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**The myeloid C-type lectin receptor
Mincle is a multifunctional sensor in
immunity and homeostasis**

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Madrid, 2019

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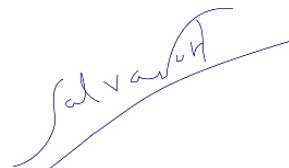
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A mi familia, a mis amigos, y a choco

***Discovery consists of looking at the same thing as everyone else
and thinking something different***

Albert Szent-Gyorgyi

Somewhere, something incredible is waiting to be known

Carl Sagan

Acknowledgements/Agradecimientos

ACKNOWLEDGEMENTS/ AGRADECIMIENTOS

Gracias a David, mi padre científico, por darme la confianza y la libertad necesarias para crecer en esta etapa, sin dejar de vigilarme de cerca. Por ser sincero conmigo, diciéndome lo bueno, pero también lo malo, por aportarme la paciencia que no tengo, por ver en mis defectos enormes virtudes “que te recordaban a ti cuando hacías tu tesis” y ver cosas en mí que yo ni siquiera imaginaba que tenía. Siempre recordaré cuando me enseñaste a inyectar subcutáneo a tu modo “tienda de campaña” cuando el laboratorio estaba aún empezando a nacer. Sin todo lo que tú has conseguido y estás consiguiendo, esta tesis no hubiese sido posible. Gracias por ayudarme a entender lo difícil que es a veces ser “el jefe”. Me voy sabiendo “que no has cortado el cordón” y eso me tranquiliza.

A Salva, y aquí tendría que hacer otra tesis sobre “la probabilidad existente de encontrar a alguien así a lo largo de la vida” o sobre “cómo ponerle palabras a algo que está en el corazón”. Gracias por ser mi amigo, mi “tor-mentor”, mi profesor, mi padre, mi paño de lágrimas, mi compañero de desayunos, mi técnico, mi PhD, mis postdoc, mi amuleto de la suerte, mi compañero de congresos, mi consejero matrimonial, mi psicoterapeuta, mi coach, y un largo etcétera. Gracias por hacer que 7 años parezcan 7 días, por aguantar “el tengo hambre” y el reggaetón, por las conversaciones variopintas sobre “pepitillas”, “*Duos habet et bene pendentes*”, criogenización *post mortem*, teoría de la panspermia, filosofía budista, gastronomía y lengua murciana, “grandes popes de la microbiota” y por poner “ojete calor” en esos días eternos de animalario y experimentos interminables desde las 6 a.m. Por ser tan “foodie” y tan “grumet” como yo, por desarrollar el “filtrado con filtros de té” para incrementar la viabilidad celular un 150 % , por inspirarme cada día a ser mejor, a saber más, a querer seguir aprendiendo, para poder algún día estar a tu altura, por pensar que soy algo que yo ni creía, por confiar en mí, por regalarme tantos momentos inolvidables, por estar ahí cuando más lo necesitaba, por alegrar mi existencia, por los paseos a Chamartín, por tu paciencia, por tu generosidad infinita y por ser tan buen científico y mejor persona.

A Ruth, porque sin ella nada de esto tendría sentido, por ser “vieja y sabia” como la “huérfana” siendo tan joven, por tu destreza, tu ilusión, tu compromiso, tu dedicación, tu sonrisa, tu optimismo, por tus ganas de aprender y dejarte enseñar, por lo fácil que ha sido trabajar contigo, por saber dónde he dejado todo sin ni siquiera decirlo, por “el sí cabe” aunque no quepa, porque el “medio de *Leishmania* no era tan naranja”, por rescatarme en momentos de pánico, por hacer de enfermera si aparezco con el dedo pequeño “en carne viva”, por los cafés y los pitillos, por los días de montaditos y sureña arreglando el mundo, por los viajes juntas, por las comidas fuera del CNIC con cualquier excusa, por llorar, reír, cantar y bailar conmigo, por ser mi amiga, por los consejos, por las confidencias, por hacer justo la mitad de mi tesis, por preocuparte de cada

detalle, por protegerme, por cuidarme, por enseñarme, porque esas manitas que tienes son increíbles, pero más increíble es tu corazón.

A los miembros pasados y presentes del laboratorio, porque cada uno de ellos han dejado un poquito de sí mismos en mí y en este trabajo. A Noelia, mi gallega, por ser como mi madre durante estos años, gracias por todos los momentos inolvidables que hemos vivido y por los que nos quedan por vivir, gracias por tu dulzura, por cuidarme, por cogerme de la mano en el verano del 2010 y no haberme soltado todavía. Nunca olvidaré los días interminables de cultivos, el congreso de Badajoz, mi primera membrana “carbonizada”, los días como “pollo sin cabeza”, la manera de pronunciar “SHIP”, los viajes nocturnos hablando del futuro en el 147, el primer día que “hablaste murciano” con ese acento tan tierno, solo puedo decir que supe lo que era la “morriña” cuando te volviste a Galicia. A Gonzalo, el hombre de los tirantes, por haber iniciado conmigo esta aventura, por las veces que cantamos por Rocio Jurado y nos reímos de todos y de todo, por hacerme ver que la opinión de los demás no es más importante que la de uno mismo. A Jaime, por ser tan buenísima persona, por momentos inolvidables cuando “cazabas” ratones que no eran tuyos en el animalario, por enseñarme que tirar la toalla nunca es una opción y que siempre encuentras unos cuantos “cilios” a los que agarrarte. A Laura, por las comidas en la terraza del CNIO los sábados de algún verano, por las conversaciones sobre bares y exposiciones molonas, por tu comprensión, tu ayuda y tus consejos siempre que los he necesitado. A Michel y Helena, por compartir conmigo estos años con sus buenos y malos momentos. A Johan, el gabacho bohemio, porque aunque nunca lo pretendió, dejó un poso en mi corazón. A Chechu, por enseñarme que la ciencia también puede ser divertida y hacerme ver que el dibujo no era lo mío. A Fran, por sus chistes que, aunque malos, te alegran un mal día, por el “soy Fran que he vuelto de vacaciones”, por enseñarme que las primeras impresiones son solo eso. A Carlos, por sus consejos en el desarrollo de la tesis. A Paula, por prestarse como “conejiillo de indias” y ser el primer estudiante de tesis al que he intentado ayudar. A Sarai, por su optimismo, por las charlas en el muelle, y por acompañarme desde casi el principio. A Joaquín, por hacerme reír y porque con su incorporación este labo es cada vez más parecido a vivir en Murcia. A Sofía, por abrir mi mente y mostrarme otra manera de ver las cosas, por quedarte a dormir en mi “mini” casa cuando la casa me parecía enorme, por parar “el cercanías” a paraguazos y por estar a mi lado durante este tiempo. A Paola, per il suo corso espresso di italiano e conversazioni che illuminano l'anima. To Steffi, to help and advise me to look for a postdoctoral position sharing with me her experience and all that I have needed. Thank you to teach me that the illusion to get a dream is stronger than the bad times, “Danke kumpel”. A Nacho, porque aunque corto, el tiempo compartido ha sido muy divertido. A Annalaura, por aparecer y llenar de “glamour y metabolómica” este labo, gracias por contribuir de manera esencial a parte de esta tesis y por saber ver más allá del estudiante de tesis y preocuparte por la

persona. A Elenita, porque eres un “ser fitness de luz”, por llenarnos de k fir y de consejos sobre nutrici3n y deporte, por la de felicidad y tranquilidad que irradias desde tu mesa.

A los laboratorios vecinos por aguantarme d a tras d a, especialmente a todos los postdoctorales que comparten despacho con Salva, y han sufrido mis visitas durante estos a os. Menci3n especial merece Magda, por ayudarme con el an lisis del metabolismo hep tico, con la b squeda de un nuevo labo y “la proposal” de la Marie Curie. Por supuesto, a To i, por el sentimiento que generas en m  cuando te veo, porque quiero ser as  de valiente, de hermosa por dentro y por fuera, de inteligente, porque siento orgullo de haber conocido a la que descubri3 “lo de los tel3meros”, por saber escuchar y no solamente o r, por todo lo que me has ense ado “de gratis” sin tener que hacerlo, por aconsejarme y por “regalarme a Andr s”. Gracias a ellas dos, a Sara, a Nuria y a Mapi, por reafirmar mi convicci3n de que este mundo est  lleno de mujeres maravillosas, luchadoras e independientes.

A la gente de Guadalupe, por alimentarme de manera desinteresada, especially to Ivanna thanks to make my days happier and to try to introduce me in the “acroyoga” world. A “los Pacos” porque ah  fue donde empez3 todo, y sin ellos hubiese sido totalmente diferente, a Cristina, Giulia y Fran por ser tan buenos compa eros, a Carolina por ser tan risue a, tan tranquila y tan hermosa, gracias por ense arme como hacer una tesis. A Marta, Olga, Danay y Maria Laura por compartir conmigo muchos de estos a os. A Noa por sacarme una sonrisa diaria m nimo. A Jose Mar a, por su sonrisa imborrable y su ilusi3n perenne. A la gente de Almudena, en especial a  ngel, gracias por estar ah  y compartir conmigo todo lo que he necesitado, a Arantxa, Nahikari y Virginia por compartir estos momentos.

A mis compa eras de ingl s a lo largo de estos a os, Ana Lopez Dorado, Carmen G3mez-Valad s, Cristina Sanchez Ramos, y Mar a G3mez Serrano, por tardes maravillosas de risas y por su apoyo incondicional.

Al Ministerio de Cultura y Deporte, as  como al CNIC, por la financiaci3n recibida para realizar este trabajo. A todas las personas de las unidades, porque sin ellos este trabajo no hubiese sido posible, especialmente a la gente de Cel3mica, Raquel, Mariano, Ligos y Elena, por su paciencia y su dedicaci3n, a la gente de Microscop a y Gen3mica por ayudarme cuando lo he necesitado. Al personal del animalario, a John y Antonio por su inestimable ayuda y en especial a Iv n, por ser el mejor t cnico de animalario y adem s una persona incre ble, por estar ah  para todo. A Mari  ngeles y Laura Grau por su ayuda. A la gente de la cafeter a, especialmente Elena, Jose y  ngel por poner a mis d as siempre una sonrisa.

A todos mis colaboradores, por su contribuci3n inestimable a este trabajo. To the Powrie’s lab, for three unforgettable months. Especially to Camille, “mi peque a bonita chica”, for being the light of the dark Oxford, for teach me, for introduce me to the “French family”, for stay with me

in the distance, for help me every day with everything, for make me really happy, “we are the magic couple” toujours ensemble ami.

A mis profesores, por enseñarme y desatar en mí un deseo inmenso de descubrir y entender el mundo que me rodea. Gracias a José Iborra Ramírez, por animarme a estudiar Biología y a David Martín Oliva por brindarme la primera oportunidad de “cacharrear” en un laboratorio.

A mis compañeros de la Universidad, especialmente a Fran por convencerme de que venir a Madrid aquel verano del 2010 era una buena idea y a Isra, por esa semana de ensueño en Atenas. A mis amigos, los de Madrid, a Checa por sacarme a entrenar y ponernos “duros como piedras”, a Montse por ser mi amiga, mi masajista Thai, mi pepito grillo, una loca con la que “quemar Madrid” un finde y una mujer tranquila con la que tomar unos vinos en Chueca, gracias por estar a mi lado cuando más lo he necesitado, a Santi por quererme y empatizar conmigo desde su “hombría”, a Carlos y Laura por sacarme a cenar y hacer terapia comiendo. A Santi y a Marcos por encargarse de choco todos los mediodías y durante mis viajes, a Desi y su mamá por cuidarme y ayudarme en el día a día, a Andrés por poner cordura a este “caos de persona”, gracias a todos por ser mi pequeña pero increíble familia aquí. A mis amigos de Caravaca, porque sin ellos podría haber perdido aún más la cabeza, a mi grupo “sin remordimientos” por estar a mi lado desde hace casi 20 años, por los viajes juntas, por los días de playa y por venir a recargarme las pilas cuando más lo necesitaba. A mi Noe, porque desde párvulos me acompañas, por las conversaciones de los Domingos sacando a choco, como si 400 km no fuesen nada, por comprenderme y por estar a mi lado. Vosotras me hacéis mejor cada día.

A mi familia, por pensar que soy una “eminencia” en algo de “los bichos del intestino”, por hablar de mi con un orgullo inmenso, porque sin vosotros nada de esto hubiese sido posible. A mi padre y a mi madre, por haberos sacrificado por mí día tras día, por acompañarme en la distancia y sufrir cuando yo he sufrido. A mis abuelos, en especial a mi Yaya por cocinarme “migas” cuando tengo antojo, y hablar conmigo como si fuésemos quinceañeras. A mis tíos y mis primos por estar siempre apoyándome. A mi “Kari” por acompañarme, por quererme, por tus visitas a Madrid, por las exposiciones, los restaurantes molones, las compras y por darme todo sin esperar nada a cambio. A Gamboa, mi camionero de cabecera, por hacer de chófer desde que me mudé a Granada hasta ahora.

Y por último y no menos importante, a Choco, porque, aunque no hablas, dices más que muchas personas, por esperarme con tu carita de lápiz cuando llego del trabajo, por las carreras en el parque, por las duchas forzosas y por todos los momentos que me regalas.



Summary

SUMMARY

C-type lectin receptors (CLRs) are versatile platforms that sense a diversity of endogenous and exogenous ligands that may trigger differential responses. A prototypical example of this adaptability is the myeloid CLR Mincle. Classically viewed as an activating receptor, Mincle responds to several “non-self” ligands derived from pathogens contributing in most cases to the generation of immunity against the infection. However, Mincle-sensing of “non-self” ligands can also lead to immunosuppression directly favouring an anti-inflammatory response, or indirectly inhibiting heterologous receptors signalling. Here, we have found that human and mouse Mincle bind to a ligand released by *Leishmania*, a eukaryote parasite that effectively evades the immune system. Mincle-deficient mice had less pathology and lower parasite burden compared to wild-type mice after *Leishmania major* infection. Mincle deficiency enhanced adaptive immunity against the parasite, correlating with increased activation and migration of Mincle-deficient dendritic cells (DCs). *Leishmania* triggered a Mincle-dependent inhibitory axis characterized by SHP1 coupling to the FcR γ chain. In conclusion, *Leishmania* shifts Mincle to an inhibitory ITAM configuration that favours pathogen-mediated immune escape impairing DC activation.

On the other hand, we have found that Mincle detects mucosa-associated commensals. Interaction of commensals with Mincle triggered DC activation through the classical ITAM configuration characterized by Syk coupling to the FcR γ chain. Mincle sensing of commensals in Peyer’s patches induced IL-6 and IL-23p19 expression and regulated intestinal Th17 cells and IL-17-secreting innate lymphoid cells (ILCs). These cells produce IL-17 and IL-22 that are critical for maintenance of the intestinal barrier function. Consequently, Mincle-deficient or CD11c-Cre Syk^{flox/flox} mice showed impaired production of intestinal antimicrobial peptides and IgA, resulting in increased systemic translocation of gut microbiota and liver inflammation. Thus, sensing of commensals by Mincle reinforces intestinal immune barrier and promotes host-microbiota mutualism, preventing systemic inflammation. Our work supports the notion that Mincle can couple to an activating or to an inhibitory ITAM configuration depending on the nature of the ligand.

Resumen

RESUMEN

Los receptores de lectina tipo C (CLRs, del inglés C-type lectin receptors) son estructuras versátiles que reconocen una gran variedad de ligandos endógenos y exógenos que van a generar diferentes respuestas. Un ejemplo destacable de esta adaptabilidad, es el CLR mieloide, Mincle. Tradicionalmente considerado un receptor activador, Mincle responde a varios ligandos “no propios” derivados de patógenos, contribuyendo en la mayoría de los casos a la generación de inmunidad frente a la infección. Sin embargo, el reconocimiento de ligandos “no propios” por parte de Mincle puede también generar inmunosupresión de manera directa induciendo una respuesta anti-inflamatoria o indirectamente, inhibiendo la señalización de receptores heterólogos. En este trabajo, hemos encontrado que Mincle humano y de ratón, reconoce un ligando excretado por *Leishmania*, un parásito eucariota capaz de evadir eficientemente la respuesta inmunológica. Los ratones deficientes para Mincle tenían menos patología y carga parasitaria comparados con animales de genotipo silvestre tras la infección con *Leishmania major*. La deficiencia de Mincle aumentaba la respuesta adaptativa frente al parásito, lo que correlacionaba con una mayor activación y migración de las células dendríticas (DCs, del inglés dendritic cells) deficientes para Mincle. *Leishmania* inducía a través de Mincle una cascada de señalización inhibitoria caracterizada por la unión de SHP1 a la cadena de FcR γ . En conclusión, *Leishmania* cambia Mincle hacia una configuración inhibitoria del ITAM que favorece la evasión de la respuesta inmunológica por parte del parásito, impidiendo la activación de las DCs.

Por otro lado, hemos encontrado que Mincle reconoce los comensales asociados a la mucosa intestinal. La interacción de Mincle con los comensales conducía a la activación de las DCs, a través de la configuración clásica del ITAM, caracterizada por la unión de Syk a la cadena FcR γ . El reconocimiento de los comensales vía Mincle en las placas de Peyer inducía la expresión de IL-6 e IL-23p19 y regulaba las células Th17 y las ILCs (del inglés innate lymphoid cells) productoras de IL-17 en el intestino. Estas células producen IL-17 e IL-22, ambas críticas para el mantenimiento de la barrera intestinal. En consecuencia, los ratones deficientes para Mincle o CD11c-Cre Syk^{flox/flox} mostraron menor producción de péptidos antimicrobianos e IgA, lo que conllevaba una mayor extravasación de los comensales e inflamación hepática. Por lo que, la detección de los comensales vía Mincle refuerza la barrera intestinal a nivel inmunológico, y promueve el mutualismo hospedador-microbiota, evitando la inflamación sistémica. Nuestros hallazgos respaldan la noción de que Mincle puede acoplarse a un ITAM con configuración activadora o inhibitoria dependiendo de la naturaleza del ligando.

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Acronyms

LYST OF ACRONYMS

ABX: Antibiotics

ALT: Alanine transaminase

AST: Aspartate transaminase

Bcl10: B cell lymphoma/leukemia 10

BSA: Bovine serum albumin

CARD9: Caspase recruitment domain-containing protein 9

CLRs: C-type lectin receptors

CRD: Carbohydrate recognition domain

CTLD: C-type lectin-like domain

DAG: Diacylglycerides

DAMPs: Damage-associated molecular patterns

DAP12: DNAX-activation protein 12

DCIR: Dendritic cell immunoreceptor

DCs: Dendritic cells

DC-SIGN: Dendritic cell-specific ICAM-grabbing non-integrin

Dectin-1: Dendritic-cell-associated C-type lectin-1

Dectin-2: Dendritic-cell-associated C-type lectin-2

dLNs: Draining lymph nodes

DNGR-1: DC, NK lectin group receptor-1

F-actin: Filamentous actin

FBS: Fetal bovine serum

FcR γ : Fc receptor γ

FFA: Free fatty acid

GBA1: β -glucocerebrosidase

GMCSF: Granulocyte macrophage colony stimulating factor

i.d: Intradermal

IECs: Intestinal epithelial cells

IFN γ : Interferon- γ

IgA: Immunoglobulin A

ILCs: Innate lymphoid cells

IRF1: Interferon regulatory factor 1

ITAM: Immunoreceptor tyrosine-based activating motif

ITIM: Immunoreceptor tyrosine-based inhibitory motif

LP: Lamina propria

LPS: Lipopolysaccharide

LysoDC: Lysozyme-expressing dendritic cell

LysoMac: Lysozyme-expressing macrophage

M_{Ag}H: Macrophage antigen H

MAMPs: Microbe-associated molecular patterns

MAPK: Mitogen activated protein kinases

MCL: Macrophage C-type lectin

MFI: Mean fluorescence intensity

Mincle: Macrophage-inducible C-type lectin

MMR: Macrophage mannose receptor

moDCs: Monocyte-derived DCs

NF- κ B: Nuclear factor- κ B

NFAT: Nuclear factor of activated T-cells

NK: Natural killer cells

NO: Nitric oxide

NOD: Nucleotide-binding oligomerization domains

O/N: Overnight

p.i: Post-infection

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate buffered saline

PFA: Paraformaldehyde

PKB: Protein kinase B

PPs: Peyer's patches

PRRs: Pattern recognition receptors

RegIII γ : Regenerating islet-derived protein 3 gamma

RIG: Retinoic acid-inducible gene

ROR γ t: Retinoic acid receptor (RAR)-related orphan receptor gamma t

ROS: Reactive oxygen species

rRNA: Ribosomal RNA

RT: Room temperature

SAMPs: Self-associated molecular patterns

SAP-130: Sin3-associated protein 130

SED: Subepithelial dome

SFB: Segmented filamentous bacteria

SHP-1/-2: Src homology region 2 domain-containing phosphatase -1/-2

SIGNR3: Specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3

SLA: Soluble *Leishmania* antigen

SLPs: Surface layer proteins

SN: Supernatant

SPF: Specific pathogen free

Syk: Spleen tyrosine kinase

TDB: Trehalose-6,6-dibehenate

TDM: Trehalose-6,6-dimycolate

Tfh: T follicular helper cells

Th: CD4⁺ T helper cells

TNF α : Tumour necrosis factor- α

Treg: CD4⁺ regulatory T cells

Tx-100: Triton X 100

β -GlcCer: β -glucosylceramide

Introduction

1. INTRODUCTION

1.1 The immune system at the barriers

The immune system has evolved to react to complex environmental clues. These signals are continuously present at barrier tissues, such as skin and the gastrointestinal tract. These barrier tissues have site-specific beneficial microorganisms referred to as the microbiota, while at the same time, are the entry for most of the challenges, including pathogenic microorganisms (**Fig. 1.1**). In these conditions, the immune system has to work to eliminate pathogenic microbes and foreign substances, avoiding damage of self-tissues and facilitating the symbiosis to resident microbiota (Belkaid and Artis, 2013). The immune system has cellular and humoral components. These components belong to the innate or adaptive arms of the immune system. The innate arm is the first line of contact with the surrounding world. Lymphoid and myeloid cells composed the innate immune system, including innate lymphoid cells (ILCs), natural killer cells (NK), neutrophils, eosinophils, basophils, mast cells, macrophages and dendritic cells (DCs) (Gasteiger et al., 2017, Riera Romo et al., 2016). Myeloid cells, mainly macrophages and DCs, are key cells modulating the generation of the adaptive response, mainly B and T cell responses (**Fig. 1.1**). There are at least three types of mature T cells: CD4⁺ helper (Th) or regulatory T cells (Treg) and CD8⁺ cytotoxic T cells (Tc). Additional layers of complexity come from the existence of several subtype of CD4⁺ Th cells (Th1, Th2 and Th17) and Treg (natural or inducible), which can differ from each other in their origin, pattern of cytokine production and functions (Nicholson, 2016).

1.2 Myeloid-cells Pattern Recognition Receptors (PRRs)

To influence the adaptive immune response, myeloid cells are equipped with a variety of germ line-encoded receptors, called pattern recognition receptors (PRRs) (**Fig. 1.1**). Charles Janeway hypothesised the existence of these receptors as sensors of pathogen-associated molecular patterns (PAMPs) on pathogens as inducers of immunity (Janeway, 1989). However, PAMPs are not exclusive to pathogens, and are present in the resident microbiota (Chu and Mazmanian, 2013). In addition, these receptors can also recognize endogenous damage-associated molecular patterns (DAMPs), host cellular components released upon cell death (Jounai et al., 2012). DAMPs/PAMPs recognition by PRRs, leads to innate cell activation, including degranulation and phagocytosis, but also affecting antigen presentation and co-stimulation, which in turn shapes the nature of the adaptive response (Iwasaki and Medzhitov, 2010).

PRRs families comprise transmembrane and intracellular receptors. The former include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which are found at the cell surface or in endocytic compartments, where they recognize microbial signatures and modulate antigen presentation as well as cytokine/chemokine production. The latter contains nucleotide-binding

oligomerization domains (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG-I)-like receptors (RLRs) and DNA sensors which are located in the cytoplasm, where they detect the presence of intracellular pathogens and induce IL-1 β or type I IFN production, respectively (Brubaker et al., 2015) (Fig. 1.1).

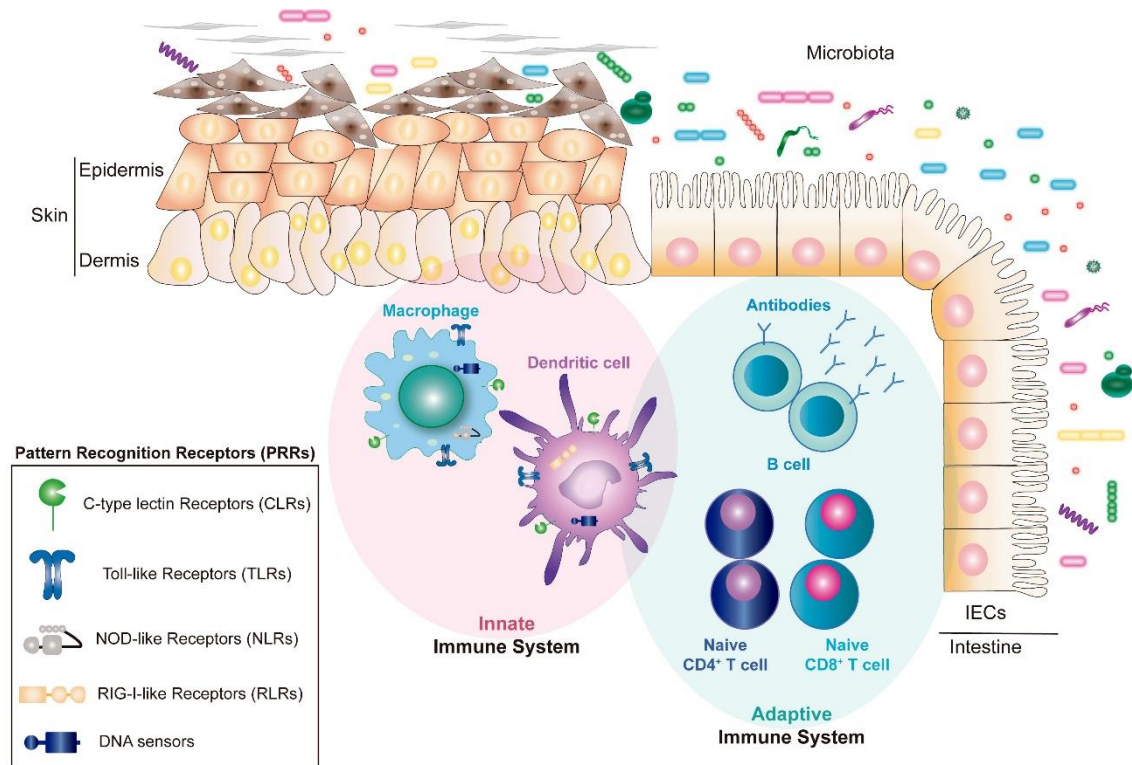


Figure 1.1 Components of the innate and adaptive immune system at the barrier tissues. Top: the physical barriers, including the skin, and the intestine, together with their resident microbiota are shown. Left: representation of the innate immune myeloid cells (macrophages and dendritic cells) equipped with several pattern recognition receptors (PRRs). Right: the adaptive cellular components (CD4⁺, CD8⁺ T cells and B cells) are shown. NOD, nucleotide-binding oligomerization domains; RIG-I, retinoic acid-inducible gene; IECs, intestinal epithelial cells.

1.3 Myeloid C-type lectin Receptors (CLRs)

1.3.1 Families of myeloid CLRs

C-type lectin receptors (CLRs) are a superfamily of proteins that possess one or more C-type lectin-like domain (CTLD). The CTLD is a conserved structural motif arranged as two protein loops stabilized with intra-chain disulphide bridges. The second loop is more flexible than the first and generally contains the ligand-binding site. CTLD often mediates glycoconjugates binding, in a Ca²⁺-dependent manner through conserved residues present in a module termed carbohydrate recognition domain (CRD). This includes the EPN (Glutamic acid–Proline–

Asparagine) or the QPD (Glutamine–Proline–Aspartic acid) motifs, which determine specificity for mannose-based or galactose-based ligands, respectively. However, CTLD may also recognize proteins, lipids or even inorganic ligands. CLRs superfamily can be classified into 17 groups (I-XVII) based on their phylogeny and their structural domain organization. Myeloid CLRs, express by macrophages and DCs, mainly belong to group II, V and VI. Group II and V are type II transmembrane proteins with a short cytoplasmic tail, a transmembrane domain, and an extracellular single CTLD, which in the group II possesses a Ca²⁺-dependent CRD motif, but in the group V lacks the Ca²⁺ or carbohydrates binding sites. Group VI are type I transmembrane receptors with an extracellular domain encompassing an N-terminal ricin-like domain, a fibronectin type 2 domain and 8 or 10 CTLDs. The extracellular domain is linked to a transmembrane region and a short cytoplasmic domain (Brown et al., 2018) (Fig. 1.2).

Apart of this classification based on structural differences present in their extracellular domain, myeloid CLRs can be grouped into four categories, depending on the presence of different intracellular signalling motifs (Sancho and Reis e Sousa, 2012) (Fig. 1.2) :

1. Immunoreceptor tyrosine-based activating motif (ITAM)-coupled CLRs, which have YxxL tandem repeats (being “Y” tyrosine , “x” any aminoacid and “L” leucine) in their intracellular tail or can interact with ITAM-containing adaptor proteins, as Fc receptor γ (FcR γ) chain or DNAX-activation protein 12 (DAP12). Mincle (*CLEC4E*) and Dectin-2 (*CLEC6A* in human, *Clec4n* in the mouse) are representative examples of this group; both receptors use the FcR γ chain adaptor for their signalling.
2. Hemi-ITAM (hemITAM)-bearing CLRs, which contain a single tyrosine within an YxxL motif in their cytoplasmic domain, Dectin-1 (*CLEC7A*) and DNGR-1 (*CLEC9A*) are prototypical examples of this category.
3. Immunoreceptor tyrosine-based inhibitory motif (ITIM)-bearing CLRs, some examples are human DCIR (*CLEC4A*) and MAgH (*CLEC12B*).
4. CLRs lacking these typical signalling motifs, DC-SIGN (*CD209*) and MMR (*MRC1*) are part of this group.

ITAM- or hemITAM-coupled CLRs are considered activating receptors that couple to the spleen tyrosine kinase (Syk). Phosphorylation of the tyrosine(s) in the ITAM or hemITAM motifs generates docking sites for the Src-homology 2 domain (SH2) domains of Syk, which undergoes a conformational change permitting auto-phosphorylation and activation. Syk activation usually leads to the assembly of a ternary protein complex consisting of the caspase recruitment domain-containing protein 9 (CARD9), B cell lymphoma/leukaemia 10 (Bcl10), and the mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT-1). The formation of Card9/Bcl10/Malt1 results primarily in the induction of nuclear factor- κ B (NF- κ B)-dependent responses, which leads to myeloid cells cytokine production and T cell priming. In addition, there are other pathways that can also be activated downstream of particular CLRs,

as mitogen activated protein kinases (MAPK) (Slack et al., 2007), nuclear factor of activated T-cells (NFAT) through phospholipase C- γ 2 (Goodridge et al., 2007, Xu et al., 2009) and a Syk-independent non-canonical NF- κ B activation relying on the activation of the V-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1) kinase (Gringhuis et al., 2009). In contrast, ITIM-coupled CLR s do not have any activity *per se* but through the recruitment of tyrosine phosphatases, such as Src homology region 2 domain-containing phosphatase (SHP)-1 or -2, can mediate negative regulation of kinase-associated receptors, for example the Syk-coupled CLR s (Fig. 1.2). For this reason, these CLR s are ideal targets for pathogen immune escape. Finally, CLR s without a clear signalling motif cannot induce myeloid cell activation by themselves, but some of them are involved in endocytosis, thus contributing to antigen processing and presentation to T cells and can modulate the signalling of heterologous receptors (Del Fresno et al., 2018).

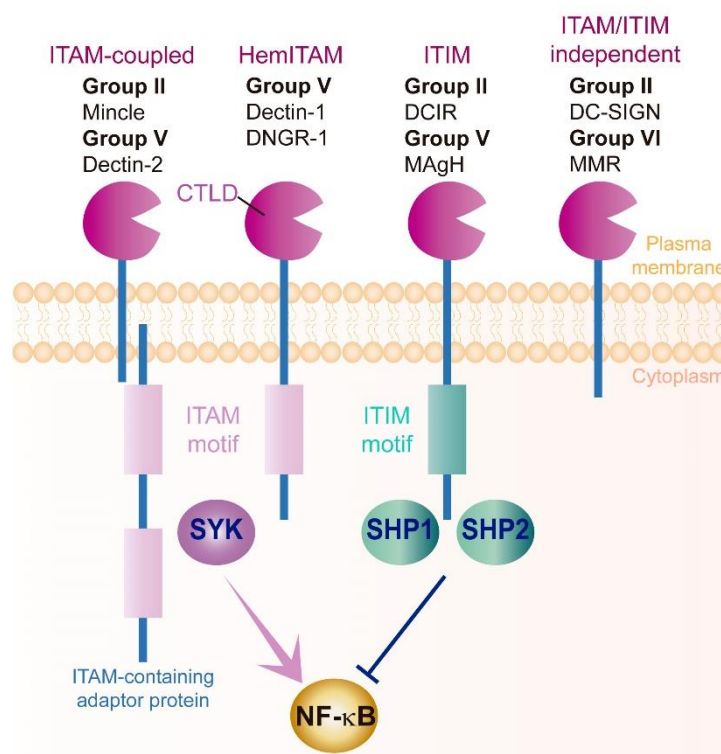


Figure 1.2 Families of myeloid C-type lectin Receptors (CLR s). Schematic representation of four CLR s categories based on their cytoplasmic signalling motifs. The name and the group number, based on structural features of the extracellular domain, of some prototypical examples of each category are shown. From the left to the right: Immunoreceptor tyrosine-based activating motif (ITAM)-coupled CLR s, signal via Syk through association with ITAM-containing adaptor proteins as Fc γ R chain or DAP-12, hemi-ITAM-(hemITAM)-bearing CLR s, signal via Syk through a single tyrosine-based motif in their cytoplasmic tail, immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing CLR s, possess an ITIM motif that can recruit phosphatases SHP-1 and SHP-2 and ITAM-ITIM-independent CLR s, do not signal through Syk

or phosphatases. Mincle, macrophage-inducible C-type lectin; Dectin-2/1; dendritic-cell-associated C-type lectin-2/1; DNGR-1, DC, NK lectin group receptor-1; DCIR, dendritic cell immunoreceptor; MAgH, macrophage antigen H; DC-SIGN; dendritic cell-specific ICAM-grabbing non-integrin; MMR, macrophage mannose receptor; Syk, spleen tyrosine kinase; CTLD, C-type lectin-like domain; (SHP)-1 or -2, src homology region 2 domain-containing phosphatase; NF- κ B, nuclear factor- κ B

1.3.2 Ligands characteristics influence myeloid CLR's signalling

Apart of the mentioned intracellular motif borne by myeloid CLR's, the nature and the state of the ligand influence CLR's signalling. Monomeric or oligomeric state of the ligand can affect how the ligand interact with the CLR. Soluble ligands for CLR's are poor activators, whereas the same ligands in plated form or particulate ligands, promote an efficient activating signalling (Iborra and Sancho, 2015). As described for other PRR's, CLR's are frequently promiscuous receptors that can bind more than one ligand, each with a distinct affinity or avidity for the receptor. Ligand affinity and avidity can affect the quantity and duration of signals through the ITAM domain, resulting in differential responses (Hamerman et al., 2009, Yamasaki et al., 2004). In this sense, the prototypical example of the differential effect of low affinity and avidity is the ITAM-containing adaptor protein FcR γ chain. Upon binding of a high-avidity ligand, FcR γ chain becomes fully phosphorylated and associates with the kinase Syk triggering an activating signal that contributes to immunity (**Fig. 1.3A**). However, as described for the Fc α RI receptor, which associates with the FcR γ chain for signalling, upon interaction with low affinity or avidity ligands, FcR γ chain is partially phosphorylated and recruits SH2-containing phosphatases, a configuration termed inhibitory ITAM, being able to inhibit signals through heterologous receptors (Pasquier et al., 2005, Kanamaru et al., 2008, Blank et al., 2009) (**Fig. 1.3B**). Accordingly, the aforementioned ITAM-associated CLR's that couple to the FcR γ chain, namely Mincle and Dectin-2, would thus behave similarly to ITIM-coupled CLR's. They could respond to low-valency ligation by soluble extracellular ligands, in a similar manner to the Fc α RI receptor, recruiting phosphatases and inhibiting heterologous activator receptors, dampening immunity (Iborra and Sancho, 2015) (**Fig. 1.3B**).

