < 0.05$.

3. Results

3.1. Granulomas of calves naturally infected by *Mycobacterium bovis* exhibit high numbers of bacteria

Formalin-fixed paraffin-embedded lymph node sections from 25 Holstein Friesian cattle naturally infected with *M. bovis* were used. IHCs were performed on these tissues to identify cell populations, cytokines, and the presence of mycobacteria. A total of 3,439 granulomas were analyzed, of which 31.3% (1,077) were from adult cattle, and 68.6% (2,362) were from calves. Using the Ziehl–Neelsen staining, we had previously observed more AFB in granulomas of the mediastinal lymph nodes of calves compared with adult cattle. To

TABLE 1 Immunohistochemical reagents and technical procedures.

Antibody and dilution	Antibody type	Supplier	Primary antibody incubation	Epitope demasking	Buffer
MAC387 1/500	Mouse monoclonal IgG1	Bio-rad MAC387	O/N 4°C	Proteinase K	PBS
CD79 1/50	Mouse monoclonal IgG1	Dako HM57	O/N 4°C	Proteinase K	PBS
CD3 1/50	Rabbit polyclonal IgG	Biocare medical SP7	O/N 4°C	Citric acid buffer, pH 6.0	TBST
Vimentin 1/100	Rabbit polyclonal IgG	Biocare medical CRM 312	45 min	Citric acid buffer, pH 6.0	TBST
Smooth Muscle Actin 1/100	Mouse monoclonal IgG1	Biocare medical SP9	45 min	Citric acid buffer, pH 6.0	TBST
Anti-*Mycobacterium* 1/100	Rabbit polyclonal	Biocare medical CP 140	O/N 4°C	Citric acid buffer, pH 6.0	TBST
TGF-β 1/100	Mouse monoclonal IgG1	Gene Tex TB21	O/N 4°C	Citric acid buffer, pH 6.0	TBST
TNF-α 1/100	Mouse monoclonal IgG1	Gene Tex CC327	O/N 4°C	Citric acid buffer, pH 6.0	TBST
iNOS 1/500	Rabbit polyclonal	Millepore 06–573	45 min	Citric acid buffer, pH 6.0	TBST
IFN-γ 1/100	Mouse monoclonal IgG1	Gene tex CC330	O/N 4°C	Citric acid buffer, pH 6.0	TBST
WC1 1/500	Mouse monoclonal IgG1	Invitrogen, CC15	O/N 4°C	Citric acid buffer, pH 6.0	TBST

O/N = 18–20 h in a refrigerator (+4°C).
TBST = 0.005 mM Tris-buffered saline, pH 7.6 with 0.05% Tween 20.
PBS: 138 mM NaCl, 3 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂HPO₄, pH 7.4.

validate this result and quantify the number of bacteria, we performed IHC using a polyclonal anti-mycobacterium antibody; we confirmed higher immunostaining in granulomas from calves than in those from adult cows as well stage comparison presented the same pattern (Figures 1A,B; Supplementary Figure 1). The higher sensitivity of this technique allowed us to detect not only the presence of bacilli, but also cellular remains in the forms of vacuoles and cytoplasmic dust that are possibly associated with cell debris due to mycobacterial processing and phagocytosis. This staining was observed mainly in the cytoplasm of macrophages (MΦs), epithelioid MΦs, and multinucleated giant cells (MGCs). Positive staining was scarcely found in granulomas from adult cattle, which presented necrotic centers with mineralization and were circumscribed by a connective tissue capsule; in these types of lesions, the positive immunostaining was only observed in the MGCs (Figure 1C). In calf granulomas, positive staining was extracellular and predominant in necrotic areas (Figure 1D). Interestingly, the cytoplasm of different types of cells outside the granulomas had cytoplasmic dust immunolabeling in both groups. These results suggested that the higher bacteria burden found in granulomas of calves compared with adult cattle may be correlated with the type of immune response. Therefore, we sought to identify the major cell populations and cytokines associated with the immunopathology of tuberculosis.

3.2. Calf granulomas do not develop a fibrous capsule

One of the main findings of the histopathological analysis in the tissue stained with Masson's trichrome was the absence of a connective tissue capsule in the granulomas of calves, even in the presence of necrosis and calcification. Fibroblasts and myofibroblasts have been reported as the main cell populations that form the connective tissue capsules in the granulomas caused by *M. bovis*; these capsules are mainly composed of type I collagen (8). We observed greater vimentin (fibroblasts) and α-SMA (myofibroblast) immunolabeling in adult cattle compared with calves (Figure 2). Vimentin staining was identified in epithelioid MΦs, MGCs, and mostly in cells with fibroblast characteristics, which were interspersed in the cellular area of the granulomas. Positive vimentin fibroblasts formed cell layers with different thicknesses comprising the capsule of connective tissue around granulomas in stages III and IV of adult cattle; this distribution

FIGURE 1
Granulomas of calves *Mycobacterium bovis* naturally infected show higher mycobacteria immunolabeling compared to adult cattle. **(A)** Average number of pixels positive for the IHC anti-mycobacterium staining in granulomas and stages from adult and young cattle, respectively. **(B)** Mann–Whitney test *$p<0.05$, **$p<0.01$, and ***$p<0.001$. **(C,D)** Microphotograph of IHC anti-mycobacterium, 40×. **(C)** Stage IV adult bovine granuloma with faint positive brown areas around the necrosis, close-up showing cells with macrophagic morphology and a giant cell with a cytoplasmic mark. Granuloma from calf showing a large amount of positive staining in the necrotic area: the enclosure shows cytoplasmic and extracellular immunolabeling.

FIGURE 2
Absence of connective tissue capsule in granulomas of calves naturally infected by *Mycobacterium bovis* is associated with a lower number of fibroblasts and myofibroblasts. **(A,B** and **E,F)** Expression of vimentin and α-SMA immunolabeling was quantified in the granulomas and stages of adults and calves, Mann–Whitney test *$p<0.05$, **$p<0.01$, and ***$p<0.001$, respectively. **(C,D)** IHCs of vimentin in late granulomas show abundant staining mainly in the connective tissue capsule of granuloma from adult cattle compare with calf granuloma, respectively. **(G,H)** IHCs of α-SMA in late granulomas show myofibroblast forming the connective tissue capsule in granuloma from adult cattle. However, granuloma from a calf showing mixed positive cells around the necrosis without forming a connective tissue capsule.

of fibroblasts around the lesions was absent in calf granulomas (Figures 2C,D). Immunostaining of α-SMA was found in cells with fibroblast morphology. Initial granulomas from both groups showed positive α-SMA interspersed with epithelioid MΦs and lymphocytes, showing more positive cells in calf granulomas stage I (Figures 2E,F; Supplementary Figure 3). In adult cattle granulomas of stages III and IV, α-SMA positive cells were embedded in the connective tissue capsule, and the thickness of the capsule was related to the number of the myofibroblast layers. In late granulomas from calves, the positive cells were irregularly distributed and interspersed with the other cells without forming a capsule around the lesion (Figures 2G,H).

3.3. *Mycobacterium bovis* granulomas from calves and adult cattle have different cell proportions

Granulomas from calves have more bacteria, no connective tissue capsules, and a lower number of fibroblasts and myofibroblasts than those from adult cattle. Taken together, these results suggest a functional difference in the immune response that is probably related to the type of cells that form the granulomas. We used IHC to identify the main cell populations of granulomas from adult cattle and calves. Granulomas from adult cattle showed more MAC387 (MΦs/monocytes), WC1 (γδ T cells), CD79+ (B lymphocytes) cells, and fewer CD3+ (T lymphocytes) cells compared to granulomas from calves In addition, we evaluated the differences between stages of granulomas, and we observed the same pattern (Figure 3; Supplementary Figure 3). Adult's granulomas showed more MΦs,

epithelioid MΦs, and MGCs than calves' granulomas (Figures 3A,D). γδ T+ cells were present in granulomas, as well as in the parenchyma of the lymph nodes. An increasing number of positive cells correlated with the granuloma stage in adult cattle; stages III-IV showed many positive cells in the cellular area and around the connective tissue capsule in adult's granulomas, whereas few γδ T+ cells were observed in all granuloma stages from calves (Figures 3E,H). Adult's granulomas showed more B lymphocytes located between the cellular area of the initial stages and around the lesions with calcification and necrosis that calves' granulomas. Interestingly, B cells niches were found in some late granulomas in both groups (Figures 3I,L). Finally, T lymphocytes were observed in both groups surrounding the tissue capsule; they were interspersed in the initial stages and surrounding the necrosis area in later stages. A slight increase of these cells was observed in calves compared to adult's granulomas (Figures 3M,P).

3.4. A higher proinflammatory response was observed in granulomas from calves compared to adults

We hypothesized that the differences in cell populations observed in the granulomas of calves and adult cattle are related to a distinct type of immune response. To further explore this hypothesis, we evaluated cytokines and inflammatory mediators associated with tuberculosis immunopathology in humans and cattle, such as gamma interferon (IFN-γ), an inducible form of nitric oxide synthase (iNOS), transforming growth factor-beta (TGF-β) and tumor necrosis factor

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FIGURE 3

Cell population differs between granulomas from calves and adult cattle naturally infected with *Mycobacterium bovis*. **(A–P)** Average expression and representative images of immunolabeling for MAC387 (macrophages), WC1 (γδ T cells), CD79 (B cells), and CD3 (T cells), respectively, in granulomas and stages from adult cattle and calves, Mann–Whitney test *p<0.05, **p<0.01, and ***p<0.001. **(C)**, 100× Stage II granuloma from an adult showing abundant MAC387⁺ cells, including some MGCs. **(D)** 100× Granuloma from a calf with positive cells around a necrotic area. **(G)**, 40× Stage IV granuloma from an adult with abundant staining of WC1+cells (γδ T cells) in the center of the lesion. **(H)**, 40× Granuloma from a calf with a few WC1+cells. **(K)**, 40× CD79⁺ cells around the granulomas; stage IV granuloma staining around the lesion. **(L)**, 40× Granuloma from a calf, with some CD79⁺ cell aggregates around the necrotic area. **(O,P)**, 40× Intense staining of CD3⁺ cells around the necrosis interspersed between the rest of the cells that form the granuloma in adults and calves, respectively. ND, not determined (because of lack to adult granulomas stage II).

α (TNF-α) (4, 15, 16). Although proinflammatory cytokines have been associated with mycobacterium infection control, we observed more immunolabeling of IFN -γ, iNOS, and TNF-α and less TGF-β in the calf granulomas compared to adult granulomas. The same pattern was observed in stages of granulomas between calves and adult cattle (Figure 4; Supplementary Figure 4). Intracellular IFN-γ staining was observed mainly in lymphocytes. In both groups, initial-stage granulomas presented IFN-γ⁺ cells mixed with epithelioid MΦs, whereas late-stage granulomas showed IFN-γ staining in areas surrounding the necrosis. Interestingly, there was a higher number of IFN-γ⁺ cells in stage III granuloma of adult cattle compared to calves (Figures 4A,D; Supplementary Figure 4A). A higher expression of iNOS was detected in granulomas and stages of calves compared to adults. The staining was generally observed in the cytoplasm of

FIGURE 4
Granulomas from calves show a more proinflammatory response than adults. (A–P) Average expression and representative images of immunolabeling for IFN-γ, the inducible form of nitric oxide synthase (iNOS), TNF-α, and TGF-β, respectively, in granulomas from adults and calves; Mann–Whitney test *$p<0.05$, **$p<0.01$, and ***$p<0.001$. (C,D), 100× Initial granulomas showing IFN-γ⁺ cells with lymphocyte morphology in the center of the lesion. (G,H), 40× iNOS staining around necrosis, positive cells with macrophage morphology, and some MGCs with different staining intensities from adults and calves, respectively. (K), 40×; (L), 100× TNF-α⁺ cells and extracellular staining around necrosis, positive cells have an epithelioid MΦs morphology, and some MGCs show different staining intensity in adults and calves, respectively. (O,P), 40× TGF-β staining is observed extracellularly and in the cytoplasm of epithelioid MΦs, some MGCs, and fibroblast cells.

epithelioid MΦs and MGCs, although not all giant cells present in the same granuloma were positive (Figures 4E,H). TNF-α immunolabeling was greater in granulomas of calves observed in the cytoplasm of epithelioid MΦs, MGCs, and some fibroblasts found in cellular areas of granulomas and extracellularly, with greater intensity around necrotic sections and less intensity in the periphery of the lesion (Figures 4I,L). Finally, TGF-β, an anti-inflammatory cytokine, was observed in the extracellular and cytoplasmic area of epithelioid MΦs and MGCs from both groups. High TGF-β staining was observed in fibroblasts that form the connective tissue capsule in stage III-IV granulomas from adult cattle compared to calves (Figures 4M,P). All these results suggest an exacerbated proinflammatory process that causes an inability to control the infection by *M. bovis* in naturally infected cattle.

3.5. Concentration of INF-γ and gamma delta T cells is granuloma stage-dependent

When analyzing the granulomas by stage and did the comparison between adult and calves, a variation in the labeling of INF-γ and γδ T cells was observed. Although the global average of INF-γ is higher in granulomas of calves, in adult cattle we observed higher concentration of this cytokine in stage III granulomas. The immunolabeling of γδ T cells is higher in adults compare to calves. It is interesting to note that the highest concentration of γδ T cells is observed in stage IV granuloma. The amount of INF-Y does not correlate with the expression of γδ T cells observed in these lesions (Figures 5A,B).

4. Discussion

Granulomas are the characteristic lesions of bovine tuberculosis. The development, morphology, and fate of this structure depend on several factors, including chronic stimulation by the virulent mycobacteria and the host's immune response associated with the type of cell population, cytokines, chemokines, and cell activation. The immune response and morphological characteristics of *M. bovis* granulomas have been studied mainly in cattle older than 6 months of age (4). However, very little information has been reported on the immunology of tuberculosis in young animals (17).

Our results evidenced differences in the histological structure, number of bacteria, and immune response in granulomas from calves and adult cattle. In summary, granulomas in calves have more bacteria, no connective tissue capsules associated with disorganized structure; as well as fewer fibroblasts, myofibroblasts, epithelioid MΦs, MGCs, γδ T cells, B cells, and TGF-β immunereactivity than adult cattle. Taken together, these data suggest an exacerbated proinflammatory process that is inefficient in the control of *M. bovis* infection in naturally infected cattle (Figure 6). In a previous study from our group, we observed histological differences in granuloma architecture, such as the absence of the connective tissue capsule, more necrosis, and a greater number of AFBs in the granulomas of calves compared to adult cattle (9). To better understand the immune response and the number of bacteria found in these lesions, we used IHC and digital pathology analysis. We confirmed higher mycobacterium immunolabeling in granulomas from calves, mainly in extracellular and necrotic areas and in the cytoplasm of epithelioid MΦs and MGCs.

Our observations differ from previous reports in cattle experimentally infected with virulent strains of *M. bovis*, where the number of AFBs is low and located in the cytoplasm of giant cells (4, 18). However, we found a similar result in granulomas from adult cattle, where positive immunolabeling was mainly found in the cytoplasm of epithelioid MΦs and MGCs. In some lesions, it was impossible to detect positive staining, especially in stage III and IV granulomas. In monkeys, these types of lesions are capable of sterilizing mycobacteria in latent infections (19).

One important feature of this study was the use of polyclonal antibodies in the IHC since the protocol not only stained bacilli but also cellular remains. These remains were observed as vacuoles and cytoplasmic dust, possibly associated with cell debris due to mycobacteria processing and phagocytosis. Interestingly, both groups also showed mycobacteria immunolabeling in cells outside the granuloma. This had already been noted in previous studies, suggesting that mycobacteria are present outside the lesion, probably as the remains of phagocytosed bacteria (20–22).

The presence of a fibrotic capsule around the granuloma is a hallmark of bovine tuberculosis. The capsule is mainly composed of type I collagen, produced by fibroblasts and myofibroblasts. Using Masson's trichrome staining, we previously observed fibrosis in granulomas from adult cattle, in agreement with previous reports (8, 23). However, the capsule was absent in the calf granulomas. To confirm whether calf lesions lacked fibroblasts, we performed IHCs of vimentin and α-SMA; as expected, immunolabeling was detected in fibroblasts and myofibroblasts forming the surrounding fibrous tissue capsule in late granulomas and intercalated in the cellular area in stage I and II granulomas of adult cattle. Surprisingly, vimentin and α-SMA immunolabeling of early-stage granulomas from calves was similar to that of adult cattle. Nevertheless, γδ T+ cells were d lesions with necrosis and calcification showed disorganized fibroblasts and myofibroblasts that did not form a capsule around the lesion. The amount of fibrosis correlated with the presence of TGF-β in granulomas and the differentiation of fibroblasts into collagen-producing myofibroblasts (24, 25). Using IHC, we observed less TGF-β in the

FIGURE 5
INF-γ and gamma delta T cells form granulomas have stage dependent concentration. **(A)** Average expression of immunolabeling for IFN-γ and **(B)** WC1 (γδ T cells), respectively, in granuloma stages from adult cattle and calves. Mann–Whitney test *p <0.05, **p <0.01, and ***p <0.001. ND, not determined (because of lack to adult granulomas stage II).

FIGURE 6
Propose schematic model of morphology and immune activity in granulomas from calves and adult cattle naturally infected with *Mycobacterium bovis*. **(A,B)**, 5× H&E staining of lymph node sections with infection-induced granulomas. **(A)** Adult bovine lymph node with different stage II and IV granulomas found in the lymph node medulla and cortex with the classic structure. **(B)** Calf lymph node, showing granulomatous lesions with a large amount of necrosis and absence of the connective tissue capsule. **(C)** Diagram summarizing the differences identified in granulomas, showing fewer MGCs, epithelioid MΦs, γδ T cells, B cells, fibroblasts, TGF-β and more iNOS, necrosis, T cells, IFN-α+cells, TNF-α, and mycobacteria cells in granulomas from calves compared to adult cattle.

granulomas of calves compared to those of adult cattle. This cytokine has been associated with the development of fibrosis in granulomas of cattle infected by *M. bovis* (8). The function of fibrotic capsules in the pathogenesis of tuberculosis and their formation around granulomas are incompletely understood, but some studies have associated them with the chronicity of the lesion, better control of bacteria, limitation of tissue damage, and latent infection (19, 25–27). In bovine tuberculosis, the fibrotic capsule is characteristic of stage III-IV granulomas and is composed mainly of type I collagen produced by fibroblasts. In naturally infected cattle, the thickness of the connective tissue capsule has been associated with fewer bacteria in granulomas (25). The absence of capsules in granulomas from calves with high bacterial burden suggests that capsules protect cattle naturally infected with *M. bovis*, but the factors that determine the formation and deposition of fibrous tissue in the external part of the lesions are still unclear. Interestingly, recent studies suggest that the fibroblasts forming a connective tissue capsule may be MΦs that undergo macrophage-myofibroblast transition (28). Our IHC

results showed that MAC387, a protein found in MΦs and monocytes, was present in the cytoplasm and membrane of fibroblasts that formed the connective tissue capsule. This observation suggests the possibility of macrophage-myofibroblast transition in cattle granulomas.

Granulomas with higher bacterial burdens and no peripheral fibroblasts have been found in active tuberculosis infections in monkeys and humans associated with more proinflammatory cytokines (6, 19). In this study, higher immunolabeling of IFN-γ, TNF-α, and iNOS with fewer TGF-β was observed in granulomas from calves compared with adult cattle, suggesting a greater proinflammatory response. The high amount of IFN-γ in the calf granulomas suggests abundant CD3+ T cells in response to mycobacterial antigens. Strong whole-blood IFN-γ responses have been reported in calves as early as 1 month after *M. bovis* infection, and TNF-α production has been shown in BCG-vaccinated calves (29, 30). These two cytokines are essential for activating antimycobacterial mechanisms and inducing reactive nitrogen intermediates by activated MΦs, which play a crucial role in the intracellular killing of

mycobacteria. However, despite higher iNOS production in granulomas from calves, they presented fewer epithelioid MΦs and MGCs compared to adult cattle. The cytotoxic activity induced by high iNOS concentrations might explain this contradictory result (31). Another possibility is that MΦs present in calf lesions were mainly derived from circulating blood. These MΦs are more proinflammatory and short-lived, and they depend more on glycolysis to produce energy than resident tissue MΦs (32). This finding is consistent with the idea that the MAC387 antibody detects an epitope on the calcium-binding protein MRP14 found in monocytes/MΦs that have recently infiltrated acutely inflamed tissues (33, 34). Similarly, we identified more MAC387⁺ cells in the uninjured tissue surrounding lymph node granulomas of calves compared to adult cattle, suggesting that the MΦs and monocytes detected in calves are mostly from the bloodstream and, therefore, more proinflammatory. We demonstrated that granulomas from calves present a more proinflammatory response than those of adult cattle; this type of microenvironment is associated with less TGF-β, possibly resulting in the lack of connective tissue observed. Granulomas are dynamic, spatially organized structures with a proinflammatory center that may present necrosis and a periphery of cells with a proinflammatory profile. From this study, we can infer that the granulomas of young bovines infected with *M. bovis* have a reduced anti-inflammatory response, which is why they lack adequate encapsulation of fibrous tissue.

The role of B cells and γδ T cells in the pathogenesis of bovine tuberculosis is incompletely understood. However, the presence of B cells in granulomas has been associated with better control of the infection since they are numerous in the granulomas with fewer bacteria (23). In this study, CD79⁺ lymphocytes were observed among the rest of the cells and around the lesion in different granuloma stages. Multifocal aggregates of CD79⁺ cells were also observed in some late stages. Finally, more CD79⁺ cells were found in the granulomas of adult cattle compared with those of calves (which had the highest bacterial burden). This result agrees with the finding that granulomas with more B lymphocytes tend to have fewer mycobacteria. Conversely, γδ T cells play a critical role in connecting innate and adaptive immunity in response to *M. bovis.* In peripheral blood, γδ T cells represent up to 70% of the lymphocytes in young animals and decline to an average of 10–20% in adult bovines (35). Moreover, WC1+ γδ T-cell from neonatal calves express high levels of INF-γ in response to IL-12 and IL-18 compared with adult animals (36). The higher percentage of T cells in calves suggests that they have an important participation in the immune system. In granulomas, γδ T cells are the first to arrive at the infection sites; they have been observed as early as 7–15 days after experimental infection with *M. bovis,* suggesting that they play a role in granuloma formation. Likewise, it has been reported that the number of γδ T cells is positively correlated with the stage of the granuloma, which agrees with our observations that the number of γδ T cells was higher in the granulomas of adult bovines, it is interesting to note that the highest concentration of γδ T cells is observed in stage IV. Although adult granulomas showed a higher concentration of γδ T cells, this does not correlate with the expression of INF-γ observed in these lesions (37, 38). Surprisingly, although these cells are increased in the circulation of calves, we found a small number in the granulomas. A possible explanation for this result is that γδ T cells in young cattle remain mainly in the bloodstream and are less present in the interstitium.

These observations highlight the involvement of γδ T cells in the pathogenesis of tuberculosis in young animals.

One limitation of this study is that several factors that could affect the type of lesion remain unknown, including the grade of bacterial virulence, route of infection, bacterial dose, and date of infection. It is widely recognized that the pathology of bovine tuberculosis is multifactorial. However, this study emphasizes age as an important factor in the type of immune response and granuloma formation in cattle naturally infected with *M. bovis*. Granulomas from calves displayed a greater number of bacteria, lacked the connective tissue capsule, were associated with fewer and more disorganized fibroblasts and myofibroblasts, showed a predominance of proinflammatory cytokines (IFN-γ, TNF-α, and iNOS), and had fewer epithelioid MΦs, MGCs, γδ T cells, B lymphocytes, and TGF-β, compared to the granulomas from adult cattle. Our results suggest that calves have active-like tuberculosis with an exacerbated proinflammatory response that may be associated with more necrosis and a lower microbicidal capacity, making them more permissive to infection and dissemination of mycobacteria. This study highlights the importance of understanding the immune response and pathogenesis of bovine tuberculosis in young animals.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Ethics and Animal Welfare Committee of the Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México (CICUA, FMVZ-UNAM), and complied with the Mexican guidelines for animal research (JAGP-074).

Author contributions

JC-U and JAG-P conceived the experiments and wrote the original draft. JAG-P provided resources, project administration, and funding acquisition. JC-U and MAB-A collected and prepared samples. RH-P and CL-M advised on field data acquisition and analysis and provided scientific guidance during the experiment and drafting of the manuscript. JC-U, MJ-R, and GB-G performed the experiments and analyzed the data. SH-Y performed validation, writing-review, and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1105716/full#supplementary-material

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Supplementary Material

Supplementary Figures and Tables

Group	Case number	Age	Sex	Cause of death
Adults	1	4 Years	F	Infertility
	2	3 Years	F	Feedlot bloat
	3	3 Years	F	Traumatic pericarditis
	4	3 Years	F	Tibial fracture
	5	5 Years	F	Feedlot bloat
	6	5 Years	F	Infertility
	7	3 Years	F	Unknown
	8	5 Years	F	Feedlot bloat
	9	3 Years	F	Infertility
	10	2 Years	F	Traumatic pericarditis
	11	5 Years	F	Unknown
	12	5 Years	F	Feedlot bloat
	13	3 Years	F	Traumatic pericarditis
	14	5 Years	F	Unknown
	15	1 Year	F	Chronic feedlot bloat
Calves	16	4 Months	M	Pneumonia
	17	8 Days	F	Cardio-respiratory failure.
	18	1 Month	F	Diarrhea and dehydration
	19	1.5 Months	F	Pneumonia
	20	3 Months	F	Pneumonia
	21	3 Months	F	Feedlot bloat
	22	2 Months	F	Peritonitis
	23	1 Month	F	Acute respiratory failure
	24	2.5 Months	F	Pneumonia
	25	3.5 Months	F	Acute respiratory failure

F, female and M, male

Supplementary table 1: Causes of death in studied cattle.