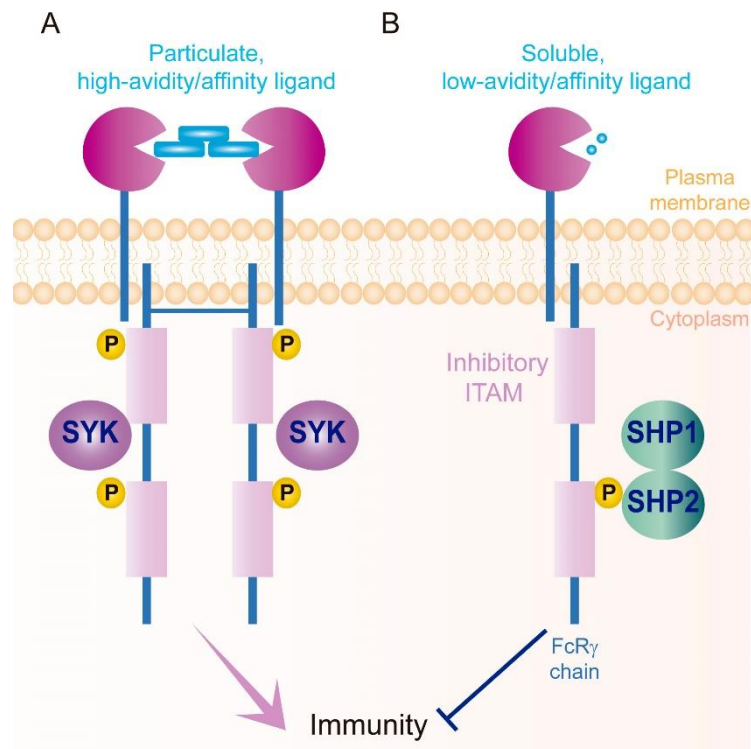


Figure 1.3 Model of how nature of the ligand could influence myeloid CLR signalling.

(A) Upon binding of a particulate, high-avidity/affinity ligand, the FcR γ chain ITAM is fully phosphorylated and associates with Syk kinase, which triggers an activating signal promoting the development of immunity. (B) Soluble low-avidity/affinity ligands could induce hypophosphorylation of the ITAM domain in the FcR γ chain associated with the CLR, termed “inhibitory ITAM”, which preferentially binds tyrosine phosphatases, SHP-1 or -2 (mechanism described for Fc α RI receptor detailed above). Syk, spleen tyrosine kinase; (SHP)-1 or -2, src homology region 2 domain-containing phosphatase; ITAM, immunoreceptor tyrosine-based activating motif. They symbol P indicates a phosphorylated motif.

1.4 Sensing of self and non-self by myeloid CLR

Myeloid CLR usually are promiscuous and versatile receptors, some can detect endogenous (self) ligands, other recognize exogenous microbiota- or pathogen-derived (non-self) ligands and many of them can act as dual receptors sensing non-self and self-ligands. Self-ligands can be classified as “non-dangerous self” also known as “self-associated molecular patterns” (SAMPs), “dangerous-self” or DAMPs released or exposed upon cell death, and “altered-self”, which are neoglycans derived from transformed cells (Varki, 2011, Matzinger, 1994, Matzinger, 2002, Eggleton et al., 2008). On the other hand, non-self ligands can be present in pathogen microorganisms (PAMPs) but also in the commensal microorganisms that form the microbiota, which we can call microbe-associated molecular patterns (MAMPs) (Chu and Mazmanian, 2013).

Myeloid CLR recognition of different kinds of self-ligands might contribute to immunity or to homeostasis. On the other hand, non-self ligand detection by myeloid CLR either could drive immunity and inflammation to fight pathogens, or could be used by pathogens for immune evasion, or even could promote tolerance to commensals microorganisms at barrier tissues such as the skin or the intestine.

1.4.1 Sensing self to trigger immunity

Myeloid CLR respond to “dangerous-self” ligands released or exposed upon cell death in the presence or not of infection. One example is the hemITAM-bearing CLR DNGR-1 (*CLEC9A*). DNGR-1 recognizing actin filaments (F-actin) (Ahrens et al., 2012, Zhang et al., 2012, Hanc et al., 2015, Schulz et al., 2018) senses tissue damage concomitant with viral infections facilitating antigens cross-presentation to CD8⁺ T cells (Zelenay et al., 2012, Iborra et al., 2012, Iborra et al., 2016). Then, damaged infected cells expose the actin filaments and DNGR-1 favours host responses after detecting this danger signal on infected cells (Fig. 1.4A).

1.4.2 Sensing self to promote homeostasis

Self-ligand recognition by myeloid CLR can also promote homeostasis, preventing immunopathology. An example of this CLR is the ITIM-bearing CLR DCIR (*CLEC4A* in human, *Clec4a2* in mouse), which recognizes self-glycoproteins and maintain immune system homeostasis, as consequence its deletion causes autoimmune diseases in aged mice (Fujikado et al., 2008, Bloem et al., 2013, Maruhashi et al., 2015) (Fig. 1.4B).

1.4.3 Sensing non-self to drive immunity

Pathogens derived ligands interaction with myeloid CLR can drive the activation of the immune system to fight against pathogens. The archetypal example of this situation is Dectin-1 (*CLEC7A*), which recognizes β -glucans present in the cell wall of many fungi (Brown, 2006). Myeloid Dectin-1 engagement leads to Syk mediated -NF- κ B, -MAPK and -NFAT signalling activation, affecting phagocytosis, respiratory burst, and the production of various inflammatory mediators, including the pro-inflammatory cytokines IL-6 and IL-23, contributing to antifungal adaptive immunity (Tang et al., 2018) (Fig. 1.4C).

1.4.4 Sensing non-self to promote tolerance to commensal microorganisms

Sensing non-self MAMPs by myeloid-CLR is important to promote tolerance to commensal microorganisms at the barrier tissues such as the intestine or the skin. Remarkably, in this context, myeloid CLR have to deal with both pathogens and commensals microorganisms (Swiatczak and Cohen, 2015). The hemITAM-bearing CLR, SIGNR3 (mouse *Cd209d*)

recognizes fungi present in the intestinal commensal microbiota, SIGNR3 deficient mice have more severe colitis compared to control mice, indicating that fungi recognition by SIGNR3 promotes tolerance to the microbiota (Eriksson et al., 2013). In addition, the recognition of surface layer proteins (Slps) from *Lactobacillus acidophilus* by SIGNR3 contributes to mucosal barrier function (Lightfoot et al., 2015) (Fig. 1.4D). Conversely, its human homolog DC-SIGN (*CD209*) can recognize *Lactobacillus reuteri* and *casei*, modulating DC activation to drive the development of Treg cells that promote the tolerance to commensals microorganisms (Smits et al., 2005).

1.4.5 Sensing non-self to support immune evasion

Myeloid CLR-recognition of pathogens is key to trigger an effective immune response, but the fact that self-ligands recognition by myeloid CLR can promote homeostasis, opens up the possibility that pathogens have evolved to mimic self-ligands to escape host immunity (Martinez-Lopez et al., 2018). A good example of this immune escape mechanism is the mouse homolog of the CLR, DC-SIGN, SIGNR3, which is targeted by *Leishmania infantum* to inhibit the microbicide response mediated by the Syk-coupled CLR, Dectin-1 and the CLR, macrophage mannose receptor (MMR), favouring parasite resilience (Lefevre et al., 2013) (Fig. 1.4E).

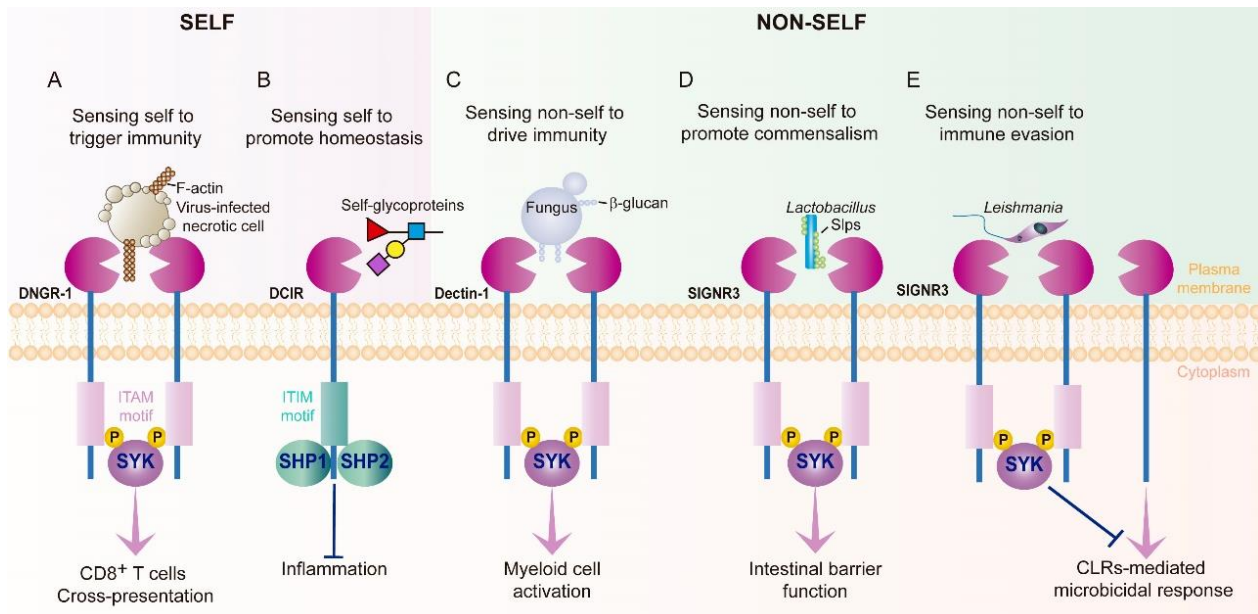


Figure 1.4 Self and non-self sensing by myeloid CLR Sensing self (A) to trigger immunity: necrotic cells infected with virus (for example vaccinia virus) can be detected by DNGR-1 on DCs (B) to promote homeostasis: self-glycoproteins recognition by DCIR prevents inflammation. Sensing non-self (C) to drive immunity: Dectin-1 detects β -glucan on some fungi (for example *Candida albicans*) mediating myeloid activation such as cytokine production (D) to promote tolerance to commensal microorganisms: recognition of surface layer proteins (Slps)

from *Lactobacillus acidophilus* by SIGNR3 contributes to mucosal barrier function (E) for pathogen immune evasion: upon *Leishmania infantum* infection, Dectin-1 and MMR induce a microbicide response. SIGNR3 promotes parasite survival by inhibiting this response. F-actin, filamentous actin; DNGR-1, DC, NK lectin group receptor-1; DCIR, dendritic cell immunoreceptor ; Dectin-1, dendritic cell-associated lectin-1 ; SIGNR3, specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3; MMR, macrophage mannose receptor ; Syk, spleen tyrosine kinase; (SHP)-1 or -2, src homology region 2 domain-containing phosphatase; ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif. The symbol P indicates a phosphorylated motif.

In light of the presented examples, we could hypothesize that activating receptors could mainly sense non-self ligands to trigger the immune response while ITIM-bearing CLR s could preferentially bind self –ligands to dampen inflammation. However, the current general view is that most CLR s are adaptable structures that can bind both endogenous and exogenous ligands, resulting in distinct functional outcomes that are not strictly determined by their intracellular bearing motif (Del Fresno et al., 2018 8903).

1.5 The myeloid CLR Mincle is an adaptable sensor

A good example of that adaptability is the myeloid CLR Macrophage inducible C-type lectin, Mincle (*CLEC4E*). Mincle is a classic dual receptor for both self and non-self ligands.

1.5.1 Mincle, its origin, pattern of expression, molecular signalling and structure

Mincle was identified in 1999, as a transcriptional target of nuclear factor interleukin-6 (NF-IL6, also called C/EBP β) in macrophages (Matsumoto et al., 1999). Mincle is encoded by a chromosome 6 gene in mouse (chromosome 12 in humans), clustered with closely related CLR s of the Dectin-2 family (Dectin-2, DCIR, DCAR, and BDCA-2) (Graham and Brown, 2009). Mincle is expressed on monocytes, macrophages, neutrophils, DCs, and some subset of B cells (Richardson and Williams, 2014, Matsumoto et al., 1999, Flornes et al., 2004, Behler et al., 2015, Lee et al., 2012, Kawata et al., 2012). The expression of Mincle in the steady-state condition is very low; however, its expression is induced by inflammatory stimuli including lipopolysaccharide (LPS), tumour necrosis factor- α (TNF α), IL-6, and interferon- γ (IFN γ) (Matsumoto et al., 1999 , Richardson and Williams, 2014). This induction involves NF-IL6 and myeloid differentiation primary response 88 (MYD88) (Matsumoto et al., 1999, Schoenen et al., 2014, Kerscher et al., 2016a). In particular, TLR4 stimulation in mouse macrophages (Lee et al., 2012) or TLR9 stimulation in human B cells (Kawata et al., 2012), induces Mincle expression. In contrast, Mincle expression can be downregulated by IL-4 in a STAT-6-dependent manner, and LPS prevent this suppression (Ostrop et al., 2015, Hupfer et al., 2016).

As mentioned above, Mincle is a type-II transmembrane molecule with an extracellular CRD motif belonging to the CLR group II (Balch et al., 1998). Extracellular ligands binding can

occur at the CRD motif or in distinct sites, as described. Within its CRD Mincle contains an EPN motif (Glu–Pro–Asn), which suggests mannose/fucose/N-acetyl glucosamine/glucose specificity (Lee et al., 2011). Intracellularly, Mincle is an ITAM-coupled CLR that interacts through a positively charged arginine located in its transmembrane region with the ITAM-containing adaptor protein FcR γ chain, being this interaction critical for its signalling (Yamasaki et al., 2008). This receptor can form heterodimers with another myeloid CLR called macrophage C-type lectin (MCL;*CLEC4D*) which signals through Syk and interacts with the adaptor FcR γ chain (Graham et al., 2012) (Miyake et al., 2013), but it does not contain an arginine within the transmembrane domain and uses Mincle to interact with the adaptor protein (Lobato-Pascual et al., 2013, Miyake et al., 2015). The stalk region from MCL is needed for Mincle translocation to the cell surface after exposure to pro-inflammatory stimuli (Miyake et al., 2015). In contrast to Mincle, MCL is constitutively expressed by macrophages and DCs and MYD88 is critical for its surface translocation (Miyake et al., 2013, Kerscher et al., 2016a). Canonical Mincle signalling involves Syk kinase and extracellular signal–regulated kinase (Erk) activation, assembly of the ternary protein complex (Card9/Bcl10/Malt1) and finally the activation of NF- κ B and the expression of pro-inflammatory cytokines, such as TNF α , IL-6 and chemokines, such as MIP-2 (*CXCL2*) and KC (*CXCL1*) (Hara and Saito, 2009).

1.5.2 Mincle is a multi-sensor for multiple ligands

Several structures from both the mammalian host as well as microbes have been identified as ligands for Mincle. Therefore, Mincle is a classic dual sensor able to sense self and non-self ligands from a growing list of bacteria and fungi.

1.5.2.1 Mincle-self-ligand recognition

Self-ligands for Mincle include “dangerous” ligands that promote immunity and inflammation. For example, some forms of cholesterol, including, the crystalline form, which is present within atherosclerotic plaques, that promotes inflammation via human, but not rat and mouse Mincle (Kiyotake et al., 2015). Conversely, the cholesterol sulphate form, which is present at high levels in the skin, promotes allergic skin inflammation through Mincle (Kostarnoy et al., 2017) (**Fig. 1.5A**). In addition, the host intracellular glycosphingolipid β -glucosylceramide (β -GlcCer) can signal through Mincle promoting systemic inflammation characteristic of Gaucher’s disease, an inherited genetic defect in β -glucocerebrosidase (GBA1) that leads to the accumulation of this compound. Accordingly, GBA1-deficient DCs are more potent promoting adaptive immune response than WT DCs, in a Mincle-dependent manner, suggesting that β -GlcCer is an endogenous adjuvant which acts through Mincle (Nagata et al., 2017) (**Fig. 1.5A**). Thus, Mincle is a promiscuous receptor and there are “dual” self-ligands that can promote immunity or immunosuppression depending on the context. For example, the first self-ligand identified for Mincle, the Sin3A-associated protein 130 (SAP-130). It is a component of the U2

small nuclear ribonucleoprotein (snRNP) localized at the nucleus in normal live cells (Das et al., 1999), and is released from necrotic cells, but not from live or early apoptotic cells. SAP-130 binds to Mincle CRD in a distinct site than carbohydrates in Ca²⁺- and EPN-independent manner. The interaction between Mincle and SAP130 can lead to inflammation in certain situations, like ischemia/reperfusion, ethanol-induced liver injury, obesity and to the development of experimental autoimmune uveoretinitis and hepatitis (Yamasaki et al., 2008, Arumugam et al., 2017, Suzuki et al., 2013, Zhou et al., 2016, Tanaka et al., 2014, Lee et al., 2016a, Greco et al., 2016). On the contrary, in a pancreatic tumorigenesis mouse model, Mincle-SAP-130 interaction promotes oncogenesis mediated by the tumour-infiltrating myeloid cells-derived immunosuppression (Seifert et al., 2016) (**Fig. 1.5B**). On the opposite side, the “non-dangerous” self-ligand, albumin provokes immunosuppression through Mincle after subarachnoid haemorrhage (Xie et al., 2017) (**Fig. 1.5C**).

1.5.2.2 Mincle non-self-ligand recognition

Regarding non-self ligands, Mincle is able to recognize pathogens and commensals, bacteria and fungi. From commensals microorganisms, Mincle can recognize ligands present in some commensal bacteria, such as the corynomycolic acid-containing glycolipids from *Corynebacterium* (Schick et al., 2017, van der Peet et al., 2015) and the α -glucosyl diglyceride from *Lactobacillus plantarum* (Shah et al., 2016) but how Mincle-commensals interaction impacts the generation of the immune response is starting to be explored (**Fig. 1.5D**). In addition, *Malassezia* spp., an obligatory lipophilic fungus, including *M. pachydermatis* and *M. dermatis* contain at least two glycolipids, glyceroglycolipid and mannosyl fatty acids linked to mannitol, which can act as ligands for Mincle (Ishikawa et al., 2013). *Malassezia* is part of the normal skin microbiota of many mammals (Sparber and LeibundGut-Landmann, 2017), although this fungus can elicit an inflammatory response in the skin lesions of patients with atopic/eczema dermatitis and psoriasis and can cause diseases such as tinea versicolor, atopic dermatitis, and lethal sepsis (Ashbee, 2006). It is described that Mincle-*Malassezia* interaction promotes immunity although the experimental approach employed, by intraperitoneal administration of the fungus, does not resemble its normal niche and does not provide reliable information on the potential role of Mincle in maintaining the commensalism. Although it is clear from these studies that Mincle-induced inflammation in this context may contribute to the regulation of dermatitis or sepsis triggered by *Malassezia* (Yamasaki et al., 2009, Ishikawa et al., 2013) (**Fig. 1.5E**).

Regarding pathogens, Mincle can interact with them and mediates “direct immunity” (**Fig. 1.5F, G**), “direct immunosuppression” (**Fig. 1.5H**) or “indirect immunosuppression” by inhibiting immunity mediated by other receptors (**Fig. 1.5I**). From pathogenic bacteria, the most studied ligands for Mincle are the mycobacterial glycolipids from *Mycobacterium* species, being the trehalose-6,6-dimycolate (TDM) the best characterized one (Ishikawa et al., 2009, Matsunaga

and Moody, 2009). The cell wall glycolipid, TDM (initially called cord factor) is one of the major components of complete Freund's adjuvant. TDM and its less toxic analogue, trehalose-6,6-dibehenate (TDB) (Pimm et al., 1979) were shown to signal through Mincle (Ishikawa et al., 2009, Schoenen et al., 2010). Mincle recognition of TDM by myeloid cells favours the TDM-induced granuloma formation and the generation of Th1/Th17 mediated immunity although Mincle-deficient mice form granulomas similar to WT mice and generate an adequate Th1/Th17 response upon infection with *M. tuberculosis* (Schoenen et al., 2010, Behler et al., 2015). However, recent *in vitro* studies indicate that Mincle can also promote the expression of the anti-inflammatory cytokine IL-10 in response to TDM, it requires TLR-2-mediated simultaneous signal and leads to TLR2-induced IL-12p40 downregulation (Patin et al., 2016). In addition, Mincle via p38 and eukaryotic translation initiation factor 5A (eIF5A) hypusination increases nitric oxide (NO) leading to granuloma resolution (Lee et al., 2016b). Thus, Mincle seems to play a dual role in the promotion and resolution of inflammation in response to purified TDM, but this receptor is dispensable for the response during infection due to other PRR co-stimulation. Another mycolic acid derivative compound from mycobacteria, glycerol monomycolate, and the *M. tuberculosis* metabolites, β -gentiobiosyl diacylglycerides, were identified as Mincle ligands, the first one only in the case of human but not mouse Mincle (Hattori et al., 2014, Richardson et al., 2015) (Fig. 1.F,H). *Klebsiella pneumoniae* (Sharma et al., 2014), *Streptococcus pneumoniae* (Rabes et al., 2015, Behler-Janbeck et al., 2016), *Tannerella forsythia* (Chinthamani et al., 2017), *Pneumocystis* (Kottom et al., 2017) are other reported pathogenic bacteria that through Mincle promote immunity against these pathogens although the specific ligands that bind Mincle are unknown, only identified in the case of diacylglycerol from *S. pneumoniae* (Behler-Janbeck et al., 2016) (Fig. 1.5F). Instead of promoting immunity, *Helicobacter pylori* recognition by Mincle generates an anti-inflammatory response, being this receptor employed by *H. pylori* to escape clearance by host immune system (Devi et al., 2015) (Fig. 1.5H).

Regarding pathogenic fungus, as mentioned for pathogenic bacteria, most of the specific ligands that bind Mincle are yet unidentified. *Candida albicans* (*C. albicans*) was suggested to contain ligands for Mincle, although controversy exists between studies performed with different strains of *C. albicans*. *Candida*-Mincle interaction contribute to the inflammatory response against the fungal infection (Bugarcic et al., 2008, Wells et al., 2008, Yamasaki et al., 2009)(Fig. 1.5G). In contrast, Mincle recognition of pathogenic fungus, *Fonseca pedrosoi* the causal agent of the chromoblastomycosis, a chronic skin infection, is not sufficient for host control of the infection. This interaction results in high levels of IL-10 and low level of TNF- α and IFN- γ only reverted by TLR co-stimulation (Sousa Mda et al., 2011). Interestingly, another myeloid CLR, Dectin-2 is involved in the generation of fungus-specific Th17 cells, whereas Mincle inhibits this response (Wuthrich et al., 2015) (Fig. 1.5H). Similarly, this Mincle-mediated "indirect

immunosuppression” of heterologous receptors occurs in response to *Fonsecaea monophora*. Dectin-1 and Mincle are involved in the recognition of this fungus. Dectin-1 initiates protective immunity by activating the transcription factor, interferon regulatory factor 1 (IRF1), crucial for *IL12A* transcription, which is dampened by Mincle activation. Mincle-Syk-CARD9-PKB pathway-dependent activation of the E3 ubiquitin ligase mouse double minute two (Mdm2) leads to nuclear IRF1 degradation, blocking *IL12A* transcription (Wevers et al., 2014) (Fig. 1.5H). Altogether, these examples illustrate how Mincle deals with self and non-self ligands resulting in activating or inhibitory signals. However, the correlation of sensing self with an inhibitory response and sensing non-self with an activating response is not established. In this regard, non-self signals from pathogens may mimic self-inhibitory signals to escape immune surveillance and non-self ligands recognition in the context of commensals can promote immune tolerance instead of immunity.

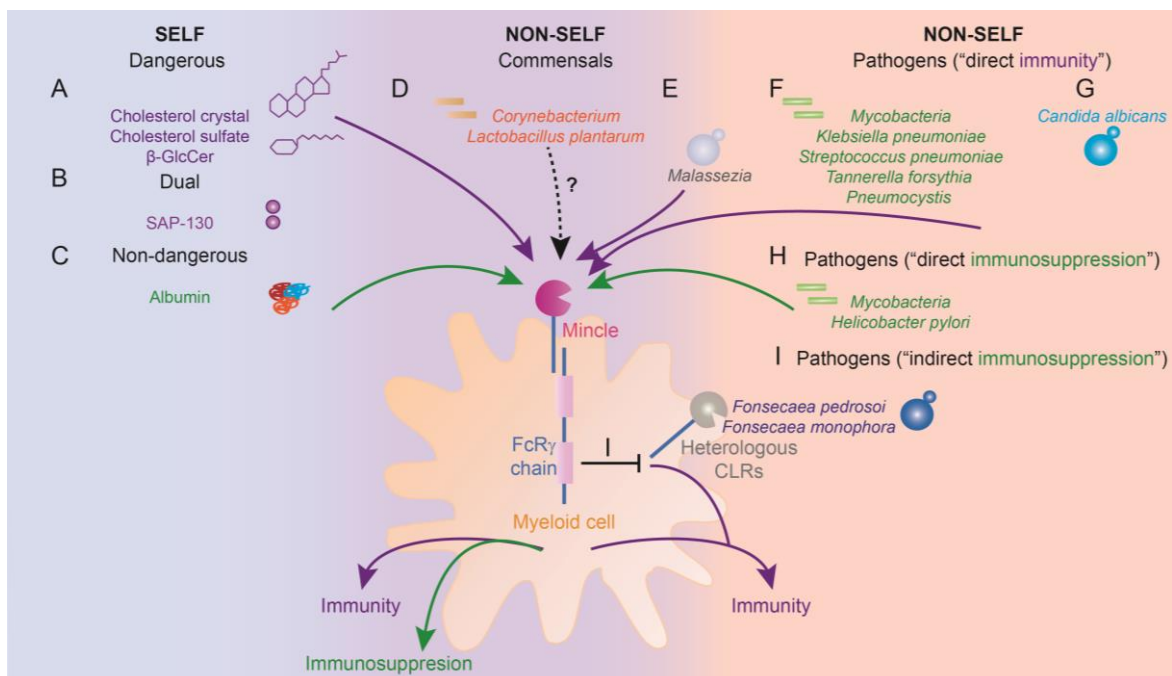


Figure 1.5 Mincle is a flexible receptor for multiple ligands. (A) Dangerous-self ligands include cholesterol crystals or sulphate, and β -GlcCer that promote inflammation; (B) Dual-self ligands, SAP-130 can either promote inflammation (ischemia/reperfusion, obesity, uveoretinitis) or immunosuppression (cancer) depending on the context; (C) Non-dangerous-self ligands such as the albumin drives immunosuppression. (D, E) Commensal non-self ligands present in bacteria and fungi, such as *Corynebacterium*, *Lactobacillus*, or *Malassezia* can be recognized by Mincle, although the triggered response has neither been explored yet (D), nor addressed in the proper context (E). (F-I) Pathogen-derived ligands can either directly promote immunity against pathogenic bacteria (F) or fungi (G), or directly promote immunosuppression by inducing an anti-inflammatory response (for example IL-10 secretion by myeloid cells) (H), or

indirectly drive immunosuppression by dampening the inflammatory signalling from heterologous CLR_s (I). β -GlcCer, β -glucosylceramide; SAP-130, Sin3-associated protein 130; FcR γ , Fc receptor γ ; CLR_s, C-type lectin receptors.

Objectives

2. OBJECTIVES

The general objective of this thesis is to investigate the dual role of the myeloid Syk-coupled CLR Mincle during recognition of non-self ligands derived from either pathogenic or commensal microorganisms. The specific objectives are the following:

1. Analyse the role of Mincle in the *Leishmania major*-induced immune response (*see also Section 4. Results-Section 4.1.1 and FIG.4.1.1*):

- Explore whether *Leishmania* contains a ligand for the CLR Mincle.
- Characterize the Mincle-*Leishmania*- induced intracellular molecular pathway.
- Analyse the contribution of Mincle-*Leishmania* interaction to DC activation and migration.
- Determine the impact of Mincle-mediated DC modulation in the generation of the *Leishmania*-specific adaptive immunity.

2. Analyse the role of Mincle in the regulation of intestinal homeostasis of commensals (*see also Section 4. Results-Section 4.2.1 and FIG.4.2.1*):

- Study the presence of Mincle ligands in the intestinal commensal microbiota.
- Characterize intracellular molecular pathway induced by detection of microbiota by Mincle.
- Analyse Mincle expression in the intestinal myeloid cells.
- Role of Mincle-microbiota interaction in DC activation.
- Effect of Mincle-mediated DC modulation in the generation of the Th17 cells and ILCs functionality in response to microbiota.
- Investigate how Mincle-microbiota interaction contributes to the maintenance of the intestinal barrier function.

Materials&Methods

3. MATERIALS & METHODS

3.1 Mice

Mouse colonies were bred at the CNIC under specific pathogen-free (SPF) conditions. Colonies included *Clec4e*^{-/-} (B6.Cg-Clec4etm1.1Cfng) mice backcrossed more than 10 times to C57BL/6J-Crl were kindly provided by the Scripps Research Institute, through R. Ashman and C. Wells (Griffiths University, Australia) (Wells et al., 2008). CD11cΔ*Syk* (Iborra et al., 2012) and *Clec4e*^{-/-} mice were generated along with WT littermates by heterozygous matings when was required. *Fcer1g*^{-/-} (B6; 129P2-Fcer1gtm1Rav/J) from The Jackson Laboratory (Takai et al., 1994), CD11cΔ*SHP1* (Abram et al., 2013) and OT-II CD4⁺ TCR transgenic mice in C57BL/6 background (B6.Cg-Tg (TcraTcrb) 425Cbn/J) were from The Jackson Laboratory. OT-II mice were mated with B6/SJL expressing the CD45.1 congenic marker to facilitate cell tracking. Bone marrow from *Clec4d*^{-/-} mice were provided by Dr. S. Yamasaki (RIMD, Japan). *Rorc*^{-/-} mice and *Aicda*^{-/-} mice were kindly provided by Dr. B. Becher (UZH, Switzerland) and Dr. A. Ramiro (CNIC, Spain), respectively. Animal studies were approved by the local ethics committee. All animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

3.2 *Leishmania* species, parasite preparation, inoculation, and quantification

In vivo experiments were carried out using different *Leishmania major* (*L. major*) lines: *L. major* Friedlin strain FV1 (MHOM/IL/80/ Friedlin) was generously provided by Dr. D. Sacks (NIH) (Iborra et al., 2011). *L. major* FV1 (MHOM/IL/80/ Friedlin) parasites expressing ovalbumin (*Leishmania*-OVA) were kindly provided by Prof. Deborah Smith and Prof. Paul Kaye (University of York) (Prickett et al., 2006). Recombinant *L. major* LV39c5 (RHO/SU/59/P) parasites expressing the red-fluorescent protein mCherry (mCherry⁺ *L. major*) were described previously (Calvo-Álvarez et al., 2012). *L. amazonensis* (IFLA/BR/67/pH.8), *L. braziliensis* (MHOM/BR/75/M2904), *L. donovani* (MHOM/IN/83/Dd8), and *L. tropica* (MHOM/SU/74/K27) were generously provided by Dr. J. Moreno (IS Carlos III, Spain). *L. infantum* (MCAN/ES/96/BCN150) was kindly provided by Dr. L.C. Gómez-Nieto (UNEX, Spain). For *Leishmania* challenge, parasites of different lines were cultured and kept in a virulent state as described (Martinez-Lopez et al., 2015). Mice were infected by intradermal (i.d) inoculation of 1×10³ or 5×10⁴ metacyclic *L. major* promastigotes into the dermis of both ears (Martinez-Lopez et al., 2015). Lesion size in the ear and number of viable parasites was determined as described (Martinez-Lopez et al., 2015). The parasite load is expressed as the number of parasites in the whole organ.

3.3 Parasite preparation of protein extracts and binding to Mincle-hFc chimera

For preparation of soluble *Leishmania* extract (SLA), approximately 10^9 promastigotes were harvested and washed twice in phosphate buffer saline (PBS). After 3 cycles of freezing and thawing, the suspension was centrifuged at $13,000\times g$ for 20 minutes at 4°C , and supernatant containing SLA was stored at -80°C . Protein concentration was estimated by the Bradford method. Freeze-thawed *L. major* parasites were prepared by 3 cycles of freezing and thawing of 10^8 stationary parasites in complete RPMI medium or PBS. Fixed and permeabilized *Leishmania* parasites were prepared by fixing 10^8 parasites with 0.5 ml of 4% paraformaldehyde (PFA) and immediate addition of 0.5 ml 1% NP-40. After incubation for 10 minutes at room temperature (RT), parasites were washed with PBS. To obtain culture supernatants, stationary promastigotes were washed 3 times in PBS, suspended at 5×10^8 parasites/ml in serum free DMEM, and incubated for 3 hours at 37°C . Culture supernatants were collected by two steps of centrifugation, first at $1,500\times g$ for 5 minutes at 4°C , followed by a second step at $2,500\times g$ for 10 minutes. Protein concentration was estimated by the Bradford method.

For dot-blot determination of Mincle ligands in *Leishmania* extracts, protein samples were applied to $0.2\ \mu\text{m}$ membranes (BioRad) using a vacuum dot blot apparatus (BioRad). To load different protein amounts in each dot, protein samples were serially diluted in PBS (1:3). Similarly, for ELISA, high-binding plates were loaded with protein samples serially diluted in PBS (1:3). Plates were incubated for 24 hours at 4°C . Later, membranes and plates were washed with PBS and incubated with blocking solution (2% defatted milk in PBS) for 120 minutes at RT, followed by incubation with Mincle-hFc chimera or control-hFc ($2\ \mu\text{g/ml}$) for 2 hours. Membranes and plates were then incubated with anti-human IgG (Fc gamma-specific) conjugated to biotin. Membranes were imaged with the LI-COR Odyssey Infrared Imaging System.

3.4 Generation and assay of B3Z cell lines expressing Mincle and FcR γ chain mutants

B3Z cells (kindly provided by N. Shastri, University of California) express a β -gal reporter for nuclear factor of activated T cells (NFAT) (Karttunen et al., 1992). B3Z cells were transduced with retroviruses expressing FcR γ chain, Syk and mouse Mincle. FcR γ chain ITAM tyrosine 65 and 76 phenylalanine mutants were generated using the QuickChange lightning site-directed mutagenesis kit (Agilent). Binding of ligands can be detected by NFAT reporter activation and induction of β -gal activity. B3Z cells were plated in 96 well plates and incubated with plated Trehalose-6,6-dibehenate (TDB) or anti-Mincle (clone 1B6) antibody in the presence or absence of *Leishmania* extract. Lysed parasites used in B3Z assays were opsonized with fresh serum from infected Balb/c mice for 2 hours at RT and washed twice with PBS. Before B3Z cell

plating, promastigotes were seeded on plates coated with 50 µg/ml poly-L-Lysine (Sigma), for 30 minutes at 37 °C. After overnight (O/N) culture, cells were washed in PBS, and LacZ activity was measured by lysis in CPRG (Roche)-containing buffer. Four hours later O.D._{595 nm} was measured relative to O.D._{655 nm} used as a reference.

3.5 Western blot analysis and immunoprecipitation

For immunoblotting, 1×10^6 CD11c⁺ GM-cells were seeded in 24 well plates for 4 hours in serum free RPMI and stimulated with dead *Leishmania* at a 10:1 parasite: GM-cell ratio. For immunoprecipitation, 1×10^7 CD11c⁺ GM-cells or 3×10^7 B3Z cells were stimulated with dead *Leishmania* at 5:1 ratio. After stimulation, cells were washed and suspended in Triton X-100 (TX-100) lysis buffer (50 mM Tris-HCl pH 7.5, 1mM EGTA, 1mM EDTA pH 8.0, 50mM NaF, 1mM sodium glycerophosphate, 5mM pyrophosphate, 0.27 M sucrose 0.5 % Triton X-100, 0.1mM PMSF, 0.1% 2-mercaptoethanol, 1mM sodium orthovanadate, and protease inhibitor cocktail (Roche)). After incubation on ice for 15 minutes, nuclei were pelleted by centrifugation for 15 minutes ($1,300 \times g$, 4°C). For immunoprecipitation assays, cell extracts were incubated O/N with dynabeads® bound to 2 µg anti-mouse SHP1 (rabbit polyclonal; Santa Cruz C-19), or 4 µg anti-HA (Miltenyi). After pull-down, beads were washed twice in ice-cold lysis buffer and eluted by boiling in SDS sample buffer. Cytosolic protein extracts or immunoprecipitated proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad Laboratories). After blocking with 5% bovine serum albumin (BSA, fraction V, Sigma), membranes were incubated O/N with antibodies to phospho-SHP1 (Tyr564; #8849), SHP1 (#3759), phospho-Syk (Tyr525/526; #2711), Syk (#2712) (Cell Signalling Technology); SHP1 (Santa Cruz, sc-7289); Mincle (Clone 1B6); or Fcεr1g (antibodies online, RB41735). In the indicated cases, phospho-Akt (Thr308) or total Akt (40D4) antibodies both from Cell signalling Technology were used. Membranes were imaged with the LI-COR Odyssey Infrared Imaging System.