Antibodies used in immunohistochemistry and number of granulomas analyzed.

Immunolabeling	Granulomas analyzed	
	Adults	Calves

MAC387	72	146
CD3	105	155
CD79	103	215
WC1	78	202
α-SMA	88	229
Vimentina	106	226
TNF-α	96	210
INF-γ	108	224
TGF-β	114	201
iNOS	116	254
Anti-mycobacterium	91	300
Total	**1077**	**2362**

Supplementary table 2: Antibodies used, and number of granulomas analyzed in each group.
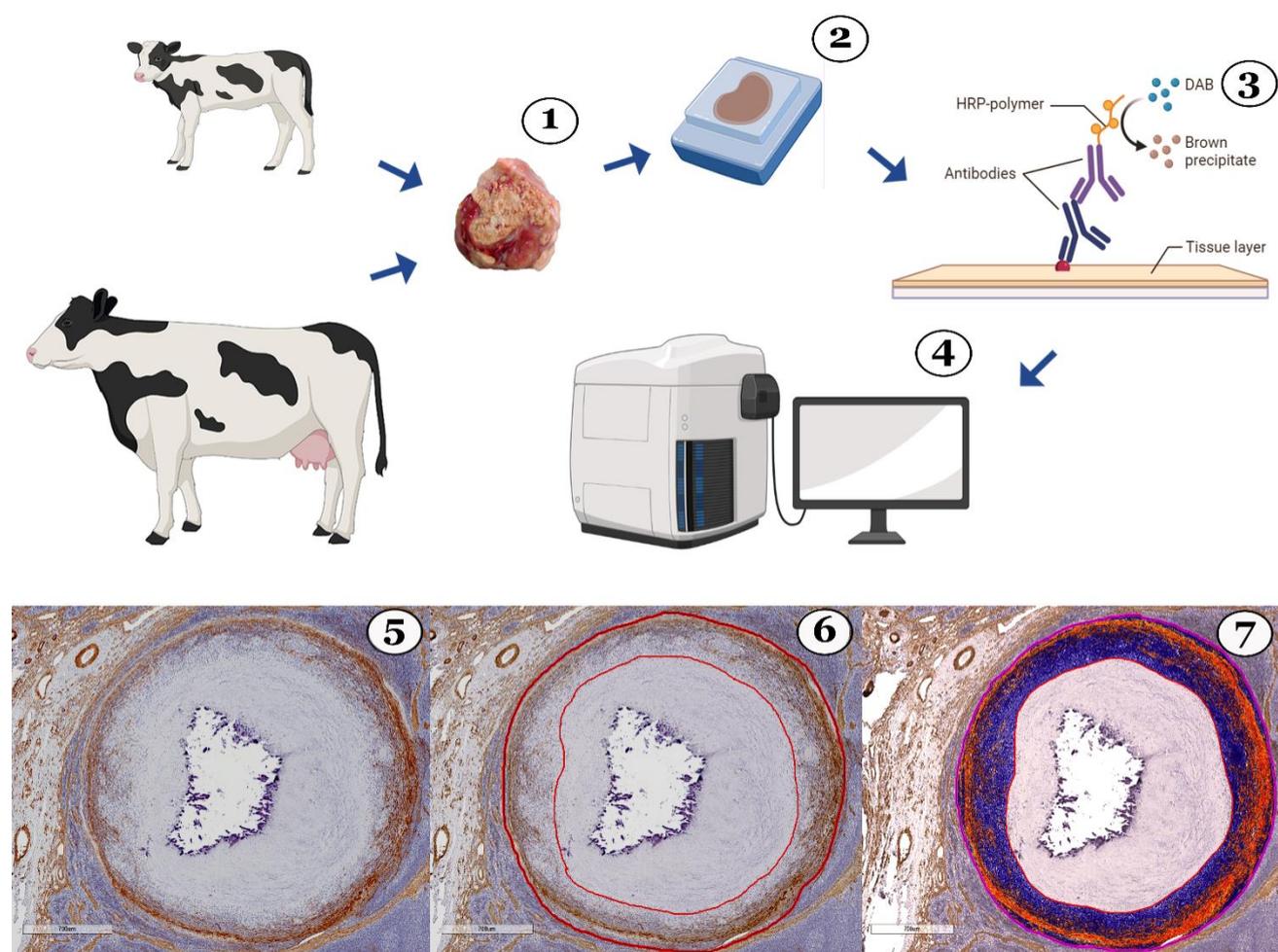
Supplementary Figure 1: Scheme summarizing experimental procedures: Lymph nodes of Holstein-Friesian dairy cattle *M. bovis* naturally infected were included [1], the tissues were embedded in paraffin blocks [2], then serial sections of 4-5 μm were cut and immunolabeled [3] then tissue sections were scanned [4], and selected images were analyzed using the ImageScope software system, finally the number of positive pixels of each immunostaining was quantified [5-7]. (Created with BioRender.com, accessed on Sep 4, 2022)
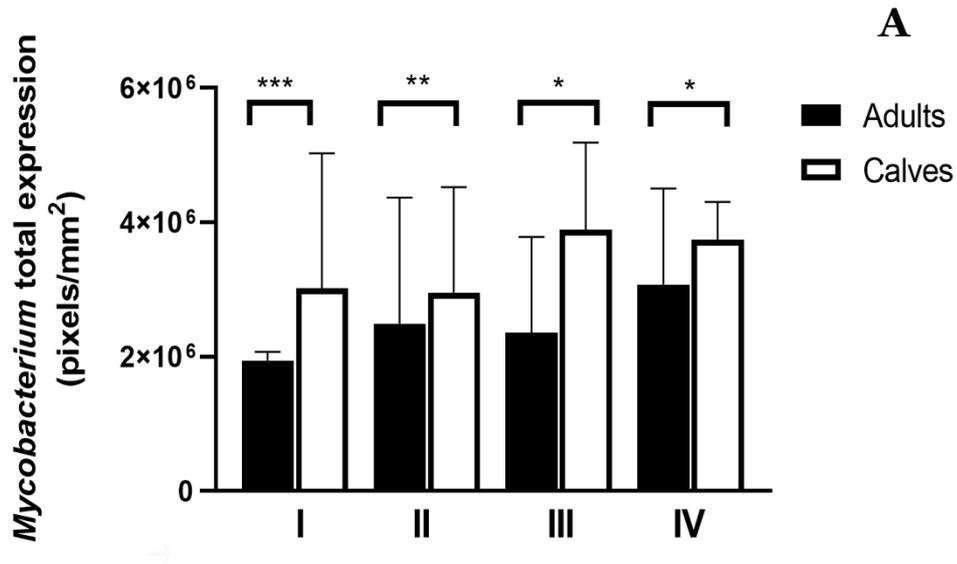
Supplementary Figure 2: Stages granulomas of calves *M. bovis* naturally infected show higher mycobacteria immunolabeling compared to adult cattle.
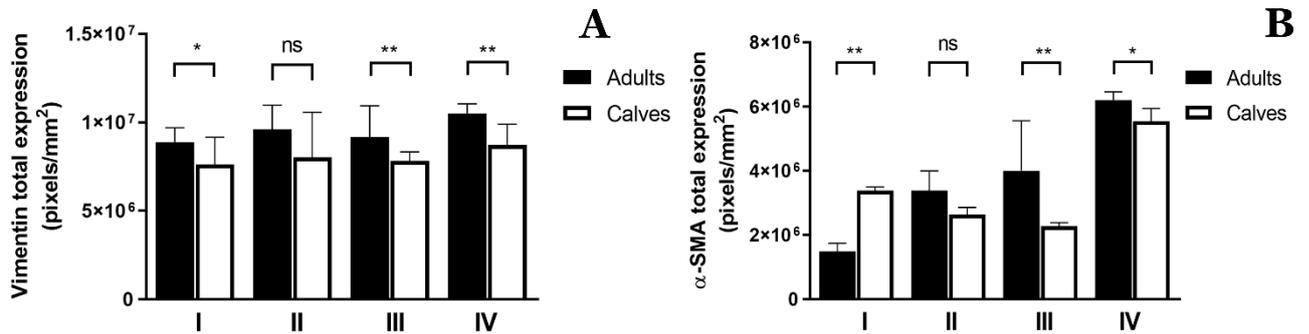
Supplementary Figure 3: Stage granulomas of calves naturally infected by *M. bovis* are associated with a lower number of fibroblasts and myofibroblasts. A and **B**) Expression of vimentin and α-SMA immunolabeling was quantified in stages granulomas of adults and young cattle, Mann-Whitney test * * * *P*< 0.001 and * * *P*< 0.01 respectively.
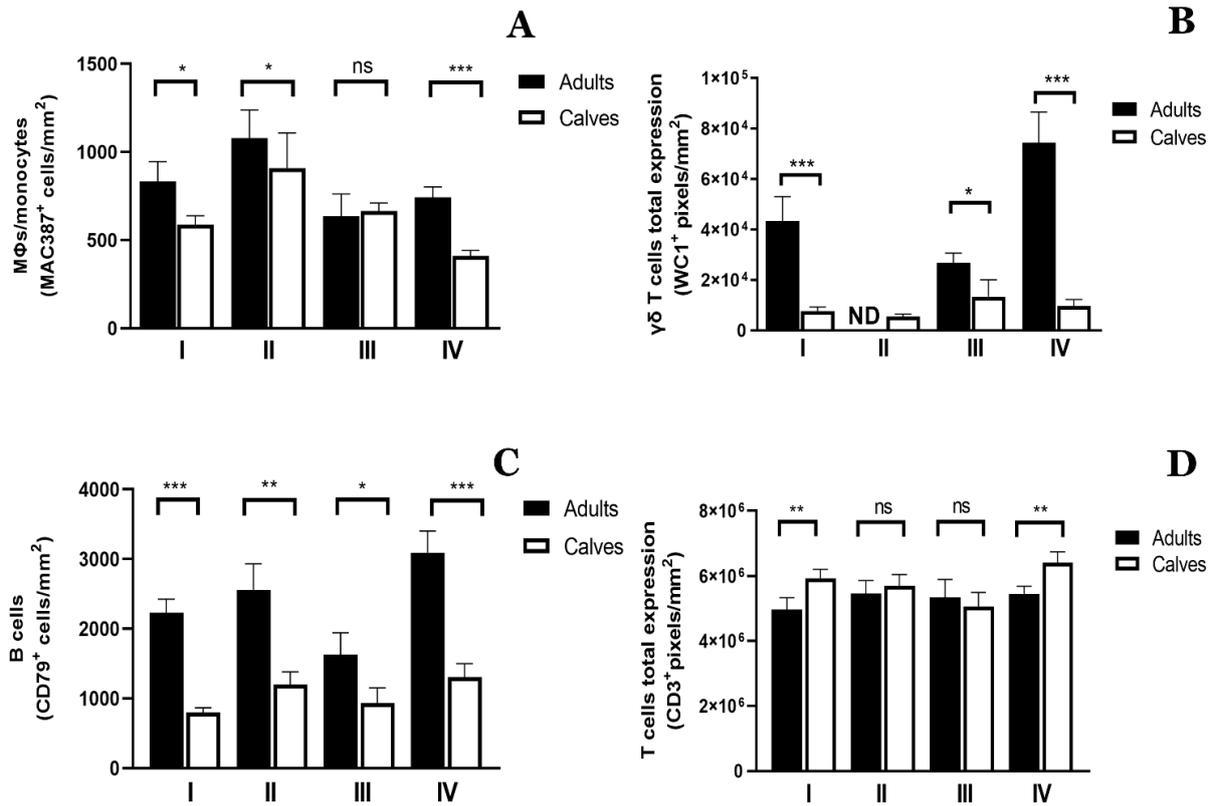
Supplementary Figure 4: Cell population differs between stages in granulomas from calves and adult cattle naturally infected with *M. bovis*. Average expression of immunolabeling in stage granulomas for MAC387 (macrophages), WC1 (γδ T cells), CD79 (B cells), and CD3 (T cells) respectively, from adult cattle and calves. Mann-Whitney test * *P*< 0.05, * * *P*< 0.01, and * * * *P*< 0.001. ND, not determined (because of lack to adult granulomas stage II).
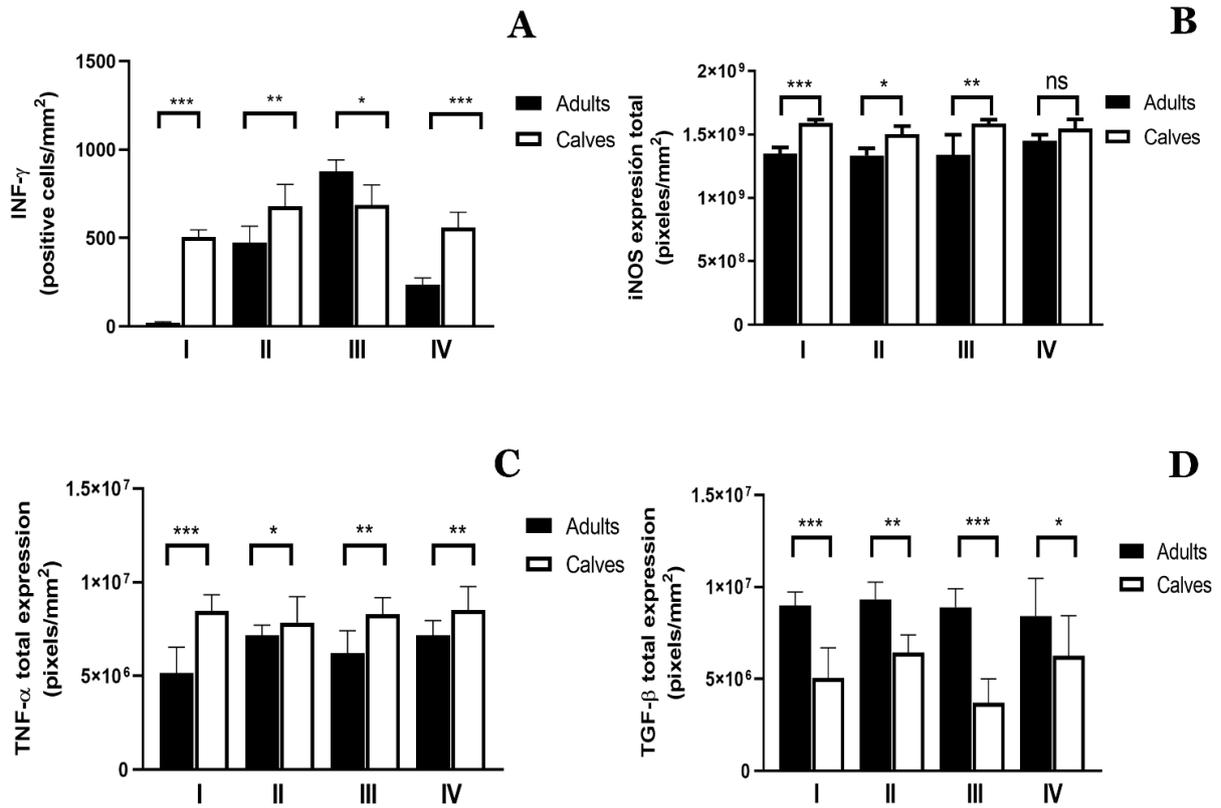
Supplementary Figure 5: Stages granulomas from calves show a more proinflammatory response than adults. A-D) Average expression of immunolabeling for IFN-γ, the inducible form of nitric oxide synthase (iNOS), TNF-α, and TGF-β, respectively, in stages granulomas from adults and calves; Mann-Whitney test * *P*< 0.05, * * *P*< 0.01, and * * * *P*< 0.001.

Atypical granuloma formation in *Mycobacterium bovis*-infected calves

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Abstract

Bovine tuberculosis is a chronic inflammatory disease that causes granuloma formation. Characterization of granulomatous lesions of *Mycobacterium bovis* (*M. bovis*) experimentally infected cattle has helped to better understand the pathogenesis of this disease. However, few studies have described granulomas found in *M. bovis* naturally infected cattle. The aim of this work was to examine granulomas from Holstein-Friesian cattle naturally infected with *M. bovis* from a dairy basin located in the central region of Mexico. Tissue samples from thirty-two cattle with lesions suggestive of tuberculosis were collected post-mortem. Fifteen of the 32 sampled animals (46.8%) were 4 months of age or younger (calves), whereas the rest (53.2%, 17/32) were over one year old (adults). Macroscopic lesions suggestive of tuberculosis were found in the mediastinal lymph node chain of all animals (32/32). From the 1,143 granulomatous lesions that were microscopically analyzed, 34.6% (396/1143) were collected from adult animals and subsequently classified according to the nomenclature suggested by Wangoo *et al.*, 2005. Surprisingly, lesions from calf tissues showed an atypical pattern which could not be fitted into the established developmental stages of this classification. Granulomatous lesions found in calves covered most of the affected organ, histologically showed large necrotic areas with central calcification, absence of a connective tissue capsule, and few giant cells. Also, there was a higher percentage of lesions with acid-fast bacilli (AFB) when compared to studied granulomas in adults. Growth of *Mycobacterium spp* was detected in 11 bacteriological tissue cultures. Genotypic identification of *M. bovis* was performed by DNA extraction from bacterial isolates, formalin-fixed and paraffin-embedded (FFPE) tissues and samples without bacterial isolation. *M. bovis* was detected by PCR in 84.3% (27/32) of the studied cases; whereas other AFB were observed in tissues of the remaining sampled animals (5/32). Our results describe atypical granuloma formation in calves 4 months of age or younger, naturally infected with *M. bovis*. These findings contribute to better understanding the physiopathology of *M. bovis* infection in cattle.

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Introduction

Bovine tuberculosis (bTB) is a disease caused by *M. bovis*. It affects a wide range of mammals including humans, creating public health problems as well as economic losses for the livestock industry, due to factors such as low milk production, organ condemnation during *post-mortem* examination, and costs of eradication programs. It has been estimated that there are over 50 million infected cattle worldwide, generating losses of close to 3 billion dollars per year [1–4]. bTB is characterized by a chronic inflammatory process, which mainly affects the lungs and lymph nodes associated with the respiratory system, characterized by formation of caseous and necrotizing granulomas. Pathological changes associated with the infection reflect an interaction between immune responses and pathogen virulence factors [5].

Granulomas are considered the pathognomonic lesions of tuberculosis. Their formation represents an attempt by the host to isolate and contain mycobacteria, as well as to limit further surrounding tissue damage by dampening chronic inflammation [6]. Granulomatous lesions caused by *M. bovis* have been extensively studied to determine mycobacterial growth control mechanisms by the host, as well as pathogen survival ability, in order to better understand pathogenesis of the disease [7]. Granulomas have been classified in *M. bovis* experimentally infected cattle according to morphological criteria such as degree of necrosis and mineralization, and presence of a connective tissue capsule [8]. Lesions and tissues surrounding granulomas have also been examined [9–11]. In addition, techniques such as immunohistochemistry (IHC) or laser capture microdissection in combination with real time quantitative polymerase chain reaction have been implemented to identify immunological cell composition of lesions [8,9]. However, little is known about macroscopic and microscopic characteristics of granulomas derived from *M. bovis* naturally infected cattle. Therefore, the aim of this study was to examine granulomatous lesions of *M. bovis* naturally infected Holstein-Friesian cattle. The four stages of granuloma formation previously described by Wangoo *et al.*, (2005) [8] were identified in 17 animals over one year of age. However, all granulomas found in tissues from 15 calves 4 months of age or younger, showed atypical structures.

Material and methods

Ethics statement

All procedures were reviewed and approved by the Ethics and Animal Welfare Committee of the Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México (CICUA, FMVZ-UNAM), and complied with the Mexican guidelines for animal research (JAGP-074).

Sample collection

Tissue samples presenting lesions suggestive of tuberculosis were collected with owner consent at post-mortem examination of animals. All cattle died from conditions that did not include tuberculosis. Animals were located at the central region of Mexico. Prevalence of bovine tuberculosis in this dairy basin is reported as being higher than 16% [12].

Macroscopic pathology

During necropsy examination the carcass was externally and internally inspected, and organ systems were removed. Samples of lymph nodes, lung tissue and individual organs presenting lesions suggestive of tuberculosis were collected. Tissues with lesions were divided in two halves; one was used for histopathological studies and the other for bacteriological cultures.

Histopathological analyses

Tissue segments presenting lesions suggestive of tuberculosis were fixed in 10% formaldehyde and paraffin-embedded. Serial sections of approximately four microns were used for staining with Hematoxylin and Eosin (H&E), Masson's trichrome, Ziehl Neelsen (ZN) and Von Kossa. Stained sections were examined under a CxL Labomed photonic microscope. Granulomatous lesions identified within H&E stained sections were classified in four developmental stages [8]. Acid-fast bacilli were quantified in ZN stained sections, using the 0–3 range scale where 0 = devoid of bacilli; 1 = 1–10 bacilli; 2 = 11–50 bacilli; and 3 = >50 bacilli [13]. Masson and Von Kossa trichromic stained sections were used to determine presence of connective tissue and degree of calcification respectively.

Bacteriological isolation

Bacteriological isolation of samples followed biosecurity conditions of Petroff's decontamination method [14]. Approximately 2 cm^2 of tissue from samples were individually macerated in a mortar with previously sterilized sand. Sand was decontaminated with 10% hydrochloric acid and 2 M sodium hydroxide. Macerates were centrifuged at 3,000 g for 20 min, supernatants were discarded and sediment was inoculated on Lowenstein Jensen and Stonebrink culture medium. Culture tubes were subsequently placed in the incubation chamber at 37°C for a period of 8–12 weeks. Media in samples were weekly assessed for microbial contamination. If contamination was deemed positive, tubes were discarded and the procedure repeated.

Extraction of genomic DNA of *Mycobacterium spp*

Genomic DNA was extracted from: (a) eleven cultures with suggestive growth of *Mycobacterium spp.*, (b) nine tissues from negative bacteriological growth cases, and (c) twelve formalin-fixed paraffin-embedded (FFPE) tissues that had histopathological lesions compatible with tuberculosis and presence of AFB. Samples were placed in 400 μl of TE solution (100 mM tris-HCl, 10 mM EDTA, pH 8), and inactivated at 80°C for 30 min. Fifty μl of lysozyme (10 mg/ml) was then added (SIGMA-Aldrich USA) and samples were incubated at 37°C for 16 h. A total of 75 μl of 10% SDS and 50 μl of K-proteinase (1 mg/ml) (SIGMA-Aldrich, USA) was subsequently added and samples were further incubated at 65°C for 10 min. Finally, 100 μl of CTAB (N-cetyl-N, N, N-trimetyl ammonium bromide) (SIGMA-Aldrich, USA) and 100 μl of 5 M NaCl was added and again incubated at 65° for 10 additional min. DNA was isolated by use of 700 μl of chloroform/isoamyl alcohol in a 24:1 ratio (SIGMA-Aldrich, USA). The liquid phase was recovered by centrifuging at 12,000 g for 5 min, and this step was repeated with one ml of chloroform/isoamyl alcohol. This phase was subsequently precipitated with 0.7 ml of absolute isopropyl alcohol (SIGMA-Aldrich, USA) and washed with 1 ml of 70% ethyl alcohol. The DNA pellet was then resuspended in 50 μl of nuclease-free water (GIBCO, Auckland, NZ) [15].

For DNA extraction from FFPE tissues, 12 micron sections were obtained by use of a microtome, which was cleaned with 70% alcohol between slices to avoid cross-contamination of samples. One ml of xylol was added to each tissue section which was then vortexed and incubated for 5 min. Xylol was subsequently decanted and tissue section was washed twice with absolute ethyl alcohol, allowed to dry and resuspended in 400 μl of TE and of lysozyme was then added continuing with the procedure previously described [15].

DNA concentration and purity were assessed by spectrometry (D.O. at 260/280 nm) using a Nanodrop spectrophotometer (ND-1000). Integrity of samples was evaluated by electrophoresis with a 0.7% agarose gel for 60 min at 80 volts.