3.6 FITC skin sensitization migration assay

Mice were inoculated in the left ear with PBS and in the right ear with *L. major* parasites (5×10^4). Ears were painted 16 hours or 14 days later with 1% FITC (Sigma-Aldrich) prepared in an inflammatory stimulating solution of acetone and dibutyl-phthalate (1:1, vol:vol) as previously described (Macatonia et al., 1987). Retromaxillary draining lymph nodes (dLNs) were harvested 24 hours after painting, and LN cells were analysed by flow cytometry.

3.7 Flow cytometry, human-Fc chimeras generation and antibodies

Samples for flow cytometry were stained in ice-cold PBS supplemented with 2 mM EDTA, 1% fetal bovine serum (FBS), 0.2 % sodium azide (FACs buffer) and the appropriate antibody cocktails (See also Table-3.1 for antibodies details). Purified anti-Fc γ RIII/II (2.4G2) or anti human Fc block (Becton Dickinson) was used to block unspecific antibody binding towards murine or human cells, respectively. Non cell-permeant Hoechst 33258 (0.1 μ M) was used as a counterstain to detect necrotic cells while human dead cells were excluded from the analysis using a live/dead fixable near-infrared dead cell stain kit (Molecular Probes). Data was acquired on a LSR Fortessa (BD Biosciences) and analysed with FlowJo software (TreeStar).

For generation of the recombinant human Mincle-hFc chimera, the cDNA encoding human Mincle extracellular domain (amino acid residues 46-219 Swiss-Prot Accession number Q9ULY5) was optimized for mammalian expression and synthesized by Life Technologies. Cysteine residue 52 was replaced by serine to reduce the level of disulphide-linked aggregation seen with the wild type sequence. The cDNA fragment was cloned into the mammalian expression destination vector pDEST12.2 (Invitrogen). The expression vector had been modified to contain regions encoding an N-terminal polyhistidine (His10) and human Fc tag. Mincle-hFc protein was directed for secretion into the medium by the inclusion of a CD33 signal sequence. Human Mincle-hFc was expressed in suspension-adapted CHO cells using polyethylenamine (Polysciences) as the transfection reagent. Recombinant Mincle fusion protein was purified from culture supernatant using Protein A (HiTrap Protein A HP column; GE Healthcare) affinity chromatography followed by size exclusion chromatography (Superdex 75 column; GE Healthcare). Mouse Dectin-1-Fc was generated by cloning the EcoRI-NotI fragment of the PCR product into the EcoRI-NotI sites of the pSecTag.Fcmut vector, which couples to human IgG1 Fc (See Table 3.2 for primers sequence). Human IgG1-hFc control was from Abcam and MCL-hFc was kindly provided by Dr. G. Brown (University of Aberdeen, UK).

3.8 Mouse cell isolation and purification

At the indicated times after *Leishmania major* (*L. major*) infection, ears were harvested from naive or infected mice. The ventral and dorsal sheets of the infected ears were separated and placed in RPMI containing 50 μ g/ml Liberase CI enzyme blend (Roche). After 90 minutes at 37°C, the tissues were cut into small pieces and homogenized. Retromaxillary LNs were removed and mechanically dissociated using tweezers and a syringe plunger. Tissue homogenates were filtered through a 70 μ m cell strainer (Falcon Products).

Small intestine lamina propria (LP) cells were isolated as previously described (Goodyear et al., 2014). Briefly, small intestine was opened longitudinally and washed with HBSS (Thermo Fisher Scientific), cut and placed into HBSS 5 mM DTT (Sigma Aldrich). Next, tissues were

washed 3 times with HBSS 2 % FBS, 5 mM EDTA, minced into fine pieces and digested in HBSS supplemented with 0.2 Wunsch units/ml Liberase TM (Sigma-Aldrich) and 200 Kunitz/ml DNase I (Biomatik). Cells were passed through a 70 µm cell strainer and washed with RPMI complete medium supplemented with 20 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol.

In the indicated experiments, liver cell dissociation was performed as previously described (Allen et al., 2017). Briefly, liver was perfused through the heart with 20 ml of PBS. The liver was extracted, minced into fine pieces and digested in HBSS containing 0.5 mg/ml of type IV Collagenase from *Clostridium histolyticum* (Sigma Aldrich) and 40 µg/ml DNase I (Biomatik) during 20 minutes at RT. The digestion was stopped with FBS. The cells were filtered through a 70 µm cell strainer and centrifuged at 54xg 2 minutes at 4 °C. The supernatant was collected and centrifuged at the same speed two times. The final supernatant (non-parenchymal cells) was centrifuged at 300xg 10 minutes at 4 °C and use for flow cytometry analysis.

Spleen and skin-LNs or Peyer's Patches (PPs) (5 per mouse/ 3 mice per sample) collected in RPMI complete medium were mechanically dissociated using tweezers and a syringe plunger. Tissue homogenates were filtered through a 70 µm cell strainer. In indicated experiments, PPs were digested as described (Bonnardel et al., 2015b) using 100 µg/ml of type 2 collagenase from *Clostridium histolyticum* (Worthington Biochemical Corporation) and 140 µg/ml of DNase I (Biomatik) for 40 minutes at RT and myeloid cells were enriched by negative selection using a cocktail of biotin-conjugated antibodies (anti-CD3, CD19, Biolegend) and Streptavidin-microbeads (Miltenyi Biotec).

Where further purification of naive CD4⁺ T cells from spleen and lymph nodes was required, cells were first enriched by negative selection using a cocktail of biotin-conjugated antibodies (anti-CD11c, CD11b, B220, MHC-II, CD8, GR1, CD16/32, BD Bioscience) and Streptavidin-microbeads (Miltenyi Biotec) and then sorted based on expression of CD44 and CD62L using a FACs Aria cell sorter. DCs from LNs and ears were purified with anti-CD11c-microbeads (Miltenyi Biotec). Where indicated, cells were labelled with CellTracer Violet (5 µM, Thermo Fisher Scientific). Doms CD11b⁺, CD8α⁺ DCs, LysoDCs and LysoMacs from PPs and CD3⁺ T cells or CD3⁻ CD90.2⁺ ILCs from small intestine LP were purified using the FACs Aria cell sorter.

3.9 Human *Leishmania*-infected spleen or skin and healthy human intestinal samples

Human samples of *Leishmania*-infected spleen or skin were collected from the BioB-HVS biobank and were used for Mincle expression analysis by immunohistochemistry (anti-human Mincle, 2A8 clone) or immunofluorescence (anti-human Mincle, 1B6 clone), respectively. Human intestinal samples were obtained from healthy controls with no known autoimmune diseases or malignancies who had been referred to the endoscopy unit for screening of

gastrointestinal diseases, although in all cases they had macroscopically and histologically normal (non-inflamed) intestines. Samples were obtained following informed consent after ethical approval (BER-CDEII-2015) from Hospital Universitario de La Princesa (Madrid, Spain). Biopsies were collected in ice-chilled complete medium and processed immediately in the laboratory by incubating them twice in HBSS containing 1 mM DTT and 1 mM EDTA for 30 minutes at 37°C with 250 rpm rotation. Intestinal biopsies were then digested in RPMI supplemented with 1 mg/ml of collagenase D (Roche) for a maximum of 90 minutes at 37°C with 250 rpm rotation. Intestinal LP cells were then passed through 100 µm cell strainers (Fisher) and washed in FACS buffer (ice-cold PBS supplemented with 2.5 mM EDTA, 5 % FBS, 0.2% sodium azide) before proceeding with the antibody staining (anti-human Mincle, 1B6 clone) and flow cytometry analysis.

3.10 M-CSF and GM-CSF Bone Marrow-derived cells generation, purification and stimulation

Bone-marrow-derived macrophages (BMDM) from the indicated genotypes were obtained from bone marrow cell suspensions after culture on non-treated 60-mm Petri dishes in complete RPMI medium supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, and 30% supernatant of the M-CSF-producing cell line L929. On day 7, preparations of BMDM, characterized as CD11b⁺ F4/80⁺ cells, were >95% pure. Cell suspensions from bone marrow of the indicated genotypes including WT, *Myd88*^{-/-} (Adachi et al., 1998), *CD11cΔSyk* (Iborra et al., 2012), *CD11cΔSHP1* (Abram et al., 2013), *Fcer1g*^{-/-} (B6; 129P2-Fcer1gtm1Rav/J) (Takai et al., 1994), *Clec7a*^{-/-} (Marakalala et al., 2013), *Clec4n*^{-/-} (Saijo et al., 2010) and *Clec4e*^{-/-} (Wells et al., 2008) were cultured on non-treated 150-mm Petri dishes as described (del Fresno et al., 2013) for *Leishmania* studies tissue culture flasks (Falcon Products) for microbiota studies, both in the presence of 20 ng/ml recombinant GM-CSF (Peprotech). GM-CSF BM-derived cells were collected on day 8 in the case of *Leishmania* studies or day 6 for microbiota studies and purified by positive selection with anti-CD11c-microbeads (Miltenyi Biotec).

GM-CSF BM-derived CD11c⁺ cells (GM-BM, 2x10⁶/ml) were stimulated by co-culture with serial dilutions of freeze-thawed (F/T) *L. major*, LPS EB (100 ng/ml, InvivoGen), plated TDB (1 µg/well, InvivoGen), zymosan (10 µg/ml, InvivoGen) or by co-culture with the indicated ratio of total microbiota from SPF mice (quantified by the optical density at 600 nm (OD_{600 nm}) together with quantification by flow cytometry) in the presence of 100 U/ml Penicillin and 100 µg/ml Streptomycin.

Activation of GM-BMs was assessed by quantifying the upregulation of MHCII, CD40, CD86, CCR7, Syk phosphorylation and/or cytokine release measured by ELISA. In the indicated

experiments, the stimulation of GM-CSF BM-derived CD11c⁺ cells was done in the presence of SHP1/2 phosphatase inhibitor (5 or 10 μ M, NSC-87877, Calbiochem).

For some experiments, GM-BMs were FACS-sorted based on the expression of MERTK and CD115 into conventional DCs (GM-DCs) and monocyte-derived macrophages (GM-Macs) as previously described (Helft et al., 2015).

3.11 Transduction of GM-CSF BM-derived cells with lentiviruses

MCL and its mutant genes were introduced into the CSII- CMV-MCS-IRES-Bsd expression vector. Human embryonic kidney (HEK) 293T cells were transfected with the expression vector together with packaging vectors (pCMV-VSV-G-RSV-Rev and pCAG-HIVgp). Culture supernatant was collected at 48–72 hours after transfection. Virus was concentrated by ultracentrifugation at 50,000 \times g for 2 hours at 20 °C. For transduction, WT, Mincle-deficient or MCL-deficient GM-BM cells were incubated with lentivirus at a multiplicity of infection empirically determined for each lentivirus that yield to successful transduction tested by blasticidin S resistance, together with 20 μ l of DOTAP liposomal transfection reagent (Sigma-Aldrich). After 16 hours, the medium was replaced with fresh culture medium. Lentivirus-infected cells were selected by culture with 10 μ g/ml blasticidin S for 3 days.

3.12 Immunofluorescence and *in vivo* imaging

For immunofluorescence of intestinal mucus or promastigotes and *L. major*-infected macrophages were adhered to coverslips coated with poly-L lysine (50 μ g/ml, Sigma) O/N at 4°C or during 30 minutes at 37°C, respectively. Coverslips were washed in PBS, and the mucus or the cells were then fixed in 2% PFA in PBS for 10 minutes at RT, washed with PBS, and permeabilized with 0.1% TX-100 in PBS solution for 10 minutes in the case of the promastigotes and *L. major*-infected macrophages. Preparations were then incubated with blocking solution (2% skimmed milk, with 0.1% TX-100 in PBS for *Leishmania* or without TX-100 for mucus analysis) for 60 minutes at RT and stained with Fc ectodomains and counterstained with DAPI in the indicated experiments to reveal nuclei and kinetoplasts. Finally, samples were covered with ProLong Gold Antifade Reagent liquid mountant (Life Technologies) and visualized under a Zeiss LSM 700 confocal microscope or by using a Leica SP8 STED super resolution microscope as indicated.

For immunofluorescence of phospho-Syk (P-Syk), purified CD11c⁺ GM-BM cells were adhered to coverslip and fixed with Fixation/Permeabilization Solution Kit (BD biosciences) after stimulation. Anti-P-Syk (Clone C87C1) antibody from Cell Signalling was added and incubated at 4°C O/N. Samples were washed and anti-P-Syk was detected using the tyramide signal amplification kit (TSA detection kit Alexa Fluor 568, Molecular Probes) as recommended by the manufacturer. For Mincle expression analysis, PPs whole mount preparations were done as

described (Rios et al., 2016) and stained with anti-CD11c antibody (Clone 3.9, Abcam), biotin anti-mouse Mincle (Clone 1B6, MBL Life Science), PE labelled anti-CD11b (Clone M1/70, BD biosciences), Alexa Fluor 647 Chicken Anti Mouse IgG (H+L) (Molecular Probes), Alexa Fluor 488 Streptavidin (Thermo Fisher Scientific) and DAPI. The samples were fixed, covered as described and visualized using a Zeiss LSM 780 confocal microscope. Finally, anti-human Mincle antibody (Clone 1B6) was used on sections of human samples of *Leishmania infantum*-infected skin.

The IVIS Spectrum *in vivo* imaging system (Perkin Elmer) was used to determine the evolution of parasitemia in real time. Mice were inoculated i.d. in the ear with mCherry⁺ *L. major* (5×10^4), and fluorescence emission was tracked as described (Calvo-Álvarez et al., 2012). mCherry⁺ *L. major*-infected animals were lightly anesthetized with isoflurane placed in the camera chamber, and the fluorescence signal was acquired for 3 seconds. Fluorescence determinations were recorded using the Xenogen *in vivo* imaging system 100. To quantify fluorescence, a region of interest was outlined and analysed using the Living Image Software Package (version 2.11, Xenogen). Results are expressed as average radiance (photons/s/sr/cm²).

3.13 Cell stimulation, antigen presentation *in vitro* and intracellular cytokine detection

For intracellular cytokine analysis, T cells from ears and/or ear LNs were restimulated by incubation for 6 hours over plated anti-CD3 (2C11, Bioxcell, 10 µg/ml) in the presence of soluble anti-CD28 (37.51, Bioxcell, 5 µg/ml), and brefeldin A (Sigma, 5 µg/ml) added for the last 4 hours of culture.

For sorted T cells or ILCs from the small intestine LP analysis, the cells were stimulated with PMA (Sigma Aldrich, 100 ng/ml) and ionomycin (Sigma Aldrich, 500 ng/mL) for 4 hours, in the presence of brefeldin A in the last 3.5 hours (5 µg/ml). Cells were then stained with the corresponding surface antibodies, fixed with 4 % PFA, and incubated with anti-IL-17, anti-IL-22 or anti-IFN-γ during permeabilization with 0.1% saponin.

For *in vitro* Th17 priming, splenic naive CD4⁺ cells were purified using MACS technology (Miltenyi Biotec) according to manufacturers' instructions. 10^4 CD4⁺ cells were incubated with plate-bound anti-CD3 (1 µg/ml) as well as soluble anti-CD28 (2 µg/ml), anti-IFN-γ (10 µg/ml), anti-IL-4 (10 µg/ml), TGF-β (2 ng/ml), IL-6 (5 ng/ml) and IL-23 (20 ng/ml). All recombinant cytokines used in this assay were from Biolegend. IL-17A and IL-22 production was assessed after 5 days by flow cytometry.

FACS-sorted Dendritic CD11b⁺, CD8α⁺ DCs, LysoDCs and LysoMacs from PPs (5×10^3), GM-DCs and GM-Macs (5×10^4) or MACS-purified GM-CSF BM-derived CD11c⁺ cells (1×10^5) were co-cultured with FACS-sorted Celltrace Violet-labelled naive CD4⁺CD62L⁺CD44⁻ OT-II T (5×10^3 or 5×10^4 , respectively), and chicken ovalbumin peptide (OVA323-339, 100 µM) in 200 µl RPMI complete medium in 96-well round-bottom plates. GM-CSF BM-derived CD11c⁺

cells, GM-DCs and GM-Macs were loaded previously with mucosa-associated commensals at a 10:1 ratio, in the presence of 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin. After 72h, cell proliferation was analysed by Celltrace Violet dilution (flow cytometry) and supernatants assessed for IL-17 or IFN-γ quantification by ELISA. For re-stimulation, cells harvested after 72 h of primary culture were stimulated with PMA and ionomycin for 6 h (intracellular cytokine detection) in the presence of brefeldin A in the last 4 hours of culture. Cells were stained for CD4, fixed with 4% PFA, and incubated with anti-IL-17, anti-IL-22 or anti-IFN-γ during permeabilization with 0.1% saponin.

For antigen presentation *in vitro* analysis, CD4⁺ OT-II cell violet-labelled transferred mice LNs were removed and cell suspensions were prepared and seeded in the presence of 10 µm class-II peptide (323-339) and brefeldin A. LN cells were stained and analysed by intracellular flow cytometry. In some experiments, T cells were purified from retroaxillary LNs of infected and healed mice and co-cultured with DCs enriched from dLNs of mice infected 48 hours before. IFN-γ release was determined in culture by ELISA. For the detection of the release of IFN-γ and IL-10 specific for *Leishmania* antigens, 3 × 10⁶ cells from dLNs were seeded in 48-well plates at 37°C for 72 hours in the presence or absence of SLA (12 µg/ml). Cytokine release was measured in culture supernatants by ELISA

For intracellular IL-12p40 and IL-6 staining *in vivo*, mice were intraperitoneally inoculated with brefeldin A (125µg/mouse). PPs were recovered 12 h after brefeldin A injection. The cells obtained were fixed and permeabilized and were stained with PE conjugated anti-IL-12p40 antibody (eBioscience) or APC anti-mouse IL-6 antibody or its isotype control antibody APC Rat IgG1, κ (Biolegend).

3.14 *Leishmania*, total or mucosa-associated commensals preparation and binding studies

To characterize the nature of the *Leishmania* ligand for Mincle, SLA and TDB were plated on ELISA plates, treated with different concentrations of NaIO₄ (Sigma) and degradation agents (Proteinase K, 50 µg/ml; trypsin, 0.05%; DNase I, 0.1 mg/ml), and tested with Mincle-Fc or human IgG1-Fc. SLA was conjugated to APC using Lightning-Link® APC technology (Innova Biosciences), and adhesion to cellular Mincle on B3z cells was analysed by flow cytometry.

For total microbiota analysis, fresh content from the whole intestine, except caecum, of SPF or germ free (GF) mice was collected from mice with ad libitum access to food and water. For mucosa-associated commensals isolation, SPF mice or mice treated with antibiotics in utero until the weaning and gavaged with *Lactobacillus plantarum* (*L. plantarum*) were starved for 18 hours prior to collecting the mucus by gentle scraping of the epithelium from the small intestine as previously described (Cohen and Laux, 1995). In both cases, the samples were vortexed, filtered by 70 µm filter, centrifuged at 600xg 1 minute to remove large particles, the supernatant was centrifuged at 13000xg for 3 minutes and the pellet was diluted in to an OD_{600nm} of 0.6 or

count by flow cytometry by using Bacteria Counting Kit (Thermo Fisher Scientific) as described (Veal et al., 2000). The analysis by flow cytometry was done as previously described (Eriksson et al., 2013). Mincle-hFc, Dectin-1-hFc generated as described, Dectin-2-hFc or Mincle-hFc (R&D Systems) and control-hFc (R&D Systems) were prepared in 10 % skimmed milk in PBS, were incubated O/N at 4°C on a rotating wheel, washed three times and labelled with PE-conjugated goat anti-hFc antibody (eBioscience). SYTO 61 red fluorescent nucleic acid stain (Life Technologies) at 2.5 mM was then incorporated and washed three time in PBS. For IgA-coated bacteria analysis, anti-mouse IgA PE (eBioscience) was added together with SYTO 61 red fluorescent nucleic acid stain.

For Mincle-hFc bound bacteria purification, anti-hFc stained bacteria were incubated with anti-PE microbeads (Miltenyi), washed twice and then purified. After MACS separation, part of the negative and the positive fractions were collected for 16S sequencing analysis.

For blocking experiments with *Leishmania* and commensal microbes, human Mincle-Fc chimera was blocked with anti-human Mincle 2F2 clone; isotype matched antibody (clone MID 15B4, rat IgM) was used as a control (Sigma Aldrich) or with TDB (InvivoGen) prior staining assay.

3.15 CellTracer Violet Labelling of *Lactobacillus plantarum*

Lactobacillus plantarum (ATCC14917) was grown O/N on MRS Broth medium (Oxoid) at 37°C in aerobic conditions to approximately OD_{600nm} of 0.5. Bacteria were labelled with CellTracer Violet (100 µg/ml, Thermo Fisher Scientific). Bacteria were incubated 10 minutes at 30°C with constant agitation, washed twice and inoculated by gavage in SPF WT mice starved for 18 hours and previously intraperitoneally injected with 3 mg of cimetidine HCl (Sigma Aldrich) and 0.02 mg of sincalide (Tebu-bio). The mucosa associated commensals were collected 6 hours later for flow cytometry analysis.

3.16 RNA isolation and quantitative RT-PCR

Total RNA from the liver parenchymal fraction, small intestine, ileum or PPs was isolated using Trizol (Thermo Fisher Scientific) in combination with RNeasy Mini Kit (Qiagen) accordingly to manufacturer's instructions. RNA was reverse transcribed to cDNA using random hexamers and High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Quantitative PCR amplification was performed with the GoTaq qPCR Master Mix (Promega) in a 7900HT Fast Real-Time PCR System (Applied Biosystem). All reactions were done in triplicate, following the manufacturer's instructions. Data were normalized to *Gadph* or *bactin* indicated in each case and displayed as relative values. PCR primers used for SYBR Green assays are shown ([See Table 3.2 for primers sequence](#)).

3.17 Enzyme-linked immunosorbent assays (ELISA)

IL-17 or IFN- γ levels were determined using the mouse IL-17A (homodimer) ELISA Ready-SET-Go (Affymetrix) or the mouse IFN- γ ELISA Set (BD OptEIA™) according to the manufacturer's instructions. IL-23 and IL-6 were quantified using matched antibody pair ELISA. Anti-mouse IL-23 p19 (5B2) together with biotinylated anti-mouse IL-12/IL-23 p40 (C17.8) both from Thermo Fisher Scientific. Anti-Mouse IL-6 (MP5-20F3) (BD Biosciences) with biotinylated anti-mouse IL-6 (MP5-32C11) (Thermo Fisher Scientific). The rest antibody pairs (IL12p40, IL-6, IL-10 and TNF- α) were from BD, and ELISAs were performed according to the manufacturer's instructions. For the later pairs, ELISAs were developed using extravidin®-alkaline phosphatase and pNPP alkaline phosphatase substrate from Sigma.

To measure total IgA, the plates were coated with goat anti-mouse IgA antibody (Bethyl Laboratories Inc) and the standard curve done by using purified mouse IgA, κ isotype control (BD Pharmingen) as previously described (Bunker et al., 2015). To analyse serum commensal bacteria-specific IgG, intestinal bacteria were prepared to coat ELISA plates, after heat-killing at 85°C for 1 hour, following a modified protocol from the previously described (Zeng et al., 2016). The plates were washed 3 times with PBS containing 0.05 % Tween 20 and blocked with PBS containing 10 % FBS for 1 hour at RT. Intestinal lavage was prepared by vortexing the intestinal content collected in 3 ml of PBS during 3 minutes. Samples were centrifuged at 8,000xg for 2 minutes and removed the supernatant for assaying. Intestinal content supernatants or mice serum (to IgG analysis) were applied to the wells after the blocking step and incubated at 4°C O/N. The antibody used for detection was goat anti mouse IgA HRP (Southern Biotech) or goat anti mouse IgG-heavy and light chain HRP (Bethyl) together with Sure Blue TMB 1-Component Microwell Peroxidase Substrate and TMB Stop Solution (Biogen). The absorbance was read at 450 nm.

3.18 Adoptive transfer experiments

CD4⁺ T cells were purified from pooled spleens and lymph nodes of OT-II CD4⁺ TCR transgenic mice or B6/SJL mice expressing CD45.1 by negative selection (Miltenyi Biotec) using a cocktail of biotin-conjugated antibodies (anti-CD11c, CD11b, B220, MHC-II, CD8, GR1, CD16/32 (BD Bioscience) and anti-CD25, CD44 (Biolegend) followed by separation with Streptavidin-microbeads (Miltenyi Biotec). Purified CD4⁺ T cells from OT-II were incubated at 5 \times 10⁶ cells/ml in PBS with 0.5 μ M CellTrace™ Violet (Invitrogen) for 10 minutes at 37°C. The reaction was stopped with 5% FBS PBS. CD4⁺ OT-II T cells (2–5 \times 10⁵) were transferred just after challenge in the ear dermis either with 5 \times 10⁴ metacyclic promastigotes of *Leishmania*-OVA, rVACV-OVA (kindly provided by J. Yewdell, NIAID, Bethesda) or dead *Leishmania*

OVA (1×10^5). Four days after adoptive transfer, the dLNs were removed to perform antigen presentation *in vitro*.

$CD4^+ CD45.1$ T cells (6×10^6) were adoptively transferred into recipient mice. IL-17 and IL-22 intracellular production by flow cytometry in PPs T cells was determined 14 days after transfer by PMA and ionomycin re-stimulation and intracellular cytokine staining followed by flow cytometry.

3.19 Antibiotic Treatment, SFB quantification and colonization with *Lactobacillus plantarum*

For ablation of intestinal bacteria, an antibiotic cocktail of 1 g/L each of Ampicillin (Normon), Neomycin sulfate (Sigma Aldrich), Metronidazole (Sanofi), 0.5 g/L Vancomycin (Pfizer) and Sucralose (Sigma Aldrich) 4 mg/ml was used as previously described (Rakoff-Nahoum et al., 2004). For selective depletion, Vancomycin alone was also used as previously described (Ivanov et al., 2008). Antibiotics were added into the drinking water on a weekly basis. For treatment from birth, breeding pairs were kept with the antibiotic cocktail. Control cages were kept on regular water plus sucralose. SFB in feces was detected by PCR (IDEXX BioResearch) or by qPCR in feces, briefly microbial DNA was isolated from mouse fecal samples using QIAamp PowerFecal Pro DNA kit (Qiagen), according to manufacturer's instructions. SFB DNA was then detected by quantitative PCR with SFB specific primers ([See Table 3.2 for primers sequence](#)) and SYBR-Green PCR master mix. SFB quantification was performed with reference to a standard curve generated with quantified plasmid DNA where SFB 16S rRNA gene has been cloned in PCR-blunt II vector (ThermoFisher) according to suppliers' instructions.

Mice treated with antibiotics in utero until the weaning, were inoculated by gavage with *L. plantarum*. One hour prior to the bacterial gavage mice were injected intraperitoneally with 3 mg of cimetidine HCl (Sigma Aldrich) and 0.02 mg of sincalide (Tebu-bio) in 100 μ l of PBS to inhibit stomach acid secretion and empty the gallbladder to improve the colonization as previously described (Iida et al., 2013). For preparation of bacterial inocula, *L. plantarum* was grown O/N on MRS Broth medium (Oxoid) at 37°C in aerobic conditions. The bacteria were suspended in PBS. Mice were gavaged with 200 μ l of inoculum (dose 1×10^6). Mice received doses every two days during the four weeks.

3.20 Analysis of bacterial translocation

Liver was aseptically removed and homogenized in sterile-filtered 0.05% NP40 1% BSA PBS before plating serial dilutions on LB agar plates, which were then incubated at 37°C in aerobic conditions without light.

3.21 Bacterial DNA isolation and 16S gene sequencing

Ultra-Deep Microbiome Prep10 (Hain Lifescience) was used accordingly to manufacturer's instructions to isolate enriched microbial DNA from fresh liver and to remove of animal host DNA. Microbial genomic DNA from bound Control-hFc, Mincle-hFc or not bound Mincle-hFc fractions or Mincle deficient mice and their WT littermates intestinal content was isolated by using QIAamp DNA Mini Kit or QIAamp DNA Stool Mini Kit (Qiagen) respectively. PCR and sequencing were performed following the 16S Metagenomic Sequencing Library Preparation guide (Illumina Inc.) The V3-V4 region of 16S rDNA was amplified using the primers recommended in this guide ([See Table 3.2 for primers sequence](#)). One PCR reaction was performed per sample and one single amplicon of approximately 460 bp was created. 2x Kappa HiFi Hot Start Ready Mix (Kappa Biosystems, Boston, MA) was used for the first amplification step. AMPure XP beads (Beckman Coulter, Brea CA) were used to purify the amplicons and to remove free primers and dimer species. Each amplicon (5 µl) were used as template in a second Index PCR. This second PCR attached dual indices and Illumina sequencing adapters to each amplicon using the Nextera XT index Kit (Illumina San Diego, CA). An additional clean-up step was performed with the AMPure XP beads. All the libraries were run in a Bioanalyzer DNA 1000 chip (Agilent Technologies Waldbronn, Germany) to verify the size of the product. Libraries were quantified by fluorometric method (Qubit dsDNA) and quality was check in the 2100 Agilent Bioanalyzer. All libraries were dilute to the same concentration, using Illumina Resuspension Buffer (RSB) and were combine an equal volume of all of them in the same pool to sequence. The sample pool (10 nM) was denatured with 0.2 N NaOH, then diluted to 6 pM and combined with 50% (v/v) denatured 6 pM PhiX, prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform, using a 2 x 300 cycle V3 kit, following standard Illumina sequencing protocols.

3.22 Processing of 16S sequencing data

Samples were analysed using BaseSpace Application 16S Metagenomics v1.0 (Illumina) to determine the bacterial taxonomical composition. This program use Illumina-curated version of the GreenGenes taxonomic database and RDP Classifier (Ribosomal Database Project). For some additional data analysis, the "Quantitative Insights into Microbial Ecology" software (QIIME version 1.9.0) was used. Processed reads were then clustered in Operational taxonomic units (OTUs) using UCLUST with a similarity threshold of 0.97 and were aligned using PyNast against 16S reference database GreenGenes version 13.8 using default parameters. Enrichment index was obtained by comparing the relative abundance of each genus in Mincle-hFc-enriched and depleted fractions. Specificity index was calculated by comparing the relative abundance of each genus in Mincle-hFc and Control-hFc enriched fractions.

3.23 Serum hepatic parameters and metabolomics profiling

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), total and indirect bilirubin were measured by using an automatic biochemical analyser (Dimension RxL Max Integrated Chemistry System, Siemens). For metabolomics profiling, liver was collected after perfusion through the heart with cold PBS. An aliquot of approx. 20 mg was immediately freeze-clamped in liquid nitrogen and stored at -80°C until lipid extraction and analysis. Liver tissue was lysed in cold MeOH:H₂O (1:1, sample:solvent 1:10 ratio) with a TissueLyser LT homogenizer (Qiagen). Afterwards, liver homogenate was split in two aliquots for the metabolomic profiling of diacylglycerides (DAG) and free fatty acids (FFA) using HPLC-ESI-MS-QTOF and GC-EI-MS-QTOF (Agilent Technologies, Germany) as previously reported (Gonzalez-Pena et al., 2017). For DAG profiling, the resulting data matrix is composed of all individual species of DAGs detected in the samples sorted by their characteristic retention time and neutral mass, and the abundance of each compound for each sample. DAGs were identified by the exact mass and by the elucidation of MS/MS spectra obtained using a LC-MS/MS analysis in product ion mode. In contrast, for FFA profiling, the resulting matrix is made of all individual species of FFAs detected in the samples sorted by their characteristic retention time and target ion, and the abundance of each compound for each sample. FFAs were identified by comparing their retention time, retention index and mass fragmentation patterns with those available in an in-house library as described (Mastrangelo et al., 2015).

3.24 Statistics

Statistical significance was determined by parametric Student's t-test, by Mann & Whitney's U test or one-way ANOVA and Bonferroni post-hoc test using GraphPad Prism (GraphPad Software) with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Antibody (anti-)	Fluorophore	Vendor
CD45	biotin, FITC, PE, PerCP-Cy5.5, V450, APC	eBioscience
CD45	APC-Cy7	Tonbo Biosciences
rat IgG1, κ (isotype)	biotin	Biolegend
BST2	APC	Biolegend
CCR7	PE, APC	eBioscience
CD103	biotin, FITC, PE, PerCP-Cy5.5, V450, APC	eBioscience
CD103	BUV395	BD Biosciences
CD115	biotin	Thermo Fisher Scientific
CD11b	biotin, FITC, PE, PerCP-Cy5.5, V450, APC	eBioscience
CD11b	FITC	BD Biosciences
CD11b	BV605, APC-Cy7	Biolegend
CD11b	APC-Cy7	Biolegend
CD11c	biotin, FITC, PE, PerCP-Cy5.5, V450, APC	eBioscience
CD11c	BUV395	BD Biosciences
CD11c	Alexa700	Biolegend
CD172 (SIRP1α)	biotin, FITC	eBioscience
CD172 (SIRP1α)	PerCP-Cy5.5	Biolegend
CD185 (CXCR5)	PE, APC	Biolegend
CD19	biotin, FITC, PE, PerCP-Cy5.5, V450, APC-Cy7	eBioscience
CD196 (CCR6)	PE	Biolegend
CD3	biotin, FITC, PE, PerCP-Cy5.5, V450, APC	eBioscience
CD4	biotin, FITC, PE, PerCP-Cy5.5, V450, APC	eBioscience
CD4	PE-Cy7	Biolegend

Antibody (anti-)	Fluorophore	Vendor
CD40	APC	BD Biosciences
CD44	biotin, FITC, PE	eBioscience
CD45	BV510	Biolegend
CD45.1	biotin, FITC, PE, PerCP-Cy5.5, V450, APC ,PE-Cy	eBioscience
CD45-R (B220)	BV421	BD Biosciences
CD62L	biotin, PE	eBioscience
CD64	PE-Cy7	Biolegend
CD86	PE	Tonbo Biosciences
CD8α	biotin, FITC, PE, PerCP-Cy5.5, V450, APC	eBioscience
CD90.2 (Thy-1.2)	APC	Biolegend
F4/80	FITC	Biolegend
Gr-1	biotin, FITC, PE, APC	eBioscience
HLA-DR	BV570	Biolegend
human IgG (Fc gamma)	biotin, PE	eBioscience
I-A^b (MHC-II)	biotin, FITC, APC	eBioscience
I-A^b (MHC-II)	AF700	Biolegend
IFN-γ	APC	BD Biosciences
IgA	biotin	eBioscience
IgA	PE	Thermo Fisher Scientific
IgG	PerCP-Cy5.5	Biolegend
IL-17A	APC-Cy7, PE	BD Biosciences
IL-17A	Brilliant Violet 605™	Biolegend
IL-22	PE ,PerCP-Cy5.5	Biolegend
Ly6C	biotin, FITC, PE, PerCP-Cy5.5, V450, APC	eBioscience
Ly6G	PE	BD Biosciences
Ly6G	BV510	Biolegend
MERTK (Mer)	PE	Biolegend
Mincle (1B6)	-	Medical& Biological Labs
PD-1	biotin ,BV421	Biolegend
Siglec-F	BV421	BD Biosciences
Streptavidin	PE, PerCP-Cy5.5 ,APC	eBioscience
Streptavidin	BV786	BD Biosciences

Table 3.1 Antibodies used in this study. From the left to the right: Antigen, fluorophore and vendor.

Gene name	Forward (5'-3')	Reverse (5'-3')
mDectin-1 CTLD	TTTCCCGAATTCTCTTGCCTTCTAATTGGAT	TTTCCCGCGGCCGAGTTCCTTCTCACAGATAC
Gadph	TGAAGCAGGCATCTGAGGG	CAGGAAGTAGGTGAGGGCTTG
Clec4e	AGTGCTCTCCTGGACGATAG	CCTGATGCCTCACTGTAGCAG
Tgfb	GCATGGCTGAACCAAGGA	AAAGAGCAGTGAGCGCTGAATC
Il6	CCGTGTGGTTACATCTACCCT	CGTGGTCTGTGTGATGACAGT
Il23a	TGCTGGATTGCAGAGCAGTAA	GCATGCAGAGATCCGAGAGA
Il12b	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
Reg3g	TCCACCTCTGTTGGGTTTCAT	AAGCTTCCTTCTGTCTCTCC
Saa1	CATTTGTTACAGAGGCTTTCC	GTTTTCCAGTTAGCTTCTTTCATGT
bactin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Acc	GATGAACCATCTCCGTTGGC	GACCCAATTATGAATCGGG
Ppara	ACAAGGCCTCAGGGTACCA	GCCGAAAGAAGCCCTTACA
Fas	GCGGGTTCGTGAAACTGATAA	GCAAAATGGGCCTCCTTGATA
Scd1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
Srebpc	GGCACTGAAGCAAAGCTGA	TCATGCCCTCCATAGACACA
Pepck	CCATCACCTCTGGAAGAACA	ACCCTCAATGGGTACTCCTTCTG
Cpt1a	CTCCGCCTGAGCCATGAAG	CACCACTGATGATGCCATTCT
G6pase	CGACTCGCTATCTCCAAGTGA	GTTGAACCACTCTCCGACCA
SFB	TGT GGG TTG TGA ATA ACA AT	GCG AGC TTC CCT CAT TAC AAG G
16SrRNA	TCGTCGGCAGCGTCAGA TGTG	GTCTCGTGGGCTCGGAGATGTGTA
	TATAAGAGACAGCCTACGGNGGCWGCAG	TAAGAGACAGGACTACHVGGGTATCTAATCC

Table 3.2 Primers used in this study. From the left to the right: Gene name, forward and reverse sequences (5'-3').