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Polymerase chain reaction (PCR)

A nested PCR was performed to amplify the mpb70/m22 genes, which identifies members of the *Mycobacterium tuberculosis* complex. In addition, an endpoint PCR of the RD9 and RD4 genes was used to differentiate *M. bovis*, following manufacturer specifications of a commercial kit (TopTaq Master Mix Kit). A total of 50 ng of DNA sample were added to each reaction. Amplification protocols were performed on a thermal cycler (Thermo-Hybaid, USA). For conventional PCR, the used primers for the mpb70 gene that amplify a product of 372 bp were: mpb70 F (5′-GAACAATCCGGAGTTGACAA-3′) and mpb70 R (5′-AGCACGCTGTCAATCA TGTA-3′). In addition, for a second reaction to obtain a 208 bp product from the same gene, the M22 F (5′-GCTGACGGCTGCACTGTCGGGC-3′) and M22 R (5′-CGTTGGCCGGGCTG GTTTGGCC-3′) primers were used. For the RD9 gene, selected primers were RD9 F (GTGTAG GTCAGCCCCATCC), RD9 I (CAATGTTTGTTGCGCTGC) and RD9 R (GCTACCCTCGACCAAG TGTT), with a product of 333 bp for *M. tuberculosis* and 206 bp for *M. bovis*. and for RD4 gene the primers were RDF (ATGTGCGAGCTGAGCGATG), RD4 I (TGTACTATGCTGACCCATGCG) and RD4 R (AAAGGAGCACCATCGTCCAC), with a product of 268 bp for *M. bovis* and *M. bovis* BCG, for the rest of the members of the Mycobacterium tuberculosis complex a product of 172 bp is amplified [16–18]. Results of the PCR reactions were separated by electrophoresis in a 2% agarose gel, with TAE solution (40 mM Tris-acetate, 1 mM EDTA pH 8), SYBR Green (S9430 SIGMA-ALDRICH) for DNA staining, and a molecular weight marker of 100–1,500 bp (Ready to use DDL-001). The reaction product was visualized using a photo documenter (Gel Logic 200 Imaging System, Kodak, UK).

Statistical analyses

For statistical analyses the PASW Statistics 18 and the GraphPad prism 7.0 programs were used. Chi square test and Spearman correlation coefficient were used to identify if bacilli quantification scale was dependent on lesion stage, and if there was a correlation between variables.

Results

Identification of extensive granulomatous lesions in calves when compared to adult cattle

Tissue samples were collected post-mortem from 32 cattle that presented with macroscopic lesions suggestive of tuberculosis, later confirmed by histopathology. Mediastinal lymph nodes of all studied cattle presented suggestive tuberculous lesions macroscopically. Also, granulomas could be seen in lungs of 50% (16/32) of the animals. Lesions exclusively located in mediastinal lymph nodes were observed in 15.6% (5/32) of cattle, whereas the remaining animals presented lesions in more than one organ. It is noteworthy that 53.2% (17/32) of the sampled animals were over one year old, while the other 46.8% (15/32), were calves with ages ranging from one week to four months (**Table 1**). Since differences in macroscopic and microscopic characteristics of lesions between these two age groups were observed, our results were accordingly divided in: a) cattle over one year old (adults) and b) one week to four-month-old animals (calves).

Different lesion degrees were identified by gross pathology in mediastinal lymph node approximately 2-fold large than the normal ones as well a slight increase in size of a lymph node was observed in some instances, without apparent pathological changes when sectioned lesions observed in the adult animal group, aggregates of epithelioid cells were however identified histologically, with intercalating lymphocytes and multinucleated giant cells. Small foci of multifocal calcification were observed in other cases, as well as necrotic areas with abundant

Table 1. Distribution of granulomatous lesions in cattle naturally infected with *M. bovi*.

Case number	Age	Sex	Organs with lesions suggestive of tuberculosis						
			Lungs	Retropharyngeal LN	Mediastinal LN	Hepatic LN	Liver	Retromammary LN	Mesenteric LN
1	4 Years	F		+	+			+	
2	4 Years	F			+				
3	2 Years	F			+				
4	3 Years	F	+		+				
5	3 Years	F	+		+				
6	5 Years	F			+			+	
7	5 Years	F			+				
8	3 Years	F	+		+	+			+
9	6 Years	F	+		+			+	
10	3 Years	F		+	+			+	
11	2 Years	F		+	+			+	
12	6 Years	F			+				+
13	4 Years	F			+				
14	5 Years	F		+	+				
15	3 Years	F			+				
16	4 Years	F			+				
17	5 Years	F		+	+				
18	4 Months	F		+	+	+	+		
19	4 Months	F		+	+	+	+		+
20	4 Months	M	+	+	+	+	+		+
21	4 Months	F	+		+				
22	3 Months	F		+	+				
23	8 Days	F	+		+				
24	1 Month	F	+		+				
25	1.5 Months	F	+		+				
26	3 Months	F	+		+				+
27	3 Months	F	+		+				+
28	2 Months	F	+		+				
29	1 Month	F	+		+				
30	2.5 Months	F	+		+				+
31	1 Month	F	+		+				
32	3.5 Months	F	+		+				

F, Female; M, Male; NL, Lymph node; +, Organ with lesions suggestive of tuberculosis.

caseous material corresponding microscopically to granulomas with extensive necrosis, mineralization and abundant connective tissue (**Fig 1A and 1B**). Interestingly, lymph nodes presenting lesions suggestive of tuberculosis were approximately 2 to 5-fold larger in calves than those found in adult animals. Cut surface of affected organs showed multiple poorly delimited lesions with extensive white-caseous necrotic areas throughout the exposed anatomical plane. Histologically, extensive areas of necrosis and calcification were observed (**Fig 1C and 1D**). Intriguingly, 80% (12/15) of the calves showed extensive granulomatous lesions characterized by solid nodules, which had multiple white-caseous necrotic surface areas that were poorly demarcated and not surrounded by a connective tissue capsule. Also, coalescence between lesions could be observed (**S1 Fig**). In 23.5% (4/17) of animals with affected lungs,
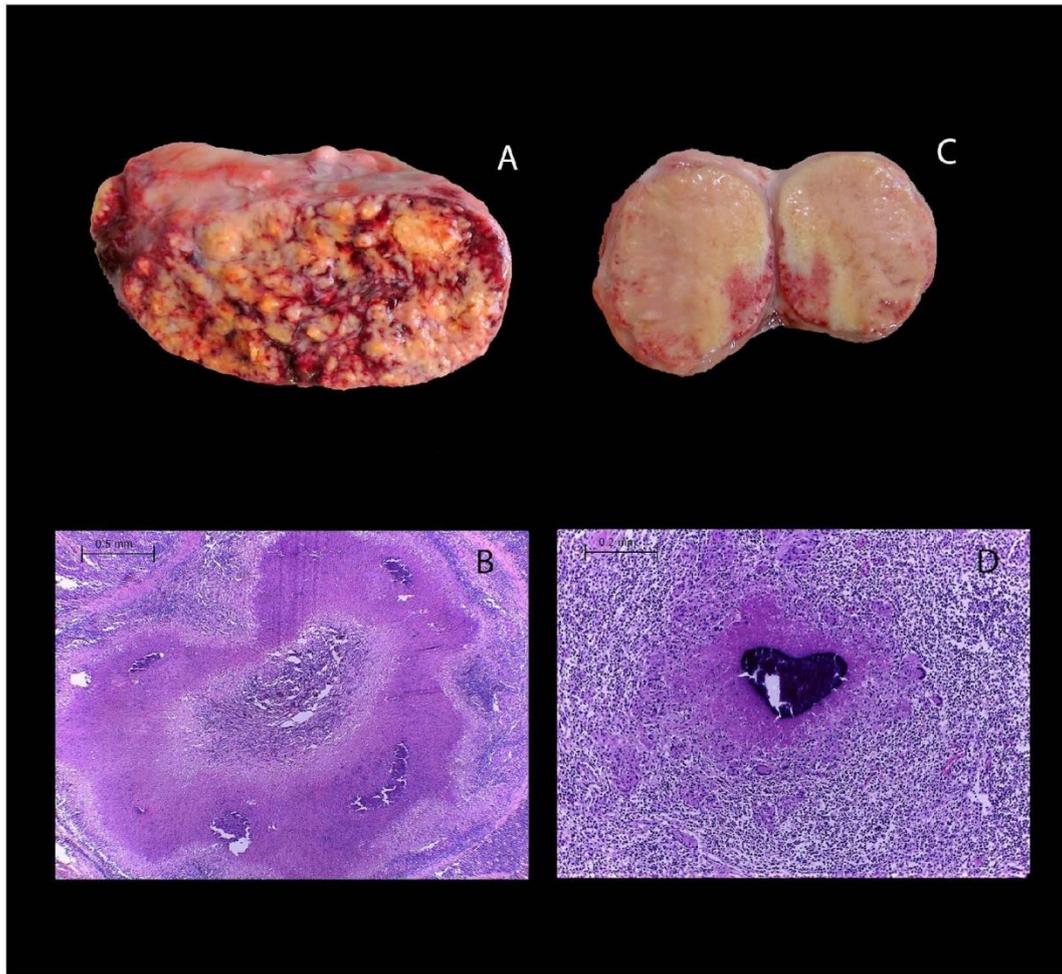
Fig 1. Extensive lesions suggestive of tuberculosis were observed in lymph nodes from calves naturally infected with *M. bovis* and compared to lesions found in adult cattle. (A) Mediastinal lymph node with severe granulomatous lymphadenitis and (B) H&E, 40x showing a granuloma encapsulated by connective tissue and abundant necrosis and mineralization from a 3-year-old Holstein-Friesian dairy cow. (C) Mediastinal lymph node with extensive areas of caseous necrosis and microhemorrhages and (D) H&E, 40x showing a granuloma without connective tissue and abundant necrosis and mineralization from a one-month-old Holstein-Friesian calf.

granulomatous lesions were identified both in pleura and parenchyma. Although lesion search followed pathologic protocols, presence of additional minute lesions in lung tissues cannot be ruled out.

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Granulomas in calves have abundant acid-fast bacilli (AFB)

A total of 203 FFPE tissues were microscopically analyzed. On average, 6.3 FFPE samples with their corresponding H&E stained slides were evaluated per animal. From the 1143 identified lesions suggestive of tuberculosis, 54.06% were found in mediastinal lymph nodes, in agreement with a greater number of macroscopic lesions being found in the same location. A greater number of lesions was observed in calves (65.35%, 747/1143), with an average of 49.8 granulomas per case, while the remaining 34.64% of lesions (396/1143) were found in adult cattle, with an average of 23.2 identified granulomas per animal (Fig 2A). In both groups a statistically significant association of a greater number of lesions with AFB was observed in comparison with the lesions without bacilli. Stage I had the highest percentage of lesions with bacilli in 41.47% (372/897) and the lowest was the stage III 9.7% (20/206) in adults and stage III-IV 9.8% (68/691) in calves (P = 0.0001). In addition, a greater number of AFB was observed within calf lesions, (55.28% of lesions with more than 50 bacilli, compared to 34.09% found in older cattle). Conversely, adult animals had a higher percentage of lesions without detectable AFB (47.9%), in contrast to only 7.4% found in calves (Fig 2B). Interestingly, identified granulomas from young animal tissues showed a lower number of multinucleated giant cells when compared to adult cattle (an average of 1.4 *vs* 14.5 cells per lesion respectively) (Fig 2C–2E). In addition, more bacilli were seen within giant cells from younger animal lesions (Fig 2F and 2G).

Calcium deposits in granulomatous lesions caused by *M. bovis* are independent of connective tissue capsule presence in calves

In adult cattle, classic granulomatous lesions showing necrotic areas with calcium deposits and a thick capsule of connective tissue surrounding the lesion were observed. However, granulomas identified in calves lacked a fibrous capsule. Absence of capsule was confirmed by Masson's trichrome staining. Indeed, no fibrous capsule surrounding any lesion from calf tissues was observed, although some granulomas showed disorganized fibroblasts within necrotic areas (Fig 3A). Calcium deposits were observed within necrotic areas of granulomas in both animal groups as black colored precipitates by Von Kossa staining. Remarkably, abundant areas of black color were seen even in the absence of fibrosis in some lesions from calves (Fig 3C and 3D).

Calves four months of age or younger showed atypical granulomas

All identified granulomatous lesions from the adult cattle group (396 in total), were classified: Stage I (initial) is characterized by aggregates of epithelioid macrophages, presence of giant Langerhans cells with interspersed lymphocytes, no necrosis and occasional neutrophil infiltrate; Stage II (Solid), mainly composed of epithelioid macrophages, there is a greater number of multinucleated giant cells than in the previous stage, lymphocyte presence and minimal caseous necrosis at the center of the lesion. Stage III (minimal necrosis), granulomas are fully encapsulated, with well-developed caseous necrosis and minimal mineralization. Stage IV (necrosis and mineralization), granulomas are surrounded by a thick capsule of connective tissue, there is extensive necrosis with areas of mineralization and adjoining epithelioid macrophages, presence of lymphocytes and multinucleated giant cells [8]. Most granulomatous lesions from adult cattle were classified as stage IV (34.3%, 136/396), presenting abundant connective tissue and calcification as shown by Masson's Trichrome and Von Kossa stains respectively. Stage I Granulomas (29.0%, 115/396) were mainly found as satellite lesions to more advanced stages (III and IV). Stage III granulomas were the least frequently observedv13.6% (54/396) (Fig 4A). Our results show a positive correlation between granuloma stage and giant

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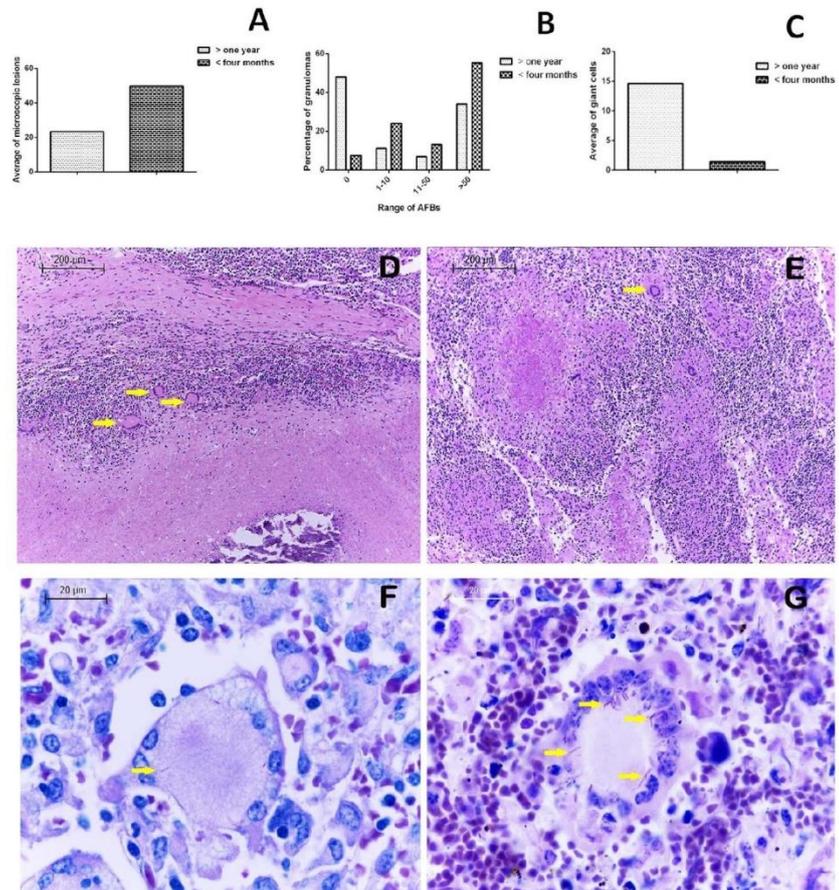
Fig 2. Calves had a greater number of granulomatous lesions and a higher bacilli scale when compared to adults. (A) Average microscopic lesions in cattle from both groups. (B) Granulomas with more than 50 bacilli are frequently seen in calves. (C) A lower average of giant cells is observed in calf lesions. (D) H&E, 40x, showing abundant giant cells (arrows) located between necrosis and fibrous capsule of a stage IV granuloma in adult cattle. (E) H&E, 400x, showing giant cells (arrows) close to an area with calcium deposits within a calf granuloma. Zielh Neelsen Stain (ZN) (F and G 1000x) (F) ZN stain showing a giant cell with intracytoplasmic bacilli in an adult bovine granuloma, and (G) ZN stain from a calf granulomatous lesion, showing a giant cell with abundant bacilli marked with arrows.

cell number (r = 0.74, p = 0.0001). Indeed, stage IV granulomas had the greater number of giant cells with an average of 32.9 cells/granuloma, followed by stage III lesions with an average of 12.4 cells, stage II with 3.8 cells and finally the lowest number of giant cells was found in stage I granulomas (0.6 giant cells/ lesion).

A total of 747 granulomatous lesions suggestive of tuberculosis were identified by histopathology in different tissues from calves. Lungs were the most affected organs (49.6% of the

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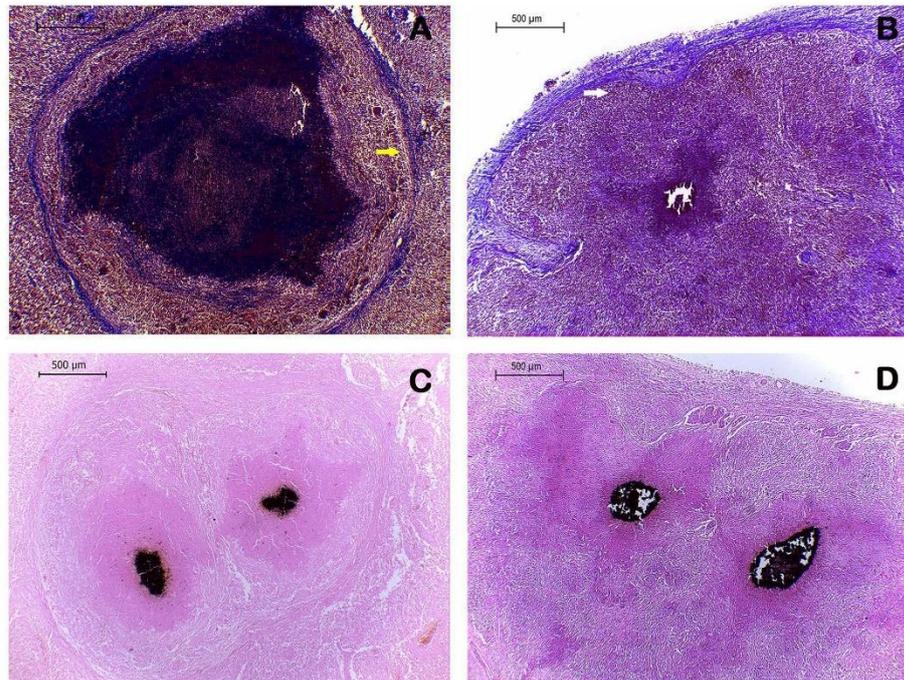
Fig 3. Calcium deposits in granulomatous lesions are independent of fibrous capsule presence in calves. (A) Masson's trichrome stained section showing a stage IV granuloma surrounded by abundant fibrous tissue (yellow arrow) in a bovine adult. **(B)** Masson's trichrome stained section showing a stage III-IV granuloma, without a fibrous capsule, but with surrounding disorganized collagen fibers in a calf. White arrow indicates connective tissue of the lymph node capsule as a reference. **(C and D)** Von Kossa staining, 200x, exhibiting stage IV (adult) and stage III-IV (calf) granulomatous lesions, with calcium deposits dyed in black.

lesions, 371/747), whereas only 4.9% of lesions were found in mesenteric lymph nodes (37/747).

Since characteristics of microscopic lesions found in animals four months of age or younger did not fully comply with the developmental stage classification proposed by Wangoo *et al.* (2005) [8], revisions were made to categorize lymph node and lung lesions in four modified stages designated as I, II, II-III and III-IV. Following this classification, stage I was the most frequently observed developmental degree of lesions from young animal tissues (47.2%, 353/747); whereas stage III-IV was the least frequently detected (9.3%, 70/747) (**Fig 4B**). Our suggested classification for granulomatous lesions from calves is briefly described below:

Stage I: Buildup of epithelioid macrophages, absence of capsule, central necrosis with cellular debris; neutrophils can be present (**Figs 4C and 5A**). **Stage II**: Numerous epithelioid macrophages with lymphocyte infiltrate; occasional giant cells and extensive necrosis and cellular debris (**Figs 4D and 5B**). **Stage II-III**: Extensive necrosis and cellular debris with poorly delimited borders, absence of a fibrotic capsule or adjoining peripheral lymphocytes, and a few macrophages or giant cells in the periphery (**Figs 4E, 5C, 5E and 5F**). **Stage III-IV**: Extensive

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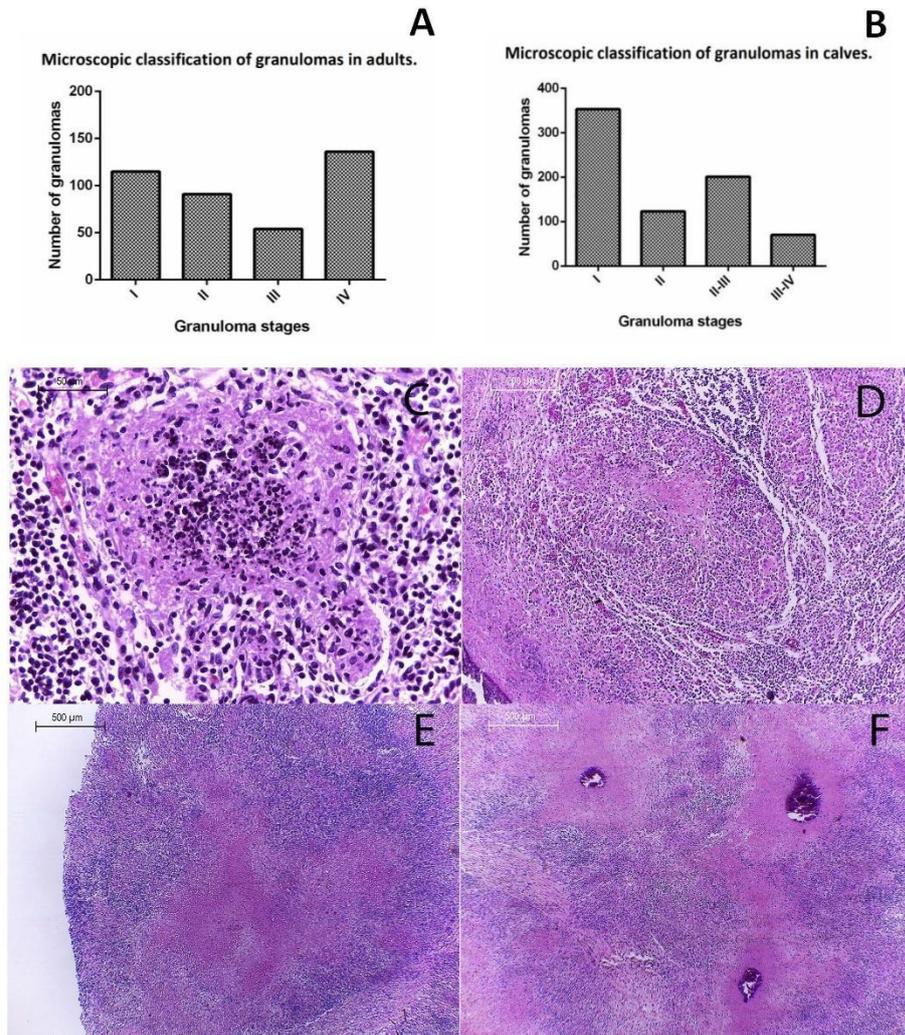
Fig 4. Histopathological classification of granulomas found in lymph nodes from calves four months of age or younger. (A) Graph of granuloma stages identified in adults, showing a higher frequency of stages I and IV. (B) Graph showing a higher frequency of stage I granulomas in calves. H&E. (C) **Stage I:** 400X, small diffuse foci of infiltrated inflammatory cells, mainly macrophages, epithelioid macrophages and abundant cellular detritus at the center of the lesion. (D) **Stage II:** 200X, Larger structure with epithelioid macrophages, multinucleated giant cells, cell debris and necrosis. (E) **Stage II-III:** 40X, large necrotic areas without borders, absence of connective tissue capsule or lymphocyte accumulation in the periphery. (F) **Stage III-IV:** 40X, lesions showing extensive necrotic areas, mineralization and cell debris without defined borders, surrounded by macrophages, absence of connective tissue capsule or lymphocytes in the periphery; lesions tend to coalesce and fuse.

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necrosis with calcification and cell debris without defined borders; numerous peripheral macrophages, absence of fibrous capsule and few lymphocytes; lesions tend to coalesce (**Figs 4F and 5D**).