Results

4. RESULTS

4.1.1 Analysis of Mincle contribution to the immunity against *Leishmania major*.

C-type lectin receptors (CLRs) are versatile receptors sensing self and non-self ligands (Iborra and Sancho, 2015). CLR-recognition of eukaryote parasites, such as *Leishmania*, together with its recognition by others PRRs, triggers a combination of activating and inhibitory pathways that can affect the final host response against the parasite (Lefevre et al., 2013, Woelbing et al., 2006, Vazquez-Mendoza et al., 2013). For example, the CLR SIGNR3 (mouse *Cd209d*) is targeted by *Leishmania infantum* in macrophages to inhibit the heterologous CLR Dectin-1(*CLEC7A*)-mediated IL-1 β secretion, favouring parasite survival (Lefevre et al., 2013). *Leishmania* genus are the causative agents of leishmaniasis, a group of neglected diseases whose clinical manifestations vary depending on the infectious *Leishmania* species but also on host factors. The life cycle of the parasite includes two developmental stages, promastigotes and amastigotes, which are well adapted to survive in the sand fly insect vector and the mammalian host respectively. The extracellular flagellated metacyclic promastigotes develop within the sand fly midgut and initiate infection after being inoculated in the skin by the bite of an infected fly. The non-motile intracellular forms, amastigotes replicate and maintain infection in the vertebrate host. In the mammalian host, *Leishmania* amastigotes reside mainly in long-lived resident macrophages (Sacks and Melby, 2001). The recognition of the parasite by host myeloid cells is key to trigger an effective *Leishmania* protective immunity (Rossi and Fasel, 2018). Clearance of *Leishmania* parasites requires IFN- γ -producing effector cells, mainly CD4⁺ Th1 cells, although other cells, such as NK cells (Bajenoff et al., 2006) and CD8⁺ T cells (Belkaid et al., 2002) can contribute to this process. IFN- γ signalling in infected macrophages promotes expression of inducible NO synthase (iNOS) and NO production that, together with reactive oxygen species (ROS) are essential to kill intracellular parasites (Green et al., 1990). Besides IFN- γ , other inflammatory cytokines, such as TNF, can activate the infected macrophages in an autocrine manner to produce NO (Bogdan et al., 1990). On the contrary, CD4⁺ Th2-related cytokines, such as IL-4, IL-13, IL-10, and antibody production are associated with alternative activated macrophages (Gordon, 2003), which favours parasite survival inside the macrophages (Kropf et al., 2005), and a non-healing phenotype (Scott et al., 1988 , Heinzl et al., 1989 , Chatelain et al., 1992, Sacks and Noben-Trauth, 2002). The priming and development of an effector Th1 adaptive immunity require IL-12 production by specific subtypes of DCs. The main source of IL-12 upon *Leishmania* infection are DCs derived from inflammatory monocytes (moDCs) and the migratory CD103⁺ DCs (Leon et al., 2007, Martinez-Lopez et al., 2015).

However, the parasite, in order to escape, target DC activation either being silent or even inhibiting DC activation, motility and migration to draining lymph nodes (dLNs) (Ponte-Sucre

et al., 2001, Jebbari et al., 2002, Revest et al., 2008, Figueiredo et al., 2012, Hermida et al., 2014, von Stebut, 2017). The outcome of this interaction, vary depending on the different DC subset involved, as they are equipped with different PRRs. In addition, several *Leishmania* species have different PAMPs and/or immune evasion strategies. In particular, low dose infection with *Leishmania major* in mice, which causes a disease that resembles human cutaneous leishmaniosis (Belkaid et al., 2000), is a poor inducer of DC activation, inhibiting migration of DCs to dLNs (Ng et al., 2008, Ribeiro-Gomes et al., 2012). DCs do eventually migrate and promote Th1 protective immunity and macrophage microbicide activity (Leon et al., 2007). The mechanisms by which *Leishmania* initially blunts DC activation and T cell priming remain ill-defined. It has been argued that they may involve uptake of apoptotic-infected neutrophils by DCs (Ribeiro-Gomes et al., 2012, Ribeiro-Gomes et al., 2015, Peters et al., 2014) or direct DCs contact with parasite products (Srivastav et al., 2012). However, the role of myeloid-CLRs, mediating *L. major*-induced DCs suppression is unknown. Here, we aim to analyse the role of Mincle to this process (**Fig. 4.1.1**). As a dual CLR, Mincle can recognize self-ligands released by dead cells and non-self ligands on the cell walls of bacteria and fungi, triggering phosphorylation of ITAM tyrosine residues in the FcR γ chain by Src-family kinases, followed by the recruitment and activation of the kinase Syk, that boosts immunity to infections and inflammation (Lu et al., 2018). Classically considered an activating CLR, Mincle has recently been associated with dampening of immunity (Seifert et al., 2016, Wevers et al., 2014, Wuthrich et al., 2015) by repressing IL12-p35 transcription through a Syk-PKB-dependent pathway in response to *Fonsecaea* (Wevers et al., 2014). Uncovering the contribution of Mincle to *L. major*-induced DC suppression will help to improve a rational design of vaccines aimed to counteract parasite virulence factors, along with the use of the most adequate adjuvants.

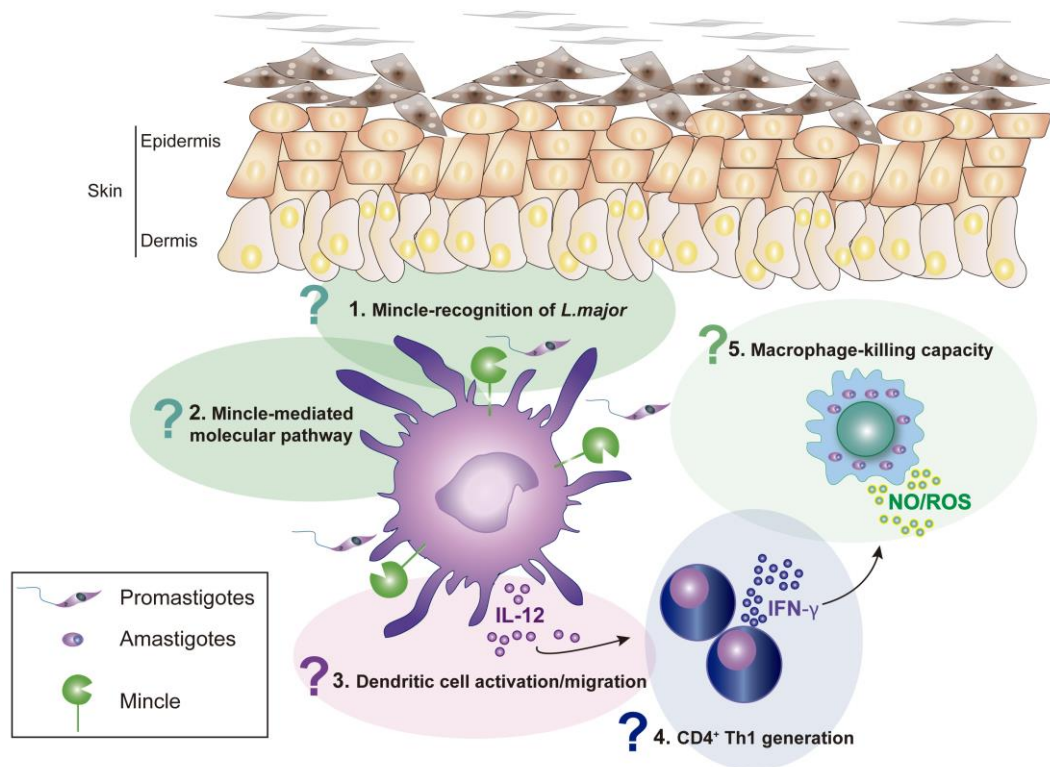


Figure 4.1.1 Contribution of the CLR Mincle to *Leishmania major*-induced immune response. Picture displaying the five specific objectives: (1- 3) Addressing *L. major*-Mincle interaction and how it could influence DCs function. (4 and 5) Analysing the effect of Mincle-*L. major* interaction on adaptive immune response and macrophage microbicide response. Mincle, macrophage inducible C-type lectin; NO, nitric oxide; ROS, reactive oxygen species

4.1.2 *Leishmania* releases a soluble proteinaceous ligand for Mincle

While screening for pathogens expressing Mincle ligands by dot blot, we found that the human Mincle ectodomain-hFc chimera (Mincle-hFc) specifically bound soluble *Leishmania major* (*L. major*) extracts from freeze-thawed promastigotes (Fig. 4.1.2 A, left). Mincle-hFc also bound to blotted supernatants from *L. major* promastigotes kept for 3 hours at 37°C to favour secretion (Fig. 4.1.2 A, right) and detected plated soluble *Leishmania* antigen (SLA) or supernatants (SN) by ELISA (Fig. 4.1.2 B); in contrast, control-hFc or macrophage C-type lectin (MCL)-hFc did not bind to plated *Leishmania* extracts (Fig. 4.1.2 C). Loss of binding upon boiling of the parasite preparations indicated that the ligand is heat-sensitive (Fig. 4.1.2 A, B). Treatment of plated *Leishmania* extract with sodium periodate, which oxidizes glycans, did not affect binding of Mincle-hFc to the *Leishmania* extract, but did inhibit the trehalose-dependent binding to TDM (Fig. 4.1.2 D).

To determine whether the ligand bound cellular Mincle, B3Z NFAT reporter cells (Karttunen et al., 1992) were transduced with a chimera comprising the extracellular human Mincle and intracellular CD3 ζ , or alternatively with the wild type (WT) mouse Mincle receptor co-transduced with the FcR γ chain and Syk. The CD3 ζ chimera responds to any multimeric ligand, whereas WT Mincle requires the Syk kinase transduction pathway to activate an NFAT reporter (Sancho et al., 2009). Plated *Leishmania* lysates triggered the Mincle-CD3 ζ reporter, but not the

WT Mincle-FcR γ -Syk or the parental cell line (Fig. 4.1.2 E). SLA did not trigger the Mincle-CD3 ζ chimera or the WT Mincle (not shown), suggesting a low valency of the soluble ligand. In contrast, SLA blocked the triggering of WT Mincle or CD3 ζ chimera by plated TDB in a dose-dependent and heat-sensitive manner (Fig. 4.1.2 F). In addition, fluorochrome-labelled SLA bound to Mincle-expressing B3Z cells, but not to the parental cell line (Fig. 4.1.2 G).

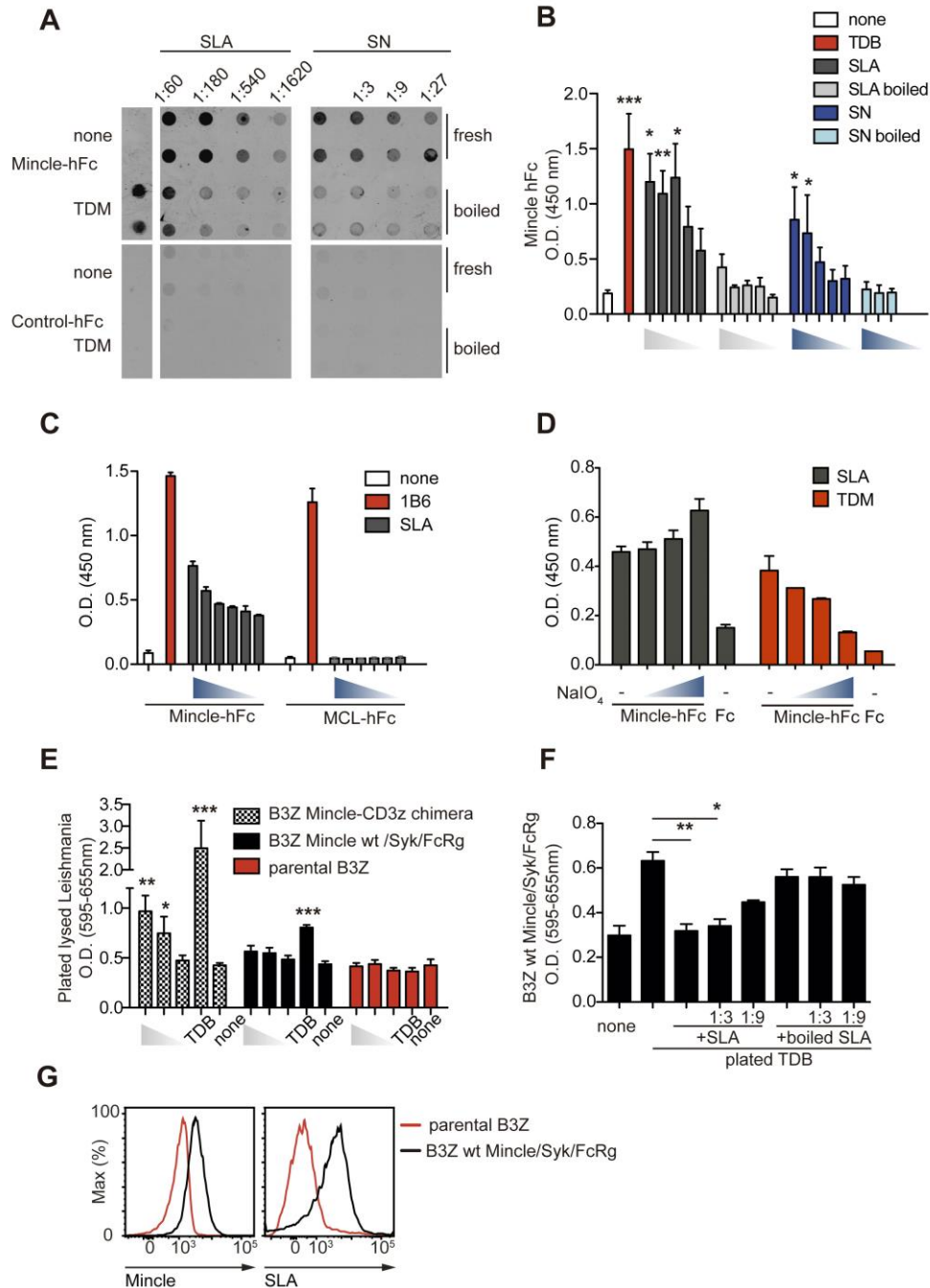


Figure 4.1.2. *Leishmania* releases a soluble ligand for Mincle. (A) Dot blots for Mincle-hFc (top) or control-hFc (bottom) with membranes spotted with fresh or boiled soluble *Leishmania* extracts (SLA) (left) or supernatants (SN) (right) from the indicated dilutions of stationary cultured parasites. Culture medium (none) or TDM were used as controls. (B) ELISA with

Mincle-hFc of different doses of SLA or supernatants (fresh or boiled) from *L. major* promastigotes and controls (none or TDB). (C) *Leishmania* extract (SLA) was plated and detected by ELISA using Mincle-, MCL- or Control-hFc. Plated 1B6 (anti-Mincle antibody) was used as a positive control for ELISA. (D) Plated *Leishmania* extract (SLA) and TDM were treated with sodium periodate and detected by ELISA using Mincle-hFc or Control-hFc. (E) NFAT reporter activity in response to 10^6 , 10^5 or 10^4 of plated lysed-*Leishmania* or TDB in B3Z cells expressing human Mincle-CD3 ζ chimera, WT mouse Mincle receptor co-expressing Syk and FcR γ , or the parental cells. (F) NFAT reporter activity in B3Z cells expressing WT mouse Mincle receptor, FcR γ , and Syk and exposed to plated TDB in the presence of the indicated dilutions of fresh or boiled SLA. (G) Staining with anti-Mincle (left) and fluorochrome-labelled SLA on control and Mincle-expressing B3Z cells. (A, C, D, G) Data are from one representative experiment of four (A) or three (C, D, G) performed. (B, E, F) Bars show arithmetic mean + SEM corresponding to three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (One-way ANOVA with Bonferroni post-hoc test).

Mincle-hFc also stained fixed and permeabilized *L. major* promastigotes, whereas Dectin-1-hFc did not (Fig. 4.1.3 A). Binding of Mincle-hFc to fixed and permeabilized *L. major* was specifically inhibited by pre-incubation of the ectodomain with 2F2 anti-Mincle antibody or with soluble TDM (Fig. 4.1.3 B). Moreover, treatment of fixed and permeabilized *Leishmania* promastigotes with proteinase K, trypsin, heat, or low pH, but not DNaseI, inhibited labeling by Mincle-hFc chimera, suggesting a proteinaceous nature of the ligand (Fig. 4.1.3 C). Notably, other *Leishmania* species were also specifically stained by Mincle-Fc (Fig. 4.1.3 D). Confocal analysis of Mincle-hFc staining in fixed and permeabilized *L. major* promastigotes revealed an intracellular granular pattern, including the flagellar pocket close to the kinetoplast, a unique site for exocytosis (Fig. 4.1.3 E). Mincle-hFc also stained the parasitophorous vacuole containing *L. major* amastigotes after uptake of the parasite by cultured M-CSF bone marrow-derived macrophages (Fig. 4.1.3 F) alongside the staining of the endogenous nuclear ligand for Mincle (Yamasaki et al., 2008). Dectin-1-hFc did not stain fixed and permeabilized promastigotes or amastigotes (Fig. 4.1.3 E, F). Thus, *Leishmania* produced a soluble proteinaceous ligand(s) for Mincle that was detected in all tested *Leishmania* species and was present at both the promastigote and amastigote stages.

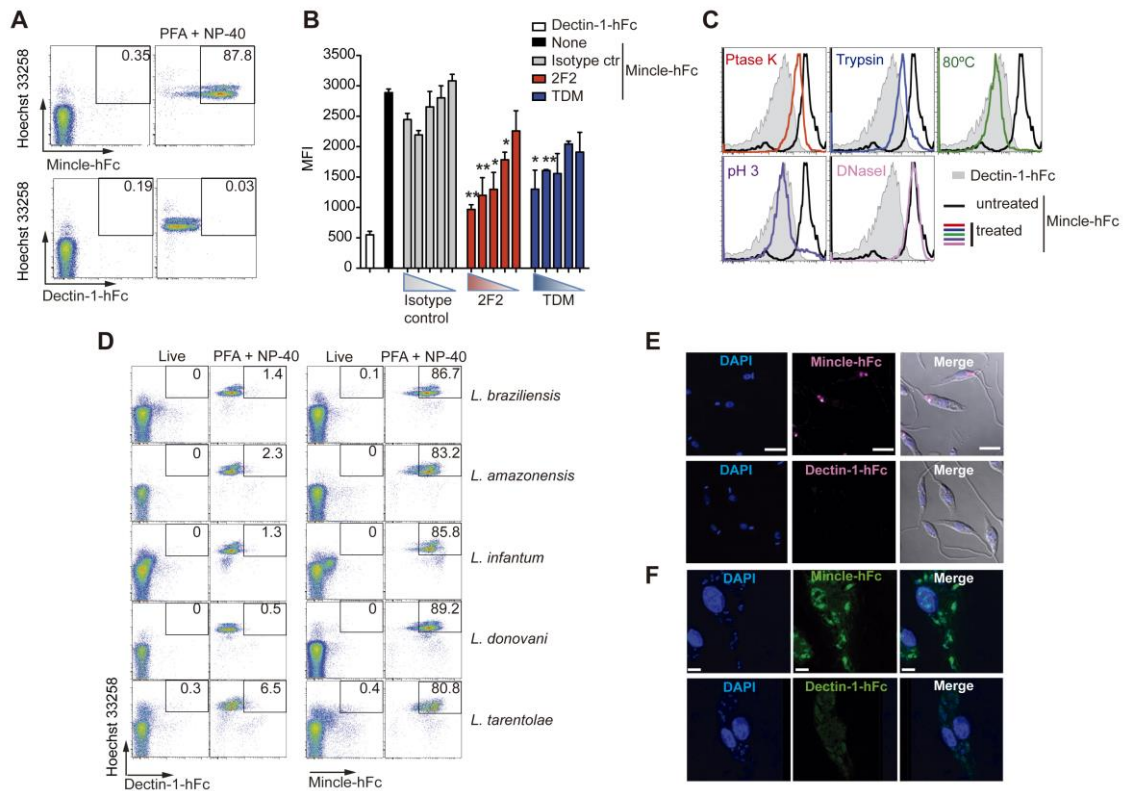


Figure 4.1.3. The *Leishmania* ligand for Mincle is proteinaceous and present at all parasite stages. (A) Mincle- and Dectin-1-hFc staining with Hoechst 33258 counterstaining in live *L. major* promastigotes or paraformaldehyde (PFA)-fixed parasites permeabilized with NP-40. (B) Mean fluorescence intensity in fixed and permeabilized *L. major* promastigotes stained with Dectin-1- or Mincle-hFc preincubated with titrated dilutions of anti-Mincle (clone 2F2), isotype control antibody (mouse IgM), or TDM (10 μ g/ml starting dose, 3 fold dilution). Bars show arithmetic mean + SEM corresponding to three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (One-way ANOVA with Bonferroni post-hoc test). (C) Fixed and permeabilized *L. major* promastigotes were subjected to the indicated treatments (colors) or untreated (black) and stained with Mincle-hFc chimera. Gray histograms show Dectin-1-hFc staining. (D) Dectin-1- or Mincle-hFc and Hoechst 33258 staining of live or fixed and permeabilized (PFA + NP40) promastigotes of different *Leishmania* species (E, F) Confocal images of Mincle- and Dectin-1-hFc staining in fixed and permeabilized *Leishmania* promastigotes (E) and MCSF bone-marrow-derived macrophages pre-incubated with promastigotes (F). Nuclei are counterstained with DAPI. Scale bar: 5 μ m. (A, C-F) Plots and images are from single representative experiments of three performed.

4.1.3 Mincle is expressed during *Leishmania* infection

The typical route of *Leishmania* infection is a skin bite by a parasite-inoculated sandfly. We therefore analysed Mincle expression in dermal cell types of WT and Mincle-deficient (*Clec4e*^{-/-}) mice after *L. major* infection. The pinnae of both ears were inoculated by intradermal (i.d.)

injection of 1000 *L. major* metacyclic promastigotes, and ear infiltrates were analysed 24 hours later and compared with dermis taken from the ears of uninfected mice. Mincle expression by myeloid cells was modest in unchallenged dermis (Fig. 4.1.4 A), but was upregulated upon *L. major* infection in tissue macrophages, neutrophils, and monocyte-derived DCs (MoDCs) infiltrating the infection site (Fig. 4.1.4 A, B), and was maintained throughout the course of infection (Fig. 4.1.4 B). Mincle staining of myeloid cells was also observed in human skin samples and serial spleen sections from patients infected with *Leishmania infantum* (Fig. 4.1.4 C, D).

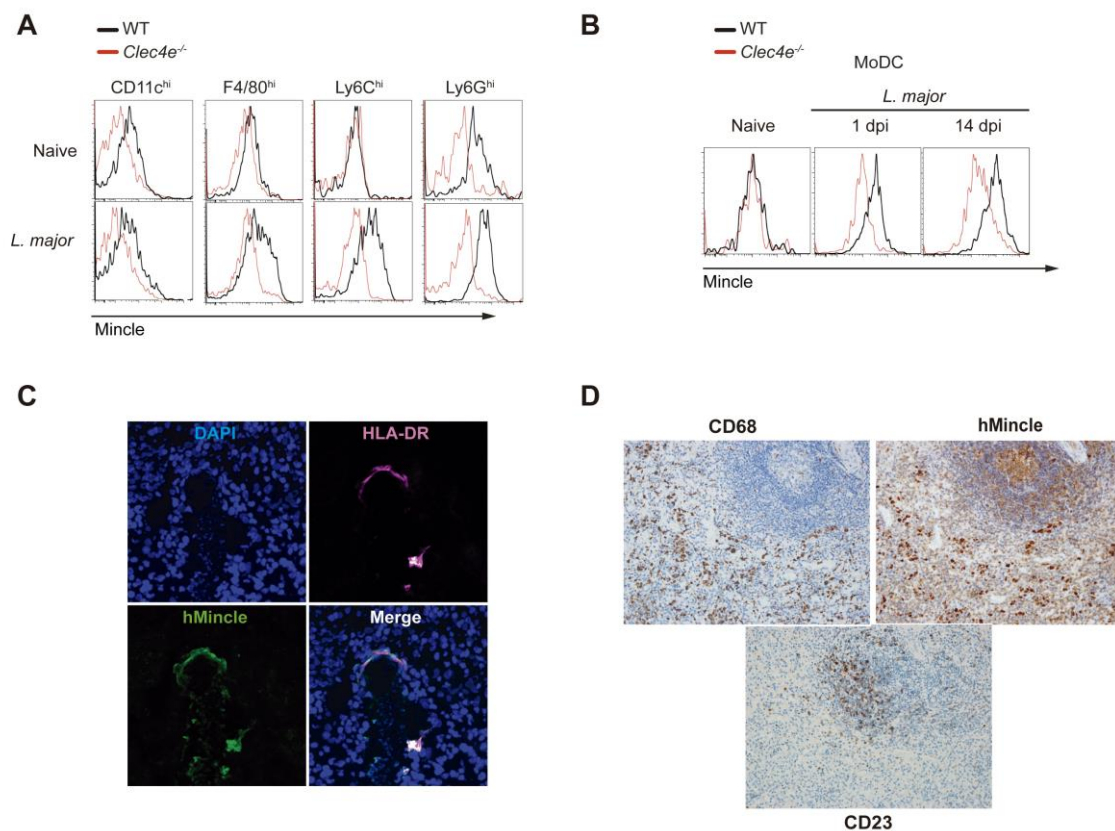


Figure 4.1.4 Mincle is expressed during *Leishmania* infection. (A and B) Expression of Mincle on the indicated cell subsets from the ear of naive or *L. major*-infected WT or *Clec4e*^{-/-} mice 1 day post-infection (p.i) (A) or on monocyte-derived DC (MoDC) at the indicated time points after infection (B). (A and B) Histograms depict representative data from three independent experiments (n=9). (C) Immunofluorescence of human skin infected with *L. infantum* and labelled with anti-HLA-DR and anti-human Mincle (1B6 clone). (D) Spleen samples from *L. infantum*-infected individuals immunostained with antibodies to human Mincle (2A8 clone), CD68, and CD23. (C, D) Single representative stainings are shown of three performed.

4.1.4 Mincle deficiency increases resistance to cutaneous leishmaniasis

To determine the contribution of Mincle to the immune response against *L. major*, we monitored cutaneous disease during a 11-week period after ear inoculation with 1000 *L. major* metacyclic promastigotes in WT or *Clec4e*^{-/-} mice. In the first 2 weeks after infection, the inflammatory pathology in *Clec4e*^{-/-} mice was similar to or greater than that in WT mice, but the response subsequently plateaued and there was no development of dermal lesions (Fig. 4.1.5 A). Therefore the third week of infection, parasite loads in the ears and dLNs of *Clec4e*^{-/-} mice were 90% lower than those of their WT counterparts (Fig. 4.1.5 B, C). Real-time tracking of i.d. ear infection with *L. major* mCherry confirmed better control of infection in *Clec4e*^{-/-} mice, with significantly lower parasite load at all times analysed (Fig. 4.1.5 D). Mincle-deficient mice thus controlled the infection earlier and more effectively than WT mice, leading to reduced pathology.

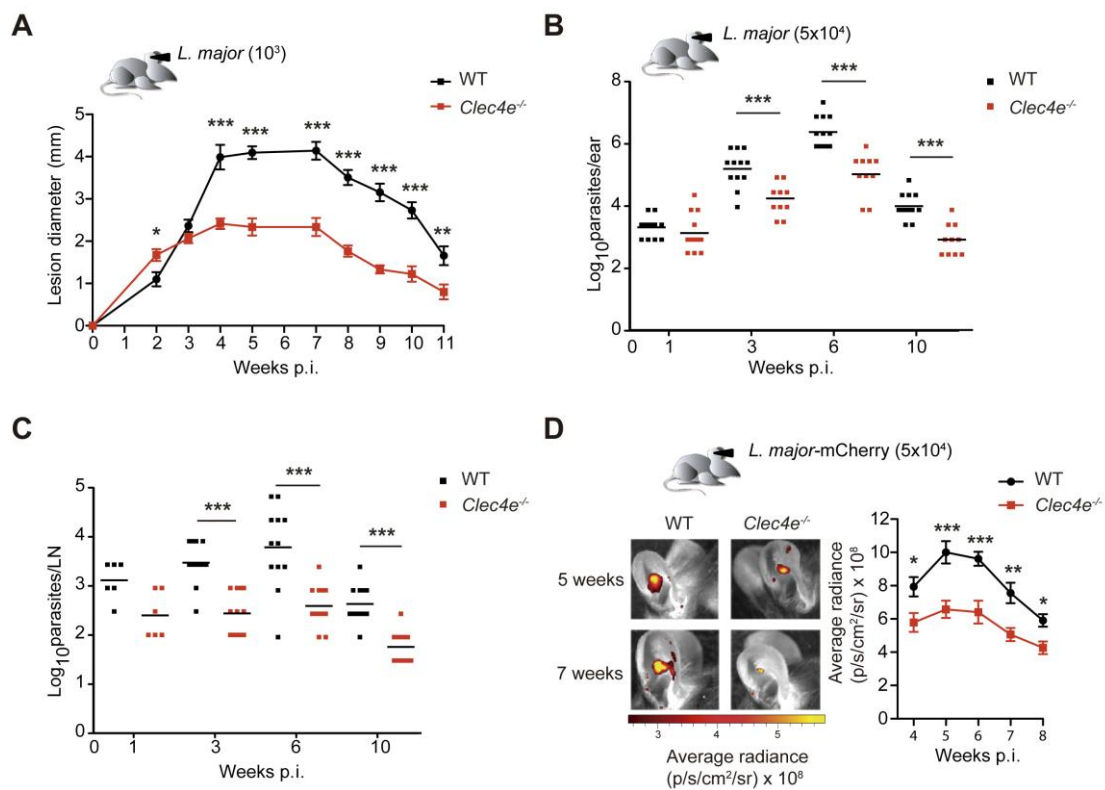


Figure 4.1.5. Mincle deficiency increases resistance to cutaneous leishmaniasis. (A) Time profiles of lesion diameter in the ear pinnae of WT and *Clec4e*^{-/-} mice infected i.d. with 1000 *L. major* parasites. Data arithmetic means \pm SEM from a representative experiment (n=16) of three performed. (B, C) Parasite load in the ear (B) and dLNs (C) of WT and *Clec4e*^{-/-} mice at the indicated times after i.d. infection in the ear with 5×10^4 *L. major* parasites. Squares show individual data and horizontal bars show arithmetic means from a representative experiment of three performed. (D) Left: *In vivo* imaging of mouse ears at the indicated times after i.d. inoculation with 5×10^4 mCherry⁺ *L. major* metacyclic promastigotes. Right: Progression of

fluorescence signal (pixel/second/cm²/sr) expressed as arithmetic mean \pm SEM (n=6). (A-D) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's t test at each time point).

4.1.5 Mincle deficiency strengthens the adaptive response to *L. major*

Polyclonal effector CD4⁺ T cells producing IFN- γ , but not CD8⁺ T cells, were significantly more abundant in the ears of infected *Clec4e*^{-/-} mice at 3, 6 and 10 weeks p.i. (Fig. 4.1.6 A, B). CD4⁺ T cells present in dLNs from infected *Clec4e*^{-/-} mice showed augmented production of IFN- γ , but not IL-10, in response to SLA (Fig. 4.1.6 C). To investigate the mechanism of the enhanced adaptive response to *L. major* in the absence of Mincle, we analysed early CD4⁺ T cell priming. As described (Ribeiro-Gomes et al., 2012, Pagán et al., 2013) infection with *L. major* expressing the model antigen ovalbumin (OVA) induced poor priming of OVA-specific CD4⁺ T cells (Fig. 4.1.6 D). Priming was boosted in *Clec4e*^{-/-} mice, with enhanced CD4⁺ T cell proliferation *in vivo* and IFN- γ production upon OVA restimulation *ex vivo* (Fig. 4.1.6 D, E). The specificity of the Mincle-dependent decrease in CD4⁺ T cell priming for *L. major* was confirmed by identical effector responses in WT and *Clec4e*^{-/-} mice upon infection with OVA-expressing vaccinia virus (Fig. 4.1.6 D, E). These data show that *Leishmania* targets Mincle to decrease priming of a CD4⁺ Th1 cell-type response against the parasite.

To determine the relevance of enhanced priming in a context of vaccination, we transferred OVA-specific CD4⁺ T cells intravenously (i.v) and subsequently injected 1 x 10⁵ freeze-thawed *L. major*-OVA i.d. into the ear. Injection of dead parasites into *Clec4e*^{-/-} mice resulted in increased numbers of OVA-specific CD4⁺ T cells producing IFN- γ upon restimulation *ex vivo* (Fig. 4.1.6 F). We next analysed whether Mincle deficiency also strengthens the function of the memory CD4⁺ T cell compartment. Vaccination with freeze-thawed *Leishmania* followed by *L. major* rechallenge 4 weeks later induced IFN- γ ⁺ CD4⁺ effector T cells in the ear of *Clec4e*^{-/-} but not WT mice (Fig. 4.1.6 G), thus generating a protective response with reduced parasitemia (Fig. 4.1.6 H). This Mincle-dependent vaccination deficiency using freeze-thawed *Leishmania* extracts in WT mice could be reverted by the use of CpG as adjuvant (Fig. 4.1.6 G, H), consistent with published findings (Walker et al., 1999). These results indicated that upon sensing *Leishmania*, Mincle inhibited the generation of effector and memory CD4⁺ T cells and impaired the adaptive response to *L. major*.

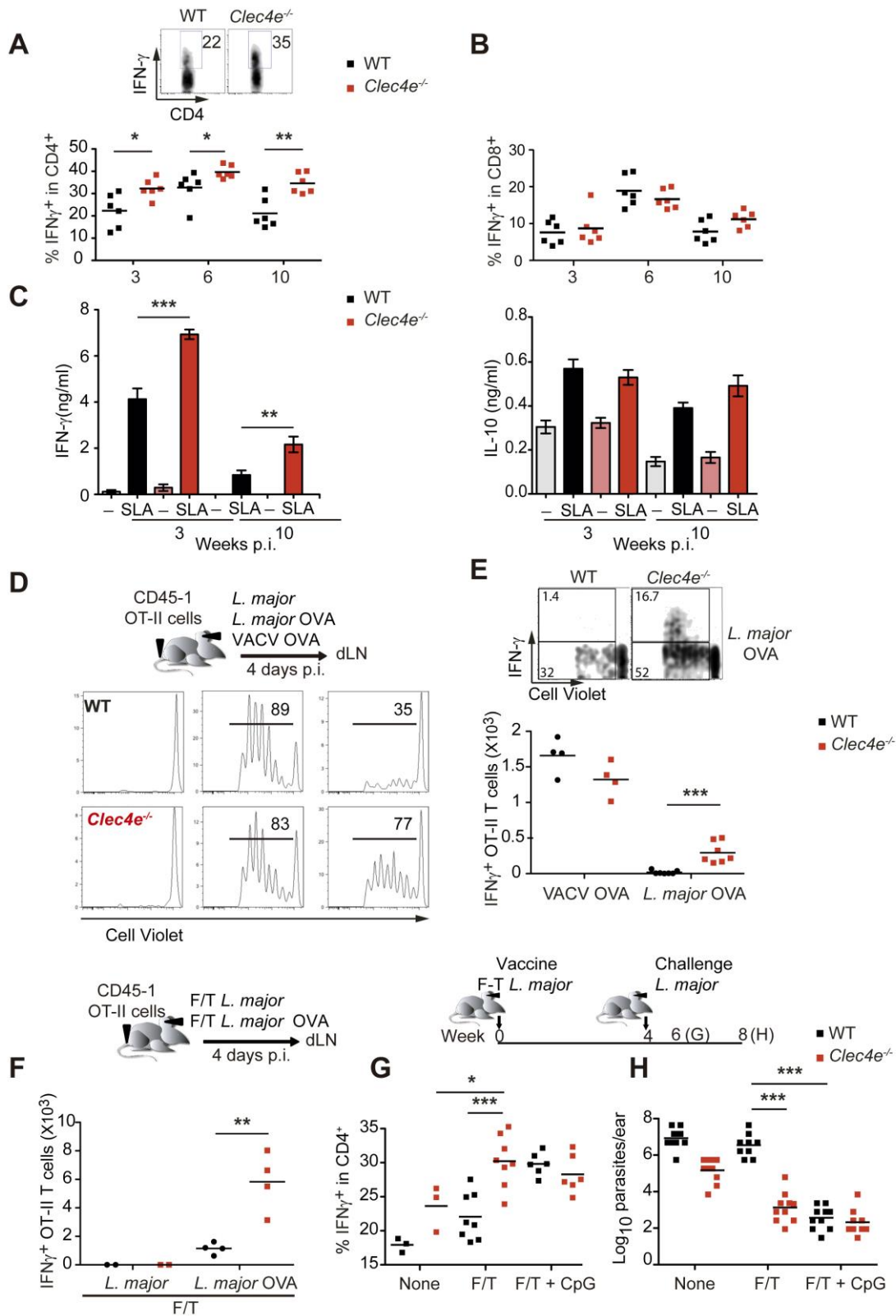


Figure 4.1.6 Increased adaptive response and enhanced CD4⁺ T cell priming during *L. major* infection in Mincle-deficient mice. (A-C) WT and *Clec4e*^{-/-} mice were infected i.d. in the ear with 5 x 10⁴ *L. major* parasites. (A) IFN- γ production in CD4⁺ T cells in response to polyclonal restimulation of ear infiltrates at the indicated times. Upper part: representative plots 3 weeks p.i. Bottom part: individual data and arithmetic means (B) Frequency of IFN- γ ⁺ in

CD8⁺ T cells in the ear stimulated as in (A). (C) IFN- γ (right) or IL-10 (left) in supernatants from dLN cells extracted at the indicated times and restimulated with SLA. Data are arithmetic means + SEM (n=6) of one representative experiment of three performed. (D-E) WT and *Clec4e*^{-/-} mice were transferred with CD45.1⁺ OT-II OVA-specific T cells labelled with Cell Violet and infected i.d. in the ear with 5 x 10⁴ particles of either *L. major*, *L. major* expressing OVA (*L. major*-OVA), or recombinant vaccinia virus expressing OVA (VACV-OVA). (D) Representative histograms showing Cell Violet dilution in OT-II cells in dLNs, 4 days p.i. (E) Representative plots of Cell Violet dilution and IFN- γ production following *ex vivo* restimulation with OVA peptide. Bottom: quantification of IFN- γ ⁺ OT-II absolute numbers in the dLNs. (F) Mice were vaccinated in the ear with 1x10⁵ F-T parasites and transferred with OT-II as in (D). Quantification of OT-II cells that were IFN- γ ⁺ in the dLNs upon *ex vivo* restimulation with OVA peptide. (G-H) WT and *Clec4e*^{-/-} mice were vaccinated i.d. in the ear with F-T *L. major* and challenged with live parasites in the same site 4 weeks later. (G) IFN- γ production in CD4⁺ effector T cells in the ear upon restimulation as in (A), assessed 2 weeks p.i. (H) Parasite load in the infected ears was evaluated 4 weeks p.i.. (A-C, E-G) Individual data and arithmetic mean of a representative experiment of three performed. *p < 0.05; **p < 0.01; ***p < 0.001: (A-F) Student's t test; (G.H) One-way ANOVA with Bonferroni post hoc test.