Bacteriological isolation and molecular genotyping of mycobacterial infection by PCR from tissues presenting lesions suggestive of tuberculosis in naturally infected animals

Suggestive growth of *Mycobacterium spp.* was observed in 55% (11/20) of the processed samples. Subsequently, we performed DNA extraction and assessed by PCR for molecular
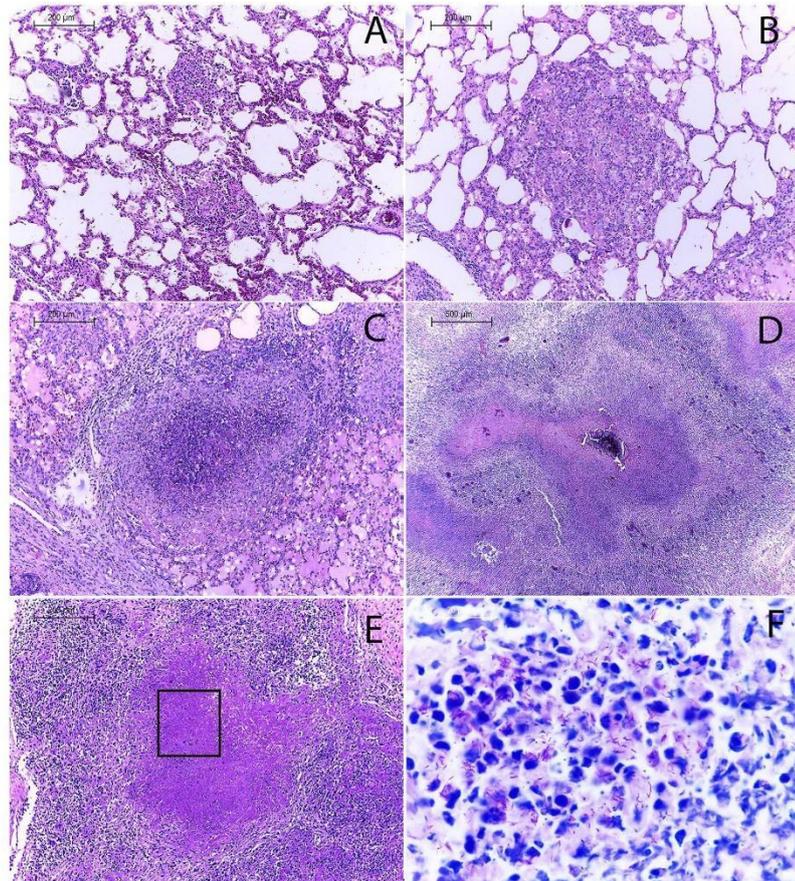
Fig 5. Histopathological classification of granulomas found in lung tissue from calves. H&E (A.-C 200x, D-E 40x F.-1000x). (A) Stage I. (B) Stage II. (C) Stage II-III. (D) Stage III-IV. (E) Close up of stage II-III. (F) Zielh Neelsen 1000x with abundant extracellular acid-fast bacilli in stage II-III.

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genotyping of *M. bovis* of: eleven isolates, from nine tissues of culture-negative cases and twelve FFPE tissues. All these cases presented macroscopic and microscopic lesions compatible with tuberculosis, as well as the presence of AFB. The mpb70 / m22, RD9 and RD4 genes were used for PCR amplification. Of these 84.3% (27/32) amplified a product of genes Mpb70 / m22 and RD9. Product amplification of gene RD4 was possible in 46.8% (15/32) samples (**Table 2**) and (**Fig 6A and 6B**).

The deletion of RD9 is observed not only in *M. africanum* and *M. bovis* but also in other mycobacteria like *M. caprae*, *M. microti* and *M. canettii*. However, with the exception of *M. bovis* those species have never been reported in Mexico.

Table 2. Bacteriological isolation and molecular genotyping of *M. bovis*.

Case number	Age	Sex	Histology compatible with tuberculosis	Presence of AFB	Bacteriological isolation	PCR genes		
						MPB70/M22	RD9	RD4
1	4 Years	F	+	+	−	+	+	−
2	4 Years	F	+	+	−	+	+	+
3	2 Years	F	+	+	+	+	+	+
4	3 Years	F	+	+	−	−	−	−
5	3 Years	F	+	+	+	+	+	+
6	5 Years	F	+	+	−	−	−	−
7	5 Years	F	+	+	−	+	+	+
8	3 Years	F	+	+	+	+	+	+
9	6 Years	F	+	+	+	+	+	+
10	3 Years	F	+	+	+	+	+	+
11	2 Years	F	+	+	+	+	+	+
12	6 Years	F	+	+	+	+	+	+
13	4 Years	F	+	+	−	−	−	−
14	5 Years	F	+	+	−	+	+	−
15	3 Years	F	+	+	−	+	+	−
16	4 Years	F	+	+	−	+	+	−
17	5 Years	F	+	+	+	+	+	+
18	4 Months	F	+	+	+	+	+	+
19	4 Months	F	+	+	+	+	+	+
20	4 Months	M	+	+	+	+	+	+
21	4 Months	F	+	+	N/A	+	+	−
22	3 Months	F	+	+	N/A	+	+	+
23	8 Days	F	+	+	N/A	+	+	+
24	1 Month	F	+	+	N/A	−	−	−
25	1.5 Months	F	+	+	N/A	−	−	−
26	3 Months	F	+	+	N/A	+	+	−
27	3 Months	F	+	+	N/A	+	+	−
28	2 Months	F	+	+	N/A	+	+	−
29	1 Month	F	+	+	N/A	+	+	−
30	2.5 Months	F	+	+	N/A	+	+	−
31	1 Month	F	+	+	N/A	+	+	−
32	3.5 Months	F	+	+	N/A	+	+	−

F, Female; M, Male; N/A, not applicable; AFB, Acid-fast bacilli; +, positive result; −, negative result.

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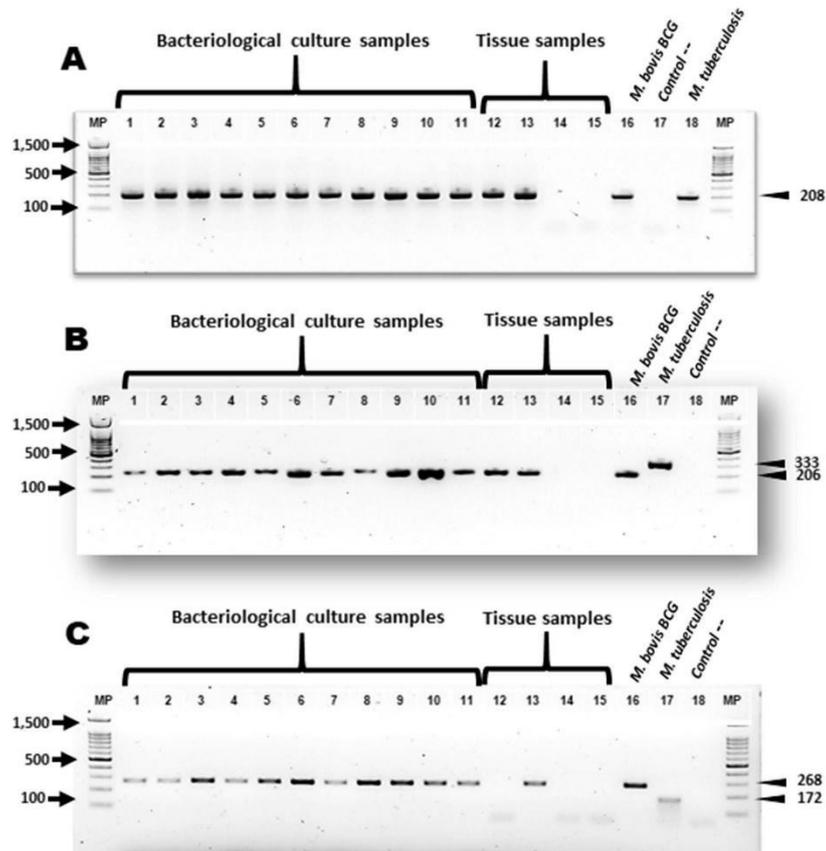
Fig 6. Molecular identification of *M. bovis* in naturally infected cattle. (A) Nested PCR amplification products of the 208 bp mpb70 gene. (B) Endpoint PCR of the 206 bp fragment from the *M. bovis* RD9 gene and the 333 bp fragment from the *M. tuberculosis* gene. (C) Endpoint PCR of the 268 bp fragment from the *M. bovis* RD4 gene and the 172 bp fragment from the *M. tuberculosis* gene **MP** molecular weight marker is 100 bp; lanes 1–11: bacteriological culture samples; lanes 12–15: negative tissue samples; and lanes 16–18: positive controls (*M. bovis* BCG and *M. tuberculosis* H37rv) and negative control.

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Discussion and conclusions

There are several studies in cattle experimentally infected by *M. bovis* that have focused on describing anatomic and pathological characteristics, including distribution of lesions and histological distinction of granulomas. However, fewer studies have detailed descriptions of granulomatous lesions in naturally infected cattle. In this study, lesions suggestive of tuberculosis from 32 Holstein-Friesian cattle naturally infected by *M. bovis* were macroscopically and microscopically evaluated. Considering characteristics of lesions, results were divided in two groups according to animal age, where the adult cattle group included animals over one year

old (53.2%, 17/32 animals), while the young animal group included calves four months of age or younger (46.8%, 15/32 animals). Interestingly, gross pathology assessment of lesions from lungs and lymph nodes from calves showed extensive consolidation with coalescent necrotic areas. Further histopathological evaluation evidenced extensive necrosis, calcification and absence of a connective tissue capsule, as well as abundant mainly extracellular acid-alcohol resistant bacilli. These findings can relate to previous reports where granulomatous lesions in lungs, liver and lymph nodes from calves under 45 days old and naturally infected by *M. bovis* where severely disseminated with extensive necrotic areas. Abundant necrosis with AFB presence in lung lesions was also microscopically identified [19–20].

Bovine tuberculosis is considered a disease that mainly affects the airways. In naturally infected cattle, lungs and associated lymph nodes are the most frequently concerned organs at post-mortem examination [20]. This study shows that mediastinal lymph nodes were involved in 100% (32/32) of the animals that presented lesions suggestive of tuberculosis, and lungs were affected in 50% of cattle (16/32). These results agree with previous reports and underline the importance of aerial passages as pathways for mycobacterial entry. A thorough pathological analysis of lungs was performed in this study, however due to the large size of these organs, some lesions could have been missed. In the four months of age or younger group 80% of animals (12/15) showed lung lesions that were easily detected macroscopically. More than 50% of the lung parenchyma was affected in some instances, likely indicating the airways as route of infection. Frequency of lung involvement in calves was unexpected since it is commonly thought that young animal lesions are mainly found in mesenteric lymph nodes consequent to intake of contaminated milk [21]. In this study, 33.3% (5/15) of calves presented lesions in mesenteric lymph nodes, and 80% (4/5) of which also had affected lungs. Therefore, possibility of a simultaneous infection through both routes cannot be ruled out. Nonetheless, *M. bovis* can also be transmitted through the congenital route, which has been previously reported in 15 to 25-day old calves [19–21]. Detection of granulomatous lesions in a young 8-day-old calf included in this study, could indicate congenital transmission as possible rout of infection. Therefore, bovine tuberculosis should be included when performing a differential diagnosis in young animal necropsies [21].

Different developmental stages of granuloma formation could be found within the same organ, during histological assessment of tissue sections in this study. Presence of different microenvironments within the same tissue has been previously suggested in studies with cattle experimentally infected with *M. bovis* [22–23]. A total of 396 microscopically identified granulomas from the adult animal group, were categorized [8]. Most were stage IV lesions, surrounded by stage I satellite granulomas. In this study, 136 stage IV granulomas were identified in adults, of which 63.9% (87/136) did not present AFB and only 17.64 (24/136) had more than 50 bacilli per granuloma. Stage IV lesions have been previously associated with a large number of bacilli in experimental infections with *M. bovis*. Conversely, in naturally infected adult animals, presence of a connective tissue capsule surrounding lesions could indicate a better control of disease development by the host [22,24–27].

Predominance of stage IV granulomas in naturally infected animals indicate a chronic process involving an anti-inflammatory immune response, that can also be related to the fibrosis that is observed surrounding lesions. Macroscopical and microscopical characteristics of lesions found in calves differed from those observed in adult tissues, precluding granuloma classification for the former age group according to stages set by Wangoo *et al.*, (2005) [8]. Revised the established categories, suggesting four modified stages (I, II, II-III and III-IV) to classify calf lesions. According to this new classification, most of the identified granulomas in calf tissues were stage I lesions (47.2%, 353/747); whereas stage III-IV granulomas were the least frequent (9.3%, 70/747). Stage III-IV lesions showed calcification and absence of a fibrous

capsule. The average number of giant cells per lesion was 1.4 compared to 14.5 cells/granuloma in stage IV lesions observed in adult cattle.

Interestingly, atypical pattern of granulomatous lesion formation has also been reported in in C3HeB/FeJ mice infected with *M. bovis*. In these rodents, lesions were characterized by extensive necrotic areas, presence of neutrophils, disorganized fibrous capsule formation, and abundant bacilli that caused death by 5 weeks post-infection [28]. Lesions that develop after natural infection of young cattle with *M. bovis*, also present traditional markers of acute infection, such as neutrophil presence and an exacerbated pro-inflammatory response. However, repeatability of these results needs to be validated in further studies under different conditions. Nonetheless, we submit an adapted classification for stages of granuloma formation in young animals naturally infected with *M. bovis*.

In conclusion, this study identified a large number of granulomatous lesions in mediastinal lymph nodes and lungs of cattle naturally infected with *M. bovis*, suggesting the airways as the main route of entry for mycobacteria during natural infection. Stage IV granulomas are the most frequently found lesion in naturally infected animals over one year old, in accordance with the chronic nature of the disease. Finally, calves four months of age or younger presented lesions with atypical macroscopic and microscopic characteristics, which were more abundant and had a greater number of associated bacilli when compared to adult cattle. These findings contribute to better understand the physiopathology of *Mycobacterium bovis* infection in cattle.

Supporting information

S1 Fig. Severe granulomatous pneumonia in calves. Lung surface sections from calves aged four months or younger with granulomatous pneumonia, showing extensive white areas lacking delimited edges, which may coalesce.
(TIF)

Acknowledgments

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Writing – review & editing: Elizabeth Morales-Salinas, Mario A. Bedolla-Alva, Rogelio Hernández-Pando.

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CASE REPORT

Open Access

Ocular tuberculosis in a calf.

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Abstract

Background: Bovine tuberculosis is a chronic inflammatory disease that causes granuloma formation mainly in retropharyngeal, tracheobronchial, mediastinal lymph nodes and lungs of bovines. The presence of these lesions in other tissues such as the eyeball is very rare and difficult to diagnose. This study describes macroscopic and microscopic pathological findings in a calf with ocular and meningeal tuberculosis.

Case presentation: March 2019, an eight-month-old Holstein Friesian calf was identified in a dairy farm located in central Mexico with a clinical cough, anorexia, incoordination, corneal opacity and vision loss. At necropsy, pneumonia, lymphadenitis, meningitis, and granulomatous iridocyclitis were observed. The histopathological examination revealed granulomatous lesions in lung tissue, lymph nodes, meninges and eyes with the presence of acid-fast bacilli associated with *Mycobacterium spp.*

Conclusion: To the best of our knowledge, this is the first report that describes macroscopic and microscopic pathological findings of ocular tuberculosis in cattle. This report highlights the importance of considering bovine tuberculosis in the differential diagnosis of corneal opacity and loss of vision in cattle.

Keywords: Bovine tuberculosis, Meningeal tuberculosis, Ocular tuberculosis, Ocular granuloma

Background

Presence of extrapulmonary tuberculosis in humans in industrialized countries has increased from 16% in 1993 to 21% in 2006 [1]. The most lethal of these presentations is meningeal tuberculosis with an estimate of more than 100,000 cases per year [2]. Central nervous system involvement is a factor for the development of ocular tuberculosis, a very rare presentation in the population [3]. Likewise, cases of ocular tuberculosis in natural infections in animals are limited. Among the species susceptible to tuberculosis are cattle affected by *Mycobacterium bovis* (*M. bovis*) with a high prevalence in different parts of the world, showing similarities in immunopathology with tuberculosis in humans, which highlights the importance of this species as a model for studying ocular presentation [4–8]. Bovine tuberculosis frequently causes granulomatous lesions in the retropharyngeal, tracheobronchial, mediastinal lymph nodes, and lungs of cattle. Other less frequently affected organs are the regional lymph nodes, spleen, liver, kidney, intestine, mesenteric lymph nodes, vertebrae, and spinal cord. However, ocular involvement has rarely been reported [6, 8]. In this case report we present for the first time the macroscopic and microscopic pathological description of ocular tuberculosis in a calf.

Case presentation

An eight-month-old Holstein Friesian calf from a stable with 220 cattle located in a complex of approximately 28,000 dairy cattle, with an intensive production system in the central area of Mexico, presented cough, anorexia, incoordination and loss of vision. The calf was referred to the Centro de Enseñanza y Diagnóstico en Enfermedades de los Bovinos of the Facultad de Medicina Veterinaria y Zootecnia at Universidad Nacional Autónoma de México, where the post mortem examination was performed. Bovines from this stable had previously been

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Fig. 1 Meningeal tuberculosis in a bovine. Bovine brain with multifocal nodular granulomas in the meninges indicated by arrows

identified with lesions compatible with bovine tuberculosis.

Body inspection at necropsy revealed a carcass with pale conjunctival and oral mucosa. On internal inspection, the cranial, intermediate, accessory, and cranioventral lobes of the lungs were hyperemic and consolidated. The mediastinal lymph nodes were enlarged and extensive areas of yellow foci with granulomatous inflammation and a central core of caseous necrosis were identified. The leptomeninges presented many white nodules corresponding to granulomas in the cerebral hemispheres located mainly at the base of the brain (Fig. 1). Corneal opacity was observed in both eyes, the ciliary processes show thickening with nodular coalescing granulomas (Fig. 2).

Tissue segments from brain, eye, lymph node, and lung were fixed in 10% formaldehyde and paraffin-embedded for routine histological staining. A granulomatous inflammatory infiltrate was observed in the leptomeninges, mainly composed of macrophages, epithelioid macrophages, and lymphocytes. In some areas, granulomas were identified with necrotic center, surrounded by epithelioid macrophages, abundant multinucleated giant cells, interspersed with lymphocytes, plasma cells and few fibroblasts; some lesions contain central mineralization surrounded by numerous epithelioid macrophages (Fig. 3a). Bacilli were identified in areas with necrosis and in the cytoplasm of macrophages by Ziehl Neelsen (ZN) staining and immunohistochemistry (Figs. 3b and 4). The eyeball showed normal anatomic loss at the level of the lens, which was replaced by granulomatous lesions with similar characteristics as the lesions found in the meninges, showing rare neutrophils and a greater amount of connective tissue around the lesions. The choroid showed multifocal granulomas with acid fast bacilli in the macrophage cytoplasm and central mineralization identified by Von Kossa stain (Fig. 5a and b). In the mediastinal lymph nodes and lungs, granulomatous lesions with the characteristics previously described were also identified.

PCR using template DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues that had histopathological lesions compatible with tuberculosis was performed [9]. We used a universal set of primers

Fig. 2 Ocular tuberculosis in a bovine. Corneal opacity in both eyes. Cross section of the eye shows anterior uvea thickening with nodular yellow coalescing granulomas indicated by arrows
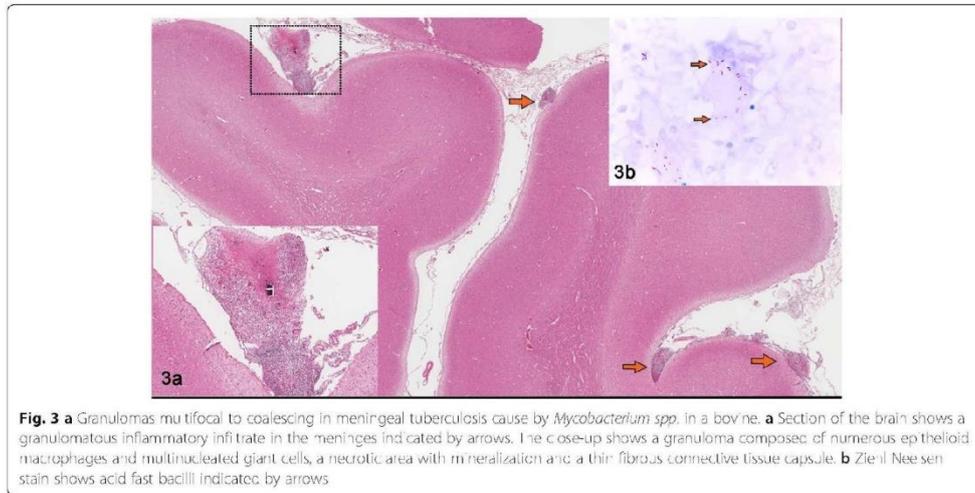
Fig. 3 a Granulomas multifocal to coalescing in meningeal tuberculosis cause by *Mycobacterium spp.* in a bovine. **a** Section of the brain shows a granulomatous inflammatory infiltrate in the meninges indicated by arrows. The close-up shows a granuloma composed of numerous epithelioid macrophages and multinucleated giant cells, a necrotic area with mineralization and a thin fibrous connective tissue capsule. **b** Ziehl Neelsen stain shows acid fast bacilli indicated by arrows

spanning for V1-V3 short variable regions of the bacterial 16S rRNA gene that amplify a product of ~ 500 bp. A nested PCR for mpb70/m22 genes was used to identify members of the *Mycobacterium tuberculosis* complex, a positive result produces amplicons of 372 bp and 208 bp of length, respectively. In addition, an endpoint PCR of the RD9 with a product of 333 bp for *Mycobacterium tuberculosis* (*M. tuberculosis*) and 206 bp for *M. bovis* was

Fig. 4 Immunohistochemistry of *Mycobacterium tuberculosis*. Shows a giant cell with cytoplasmic bacilli indicated by arrows in a section of the meninges

performed. Finally, the RD4 gene was used to amplify a product of 268 bp for *M. bovis* and 172 bp for the rest of the members of the *Mycobacterium tuberculosis* complex. All primer sequences used are described in Table 1. Electrophoresis in a 2% agarose gel with SYBR Green (S9430 SIGMA-ALDRICH) was used for separated the products of DNA and next were visualized using a photo documenter (Gel Logic 200 Imaging System, Kodak, UK). Unfortunately, despite carrying out the different PCR protocols, it was not possible to obtain amplifications from the tissues of this case.

Discussion and conclusions

According to the macroscopic findings at necropsy and laboratory tests performed, this case report describes a calf with bovine tuberculosis. The main finding was the presence of mycobacterial granulomatous lesions in the brain and both eyes. Although we attempted to identify the mycobacterium species by isolation or by polymerase chain reaction (PCR), all our efforts were negative. Therefore, we decided to use a polyclonal antibody against *M. tuberculosis* (biocare medical, CP 140) which cross reacts with members of the tuberculosis complex. Immunohistochemistry revealed a positive staining of mycobacterial antigens in the granulomatous lesions. Furthermore, *M. bovis* has previously been isolated in the calf herd and prevalence of bovine tuberculosis was greater than 16%, altogether our results suggests *M. bovis* as the etiological agent [14, 15]. The pathogenesis of ocular tuberculosis is unknown, until now it is believed that the spread of the bacteria originates from the primary site of infection to the eyeball by hematogenous
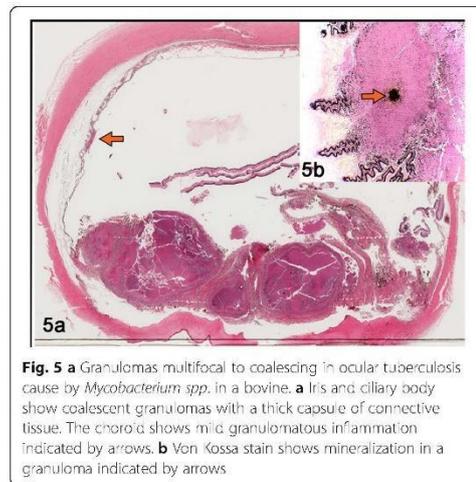
Fig. 5 a Granulomas multifocal to coalescing in ocular tuberculosis cause by *Mycobacterium spp.* in a bovine. **a** Iris and ciliary body show coalescent granulomas with a thick capsule of connective tissue. The choroid shows mild granulomatous inflammation indicated by arrows. **b** Von Kossa stain shows mineralization in a granuloma indicated by arrows

route, frequently presenting granulomas in the choroid [8]. Endogenous infection of the eyeball by *M. bovis* has been identified in adults or immunocompetent people [16, 17]. Likewise, this affectation was reported in 3.2% (9/282) of patients who presented disseminated tuberculosis when receiving immunotherapy with Bacillus Calmette-Guérin (BCG) as a treatment for bladder cancer, causing uveitis, endophthalmitis and autoimmune retinopathy. This presentation differs from destructive intraocular tuberculosis caused by *M. tuberculosis*, which mainly originates choroidal granulomas and subretinal abscesses [18, 19].

Study of ocular tuberculosis in naturally infected animals will provide a better understanding of bovine tuberculosis pathogenesis. This report highlights the importance of considering bovine tuberculosis in the differential diagnosis of corneal opacity and loss of vision in cattle.

Abbreviations
FFPE: Formalin-fixed paraffin-embedded; HE: Hematoxylin and eosin staining method; PCR: Polymerase chain reaction; ZN: Ziehl-Neelsen staining method

Authors' contributions
MABA: Responsible for the necropsy and macroscopic description. MABA, MJR and JCU: Microscopic description of the lesions. JCU and MJR: performance of immunohistochemistry. JCU and JAGP: Analysis of laboratory results and were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Table 1 The primers and sequencing used for PCR

Primers	Sequence (5' → 3')	Reference
16S-F	TTGGAGAGTTTGATCMTGGCTC	[10]
16S-R	GTATTACCGCGGCTGCTG	
MPB70-F	GAACAATCCGGAGTTGACAA	[11]
MPB70-R	AGCACGCTGTCAATCATGTA	
M22-F	GAACAATCCGGAGTTGACAA	[11]
M22-R	CGTTGGCGGGGCTGGTTTGGCC	
RD9-F	GTGTAGGTCAGCCCCATCC	[12]
RD9-I	CAATGTTTGTTGCGCTGC	
RD9-R	GCTACCCTCGACCAAGTGTT	
RD4-F	ATGTGCGAGCTGAGCGATG	[13]
RD4-I	TGTACTATGCTGACCCATGCG	
RD4-R	AAAGGAGCACCATCGTCCAC	

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