4.1.6 Mincle absence increases DCs activation and migration to dLNs after *L. major* infection

Given the increased adaptive response, we next investigated whether Mincle-deficient DCs had an enhanced ability to prime anti-*L. major* responses. DCs extracted from draining lymph nodes (dLNs) of *Clec4e*^{-/-} mice were better than WT at restimulating *L. major*-specific CD4⁺ T cells obtained from healed WT mice (Fig. 4.1.7 A). MoDCs infiltrating the dermis of Mincle-deficient mice also showed upregulation of the activation markers CD40 and CD86 and the chemokine receptor CCR7 at 20 hours and 14 days after infection (Fig. 4.1.7 B). In addition, *L. major* infection decreased the numbers of migratory DCs in a Mincle-dependent manner (Fig. 4.1.7 C). The effect of Mincle on the capacity of dermal DCs to migrate to the dLNs was further investigated in FITC skin sensitization assays. *L. major* infection inhibited migration of FITC⁺ CD11c⁺ DCs to dLNs in WT mice but not in *Clec4e*^{-/-} mice (Fig. 4.1.7 D). These results suggest that *Leishmania* sensing by Mincle impaired DCs activation in the infection site and subsequently limited their capacity to migrate to dLNs, contributing to the reduced priming to *L. major* in the presence of Mincle.

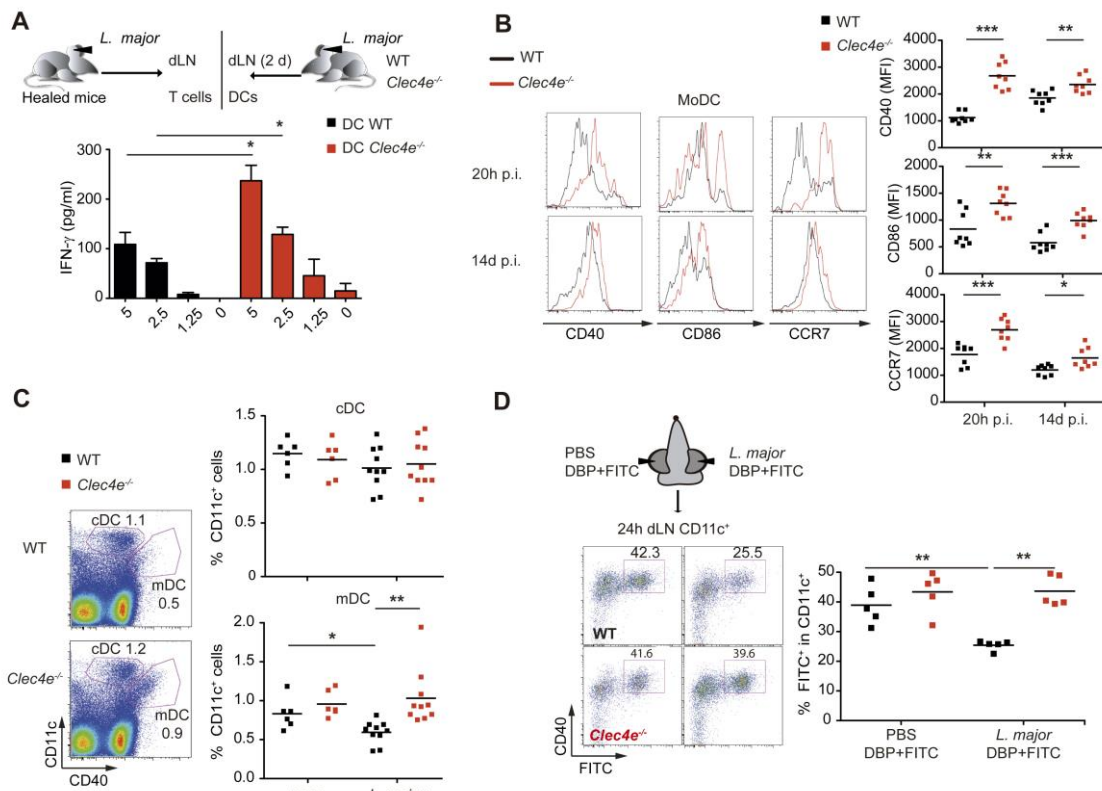


Figure 4.1.7 Enhanced DCs activation and migration to dLNs after *L. major* infection in Mincle-deficient mice. (A) IFN- γ in supernatants of T cells from healed *L. major*-infected mice after co-culture for 3 days with CD11c⁺ cells recovered from the dLNs of WT and *Clec4e*^{-/-} mice 2 days p.i. Data are arithmetic means + SEM from two independent experiments (n=6). (B) Left panels: Representative histograms of CD40, CD86 and CCR7 staining in MoDCs (CD11b⁺Ly6C⁺CD11c⁺MHCII⁺ gated cells) from ears of infected mice. Right panels: Mean fluorescence intensity (MFI) of CD40, CD86 and CCR7 expression on MoDCs. (C) Representative dot plots (Left) and frequencies (Right) of CD11c⁻ and CD40-positive cells in dLNs from uninfected mice or 24 h after *L. major* infection in the ear. (D) Ears of mice inoculated with PBS in the left ear and *L. major* parasites (10⁵) in the right ear were FITC painted and dLNs were harvested 24h later. Left: Representative plots of dLN cells gated for CD11c and stained with anti-CD40 and FITC. Right: frequencies of FITC⁺ CD11c⁺ dLN cells. (B-D) Individual data and arithmetic means corresponding to a representative experiment of two (B) or three (C, D) performed. (A-D) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's t test).

4.1.7 *L. major* promotes a Mincle- and SHP1-dependent inhibitory axis in CD11c⁺ GM-CSF-derived cells

To test whether increased DCs activation in the absence of Mincle was intrinsic, we generated GM-CSF bone-marrow-derived cells from WT and *Clec4e*^{-/-} mice. Stimulation with freeze-thawed *L. major* induced increased expression of CD40, CD86 and CCR7 in Mincle-deficient CD11c⁺ GM-CSF-derived cells (Fig. 4.1.8 A, B) suggesting an intrinsic effect. As Syk is

downstream Mincle (Yamasaki et al., 2008), we tested the absence of Syk in the CD11c compartment (CD11cCre Syk^{flax/flax}, named CD11cΔSyk mice) (Iborra et al., 2012). GMCSF bone-marrow-derived cells from CD11cΔSyk mice showed impaired activation by freeze-thawed *L. major* (Fig. 4.1.8 A, B), suggesting the possible existence of an unidentified activating Syk-coupled DCs receptor for *L. major* (Lefevre et al., 2013).

MCL and Mincle are mutually regulated and act as heterodimers for binding to TDM (Kerscher et al., 2016b, Lobato-Pascual et al., 2013, Miyake et al., 2013, Miyake et al., 2015). Consistent with these reports, GMCSF bone-marrow-derived cells from MCL-deficient (*Clec4d*^{-/-}) mice lacked expression of not only MCL but also Mincle (Fig. 4.1.8 C), Mincle expression was rescued by transduction with WT MCL or MCL^{WAA} (Fig. 4.1.8 C), which contains a mutation in calcium-binding motif of the C-type lectin (Miyake et al., 2015). The impaired expression of Mincle and MCL in *Clec4d*^{-/-} mice resulted in increased activation of CD11c⁺ GMCSF-derived cells exposed to freeze-thawed *L. major* (Fig. 4.1.8 D). Re-expression of Mincle mediated by transduction of both MCL and MCL^{WAA} correlated with impaired CD11c⁺ GMCSF-derived cells activation by *L. major* (Fig. 4.1.8 D), suggesting that regulation of Mincle expression by MCL contributes to responses to *L. major*.

Infection with *Fonsecaea* triggers Akt-dependent repression of IL12p35 transcription (Wevers et al., 2014). In contrast, freeze-thawed *L. major* did not induce Akt activation in WT mice (Fig. 4.1.8 E). We hypothesized that DCs activation by *L. major* might be antagonized by Mincle through the recruitment of SHP1 in an inhibitory ITAM (ITAMi) configuration (Aloulou et al., 2012, Ben Mkaddem et al., 2014, Hamerman et al., 2009, Pasquier et al., 2005). Consistent with this notion, treatment with the SHP1/2 phosphatase inhibitor NSC-87877 increased CD11c⁺ GMCSF-derived cells activation by *L. major* (Fig. 4.1.8 F), and NSC-87877 did not further activate Mincle-deficient DCs in response to the parasite (Fig. 4.1.8 F), suggesting that Mincle and phosphatase activity act in the same pathway.

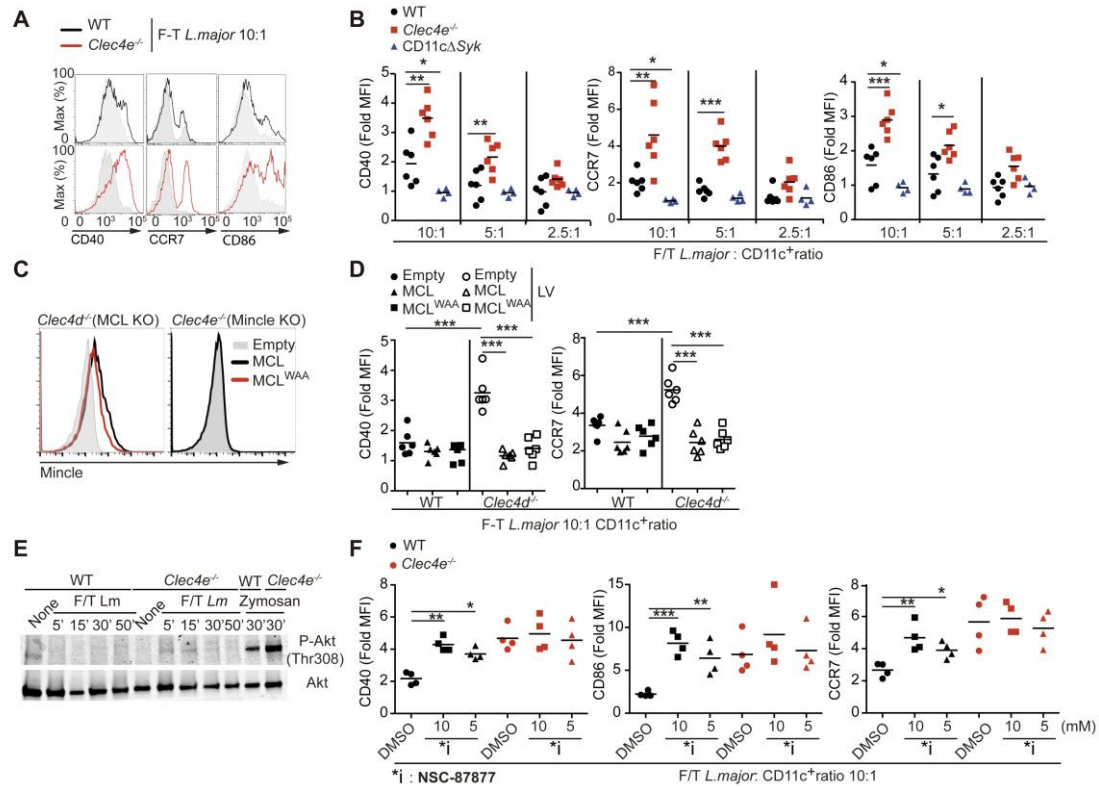


Figure 4.1.8 Mincle-MCL counter-regulation contributes to CD11c⁺ GMCSF-derived cells inhibition by *L. major*. (A) Histogram overlays for CD40, CCR7, and CD86 in CD11c⁺ GM from WT and *Clec4e*^{-/-} mice left untreated (gray histograms) or treated with freeze-thawed (F-T) *L. major*. Data are representative of three independent experiments (n=6). (B) Fold induction of MFI for CD40, CCR7 and CD86 upon F-T *L. major* treatment of CD11c⁺ GMCSF-derived cells obtained from WT, *Clec4e*^{-/-}, and CD11cΔ*Syk* mice; (C) Staining with anti-Mincle of MCL-deficient (*Clec4d*^{-/-}, left) and Mincle-deficient (*Clec4e*^{-/-}, right) GMCSF-derived cells transduced with lentiviruses expressing empty vector, MCL or MCL-WAA. Representative staining of four independent cultures and transductions. (D) Fold induction of MFI for CD40, and CCR7 upon F-T *L. major* treatment of CD11c⁺ GMCSF-derived cells obtained from WT and *Clec4d*^{-/-} CD11c⁺ GMCSF-derived cells transduced with empty vector, MCL, or MCL-WAA. (E) Western blots for P-Akt (Thr308) and total Akt in CD11c⁺ GMCSF-derived cells from WT and Mincle-deficient (*Clec4e*^{-/-}) mice treated or not (none) with F/T *L. major* or zymosan for the indicated times. A representative experiment is shown of two performed. (F) Staining of CD40, CD86, and CCR7 on CD11c⁺ GMCSF-derived cells from WT and *Clec4e*^{-/-} mice untreated or treated with F-T *L. major* (10:1 ratio to CD11c⁺ cells) in the presence or absence of the SHP1/2 inhibitor (*i) NSC-87877 at the indicated dose. (B, D, F) Panels show individual data and arithmetic mean corresponding to (D) Pooled data from three independent experiments (n = 6) or (B, F) one representative experiment of three performed. * p < 0.05; ** p < 0.01; *** p < 0.001 (B) One-way ANOVA with Bonferroni post-hoc test (D,F) Student's t test.

Supporting this conclusion, the enhanced freeze-thawed *L. major*-mediated activation seen in Mincle-deficient mice was phenocopied in GMCSF bone-marrow-derived cells from mice lacking SHP1 in the CD11c compartment (CD11c Δ SHP1) (Abram et al., 2013) (Fig. 4.1.9 A), freeze-thawed *L. major*-induced cytokine production was also higher in GMCSF bone-marrow-derived cells lacking Mincle or SHP1 (Fig. 4.1.9 B). Moreover, like *Clec4e*^{-/-} mice tested in parallel, CD11c Δ SHP1 mice displayed lower ear and LNs parasitemia in response to *L. major* infection (Fig. 4.1.9 C) and showed increased adaptive immunity (Fig. 4.1.9 D). Thus, our results suggested that Mincle inhibited CD11c⁺ GMCSF-derived cells activation through SHP1.

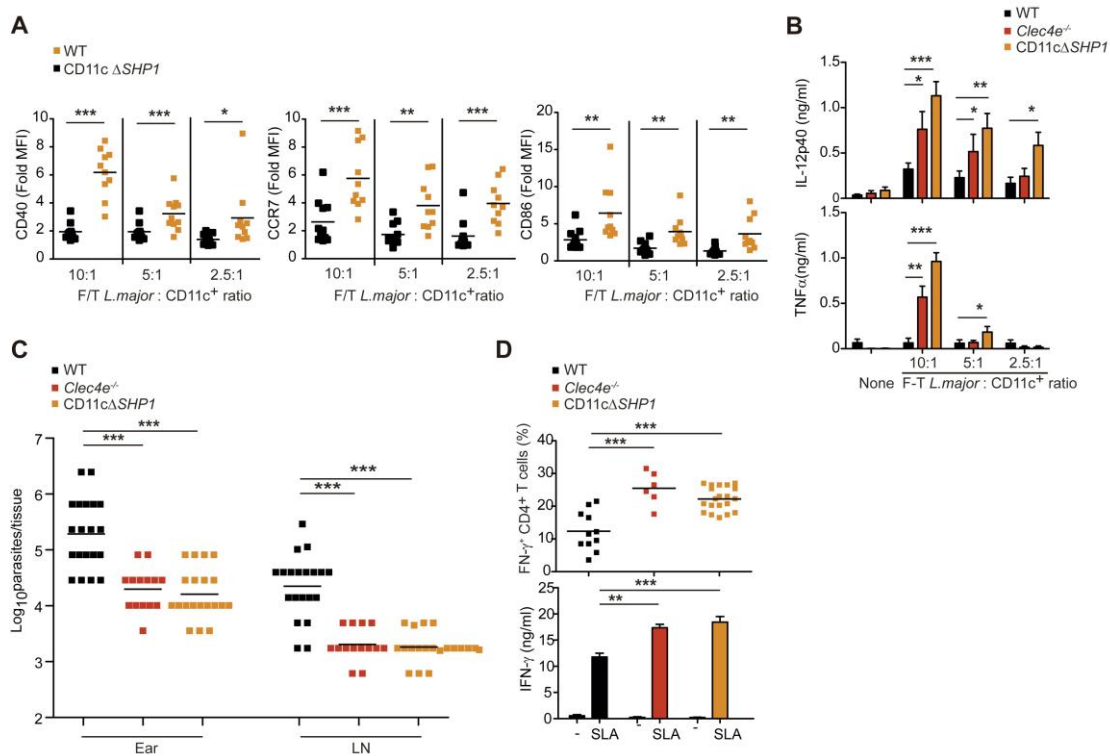


Figure 4.1.9 Mincle and SHP1 inhibit CD11c⁺ GMCSF-derived cells activation by freeze-thawed *L. major*. (A) Fold induction of MFI for CD40, CCR7 and CD86 upon F-T *L. major* treatment of CD11c⁺ GMCSF-derived cells obtained from CD11c⁺ GMCSF-derived cells from WT and CD11c Δ SHP1 mice. (B) IL-12p40 and TNF α in culture supernatants 20 hours after exposure of WT, *Clec4e*^{-/-}, and CD11c Δ SHP1 CD11c⁺ GMCSF-derived cells to different doses of F-T *L. major*. Data are arithmetic means + SEM of three independent experiments. (C, D) WT, *Clec4e*^{-/-}, and CD11c Δ SHP1 mice inoculated with 5 x 10⁴ *L. major* parasites i.d. in the ear were sacrificed 3 weeks after infection. (C) Parasite load in the infected ear and dLNs. (G) Top: intracellular IFN- γ in CD4⁺ T cells after polyclonal restimulation of ear infiltrates. Bottom: IFN- γ in supernatants after SLA restimulation of 2 x 10⁶ dLN cells. (A, C, and D) Individual data and arithmetic mean corresponding to one representative experiment of three performed. (A–D) *p < 0.05; **p < 0.01; ***p < 0.001. (B–D) One-way ANOVA with Bonferroni post-hoc test; (A) Student's t test.

4.1.8 *L. major* shifts Mincle to an inhibitory ITAM configuration that suppresses heterologous receptors

Participation of Mincle and SHP1 in the same axis was further supported by Mincle-dependent phosphorylation of SHP1 (but not SHP2) in freeze-thawed *L. major*-stimulated GMCSF bone-marrow-derived cells (Fig. 4.1.10 A). Pull-down of SHP1 in WT or FcR γ -chain-deficient GMCSF bone-marrow-derived cells revealed specific FcR γ -dependent association of SHP1 with Mincle (Fig. 4.1.10 B). Moreover, pull-down of Mincle from B3Z transfectants expressing tyrosine mutants in the FcR γ ITAM domain demonstrated that the membrane-distal tyrosine 76 was crucial for association of Mincle-FcR γ with SHP1, whereas tyrosine 65 was at least partially dispensable (Fig. 4.1.10 C), consistent with the ITAMi configuration (Ben Mkaddem et al., 2014).

We next tested the effect of the *L. major*-induced Mincle-dependent inhibitory axis on CD11c⁺ GMCSF-derived cells activation promoted by LPS. Freeze-thawed *L. major* dampened LPS-induced activation in CD11c⁺ GMCSF-derived cells and this inhibition was dependent on Mincle and Syk (Fig. 4.1.10 D). The ITAMi configuration is dependent on transient activation of Syk (Ben Mkaddem et al., 2014). We found that Syk transiently associated with Mincle in CD11c⁺ GMCSF-derived cells stimulated with freeze-thawed *L. major* in a manner dependent on the FcR γ chain (Fig. 4.1.10 E). Notably, CD11c Δ Syk CD11c⁺ GMCSF-derived cells showed impaired SHP1 recruitment to Mincle (Fig. 4.1.10 F). These results suggest that *L. major* shifts Mincle to an ITAMi configuration that suppresses heterologous activating receptors, dampening CD11c⁺ GMCSF-derived cells activation and thus impairing the induction of adaptive immune responses.



Figure 4.1.10 *L. major* promotes a Mincle/FcR γ /SHP1 axis that impairs CD11c⁺ GM-CSF-derived cells activation. (A) Western blot (WB) for P-SHP1, P-SHP2 and total SHP1, SHP2 in WT and *Clec4e*^{-/-} CD11c⁺ GM-CSF-derived cells lysed at the indicated times of stimulation with F-T *L. major*. (B) SHP1 immunoprecipitation and WB for SHP1, FcR γ and Mincle in WT and *Fcer1g*^{-/-} CD11c⁺ GM-CSF-derived cells lysed at the indicated times of stimulation with F-T *L. major*. (C) Mincle immunoprecipitation in B3Z cells transduced with mouse Mincle (*Clec4e*),

Syk, and either WT FcR γ chain or the Y76F (left) or Y65F mutants (right). WB for Mincle, FcR γ and SHP1. (D) Fold induction of MFI for CD40 and CD86 upon LPS stimulation (200 ng/ml) of F-T *L. major*-pretreated for 30 min (+) or non-pretreated (-) CD11c⁺ GMCSF-derived cells from WT, *Clec4e*^{-/-} and CD11c Δ Syk mice. ** $p < 0.01$; Student's t test comparing F-T *L. major* pretreatment and no pretreatment within each genotype. (E) Syk (upper blots) and SHP1 (lower blots) immunoprecipitation from F-T *L. major*-treated WT and *Fcer1g*^{-/-} CD11c⁺ GMCSF-derived cells and WB for Syk and Mincle (upper) or SHP1 and Mincle (lower). (F) SHP1 immunoprecipitation from F-T *L. major*-treated WT and CD11c Δ Syk CD11c⁺ GMCSF-derived cells and WB for SHP1 and Mincle. (A-F) Western Blots are from single representative experiments of at least three performed.

4.2.1 Analysis of the contribution of Mincle to the homeostasis of commensal microbiota in the intestine.

Initially described for pathogen microorganisms, PAMPs are also present in the commensal microorganisms that form the microbiota, referred as microbe-associated molecular patterns (MAMPs) (Chu and Mazmanian, 2013). Consequently, myeloid PRRs might not only contribute to generation of immunity against pathogens, but also could promote host-microbiota symbiosis after MAMPs recognition, contributing to tolerance to these commensal microorganisms.

The CLR-mediated Syk/CARD9 pathway has been recently identified as protective in the context of inflammation-associated cancer through recognition of commensal fungi (Malik et al., 2018). In addition, many myeloid CLRs can recognize ligands present in commensal microorganisms, SIGNR3 (*Cd2009d*) and its human homolog DC-SIGN (*CD209*) can recognize several ligands present in different *Lactobacillus* species (Lightfoot et al., 2015) (Konieczna et al., 2015). In addition, SIGNR3 can recognize commensal fungi (Eriksson et al., 2013). MCL (*CLEC4D*) and DCIR (*CLEC4A*) bind to intestinal microbiota to a different extent (Hütter et al., 2014). The best characterized example is Dectin-1 (*CLEC7A*)-mediated recognition of indigenous fungi and control the homeostasis of intestinal immunity by controlling Treg cell differentiation through modification of microbiota (Iliev et al., 2012, Tang et al., 2015). Most of the knowledge of CLRs recognition of microbiota is based on *in vitro* or DSS-induced colitis studies and how myeloid CLRs-microbiota interaction promotes tolerance to microbiota is still poorly described (Smits et al., 2005) (Eriksson et al., 2013). In this particular context, tolerance, is not a passive state of unresponsiveness, but implies activation of several mechanisms such as the containment of commensals inside the intestine, given that increased microbial translocation is associated with systemic inflammation (Sonnenberg et al., 2012). For that, the intestinal epithelial cells (IECs), the mucus layer, the generation of antimicrobial peptides and the

synthesis of microbe-specific immunoglobulin A (IgA) in the Peyer's patches (PPs) constitute the "mucosal firewall" (Belkaid and Hand, 2014).

This mucosal barrier is regulated by ROR γ t-dependent cells that include group 3 innate lymphoid cells (ILC3) and T helper 17 (Th17) cells, which produce IL-17 and IL-22 and modulate antimicrobial peptide secretion by IECs and IgA production in the gut ((Hirota et al., 2013, Kruglov et al., 2013). Therefore, the absence of ROR γ t-dependent cells or their mediators can lead to a breach of the intestinal barrier, commensal translocation and systemic inflammation (Lochner et al., 2011, Sonnenberg et al., 2012). ROR γ t-dependent cells function and/or generation require myeloid-recognition of commensal microbiota, segmented filamentous bacteria (SFB) is the prototypical example, and -antigen presentation or -derived cytokines such as IL-6 and IL-23 (Ivanov et al., 2006 , Persson et al., 2013, Longman et al., 2014 , Satpathy et al., 2013). In this regards, many myeloid CLRs, Dectin-1 (*CLEC7A*), Dectin-2 (*CLEC6A* in human, *Clec4n* in the mouse) and Mincle (*CLEC4E*) mainly drive IL17 production by T cells in response to pathogens, by modulation of myeloid IL-6 and IL-23 secretion (Geijtenbeek and Gringhuis, 2016, LeibundGut-Landmann et al., 2007). In the context of the intestine, there are many subsets of myeloid cells with different functions and location. Macrophages or several subsets of DCs can be located in the lamina propria (LP) and/or in gut-associated lymphoid tissues, including PPs (Bekiaris et al., 2014) Based on the expression of lysozyme and CD11b, PPs contain particular subsets of DCs (Bonnardel et al., 2015b, Bonnardel et al., 2015a, Da Silva et al., 2017). However, the particular subset of myeloid cells and the host signalling pathways linking recognition of commensal microbes to the induction of IL-17 and IL-22 production by ROR γ t⁺ cells in steady state are poorly understood. Here, we aim to analyse the role of Mincle to this process (Fig. 4.2.1). Deciphering the contribution of Mincle to commensal microorganisms-induced tolerance will open new molecular targets for many diseases that course with altered mucosal barrier or leaky gut such as obesity (Araujo et al., 2017).

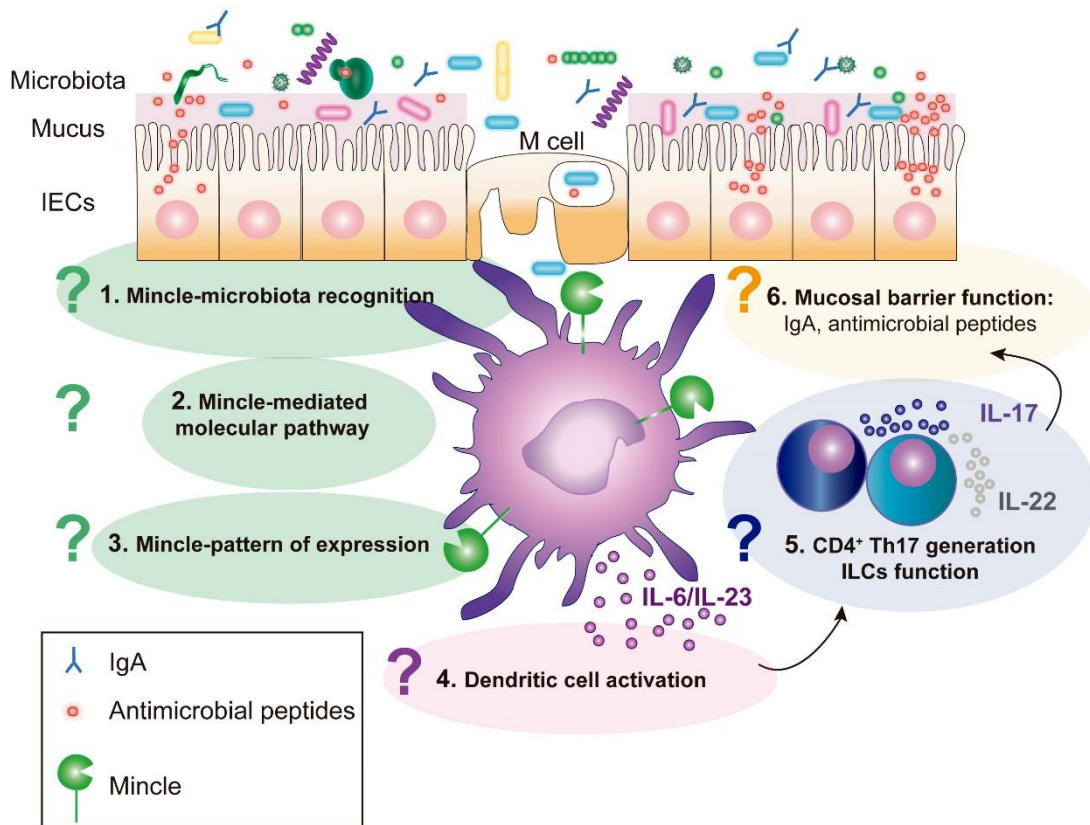


Figure 4.2.1 Contribution of Mincle to regulation of the homeostasis of commensal microbiota in the intestine. Picture displaying the six specific objectives: (1-3) Addressing microbiota-Mincle interaction and the pattern of expression of Mincle in different intestinal myeloid subsets. (4 and 5) Determining how this interaction could influence DCs function, Th17 generation and ILCs functionality. (6) Analysing the effect of Mincle-microbiota interaction mediating mucosal barrier function as tolerance mechanism. IECs, intestinal epithelial cells; ILCs, innate lymphoid cells.

4.2.2 Mincle and Syk signalling in DCs control microbiota-driven Th17 differentiation

We assessed host signalling pathways with the potential to link recognition of commensal microbes by myeloid cells with Th17 differentiation. GM-CSF bone marrow-derived CD11c⁺ cells from mice lacking MyD88 (*Myd88*^{-/-}) (Adachi et al., 1998), Syk in the CD11c⁺ compartment (CD11cCre Syk^{flax/flax}, named CD11cΔSyk mice) (Iborra et al., 2012) or WT littermates were loaded with chicken ovalbumin class-II peptide (OVA323-339) and co-cultured with naive OVA-specific (OT-II) CD4⁺ T cells in the presence or absence of specific pathogen free (SPF) gut microbiota. While the proliferation of OT-II cells did not differ upon co-culture with different GM-CSF bone marrow-derived CD11c⁺ cells (Fig. 4.2.2 A, upper part), OT-II cell capacity to produce IL-17 after priming in the presence of gut microbiota was specifically blunted in the absence of Syk but not MyD88 in CD11c⁺ cells (Fig. 4.2.2 A, bottom part).

Syk signalling following pattern recognition is characteristic of some C-type lectin receptors (CLRs). Syk binds to immunoreceptor tyrosine-based activation motif (ITAM) domains present in the Fc γ R chain (*Fcer1g*) adaptor that couples to Dectin-2 (*Clec4n* in the mouse) or Mincle (*CLEC4E*), or to hemITAMs borne by CLRs, such as Dectin-1 (*CLEC7A*) (Iborra and Sancho, 2015). Thus, we explored the ability of GM-CSF bone marrow-derived CD11c⁺ cells lacking Dectin-1 or Fc γ R chain to promote microbiota-driven Th17 differentiation from naive OT-II cells as above. Although proliferation of OT-II cells was similar between different genotypes (Fig. 4.2.2 B, upper part), microbiota-induced Th17 differentiation was dependent on the expression of the Fc γ R chain but not Dectin-1 in CD11c⁺ cells (Fig. 4.2.2 B, bottom part). We subsequently found that, upstream the Fc γ R chain, Mincle, but not Dectin-2, was needed in GM-CSF bone marrow-derived CD11c⁺ cells for microbiota-driven Th17 differentiation *in vitro* (Fig. 4.2.2 C, upper and bottom parts). Notably, exposure to microbiota induced Syk phosphorylation in GM-CSF bone marrow-derived CD11c⁺ cells in a Mincle-dependent manner (Fig. 4.2.2 D).

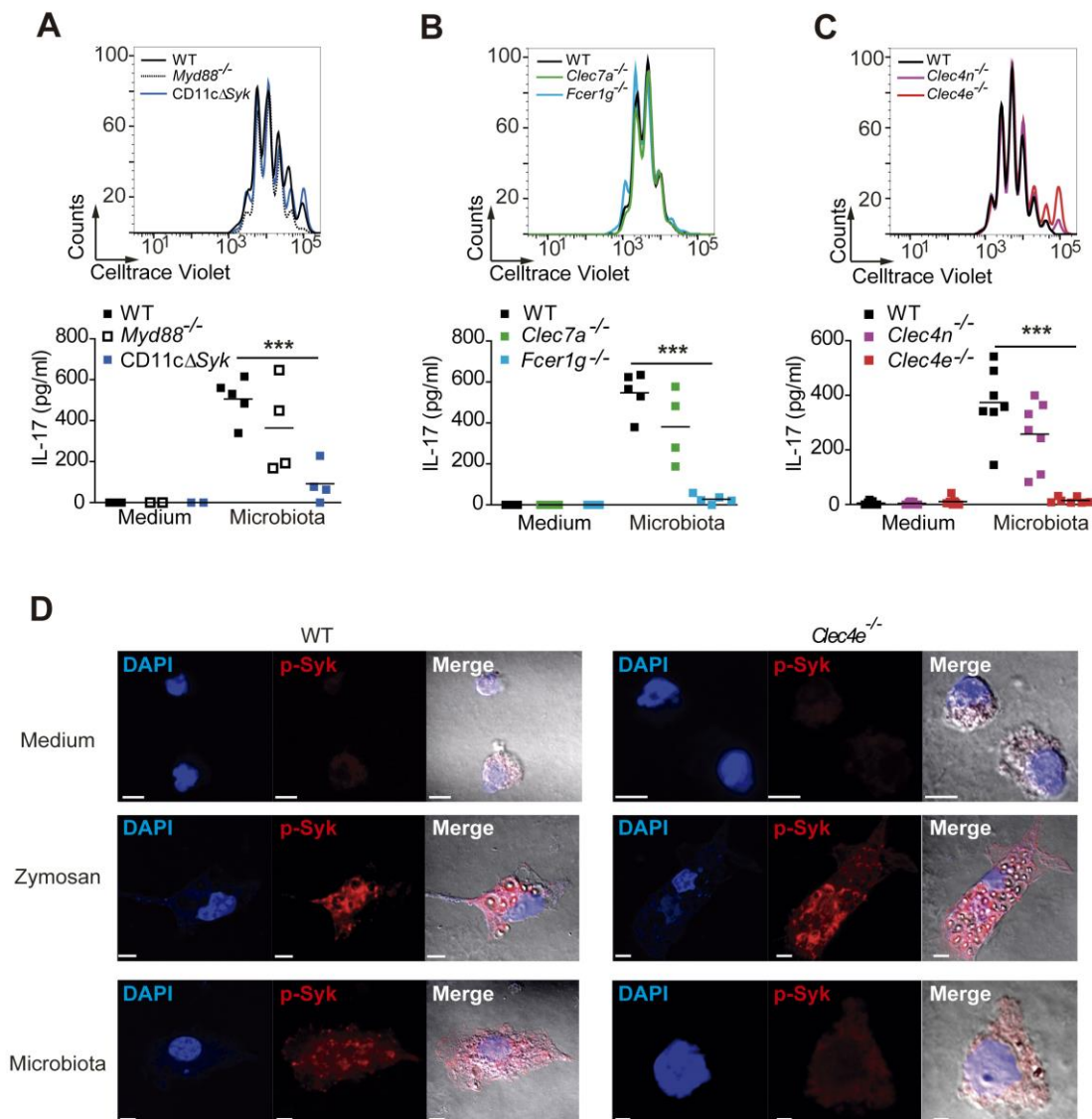


Figure 4.2.2. Mincle and Syk signalling in GMCSF bone marrow-derived CD11c⁺ cells control microbiota-driven Th17 differentiation. (A-C) Naive OT-II T cells were co-cultured with GMCSF bone marrow-derived CD11c⁺ (1:2 ratio) from the indicated genotypes previously loaded with OVA peptide and in the presence or not of microbiota (10:1 CD11c⁺ cells ratio) and IL-17 was measured by ELISA in the supernatant 3 days later. Top: Representative FACS plots showing Celltrace Violet dilution in OT-II T cells co-cultured with CD11c⁺ cells from the indicated genotypes (D) Representative confocal images of Syk phosphorylation (red) in GMCSF bone marrow-derived CD11c⁺ from WT and Mincle-deficient (*Clec4e*^{-/-}) mice, 30 minutes after stimulation or not (medium) with zymosan (10 μg/mL) or gut microbiota (10:1 CD11c⁺ cells ratio). DAPI was used for nuclear counterstaining. Merged image with DAPI, P-Syk and visible; (scale bar = 5 μm; *Clec4e*^{-/-} plus microbiota: 2 μm) (A-C) Individual data representing data generated from independent GMCSF cultures (biological replicates) in pools of at least two independent experiments. Individual data and arithmetic mean are shown. *** p < 0.001 (One-way ANOVA and Bonferroni post-hoc test); (D) One representative experiment of at least three performed.

GMCSF bone marrow-derived cells comprise conventional DCs (GM-DCs) and monocyte-derived macrophages (GM-Macs) (Helft et al., 2015). GM-Macs expressed Mincle constitutively, while intestinal microbiota stimulation induced Mincle expression in GM-DCs (Fig. 4.2.3 A) as expected (Helft et al., 2015). In addition, we found that GM-DCs efficiently primed IL-17 and IL-22 production by OT-II cells in response to microbiota and in a Mincle-dependent fashion (Fig. 4.2.3 B-D). In contrast, GM-Macs promoted IFN-γ-producing OT-II cells in a Mincle-independent manner (Fig. 4.2.3 E). These results suggest that the Mincle-FcRγ chain-Syk axis in GM-DCs drives Th17 differentiation in response to intestinal commensals.

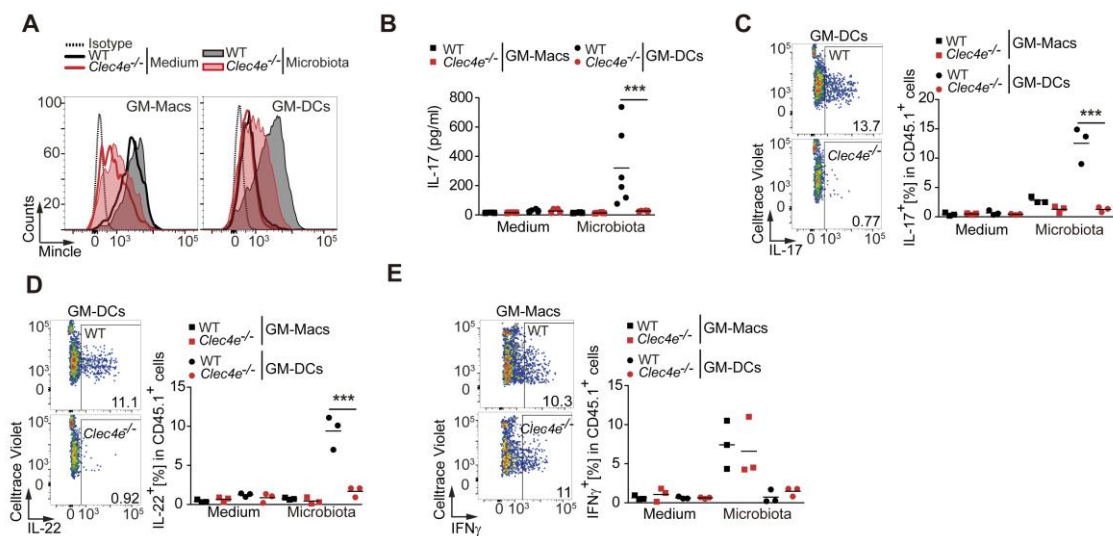


Figure 4.2.3. Mincle and Syk signalling in DCs direct microbiota-driven Th17 differentiation. (A) Representative histograms showing Mincle or isotype staining for the indicated populations. (B-E) Naive OT-II T cells were co-cultured with GM-Macs or GM-DCs (1:1 ratio), GM cells were previously loaded with OVA peptide and in the presence or not of microbiota (10:1 cell ratio) and IL-17 was measured by ELISA in the supernatant 3 days later (B). IL-17 (C), IL-22 (D) and IFN- γ (E) production after re-stimulation was measured by intracellular staining and flow cytometry in OT-II T cells from the co-cultures. Left: representative plot, right: quantification. (A) One representative experiment of at least three performed; (B-E) Individual data representing data generated from independent GMCSF cultures (biological replicates) in pools of at least two independent experiments. Individual data and arithmetic mean are shown. *** $p < 0.001$ (One-way ANOVA and Bonferroni post-hoc test).

4.2.3 Mincle senses mucosa-associated commensals

We tested whether Mincle could bind to the intestinal microbiota from our SPF mice. Mincle ectodomain-human Fc chimera (Mincle-hFc) recognized the microbiota in a dose-dependent manner (**Fig. 4.2.4 A**). Pre-incubation of Mincle-hFc with 2F2 anti-Mincle antibody or with the Mincle ligand TDB specifically prevented its binding to the microbiota (**Fig. 4.2.4 B**). In addition, Mincle-hFc did not bind to the gastrointestinal content from germ-free mice (**Fig. 4.2.4 B**). Notably, the analysis of small intestine mucosa from SPF mice revealed a more than three-fold average enrichment in Mincle-hFc labelled commensals compared to luminal-located commensals (**Fig. 4.2.4 C, D**). We additionally found that a fraction of luminal but not mucosa-associated microbiota was detected by hFc-chimeras of the Syk-coupled CLR Dectin-1 and Dectin-2 (**Fig. 4.2.4 E**). Comparative 16S sequencing analysis of Mincle-hFc-bound and -depleted microbial fractions, along with a control-hFc-bound fraction, revealed that *Lactobacillus* was one of the main genera enriched in Mincle-hFc-bound fraction (**Fig. 4.2.4 F**). Mincle-hFc stained *Lactobacillus* recovered from the small intestine epithelium of mice orally gavaged with Celltrace Violet-labelled *Lactobacillus plantarum* (*L.plantarum*) but, intriguingly, not the same bacteria growing in MRS Broth (**Fig. 4.2.4 G**). These results indicate that some phyla of mucosa-associated commensals, including *Lactobacillus*, may contain ligands for Mincle, concurring with previous published results (Shah et al., 2016).

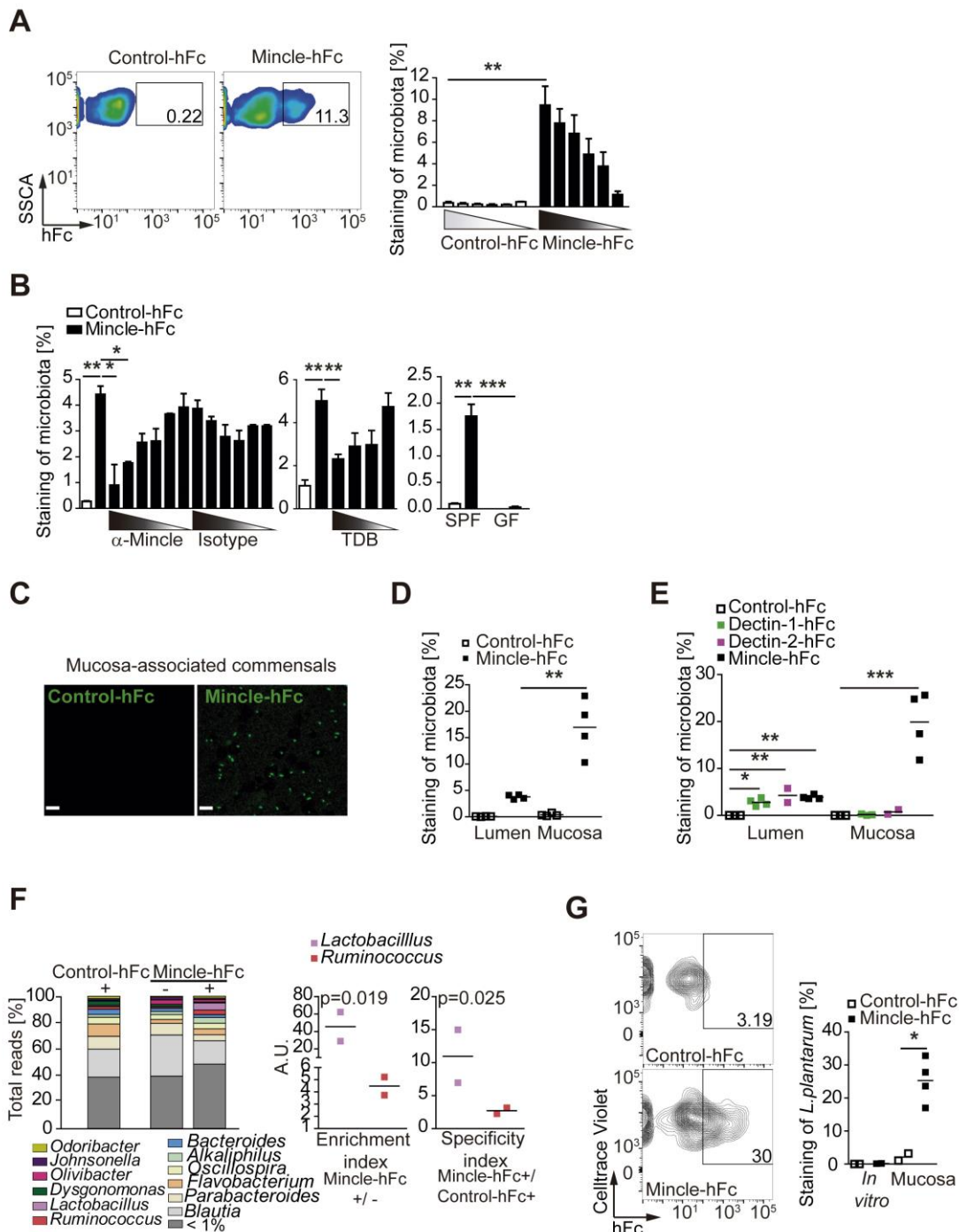


Figure 4.2.4 Mincle recognizes mucosa-associated commensals (A) Representative plots (left panels) and graph depicting the frequency of SPF microbiota stained with Control-hFc or Mincle-hFc. Arithmetic mean + SEM of pool of 3 replicates from 2 independent experiments. (B) Frequency of microbiota stained with Control-hFc or Mincle-hFc as indicated. Left: after pre-incubation with titrated dilutions of anti-human Mincle (clone 2F2) or an isotype control antibody; middle: after pre-incubation with decreasing doses of TDB (half dilution starting 1 mg/ml); right: in the intestinal content from SPF and GF mice. (C) Analysis by stimulated emission depletion super-resolution microscopy of mucosa-associated commensals from SPF mice labelled with Control-hFc or Mincle-hFc. Scale bar: 2 μ m. (D) Frequency of luminal and mucosal microbiota from SPF mice stained with Control- or Mincle-hFc by flow cytometry. (E) Frequency of luminal or mucosa-associated microbiota stained with the indicated Fc

chimeras.(F) Luminal microbiota was stained as in (A), sorted into Mincle-hFc-enriched (Mincle-hFc+), Mincle-hFc-depleted (Mincle-hFc-) and Control-hFc-enriched (Control-hFc+) fractions and analysed by 16S sequencing. Left: Relative abundance of each genus from two independent experiments. Right: Enrichment index and specificity index, calculated as explained in Methods. (G) Mucosa-associated commensals from WT SPF mice gavaged with Celltrace violet-labelled *Lactobacillus plantarum* (*L. plantarum*) or *in vitro*-grown *L. plantarum* were stained with control- or Mincle-hFc and analysed by flow cytometry. Left: representative staining. Right: frequency of bacteria positive for the indicated staining pre-gated on cell violet-positive bacteria. (B-D, G) One representative experiment of two performed. (E) Pool of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (B) Unpaired two-tailed Student's t test; (E) One-way ANOVA and Bonferroni post-hoc test.

4.2.4 Mincle is expressed in Peyer's Patches DCs

Lactobacillus exhibit a preferential binding to the follicle-associated epithelium of the Peyer's patches (PPs) (Plant and Conway, 2001). We hypothesized that Mincle-expressing cells sensing mucosa-resident commensals could be located in the gut-associated lymphoid tissue. Indeed, Mincle was expressed in PPs and FcR γ chain-dependent Mincle expression was restricted to the CD11c⁺ MHCII⁺ CD19⁻ CD11b⁺ subset in PPs (Fig. 4.2.5 A). Confocal microscopy of PPs whole mount preparations confirmed the selective Mincle expression in the CD11c⁺ CD11b⁺ immune compartment (Fig. 4.2.5 B). Concurring with previous published results (Bonnardel et al., 2015b), Mincle was mainly expressed by some Lysozyme-expressing macrophages (LysoMacs), DCs (LysoDCs) and some CD11b⁺ DCs, but not some CD8 α ⁺ DCs (Fig. 4.2.5 C). In contrast, Mincle expression in small intestine LP was mainly found on macrophages but not in CD64⁻ DC subsets (Fig. 4.2.5 D, E). Mincle expression in human intestinal samples from healthy donors was revealed in a fraction of CD45⁺ CD64⁻ CD14⁻ CD11c⁺ HLA-DR⁺ cells by flow cytometry (Fig. 4.2.5 F). These results indicate that Mincle is expressed by macrophages in PPs and small intestine LP, and some DCs in the mouse PPs.

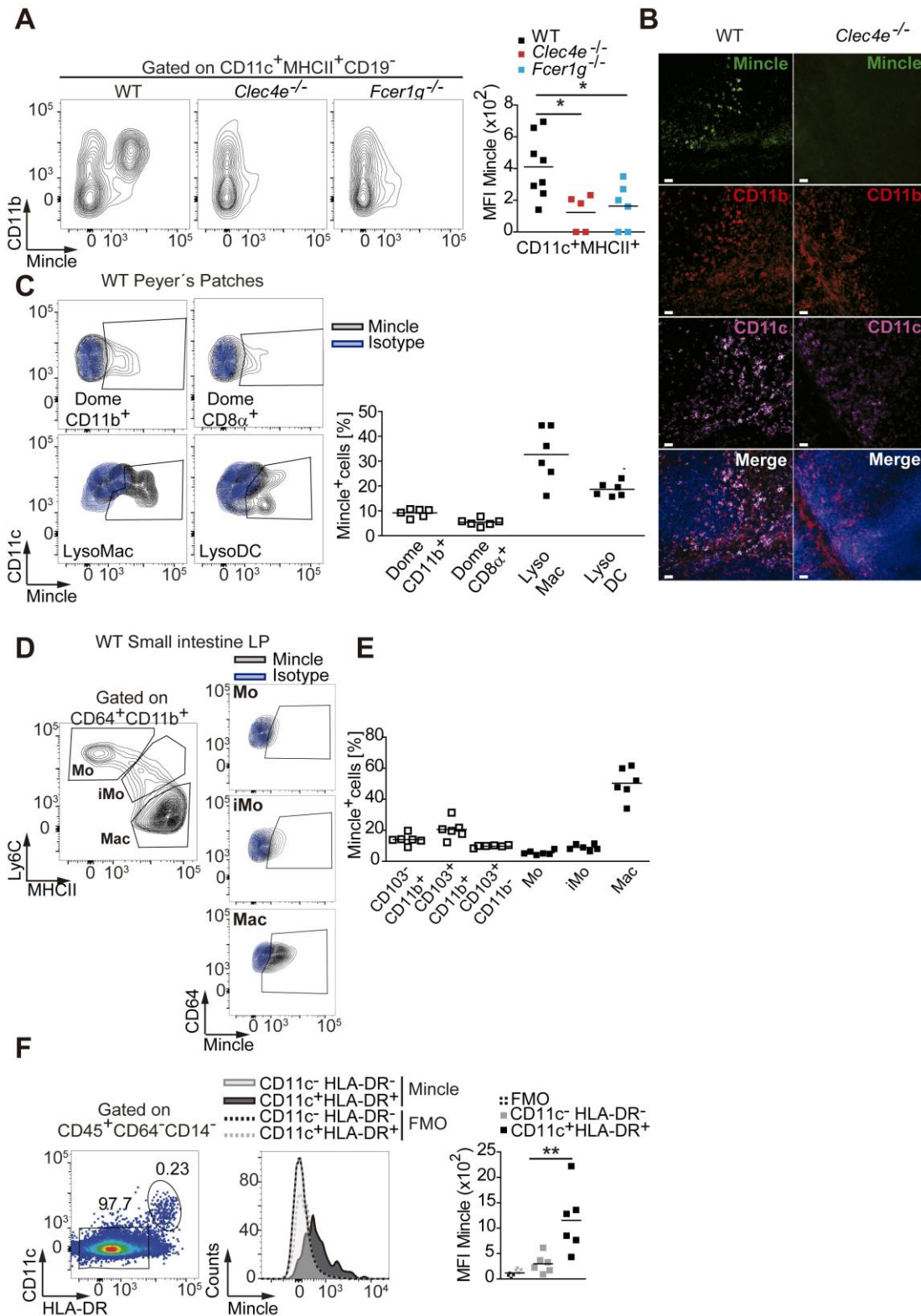


Figure 4.2.5 Mincle is expressed in Peyer's Patches DCs (A) Mincle expression in the indicated genotypes by flow cytometry in PPs. Left: Representative plots. Right: Mean Fluorescence intensity (MFI). (B) Representative confocal images showing whole mount preparations of PPs from WT and Mincle-deficient (*Clec4e*^{-/-}) mice stained with anti-Mincle antibody (clone 1B6) (green), CD11b (red) and CD11c (pink) co-stained with DAPI (blue); (scale bar = 20 μm). (C) Mincle expression by flow cytometry in the myeloid populations from

WT mice PPs. Left: Representative plot. Right: Frequency of Mincle⁺ cells in the depicted subset. (D) Left: Gating strategy used to identify the small intestine mouse LP Monocytes (Mo), intermediates Mo (iMo) and Macrophages (Mac). Right: Representative dot plot showing Mincle expression (black) or its isotype control (blue) in the indicated populations. (E) Frequency of Mincle⁺ cells in the myeloid subsets from WT small intestine LP. (F) Mincle expression in LP mononuclear cells from 3 colonic and 3 duodenal samples from healthy individuals by flow cytometry. Left: gating strategy. Middle: Mincle vs Fluorescence Minus One (FMO) expression in the CD11c⁻ HLA-DR⁻ and CD11c⁺ HLA-DR⁺ populations. Right: MFI of Mincle expression or the FMO in six independent samples. (A, C, E) At least two independent experiments have been pooled. (B, D, J) One representative experiment of two performed. (A, F) * p < 0.05; ** p < 0.01 (Unpaired two-tailed Student's t test)

4.2.5 DCs from PPs instruct Mincle- and Syk-dependent Th17 differentiation

Next, we explored the contribution of freshly isolated dome CD11b⁺ DCs, dome CD8 α ⁺ DCs, LysoMacs and LysoDCs from PPs to prime OT-II CD4⁺ T cells *ex vivo*. Dome CD11b⁺ DCs and LysoDCs had a higher capacity to induce IL-17 production by CD4⁺ T cells compared to CD8 α ⁺ DCs and LysoMacs (Fig. 4.2.6 A). Moreover, the ability of CD11b⁺ DCs and LysoDCs to promote Th17 differentiation was dependent on Mincle and Syk (Fig. 4.2.6 A, B), whereas Th1 differentiation induced by these cells was Mincle- and Syk-independent (Fig. 4.2.6 C). These results suggest that dome CD11b⁺ DCs and LysoDCs located in PPs mediate Mincle and Syk-dependent Th17 differentiation.

4.2.6 Mincle fosters IL-6 and IL-23p19 production by DCs in response to microbiota

The cytokine IL-6 plays a non-redundant role in Th17 differentiation in gut mucosa (Hu et al., 2011, Persson et al., 2013) and CD11b⁺ DCs from PPs are superior at producing IL-6 compared with other DC subsets from PPs or splenic DCs (Sato et al., 2003). Conversely, activation of ILC3s is independent of IL-6 but dependent on microbiota-induced IL-23p19 (Klose and Artis, 2016). Therefore, we explored whether both instructing signals were produced in a Mincle-dependent manner in PPs. Mincle-deficient mice exhibited reduced IL-6 and IL-23p19, but similar TGF- β and IL-12p40 transcripts in PPs compared with WT littermates (Fig. 4.2.6 D). Consistent with this, IL-6 protein production by CD19⁻ CD11c⁺ MHCII⁺ DCs in PPs was diminished in Mincle-deficient mice compared with WT littermates, whereas IL-12p40 production was not affected (Fig. 4.2.6 E, F). Accordingly, microbiota induced Mincle-dependent IL-6 and IL-23 production by GM-DCs (Fig. 4.2.6 G). These results indicate that steady state microbiota triggers Mincle-dependent IL-6 and IL-23p19 expression, which could instruct IL-17 and IL-22 production.

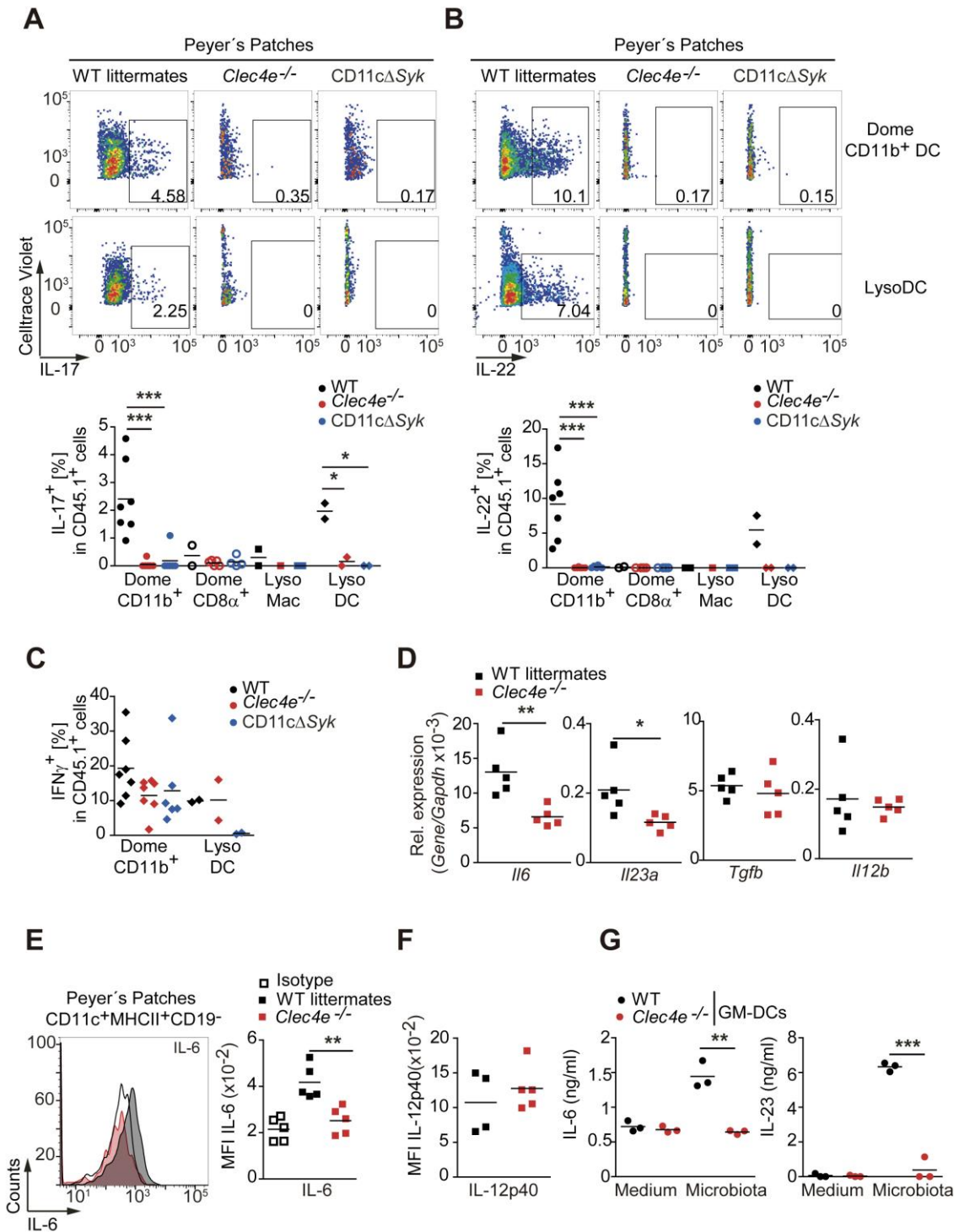


Figure 4.2.6. DCs from PPs instruct Mincle- and Syk-dependent Th17 differentiation (A-C) Naive OT-II T cells were co-cultured 3 days with dome CD11b⁺ DCs, CD8α⁺ DCs, LysoMacs or LysoDCs from the indicated genotypes, sorted from PPs (1:1 ratio) and previously loaded with OVA₃₂₃₋₃₃₉ peptide. (A, B upper part) Representative FACS plots of IL-17 (A) and IL-22 (B) intracellular staining after OT-II restimulation. (A, B bottom) Summary graph showing the percentage of IL-17⁺ (A) or IL-22⁺ (B) after PMA and ionomycin stimulation measured by intracellular staining and flow cytometry. (C) IFN-γ production after PMA and ionomycin stimulation. (D), *Il6*, *Il23a*, *Tgfb* and *Il12b* transcripts in PPs of the indicated

genotypes by qPCR normalized to *Gapdh*. (E, F) Analysis of IL-6 (E) and IL-12p40 (F) intracellular staining in CD11c⁺ MHC-II⁺ CD19⁻ cells from PPs in the indicated genotypes. (E, left) representative histograms. (E, F right) Mean fluorescence intensity (MFI) of staining. (G) ELISA of IL-6 and IL-23 production by sorted GM-DCs from WT and Mincle-deficient (*Clec4e*^{-/-}) mice untreated (medium) or stimulated with gut microbiota (10:1 DC ratio) during 12 hours. (A, B, D-G) One representative experiment of at least three performed. (C) Two independent pooled experiments. (A, B) One-way ANOVA and Bonferroni post-hoc test. (D, E, G) Unpaired two-tailed Student's t test. * p < 0.05; ** p < 0.01; *** p < 0.001.

4.2.7 Mincle and Syk in DCs are needed for intestinal IL-17 and IL-22 production

We next explored whether the absence of Mincle and Syk in the CD11c⁺ compartment could affect Th17 differentiation in the steady state intestine *in vivo*. IL-17 and IL-22 production by both CD4⁺ T cells from PPs and sorted CD3⁺ T cells from the small intestine LP was reduced both in CD11cΔ*Syk* mice (Fig. 4.2.7 A) and in Mincle-deficient mice (Fig. 4.2.7 B) compared with WT littermates, while IFN-γ production was not altered (Fig. 4.2.7 C, D). IL-17 producing T cells generated in a Mincle-dependent manner in the steady state mainly co-produced IL-10, but not IFN-γ, indicating that these T cells were non-pathogenic (McGeachy et al., 2007) (Fig. 4.2.7 E). Naive CD4⁺ T cells from both CD11cΔ*Syk* and Mincle-deficient mice differentiated normally into IL-17- and IL-22-secreting effector cells in the presence of Th17-polarizing cytokines (Fig. 4.2.7 F). Furthermore, adoptively transferred naive CD4⁺ T cells from CD11cΔ*Syk* mice or WT littermates (CD45.2⁺) similarly produced IL-17 and IL-22 in PPs of WT recipients (CD45.1) (Fig. 4.2.7 G). These results demonstrate the importance of Mincle and Syk for IL-17 and IL-22 generation in the intestine and exclude an intrinsic defect of cytokine production in the T cells of these mice.

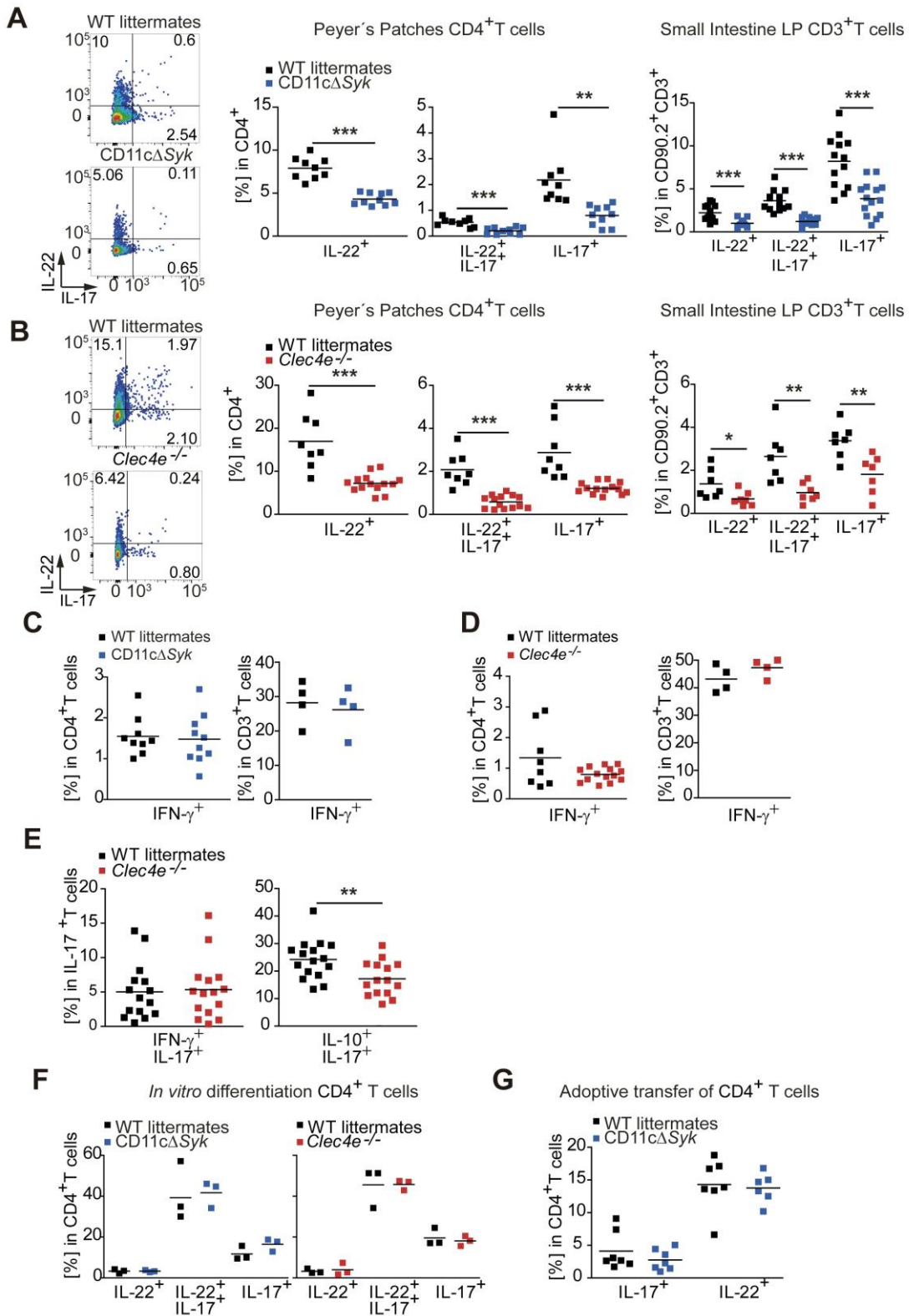


Figure 4.2.7 Mincle and Syk in DCs are needed for intestinal T cells-mediated IL-17 and IL-22 steady state production. (A, B) Left: representative plots and summary graph of IL-17 and IL-22 production by intracellular staining after PMA and ionomycin stimulation in CD4⁺ T cells from PPs; right: or small intestine lamina propria sorted CD3⁺ T cells, in the indicated genotypes. (C, D) Left: IFN-γ production by intracellular staining after PMA and ionomycin

stimulation in CD4⁺ T cells from PPs; right: or small intestine lamina propria sorted CD3⁺ T cells, in the indicated genotypes. (E) IL-17, IFN- γ and IL-10 production by intracellular staining after PMA and ionomycin stimulation in CD4⁺ T cells from PPs in the indicated genotypes. (F) IL-17 and IL-22 production by naive CD4⁺ T cells from the indicated genotypes stimulated *in vitro* on plate-bound anti-CD3 and anti-CD28 under Th17 polarizing conditions for 5 days and restimulated for intracellular staining. (G) IL-22 and IL-17 production by intracellular staining of restimulated CD45.2⁺ CD4⁺ in the PPs 14 days after adoptive transfer of 6x10⁶ naive CD4⁺ T cells purified from CD45.2⁺ WT and CD11c Δ Syk to CD45.1 recipients. (A, B, E, G) At least two independent experiments have been pooled. (C, D, F) One representative experiment of at least two performed is shown. Each symbol represents an individual mouse. The arithmetic mean for each group is indicated. ** p < 0.01; *** p < 0.001 (Unpaired two-tailed Student's t test)

Additional sources of IL-17 and IL-22 in the gut are CCR6-expressing ILC3s that are mainly located in PPs (Klose and Artis, 2016). CD11c Δ Syk and Mincle-deficient mice exhibited reduced frequencies of IL17- or IL17/IL22-producing ILCs in both PPs and small intestine LP compared with WT littermates, whereas ILCs producing only IL-22 were comparable between genotypes (Fig. 4.2.8 A, B). These results reveal that the Mincle-Syk signalling pathway regulates IL-17 and IL-22 production by both innate and adaptive cellular sources in the steady-state small intestine and PPs.

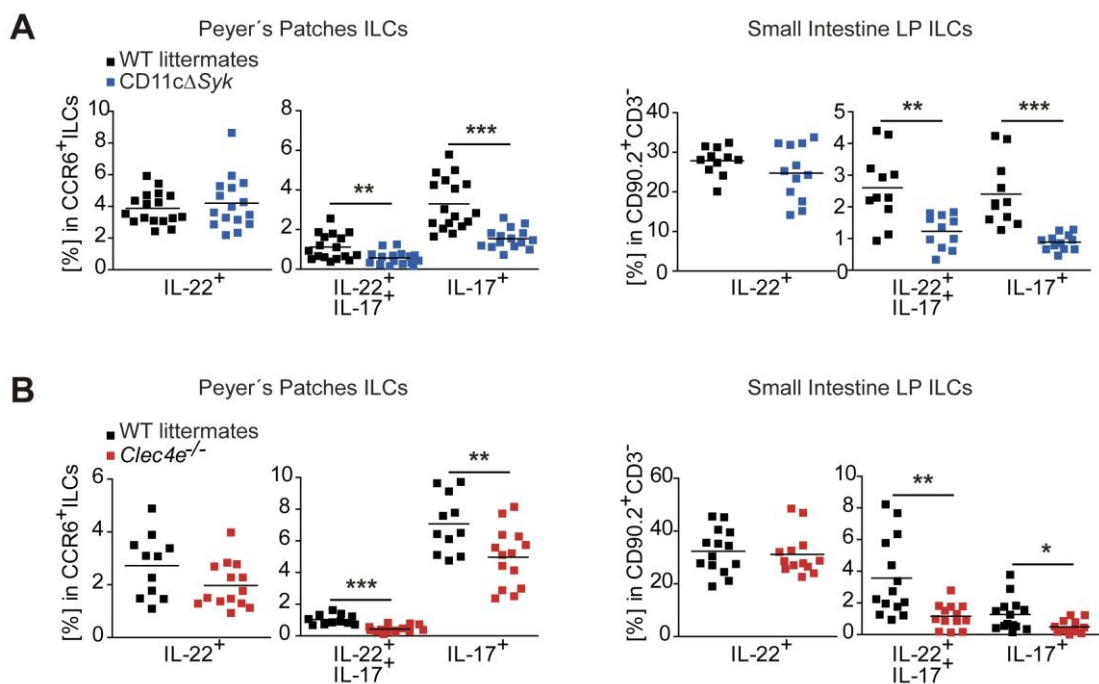


Figure 4.2.8 Mincle and Syk in DCs are needed for intestinal ILCs-mediated IL-17 steady state production. (A, B) Left: frequency of CCR6⁺ ILCs from PPs; right: from the small intestine lamina propria, producing IL-22 and IL-17 in steady state by intracellular staining in the indicated genotypes. (A, B) At least two independent experiments have been pooled. Each symbol represents an individual mouse. The arithmetic mean for each group is indicated. ** p < 0.01; *** p < 0.001 (Unpaired two-tailed Student's t test).

4.2.8 Mincle-dependent IL-17 producing-cells in PPs require commensal bacteria

Treatment with a broad-spectrum antibiotic cocktail to disrupt the gut microbiota abolished IL-17 production by T cells from PPs in WT littermates to the levels found in Mincle-deficient and CD11cΔSyk mice (Fig. 4.2.9 A, B), suggesting that PP Th17 cell development required intestinal bacteria. Given the prominent role of SFB in Th17 cell differentiation in PPs (Ivanov et al., 2009), we investigated the potential involvement of SFB in the Mincle-dependent Th17 differentiation. SFB was detected in our mouse colonies by PCR (IDEXX Bioresearch). SFB is sensitive to vancomycin treatment as demonstrated by the decrease in SFB content in feces after treatment (Fig. 4.2.9 C) (Ivanov et al., 2008). Notably, vancomycin treatment slightly reduced IL-17 production by CD4⁺ T cells from PPs of WT littermates, with the remaining IL-17 production dependent on Mincle (Fig. 4.2.9 D). These results show that vancomycin resistant bacteria mediate Mincle-dependent IL-17 production by T cells from PPs.

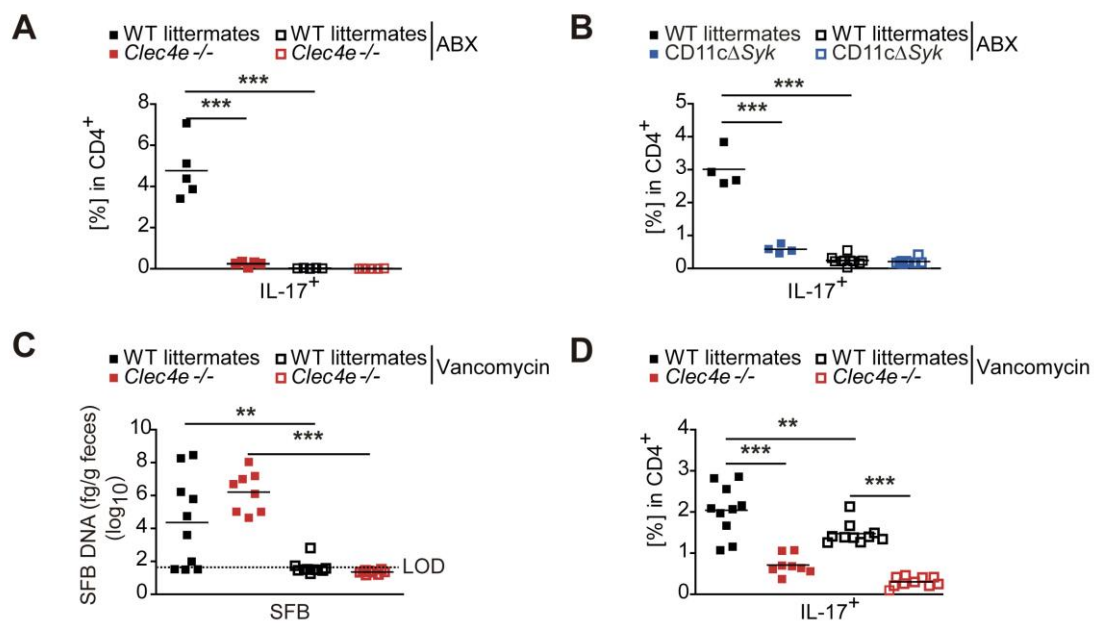


Figure 4.2.9 Mincle-dependent maintenance of IL-17 producing-cells in PPs does not require SFB. (A, B, D) Frequency of IL-17 production by intracellular staining in restimulated CD4⁺ T cells from PPs of the indicated genotypes after treatment with an antibiotic cocktail (ABX) containing ampicillin, neomycin, metronidazole and vancomycin (A,B) or vancomycin only (D) in the drinking water during 4 weeks. (C) SFB DNA was quantified by

qPCR in feces from Mincle-deficient (*Clec4e*^{-/-}) and WT littermate in steady state or after vancomycin administration as described above. The lower line indicates the limit of detection (LOD). (A, B) One representative experiment of two performed. (C, D) At least two independent experiments have been pooled. Each symbol represents an individual mouse. The arithmetic mean for each group is indicated. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (A,B,D) One-way ANOVA and Bonferroni post-hoc test. (C) Two-tailed Mann-Whitney's U test.

Given the binding of Mincle-hFc to *L. plantarum* (Fig. 4.2.4 G), we tested the ability of mucosal-associated commensals from WT mice treated with ABX during gestation and lactation and gavaged with *L. plantarum* at weaning (*L. plantarum*-enriched mucus) to induce Th17 differentiation *in vitro*. For this, naive OT-II T cells were stimulated with sorted GM-DCs from WT and Mincle-deficient (*Clec4e*^{-/-}) mice previously pulsed or not (medium) with *L. plantarum*-enriched mucus and loaded with OVA₃₂₃₋₃₃₉ peptide. We found that *L. plantarum*-enriched mucus induced IL-17 and IL-22 production by naive OT-II cells upon culture with WT but not Mincle-deficient GM-DCs (Fig. 4.2.10 A). Induction of Th17 differentiation correlated with Mincle-dependent production of IL-6 and IL-23 by GM-DCs (Fig. 4.2.10 B). Moreover, mice treated with ABX and gavaged with *L. plantarum* at weaning as indicated above showed a Mincle-dependent induction of IL-17 and IL-22 production by CD4⁺ T cells and CCR6⁺ ILCs in PPs compared with controls not gavaged with *L. plantarum* (Fig. 4.2.10 C,D), without impacting IFN- γ production (Fig. 4.2.10 E). These results suggest that mucosa-associated bacteria, including *L. plantarum*, drive Mincle-dependent IL-17 and IL-22-producing cells in PPs.

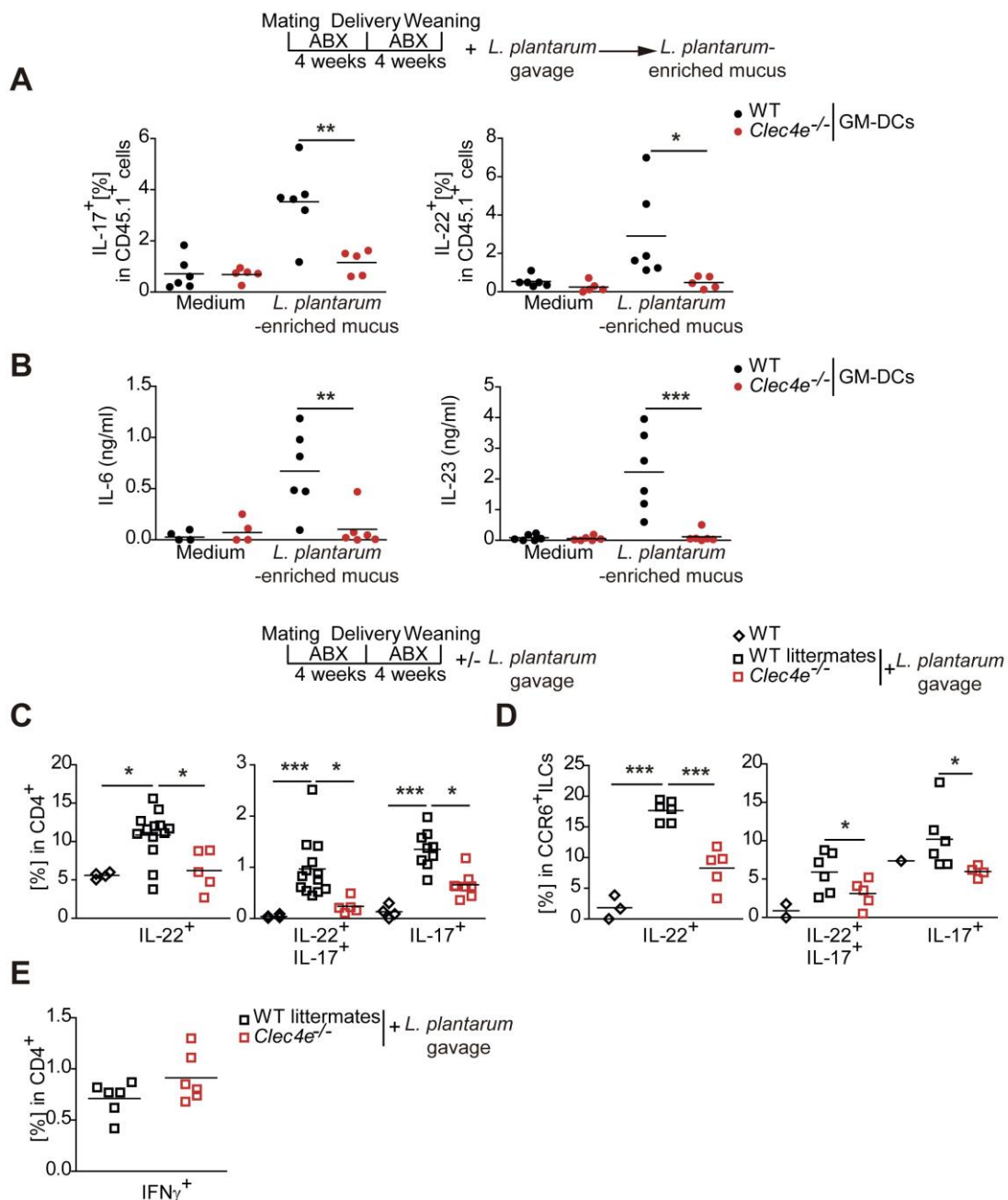


Figure 4.2.10 Mincle-dependent IL-17 producing-cells in PPs requires mucosa-associated commensal bacteria. (A) Frequency of IL-17 and IL-22 production by intracellular staining of restimulated OT-II T cells co-cultured with sorted WT and Mincle-deficient GM-DCs loaded with OVA323-339 peptide, which were pulsed or not (medium) with mucosal-associated commensals from WT mice treated with ABX during gestation and lactation and gavaged with *L. plantarum* at weaning as indicated (*L. plantarum*-enriched mucus; 10:1 DC ratio). (B) ELISA of IL-6 and IL-23 from the co-culture of GM-DC in (A). (C-E) WT littermates and Mincle-deficient (*Clec4e*^{-/-}) mice were treated with ABX during gestation and lactation and were gavaged with *L. plantarum* (1x10⁶) at weaning as indicated in the scheme (+ *L. plantarum* gavage). Frequency of IL-17- and IL-22-producing CD4⁺ T cells (C) or CCR6⁺ ILCs (D) and IFN- γ production by CD4⁺ T cells (E) from PPs. (A-C) At least two independent experiments have been pooled; (D, E) One representative experiment of two performed. Each symbol represents an individual mouse. The arithmetic mean for each group is indicated. * p < 0.05; **

p < 0.01; *** p < 0.001 (A-C) One-way ANOVA and Bonferroni post-hoc test. (D) Unpaired two-tailed Student's t test.

4.2.9 The Mincle-Syk axis contributes to the intestinal barrier function

We next explored whether the defective IL-17 and IL-22 expression in Mincle-deficient and CD11c Δ Syk mice affected the intestinal barrier function. To explore the immunological barrier, we examined the expression of antimicrobial peptides in the intestine, specifically RegIII γ , whose production relies on ROR γ t-dependent cells (Sanos et al., 2009). We found that steady-state *Reg3g* expression was reduced in the absence of Mincle or Syk in CD11c⁺ cells compared with WT littermates (Fig. 4.2.11 A). Notably, oral administration of *L. plantarum* to ABX-treated weaned mice as above promoted *Reg3g* expression in a Mincle-dependent manner (Fig. 4.2.11 B). IgA secretion can be influenced directly by Th17 cells and ILC3 (Hirota et al., 2013, Kruglov et al., 2013). Total IgA was reduced in the intestinal lumen in Mincle-deficient and CD11c Δ Syk mice compared with WT littermate controls (Fig. 4.2.11 C). Flow cytometry analysis of the frequency of IgA-coated intestinal bacteria, which was dependent on *Aicda*, revealed a specific reduction in the absence of Mincle (Fig. 4.2.11 D). Frequency of IgA⁺ plasma cells in the small intestine LP of Mincle-deficient and CD11c Δ Syk mice was decreased compared with WT littermates (Fig. 4.2.11 E), while IgG⁺ plasma cells were comparable (Fig. 4.2.11 F). This correlated with reduced percentages of PD-1^{hi} CD4⁺ T cells in PPs (Fig. 4.2.11 G), a marker of T follicular helper (Tfh) cells that promote IgA-producing germinal center B cells (Hirota et al., 2013). These differences in gut immunity did not result in significant differences in the luminal microbiota composition at the genus level between Mincle-deficient mice and their WT littermates (Fig. 4.2.11 H). These results indicate that the Mincle-Syk signalling pathway contributes to maintain a functional intestinal immune barrier, but does not play a major role in regulating intestinal microbiome composition.

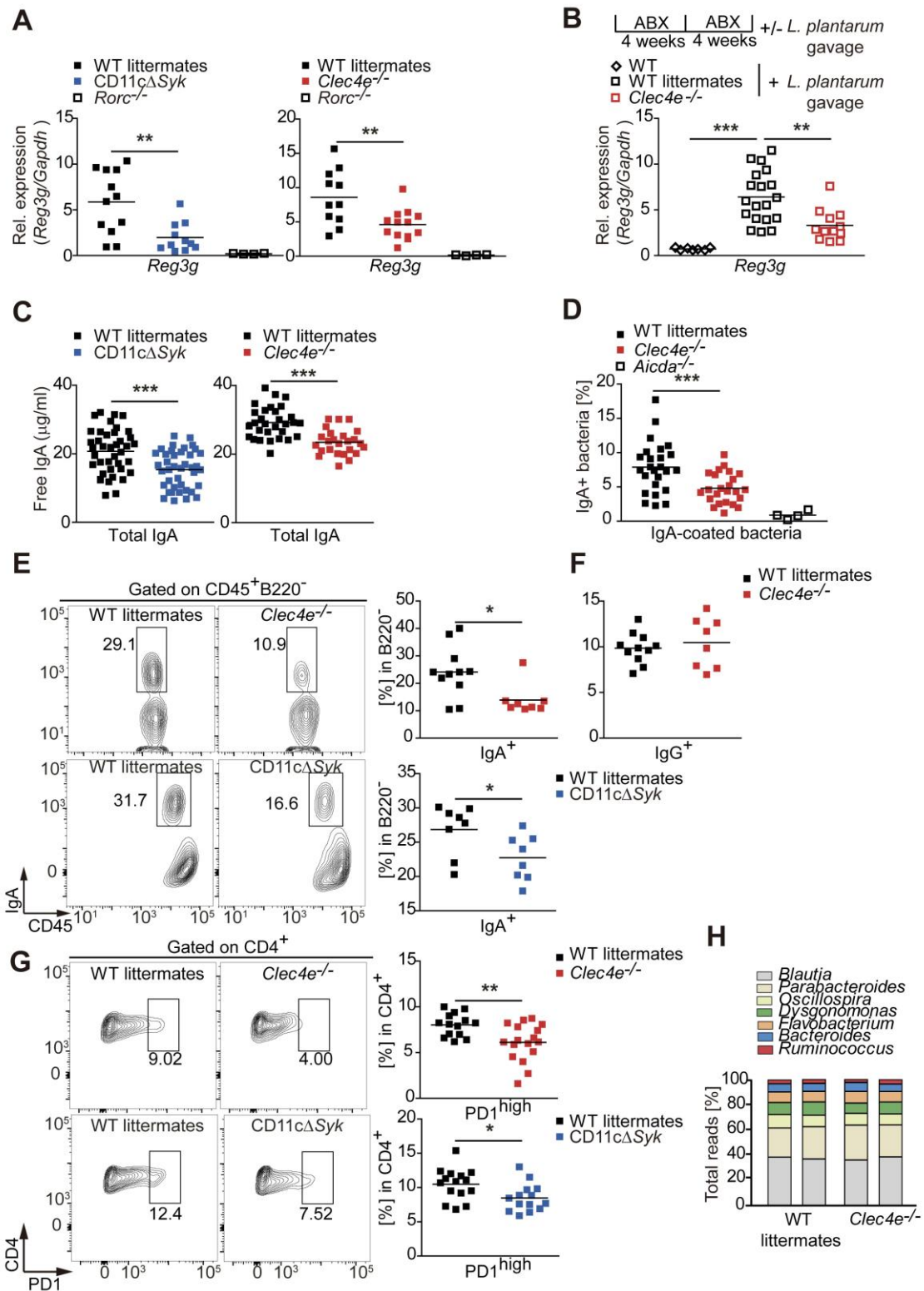


Figure 4.2.11. The Mincle-Syk axis contributes to the intestinal barrier function. (A, B) *Reg3g* transcripts were measured by qPCR normalized to *Gapdh* in the jejunum of the indicated genotypes during steady state (A) or after *L. plantarum* gavage, as described in the scheme (B). (C) Quantification of total IgA measured by ELISA in the intestinal lumen of the indicated genotypes. (D) Frequency of IgA⁺ bacteria by flow cytometry in the indicated genotypes. (E, F) Representative plots and summary graphs of CD45⁺B220⁻IgA⁺ (E) or IgG⁺ (F) plasmatic cells from the small intestine LP of the indicated genotypes. (G) Representative plots and summary

graph of CD4⁺PD-1^{high} T cells from PPs of the indicated genotypes. (H) 16S sequencing analysis of intestine luminal microbiota from Mincle-deficient (*Clec4e*^{-/-}) mice and their WT littermates. Graph showing the mean relative abundance of each genus in each sample (at least 10 mice pooled) from two independent experiments. (A-G) Data pooled from at least two independent experiments. Individual mice and arithmetic mean of each group is indicated. * p < 0.05; ** p < 0.01; *** p < 0.001(A,B) One-way ANOVA and Bonferroni post-hoc test (C-G) Unpaired two-tailed Student's t test.

1. Mincle-Syk pathway promotes commensal bacteria containment

To explore whether the functional impairment of the intestinal barrier in Mincle-deficient and CD11cΔ*Syk* mice affects microbial containment in the intestine, we first analysed the presence of live facultative aerobic bacteria in extra-intestinal organs. We found increased microbe translocation to the liver in the absence of Mincle or Syk in DCs compared to WT littermate controls (**Fig. 4.2.12 A, B**). Deep 16S rRNA sequencing revealed that most of the surplus disseminated aerobic bacteria found in the liver of Mincle-deficient mice belonged to the phylum *Proteobacteria* either following growth in LB medium or by direct sequencing of fresh liver homogenates (**Fig. 4.2.12 C**). As an additional readout for systemic dissemination of commensals (Zeng et al., 2016), we found increased intestinal bacteria-specific IgG in sera of Mincle-deficient and CD11cΔ*Syk* mice compared with WT littermates (**Fig. 4.2.12 D**).

Next, we analysed whether the presence of increased intestinal commensals in the liver of Mincle-deficient mice can lead to patho-physiological alterations. CD45⁺CD11b^{high} myeloid cells, mainly neutrophils and inflammatory monocytes, were increased in the liver of Mincle-deficient mice compared with WT littermates in an ABX-dependent manner (**Fig. 4.2.12 E**). Liver inflammation in the absence of Mincle correlated with moderate liver malfunction reflected by an increased total, but not direct, serum bilirubin, without affecting serum ALT/AST levels (**Fig. 4.2.12 F**). Some lipid metabolism-related genes such as *Scd1* (Stearoyl-CoA desaturase-1) or *Cpt1a* (Carnitine palmitoyltransferase 1) were significantly upregulated in the liver of Mincle-deficient mice (**Fig. 4.2.12 G**). This increased expression of lipogenic genes correlated with an accumulation of diacylglycerides (DAG) (**Fig. 4.2.12 H**), and some fatty acids including margaric and linoleic acids in Mincle-deficient mice (**Fig. 4.2.12 I**), as revealed by metabolomics assays. These results indicate that the intestinal immune barrier fostered by Mincle/Syk axis limits microbial translocation, preventing systemic inflammation and promoting host-microbiota mutualism.

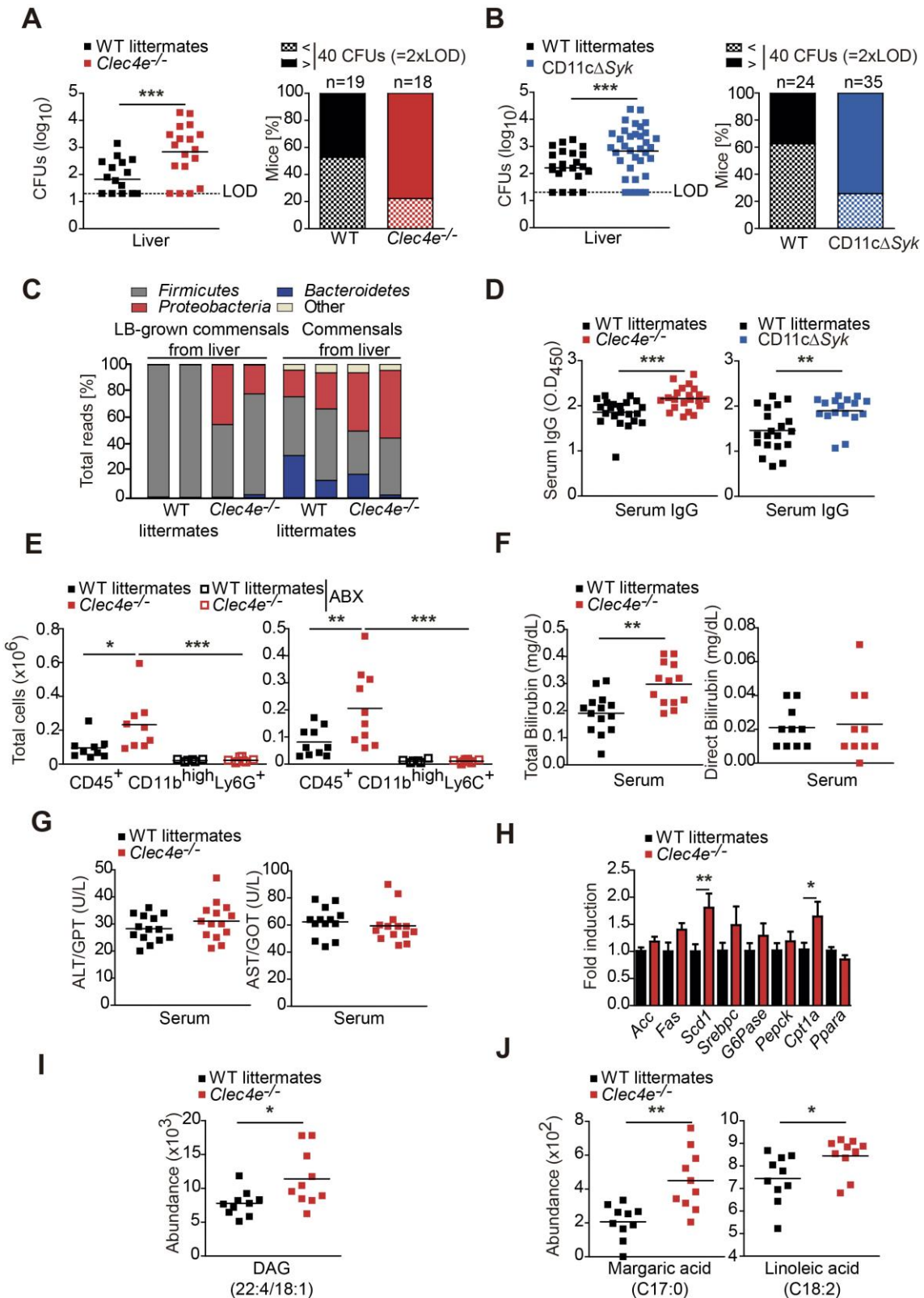


Figure 4.2.12. Mincle-Syk pathway promotes commensal bacteria containment. (A, B) Bacterial translocation into the liver of Mincle-deficient mice (*Clec4e*^{-/-}) (A) or CD11cΔSyk (B) and WT littermates. Left: bacterial load as colony forming units (CFUs) per organ, indicating limit of detection (LOD). Right: Frequencies of mice of each genotype showing more than 40 CFUs per organ (=2xLOD). (C) 16S rRNA sequencing analysis of LB-grown commensals from

the liver (left) or bacterial DNA directly extracted from the liver (right) in the indicated genotypes. The graph shows the percentage of total reads corresponding to each phylum. Each bar represents 4 pooled LB plates/mouse and 4 mice/sample of each genotype (LB-grown commensals) or 6 pooled mice per genotype (Commensals from the liver). (D) ELISA of serum IgG against intestinal bacteria in the indicated genotypes. (E) Summary graph of CD45⁺ CD11b^{high} Ly6G⁺ cells (left) or Ly6C⁺ cells (right) infiltrated in the liver of Mincle-deficient (*Clec4e*^{-/-}) and their WT littermates in steady state or after administration of an antibiotic cocktail (ABX). (F) Total (left) or direct (right) bilirubin in the indicated genotypes. (G) Alanine transaminase (ALT/GPT) (left) and Aspartate transaminase (AST/GOT) (right) analysed in serum of the indicated genotypes. (H) *Acc*, *Fas*, *Scd1*, *Srebpc*, *G6pase*, *Pepck*, *Cpt1a* and *Ppara* transcripts were analysed by qPCR and normalized to *Gapdh* in the liver of 15 mice of the indicated genotypes in 3 independent experiments. The graph shows fold induction in comparison to WT. (I, J) Individual lipid species of diacylglycerides (DAG) (I) and free fatty acid classes, Margoric (C17:0) and Linoleic (C18:2) acids (J) measured by LC-MS and GC-MS metabolomics-based profiling approach. Data presented as metabolites' abundance. (A-J) Data are pooled from at least two independent experiments. * p < 0.05 ** p < 0.01; *** p < 0.001. (A-D, F-H margoric acid) Unpaired two-tailed Student's t test; (H linoleic acid) Mann-Whitney U-test; (E) One-way ANOVA and Bonferroni post-hoc test.

Discussion

5. DISCUSSION

The skin and the gastrointestinal tract are barrier tissues that are the entrance of pathogenic microorganisms but at the same time are the niche for beneficial microorganisms that composed the commensal microbiota. In this situation, the immune system has to face a big challenge, eliminating pathogenic microbes and foreign substances, while avoiding damage of self-tissues and facilitating the symbiosis to resident microbiota (Belkaid and Artis, 2013). For that, sensing of the environmental cues is essential.

Myeloid cells are professional sensors of the environment. For that, they are equipped with a variety of germ line-encoded receptors, called pattern recognition receptors (PRRs) that allow them to detect surrounding milieu signals. Although, Charles Janeway hypothesised the existence of these receptors initially as sensors of “non-self” pathogen-associated molecular patterns (PAMPs) on pathogens as inducers of immunity (Janeway, 1989), PAMPs are not exclusive to pathogens, and are also present in the resident microbiota, referred as microbe-associated molecular patterns (MAMPs) (Chu and Mazmanian, 2013). In addition, these receptors can also detect “self” damage-associated molecular patterns (DAMPs), host cellular components released upon cell death (Jounai et al., 2012). Some of these sensors, for example several myeloid C-type lectin receptors (CLRs) are promiscuous and versatile receptors that can detect “non-self” ligands from pathogens or commensal microorganisms, while at the same time are able to sense “self” ligands coming from the host (Del Fresno et al., 2018). Detection of PAMPs/MAMPs/DAMPs may lead to myeloid cell activation and maturation, which finally shapes the nature of the adaptive response, triggering immunity or promoting homeostasis, accordingly to the initial detected signals (Iwasaki and Medzhitov, 2010).

A prototypical example of this adaptability is the myeloid CLR Mincle. Classically viewed as an activating receptor, Mincle senses and responds to several “non-self” ligands derived from pathogenic bacteria and fungi. In most of the cases, Mincle-mediated recognition of these pathogens promotes the activation of myeloid cells and the generation of immunity against the infection (Sharma et al., 2014, Rabes et al., 2015, Behler-Janbeck et al., 2016, Chinthamani et al., 2017, Kottom et al., 2017, Bugarcic et al., 2008, Wells et al., 2008, Yamasaki et al., 2009). In contrast, Mincle-sensing of “non-self” ligands can lead to immunosuppression by directly promoting myeloid cells secretion of anti-inflammatory mediators (Lee et al., 2016b, Devi et al., 2015) or by indirect inhibition of activating heterologous receptors signalling (Wuthrich et al., 2015, Wevers et al., 2014), favouring pathogen-mediated immune scape or the resolution of the inflammation.

This duality, promoting and/or inhibiting the generation of immune responses, is not restricted to Mincle-recognition of “non-self” ligands, but it is also true in response to “self” derived compounds. Upon “self” ligands recognition, Mincle can mediate immunity (Kiyotake et al.,

2015, Kostarnoy et al., 2017, Nagata et al., 2017), but also immunosuppression (Xie et al., 2017), even in response to the same endogenous ligand, as described for SAP-130 (Yamasaki et al., 2008, Arumugam et al., 2017, Suzuki et al., 2013, Zhou et al., 2016, Tanaka et al., 2014, Lee et al., 2016a, Greco et al., 2016, Seifert et al., 2016).

This versatility let us to explore the role of Mincle in two different scenarios. First, upon *Leishmania* parasite infection. Some parasites that depend on an invertebrate vector for cyclical transmission, have evolved mechanisms to persist in host myeloid cells, by evading, delaying and manipulating host immunity in order to escape host resistance and ensure their transmission. CLRs expressed on myeloid cells might be critical for *Leishmania* recognition and can be targeted to sabotage the host immune response (Martinez-Lopez et al., 2018). The second one in response to commensal microbiota. These microorganisms are well adapted to survive inside the host, and have evolved mechanisms to prevent the generation of immunity and promote the mutually beneficial relationship; myeloid sensing of the gut microbiota is essential in this process (Belkaid and Hand, 2014, Swiatczak and Cohen, 2015).

For the first objective, we used the parasite *Leishmania major* (*L. major*). *Leishmania* parasites replicate silently in the skin for several weeks after inoculation (Belkaid et al., 2000), suggesting that they might actively dampen DC recognition or activation (Srivastav et al., 2012), to establish a functional immune privilege in the skin (Peters and Sacks, 2006). During this “silent” infection, myeloid CLRs as SIGNR3 (mouse Cd209d) can be targeted by *Leishmania* parasites to escape the host immune response by inhibiting the heterologous CLR Dectin-1(CLEC7A)-mediated inflammation (Lefevre et al., 2013). Accordingly, we have found that *L. major* parasites release a soluble ligand that binds Mincle to dampen the host immune response. Importantly, the identified ligand in *Leishmania* was mainly located in the flagellar pocket, a unique site for exocytosis, and was detected in the supernatant from *L. major* parasite, which fits with the described role of *Leishmania* secreted microvesicles mediating immunosuppression for *Leishmania* invasion by the activation of tyrosine phosphatases, as SHP-1 in macrophages (Silverman and Reiner, 2011).

Mincle-*Leishmania* interaction changes the intracellular configuration of the receptor. Upon interaction with *L. major*, the ITAM-containing adaptor molecule, FcR γ chain changes to a configuration termed inhibitory ITAM instead of interacts with kinase Syk and generates an activating signal to promote immunity (Yamasaki et al., 2009). This inhibitory ITAM signalling pathway is characterized by partial phosphorylation of FcR γ chain and the ability to recruit phosphatases, inhibiting signals through heterologous receptors (Pasquier et al., 2005, Kanamaru et al., 2008). We found that Mincle-mediated inhibitory ITAM promotes the recruitment of the src homology region 2 domain-containing phosphatase (SHP)-1, suppresses DC activation by heterologous activating receptors concomitantly sensing *L. major*. Mincle

deficiency thus favoured stronger DCs activation in response to *L. major* infection, manifested in higher expression of costimulatory molecules, better migration, and priming of a Th1 cell response to parasite antigens. Increased Th1 cell-type immunity correlated with reduced parasite load and pathology in Mincle-deficient mice (Fig. 5.1).

These results reveal a new mechanism of Mincle-mediated immunosuppression, based on the inhibitory ITAM configuration and SHP1-inhibitory pathway, in response to “non-self” ligands, derived from *L. major*. This is different from a previous pathway described for Mincle-mediated inhibition of heterologous receptors in response to *Fonsecaea monophora* (*F. monophora*). In response to this fungus, Mincle mediates PKB pathway-dependent ubiquitin ligase activation, which leads to loss of Dectin-1 mediated-nuclear IRF1 activity, blocking IL12A transcription (Wevers et al., 2014). These results suggest that different pathogens can use Mincle to activate several intracellular pathways to dampen heterologous receptors-mediated immune response.

To explain how it is possible, we can speculate that the state of the ligand can affect how the ligand interact with the CLR and the downstream signalling generated upon its recognition. Soluble and/or monomeric ligands, as is the case of *L. major*, are poor activators, whereas particulate/oligomeric ligands, as could be the case of *F. monophora*, promote an efficient activating signalling (Iborra and Sancho, 2015). Ligand affinity and avidity can affect the quantity and duration of signals through the ITAM domain, resulting in differential responses (Hamerman et al., 2009, Yamasaki et al., 2004). As described for the Fc α RI receptor, which associates with the Fc γ chain for signalling, interaction with multimeric ligands stimulates cell activation by recruiting Syk, similar what happens upon *F. monophora* detection via Mincle. In contrast, upon interaction with low affinity or avidity ligands, Fc γ chain changes to inhibitory ITAM, being able to inhibit signals through heterologous receptors (Pasquier et al., 2005, Kanamaru et al., 2008, Blank et al., 2009). It fits with the observed results for Mincle-mediated inhibitory ITAM configuration formed in response to soluble extracellular ligand from *L. major* parasites, which is able to inhibit DC activation upon LPS challenge in a Mincle-dependent manner.

The inhibitory ITAM requires transient Syk activation (Ben Mkaddem et al., 2014). We found transient Syk association with Mincle following *L. major* stimulation and that Syk is required for SHP1 recruitment to Mincle. However, we found that the overall response of CD11c Δ Syk DCs to *L. major* was impaired, maybe due to Syk involvement for other PRRs intracellular signalling, as described for Dectin-1 (Lefevre et al., 2013).

Mincle collaborates with MCL for immune responses against TDM (Miyake et al., 2013, Miyake et al., 2015). Both receptors form a heteromeric complex that facilitates signalling (Lobato-Pascual et al., 2013) and mutually regulate their expression in response to microbial challenge (Kerscher et al., 2016a). We found that MCL does not recognize *L. major* and *L. major* ligand triggers SHP-1 phosphorylation via Mincle in the absence of MCL, indicating that

MCL is not directly involved in this process. It is possible that recognition of *L. major* by Mincle (in homo or heteromeric configuration) could trigger a weaker signal than TDM recognition by Mincle-MCL heteromer, and this weaker signalling may favour the inhibitory ITAM configuration.

Our results together with previous studies disarm the initial naive notion that activating receptors could mainly sense non-self ligands to trigger the immune response while ITIM-bearing CLRs could preferentially bind self-ligands to dampen inflammation. Supporting the new general view that most CLRs are adaptable structures that can bind both endogenous and exogenous ligands, resulting in distinct functional outcomes, not totally but partially depending on their intracellular bearing motif (Del Fresno et al., 2018).

For the second objective, we have assessed the role of Mincle in handling the response to “non-self” MAMPs (Chu and Mazmanian, 2013) from commensal microorganisms. We focus our study in the intestinal microbiota, which is the largest population of microorganisms that inhabit the body (Thursby and Juge, 2017). The intestinal microbiota contributes to host metabolism, resistance to pathogen colonization and host immune system development and homeostasis (Baumler and Sperandio, 2016, Honda and Littman, 2016). However, its containment is essential and increased microbial translocation is associated with systemic inflammation (Sonnenberg et al., 2012). The intestinal epithelial cells (IECs), the mucus layer, the generation of antimicrobial peptides and the synthesis of microbe-specific immunoglobulin A (IgA) in the Peyer’s patches (PPs) constitute the “mucosal firewall” (Belkaid and Hand, 2014). This barrier is regulated by ROR γ t-dependent cells that include group 3 innate lymphoid cells (ILC3) and T helper 17 (Th17) cells, which produce IL-17 and IL-22 regulating the expression of antimicrobial peptides and mucin genes, and influencing IgA responses (Hirota et al., 2013, Kruglov et al., 2013). The intestinal microbiota critically contributes to the generation and function of these cells (Ivanov et al., 2008, Sanos et al., 2009, Satoh-Takayama et al., 2008) through myeloid cell-mediated antigen presentation or -derived cytokines such as IL-6 and IL-23 after MAMPs-PRRs interaction (Ivanov et al., 2006, Persson et al., 2013, Longman et al., 2014, Satpathy et al., 2013). The absence of these cells or their mediators can lead to a breach of the intestinal barrier, commensal translocation and systemic inflammation (Lochner et al., 2011, Sonnenberg et al., 2012). The specific myeloid cell subset, as well as the innate sensors involved in this process were unknown.

In this regard, most of the myeloid Syk-coupled CLRs, including Dectin-1 (*CLEC7A*), Dectin-2 (*CLEC6A* in human, *Clec4n* in the mouse) and Mincle (*CLEC4E*) mainly drive IL-17 production by T cells in response to pathogens, by modulation of myeloid IL-6 and IL-23 secretion (Geijtenbeek and Gringhuis, 2016, LeibundGut-Landmann et al., 2007). In addition, the CLRs-mediated Syk/CARD9 pathway has been recently identified as protective in the

context of inflammation-associated cancer through recognition of commensal fungi (Malik et al., 2018) and many myeloid CLRs has been described recognize ligands from the intestinal commensal bacteria and fungi (Lightfoot et al., 2015, Konieczna et al., 2015, Eriksson et al., 2013, Hütter et al., 2014). The best-characterized example is Dectin-1(*CLEC7A*)-mediated recognition of indigenous fungi and the control of Treg cell differentiation through modification of microbiota (Iliev et al., 2012, Tang et al., 2015). However most of the knowledge is based on *in vitro* or DSS-induced colitis studies and how myeloid CLR-microbiota interaction promotes tolerance to microbiota is still poorly understood (Smits et al., 2005, Eriksson et al., 2013).

Therefore, we investigated whether Mincle could contribute to intestinal microbiota sensing by myeloid cells to promote functional responses in ROR γ ⁺ cells under homeostatic conditions.

We found that Syk kinase, used by Mincle for its signalling (Yamasaki et al., 2008), but not MyD88, the adaptor molecule needed for most TLR signalling (Kawasaki and Kawai, 2014), is a non-redundant innate signal for driving Th17 differentiation in response to intestinal microorganisms recognition by DCs. Consistent with previous studies revealing a critical role for Syk-coupled CLRs driving Th17 generation after infection (Geijtenbeek and Gringhuis, 2016, LeibundGut-Landmann et al., 2007), as well as, with the reported role of MyD88 in T cells but not in DCs, in Th17 commitment (Hu et al., 2011, Shaw et al., 2012). We provide evidence that sensing of “non-self” mucosal-associated commensal bacteria by Mincle in myeloid Lysozyme⁺ DCs and CD11b⁺ DCs in PPs induces Syk-dependent IL-6 and IL-23, cytokines that stimulate intestinal T cells and ILCs to produce IL-17 and IL-22. Absence of a functional Mincle-Syk axis in DCs leads to reduced IL-17 and IL-22 production and impaired intestinal immune barrier function, resulting in increased systemic translocation of microbiota, hepatic inflammation and metabolic alterations.

Our results indicate that commensal bacteria containing Mincle ligands have a preferential location close to the intestinal mucosa, and it could explain their ability to interact with myeloid cells and modulate IL-17 and IL-22 production. It is in agreement with previous publication showing that the differential spatial distribution of commensal microbes in the intestine influences their capacity to shape host immune responses (Ivanov et al., 2008, Fung et al., 2014, Atarashi et al., 2015). In contrast, ligands from commensal microorganisms for other Syk-coupled CLRs, as Dectin-1 and Dectin-2, are preferentially luminal and not associated to the intestinal mucosa, supporting the non-redundant role of Mincle mediating the recognition of mucosa-associated commensals. The 16S rRNA sequencing of bacteria enriched by the extracellular domain of Mincle indicates that the small intestine adherent *Lactobacillus* (Donaldson et al., 2016) contains a ligand for Mincle. The exposition of the ligand for Mincle seems to require transit through the intestine, resembling the described structural adaptations in the cell surface composition of bacteria upon different environmental situations (Sengupta et al., 2013).

Our results concur with the described ability of the *Lactobacillus plantarum* (*L. plantarum*) glycolipid GL1 to signal through Mincle (Shah et al., 2016) and with previous reports indicating that *Lactobacillus* activates Syk in DCs (Weiss et al., 2012). In addition, β -gentiobiosyl diglycerides recognized by Mincle in the commensal fungus *Malassezia* have a similar structure than the glycolipid anchor of lipoteichoic acid, a major constituent of the cell wall of gram-positive bacteria; although lipoteichoic acid does not signal through Mincle, suggesting that could exist a common ligand for Mincle in the commensal microorganisms (Ishikawa et al., 2013). In contrast, we cannot exclude the possibility that not only the described but others ligands from *Lactobacillus* can be sensed by Mincle, as it has been described for *Lactobacillus reuteri* surface mucus adhesins or the *Lactobacillus acidophilus* surface layer proteins, which interact with DC-SIGN or Dectin-2 and SIGNR3 respectively (Bene et al., 2017, Lightfoot et al., 2015).

Our results indicate that Mincle is expressed in Lysozyme⁺-DCs (LysoDCs) and -macrophages (LysoMacs) and, to a lesser extent, in CD11b⁺ DCs. These myeloid cell subsets are efficient in the capture of antigens and essential to drive the generation of the mucosal immune response (Da Silva et al., 2017). Moreover, CD11b⁺ DCs and LysoDCs, but not LysoMacs, from PPs induce Th17 cell differentiation from naive T cells in a Mincle and Syk-dependent fashion, consistent with their described T cell priming capacity (Da Silva et al., 2017). Similarly, Mincle expression by DCs, but not macrophages, contributes to the generation of splenic immunity during *Mycobacterium bovis* infection in mice (Behler et al., 2015). Some *Lactobacillus* species have preferential association with the follicle associated epithelium of PPs (Plant and Conway, 2001), suggesting that PPs could be the place where these myeloid cells could sense *Lactobacillus* via Mincle.

Intestinal microbiota recognition by myeloid cells innate sensors is critical for IL-17 and IL-22 production by ROR γ t-dependent cells (Ivanov et al., 2008, Sanos et al., 2009, Satoh-Takayama et al., 2008). *L. plantarum*, which possesses a ligand for Mincle (Shah et al., 2016) is able to induce Mincle-dependent Th17 differentiation both *in vitro* and in bacterial microbiota-depleted mice (treated with wide-spectrum antibiotics). Mincle-mediated Th17 generation seems to be independent of the described segmented filamentous bacteria (SFB)-induced Th17 cells (Ivanov et al., 2008, Gaboriau-Routhiau et al., 2009, Ivanov et al., 2009), although we cannot rule out the contribution of SFB during the initial priming of these Th17 cells. These results suggest that commensals location or cell-specific expression of different PRRs can modulate several pathways of T cell differentiation.

Mechanistically, our data indicate that Mincle/Syk pathway promotes IL-6 and IL-23p19 production by PPs DCs, upon mucosa-associated commensal recognition. It may explain the selective impact in the differentiation of Th17 but not Th1 cells. On the other hand, the

regulation of IL-23p19 may affect ILCs function, which requires IL-23R signalling for IL-22 and/or IL-17 secretion (Longman et al., 2014, Satpathy et al., 2013).

Consequently, in the absence of the Mincle or Syk in CD11c⁺ cells, we found reduced production of IL-17 and IL-22, affecting the intestinal barrier. At the level of antimicrobial peptides secretion, we found impaired *Reg3g* expression, which is regulated by IL-22 (Sanos et al., 2009). In addition, we found impaired IgA generation, which correlated with a reduction in the frequency of IgA⁺ plasma cells in the small intestine and the proportion of T follicular helper cells (Tfh) in PPs in the absence of Mincle and Syk in CD11c⁺ cells. These results concur with previous published studies indicating that Tfh differentiation requires interaction with CD11b⁺ DCs and IL-6 (Krishnaswamy et al., 2017) and the described contribution of the Th17 to Tfh cells in PPs (Hirota et al., 2013).

IL-17 and IL-22 deficiency in the absence of Mincle or Syk in CD11c⁺ cells correlates with increased peripheral dissemination of commensal bacteria as described previously that occur in the absence of ROR γ t-dependent cells or their mediators (Lochner et al., 2011, Sonnenberg et al., 2012). Commensal translocation correlates with higher liver inflammatory infiltration in Mincle-deficient mice, concurring with a recent report indicating that *Lactobacillus*-dependent IL-22 secretion by ILCs can prevent liver inflammation by promoting the intestinal barrier (Nakamoto et al., 2017). Interestingly, we found that excess liver inflammation driven by commensal translocation is associated to moderate liver malfunction and changes in the hepatic lipid metabolism, consistent with previous studies suggesting that increased intestinal permeability, bacterial translocation and inflammation can affect hepatic metabolism (Talukdar et al., 2012, Pierantonelli et al., 2017). Our results together with previous studies change the initial view of the ITAM-coupled CLR as activating receptors, sensing non-self ligands in pathogens to trigger the immunity; showing myeloid CLR as adaptable structures that can bind exogenous ligands not only in pathogens but also in commensal microorganisms promoting the mutual beneficial relationship and preventing systemic inflammation (Iborra and Sancho, 2015) (Fig. 5.2).

Altogether, our results are relevant to consider for therapeutically exploitation of Mincle. Several synthetic ligands of Mincle have been generated to specifically enhance immune response in vaccines preparations (Ostrop et al., 2015, Korsholm et al., 2014). For vaccine design, the presence of the *Leishmania* ligand for Mincle may contribute to the low effectiveness of candidate vaccines based on whole killed *Leishmania* or attenuated parasites (Duthie et al., 2012). Our results indicate that blocking Mincle or SHP1 during a vaccination setting may improve vaccine efficiency by avoiding *Leishmania*-Mincle-dampening of the adaptive response, although only a few drug-like molecules have been developed for CLR, high *in silico* druggability scores were found (Lang et al., 2011, Aretz et al., 2014). On the other hand, the elucidation of the new Mincle and Syk signalling pathway sensing mucosa-associated

bacteria that prevents systemic inflammation and its metabolic consequences, allows us to speculate with the possibility to target Mincle-expressing DCs with *Lactobacillus*-derived glycolipids to promote the functionality of the intestinal barrier for diseases associated with increased bacterial translocation, such as the metabolic syndrome (Burcelin, 2016).

Altogether, our findings suggest that Mincle can couple to an activating ITAM or to an inhibitory ITAM configuration depending on the nature of the ligand, and that the same activating ITAM does not predispose the response of the coupled CLR. If not the CLR-mediated response depend on the context, for example, signals derived from healthy or infected tissue, the level and localization of their expression, by their interaction and their collaborative or conflicting signalling with others PRRs, can promote immunity or tolerance to commensal microorganisms. This idea could apply to other ITAM-coupled CLRs with a diverse ligand range or that can heterodimerize with multiple receptors (Ostrop and Lang, 2017) (Fig. 5.1 and 5.2).

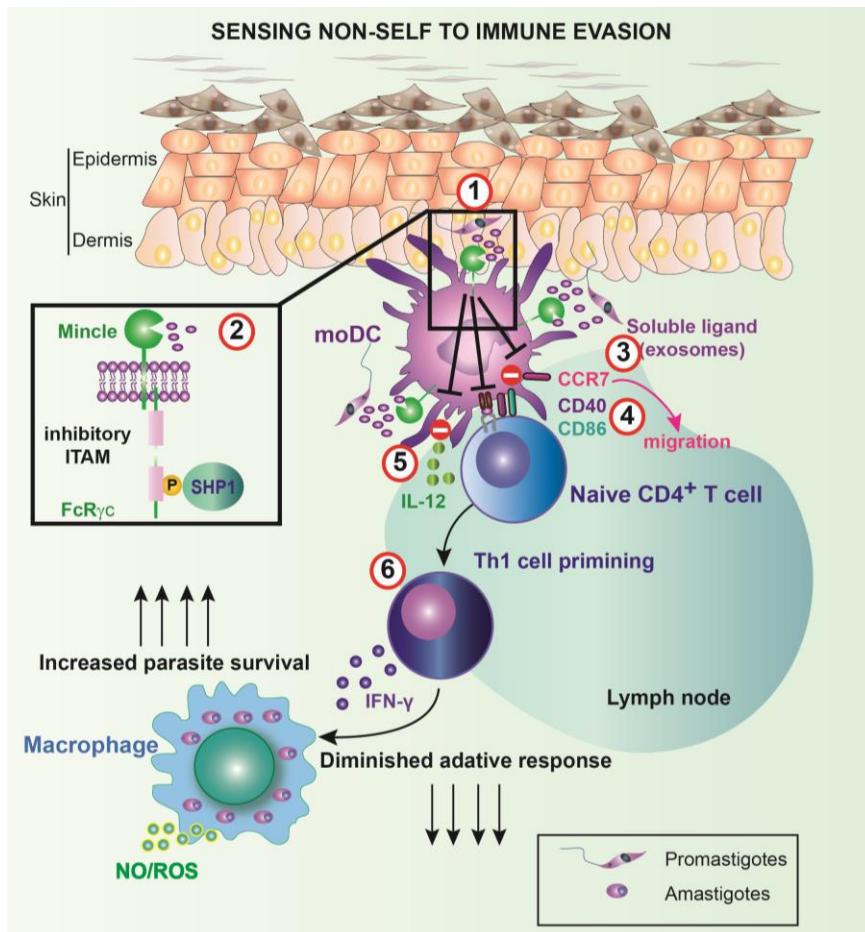


Figure 5.1 Proposed model for Mincle-mediated non-self recognition to immune evasion
 Upon binding of soluble low-avidity/affinity ligand from *Leishmania major* (1), there is a hypo-phosphorylation of the ITAM domain in the FcR γ chain (FcR γ c) associated with Mincle, termed “inhibitory ITAM”, which preferentially binds the tyrosine phosphatase SHP-1 (2), dampening the activation of moDC (3) at the level of migration (4), costimulatory molecules (5)

and cytokines production (5) suppressing the protective Th1 immune response (6). Mincle, macrophage inducible C-type lectin; ITAM, immunoreceptor tyrosine-based activating motif; moDC, monocyte-derived dendritic cells; NO, nitric oxide; ROS, reactive oxygen species; (SHP)-1, src homology region 2 domain-containing phosphatase. The symbol P indicates motif phosphorylation.

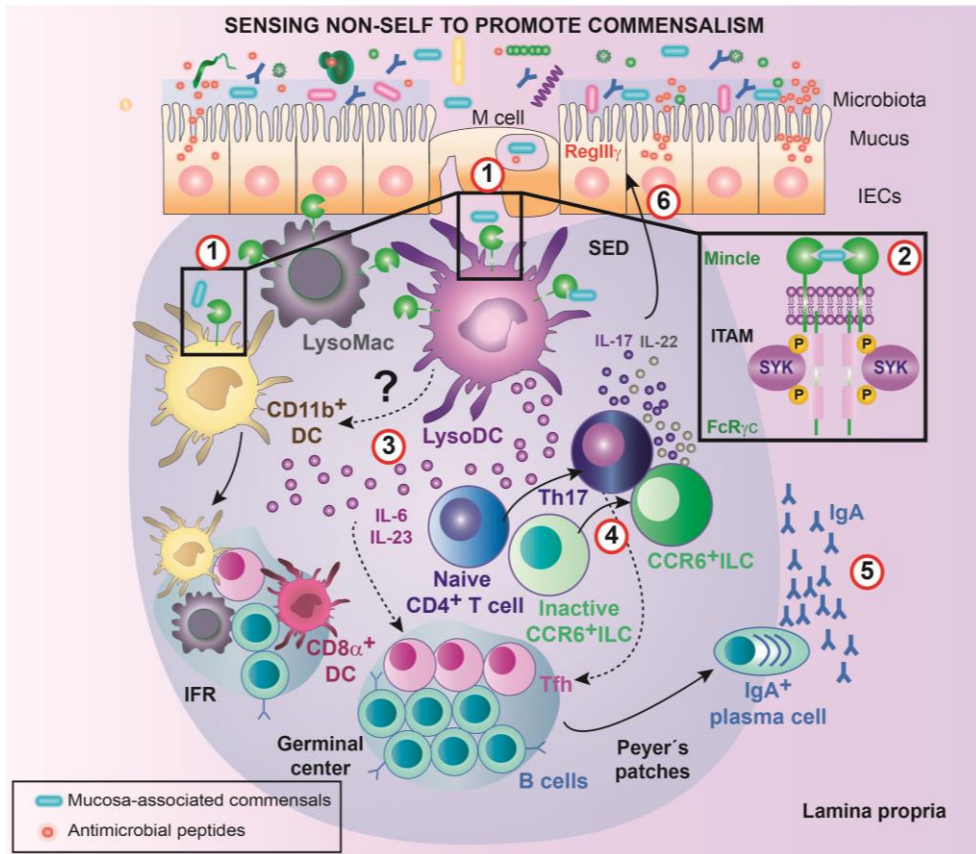


Figure 5.2 Proposed model for Mincle-mediated non-self recognition to promote commensalism. Upon recognition of *Lactobacillus* and/or other mucosa-associated commensals (1), the FcR γ chain ITAM is fully phosphorylated and associates with Syk kinase (2), which triggers an activating signal (IL-6 and IL-23 production) in PP DCs (3) contributing to mucosal barrier function at the level of Th17 and ILC activation (4), IgA production (5) and antimicrobial peptides secretion (6). IECs, intestinal epithelial cells; LysoDC, lysozyme-expressing DCs; LysoMac, lysozyme-expressing macrophages; IFR, interfollicular regions, SED, subepithelial dome; Tfh, T follicular helper cells; ILC, innate lymphoid cell; Syk, spleen tyrosine kinase; RegIII γ , regenerating islet-derived protein 3 gamma. The symbol P indicates motif phosphorylation.

Conclusions

6. CONCLUSIONS

1. *Leishmania* releases a soluble ligand that binds human and mouse Mincle
2. Mincle deficient mice show enhanced parasite-specific immune response with reduced parasite burden and pathology
3. *Leishmania* inhibits activation and migration of DCs in a Mincle-dependent manner
4. *Leishmania* shifts Mincle intracellular configuration in DCs, to an inhibitory FcR γ chain /SHP1 axis able to inhibit heterologous receptors-mediated signalling
5. Mucosa-associated commensals contain ligands for human and mouse Mincle
6. Mincle deficient and CD11c Δ Syk mice have diminished intestinal IL-17 and IL-22 with reduced barrier function and increased commensals extravasation
7. Mincle-commensals interaction triggers IL-6 and IL-23p19 in Peyer's patches
8. Lysozyme-expressing DCs and CD11b⁺ DCs from PPs prime a microbiota- and Mincle-Syk-dependent Th17 immunity
9. Intestinal Th17 cells and ILCs producing IL-17 and IL-22 require Mincle and Syk in CD11c⁺ cells
10. Sensing commensals by Mincle promotes intestinal barrier function, including IgA and antimicrobial peptide production, thus limiting microbial translocation and liver inflammation

Conclusiones

7. CONCLUSIONES

1. *Leishmania* excreta un ligando soluble capaz de unirse a Mincle humano y de ratón
2. Los ratones deficientes para Mincle tienen una mayor respuesta inmunitaria frente al parásito, acompañada de una menor carga parasitaria y menor patología
3. *Leishmania* inhibe a través de Mincle la migración y activación de las DCs
4. *Leishmania* cambia la configuración intracelular de Mincle en las DCs hacia un eje inhibitorio FcR γ c /SHP1 capaz de parar la señalización mediada por receptores heterólogos
5. Los microorganismos comensales asociados a la mucosa intestinal contienen ligandos para Mincle humano y de ratón
6. Los ratones deficientes para Mincle y CD11c Δ Syk tienen menor cantidad de IL-17 e IL-22, acompañado de una disfunción de la barrera intestinal y diseminación sistémica de los organismos comensales
7. La interacción entre Mincle y los microorganismos comensales induce la producción de IL-6 e IL-23p19 en las PPs.
8. Las DCs de las PPs que expresan lisozima y las DCs CD11b⁺ usan el eje de señalización Mincle/ FcR γ /Syk para promover la producción de IL-17 en respuesta a la microbiota
9. La producción de IL-17 e IL-22 por parte de las células intestinales Th17 e ILCs, requiere la expresión de Mincle y Syk en las células CD11c⁺
10. El reconocimiento de los microorganismos comensales mediado por Mincle favorece la funcionalidad de la barrera intestinal, a nivel de IgA y de péptidos antimicrobianos, limitando la diseminación de los comensales y la inflamación hepática

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8. BIBLIOGRAPHY

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Annexes

9. PUBLICATIONS RELATED TO THIS THESIS

- **Martínez-López M**, Iborra S, Conde-Garrosa R, Mastrangelo A, Danne C, Mann ER, Reid DM, Gaboriau-Routhiau V, Chaparro M, Lorenzo MP, Minnerup L, Saz-Leal P, Slack E, Kemp B, Gisbert JP, Dzionek A, Robinson MJ, Rupérez FJ, Cerf-Bensussan N, Brown GD, Bernardo D, LeibundGut-Landmann S, Sancho D. Microbiota Sensing by Mincle-Syk Axis in Dendritic Cells Regulates Interleukin-17 and -22 Production and Promotes Intestinal Barrier Integrity. **Immunity** (in press)
<https://doi.org/10.1016/j.immuni.2018.12.020> (2019)
- **Martínez-López M**, Iborra S, Soto M, Sancho D. *Leishmania* Hijacks Myeloid Cells for Immune Escape. **Frontiers in Microbiology** (9):883 (2018)
- del Fresno C, Iborra S, Saz-Leal P, **Martínez-López M**, Sancho D. Flexible Signalling of Myeloid C-Type Lectin Receptors in Immunity and Inflammation. **Frontiers in Immunology** (9): 804 (2018)
- Iborra S*, **Martínez-López M***, Izquierdo HM, Abram CL, Conde-Garrosa R, Campos-Martín Y, Reguera RM, Kemp B, Robinson MJ, Soto M, Lowell CA, Sancho D. *Leishmania* Uses Mincle to Target an Inhibitory ITAM Signalling Pathway in Dendritic Cells that Dampens Adaptive Immunity to Infection. **Immunity** (45):788-801 (2016)

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10. OTHER PUBLICATIONS

- Jimenez-Carretero D, Ligos JM, **Martínez-López M**, Sancho D, Montoya MC. Flow Cytometry Data Preparation Guidelines for Improved Automated Phenotypic Analysis. **The Journal of Immunology** 200 (10): 3319-3331 (2018)
- Danne C, Ryzhakov G, **Martínez-López M**, Ilott NE, Franchini F, Cuskin F, Lowe EC, Bullers SJ, Arthur JSC, Powrie F. A Large Polysaccharide Produced by *Helicobacter hepaticus* induces an Anti-inflammatory Gene Signature in Macrophages. **Cell Host & Microbe** 22(6): 733–745.e5 (2017)
- Iborra S, **Martínez-López M**, Khouili SC, Enamorado M, Cueto FJ, Conde-Garrosa R, Sancho D. Optimal Generation of Tissue-Resident but Not Circulating Memory T Cells during Viral Infection Requires Crosspriming by DNGR-1+ Dendritic Cells. **Immunity** (45):847-860 (2016)
- Sánchez-Paulete A, Cueto FJ, **Martínez-López M**, Labiano S, Morales-Kastresana A, Rodríguez-Ruiz ME, Jure-Kunkel M, Azpilikueta A, Aznar MA, Quetglas JI, Sancho D, Melero I. Cancer Immunotherapy with Immunomodulatory Anti-CD137 and Anti-PD-1 Monoclonal Antibodies Requires BATF3-Dependent Dendritic Cells. **Cancer discovery** (6):71-79 (2015)
- **Martínez-López M**, Iborra S, Conde-Garrosa R, Sancho D. Batf3-dependent CD103 (+) Dendritic cells are major producers of IL-12 that drive local Th1 immunity against *Leishmania major* infection in mice. **European Journal of Immunology** (45):119-129 (2014)
“The role of different dendritic cells (DCs) subsets in the generation of immunity against *Leishmania major* (*L.major*) infection is debated. We found that skin-resident CD103⁺ DCs are key IL-12 producers and therefore crucial for maintenance of local Th1 immunity against *L. major* infection”
- Iborra S, Izquierdo HM, **Martínez-López M**, Blanco-Menéndez N, Reis e Sousa C, Sancho D. The DC receptor DNGR-1 mediates cross-priming of CTLs during vaccinia virus infection in mice. **Journal of Clinical Investigation** 122(5): 1628-1643 (2012)

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Batf3-dependent CD103⁺ dendritic cells are major producers of IL-12 that drive local Th1 immunity against *Leishmania major* infection in mice

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The role of different DC subsets in priming and maintenance of immunity against *Leishmania major* (*L. major*) infection is debated. The transcription factor basic leucine zipper transcription factor, ATF-like 3 (Batf3) is essential for the development of mouse CD103⁺ DCs and some functions of CD8 α ⁺ DCs. We found that CD103⁺ DCs were significantly reduced in the dermis of Batf3-deficient C57BL/6 mice. Batf3^{-/-} mice developed exacerbated and unresolved cutaneous pathology following a low dose of intradermal *L. major* infection in the ear pinnae. Parasite load was increased 1000-fold locally and expanded systemically. Batf3 deficiency did not affect *L. major* antigen presentation to T cells, which was directly exerted by CD8 α ⁻ conventional DCs (cDCs) in the skin draining LN. However, CD4⁺ T-cell differentiation in the LN and skin was skewed to nonprotective Treg- and Th2-cell subtypes. CD103⁺ DCs are major IL-12 producers during *L. major* infection. Local Th1 immunity was severely hindered, correlating with impaired IL-12 production and reduction in CD103⁺ DC numbers. Adoptive transfer of WT but not IL-12p40^{-/-} Batf3-dependent DCs significantly improved anti-*L. major* response in infected Batf3^{-/-} mice. Our results suggest that IL-12 production by Batf3-dependent CD103⁺ DCs is crucial for maintenance of local Th1 immunity against *L. major* infection.

Keywords: Adaptive immune response · Batf3 · Dendritic cells · IL-12 · *Leishmania major*



Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Leishmania major (*L. major*) intradermal infection in C57BL/6 mice mimics human cutaneous leishmaniasis. Infection is brought under control by an adaptive Th1 CD4⁺ T-cell response after a few weeks of development of cutaneous lesions [1]. To trigger adaptive immunity, dendritic cells (DCs) should provide three signals to naive T cells: pathogen-derived peptides bound to MHC

molecules, costimulation, and a polarizing signal that is mediated by soluble or membrane-bound factors [2]. Priming and maintenance of effector T cells against *Leishmania* require the coordinated action of different DC subsets [3, 4] but the overall contributions of these subsets is debated. Monocyte-derived DCs from the skin migrate to the draining LNs (dLNs) after uptake of the parasite and prime the generation of Th1 adaptive immunity [5]. Earlier reports showed that CD8 α ⁻ Langerin⁻ DCs form the basis of the protective immune response and that Langerhans cells and dermal DCs (dDCs) migrate poorly to LNs and play only a minor role in early CD4⁺ T-cell activation [6, 7] and Langerhans cells play rather a negative role [8]. Infection of diphtheria toxin-treated

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Langerin-DTR mice revealed that early CD8⁺ T-cell proliferation is affected by depletion of Langerin⁺ dDCs, with the CD4⁺ T-cell response dependent on Langerin⁺ dDCs [9].

Basic leucine zipper transcription factor, ATF-like 3 (Batf3) is a transcription factor essential for the development of the CD103⁺ subset of DCs [10–13]. In contrast, numbers of CD8 α ⁺ conventional DCs (cDCs) in skin-dLNs are not significantly affected by Batf3 deficiency in the C57BL/6 background, although they are partially impaired in function, for example CD8 α ⁺ cDCs show deficient cell-associated cross-presentation [11–13]. Batf3^{-/-} mice have been used to study the role of both DC subsets in several models of infection [10, 14–16].

Using a model of low dose intradermal (i.d.) infection with *L. major* in the ear pinnae [1], we show that Batf3 deficiency leads to an exacerbated and unresolved pathology, with a 1000-fold increase in local parasite load. A recent report has shown enhanced susceptibility of Batf3^{-/-} mice to *L. major*, concomitant with a skewed cytokine production by T cells in the dLNs [17]. The authors associated this phenotype with a defective function of CD8 α ⁺ cDCs, although the mechanism was not investigated in detail [17]. Our data extend this analysis and show that Batf3 deficiency does not affect priming of T-cell responses in the dLNs, which is directed by CD8 α ⁻ Batf3-independent cDCs. We find that CD103⁺ DCs are the main suppliers of IL-12 during *L. major* infection, which is impaired in Batf3^{-/-} mice. Transfer of WT but not IL-12p40^{-/-} Batf3-dependent DCs significantly improved

anti-*L. major* responses in infected Batf3^{-/-} mice. These data point to CD103⁺ DCs as crucial providers of IL-12 for local maintenance rather than priming of Th1 immunity.

Results

Batf3^{-/-} mice develop an exacerbated *L. major* cutaneous pathology with increased neutrophilia

To assess the role of Batf3-dependent DCs in generation of immunity against *L. major*, we monitored the cutaneous pathology induced by i.d. injection of 1000 *L. major* metacyclic promastigotes in Batf3^{-/-} mice. These animals presented an exacerbated pathology that was established early on the 2nd week postinfection (p.i.) and maintained during the course of the infection, without apparent resolution (Fig. 1A and Supporting Information Fig. 1A and B). A similar pathology was provoked with a moderate dose of parasite (5×10^4), which was used in subsequent experiments (Supporting Information Fig. 1C).

One advantage of the i.d. ear model is the possibility to conduct local analysis of infection, parasite load, and the ongoing immune response. We found that infected WT mice readily controlled parasite load in the ear from the 3rd week p.i. (Fig. 1B). In contrast, Batf3^{-/-} mice were unable to control local parasite load at any time point analyzed (Fig. 1B, left panel), resulting in

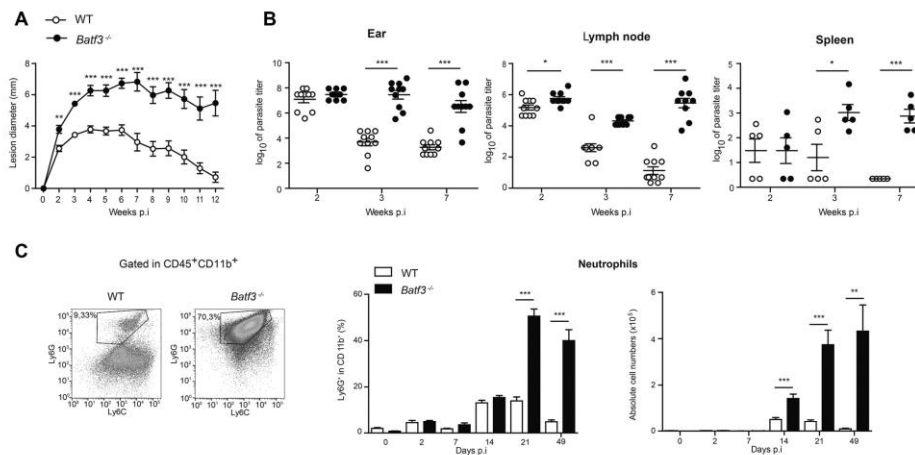


Figure 1. Batf3-deficient mice develop an exacerbated *L. major* cutaneous pathology with neutrophilia. (A) Pathology (the lesion diameter measured with a digital calliper) in WT and Batf3^{-/-} mice was tracked for 12 weeks after i.d. infection in the ear pinnae with 1000 *L. major* parasites. Data are shown as arithmetic mean \pm SEM of 20 samples and are from one experiment representative of three independent performed. (B–D) WT and Batf3^{-/-} mice were i.d. infected in the ear with 5×10^4 *L. major* parasites. (B) Parasite load in the ear, dLNs, and spleen in WT and Batf3^{-/-} mice at different time points p.i. Data are shown as arithmetic mean \pm SEM (horizontal lines and whiskers) of individual data ($n = 5$ mice, each circle represents one sample) corresponding to one experiment representative of three performed. (C) Left: Representative plots showing analysis of myeloid cell infiltrates in the ear at day 21 p.i. Right: Analysis of frequency and absolute numbers of neutrophils (CD11b⁺ Ly6G⁺) in the ears at different time points p.i. Data are shown as arithmetic mean \pm SEM of ten samples and are pooled from three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ unpaired two-tailed Student's *t* test.

an average 1000-fold higher *L. major* titer at 3 and 7 weeks. This lack of local containment resulted in systemic expansion of the parasite [18, 19], leading to higher titers in the dLNs and spleen of *Batf3*^{-/-} mice at 3 and 7 weeks p.i. (Fig. 1B, middle and right).

Lesions in *Batf3*^{-/-} mice persisted at time points at which WT mice were healing or had already completely resolved the wound (Fig. 1A and Supporting Information Fig. 1A and B). At 3 and 7 weeks p.i., the myeloid-cell infiltrate in the ears of infected *Batf3*^{-/-} mice was much greater than in WT mice (Fig. 1C). Accordingly, absolute numbers of infiltrating neutrophils in the ear were significantly higher than in WT mice, reaching an eight-fold difference by the 3rd week p.i. that was maintained throughout the infection (Fig. 1C). These data show that *Batf3* is essential for resistance to *L. major* infection.

T-cell response priming to *L. major* is mainly driven by *Batf3*-independent DCs

Uncontrolled parasite load suggested a major role for *Batf3* in the adaptive response to *L. major* infection, prompting us to examine whether antigen presentation was affected in the absence of *Batf3*. To test the priming of the antigen-specific response, we took advantage of *L. major* expressing OVA (*L. major*-OVA) [20]. WT and *Batf3*^{-/-} mice were transferred with fluorescently labeled (CellTrace™ Violet) OVA-specific CD4⁺ (OTII) or CD8⁺ (OTI) T cells from CD45.1 donor mice and subsequently infected i.d. in the ear. Analysis of OTII cell proliferation did not reveal any difference in early priming of CD4⁺ T-cell responses in the dLNs (Fig. 2A). OTI proliferation to *L. major* OVA infection was also unaffected (Fig. 2B).

To assess the relative contribution of different DC subsets to antigen presentation once the infection is established, CD8 α ⁻ cDCs, CD8 α ⁺ cDCs, and CD103⁺ migratory (mDCs) were isolated from the skin dLNs 2 weeks p.i. and exposed ex vivo to polyclonal T cells from *L. major* infected and healed mice. CD8 α ⁻ cDC from skin-dLNs of infected mice induced polyclonal IFN- γ production by both CD4⁺ and CD8⁺ T cells (Fig. 2C and D). In contrast, CD8 α ⁺ cDCs and CD103⁺ mDCs induced a poor response. Altogether, these results show that antigen presentation during *L. major* i.d. infection is not significantly affected in the absence of *Batf3* and suggest that *Batf3*-independent CD8 α ⁻ cDCs are the main DC subset involved in antigen presentation in the dLNs for induction of T-cell immunity against *L. major*.

Batf3 deficiency impairs local Th1 immunity and skews adaptive response to *L. major*

To study how *Batf3* deficiency could be affecting immunity against *L. major*, we analyzed T-cell responses in the dLNs of infected mice following restimulation with freeze-thawed *Leishmania* ex vivo. *Batf3* deficiency did not significantly affect IFN- γ production by restimulated T cells, whereas IL-10 and IL-4

production were significantly increased at the 2nd and 3rd week p.i. (Fig. 3A). Increased Th2 response in *Batf3*^{-/-} mice was however moderate when compared with BALB/c mice examined in parallel (Supporting Information Fig. 2A).

Taking advantage of the i.d. ear model, restimulation of effector T cells isolated from the infection site revealed a very significant and consistent reduction in the frequency of IFN- γ -producing CD4⁺ T cells in *Batf3*^{-/-} mice at the time-points explored (Fig. 3B). Ear infiltrates from *Batf3*^{-/-} mice contained significantly higher numbers of FoxP3⁺ CD4⁺ CD25⁺ T cells (Fig. 3C). *Batf3*-deficiency impaired local Th1-cell responses to a similar extent as BALB/c mice, whereas skewing toward Th2- or Treg-cell differentiation was not as pronounced as in BALB/c mice (Supporting Information Fig. 2B and C). These results suggest that the most significant effect of *Batf3* deficiency in the adaptive response to the parasite is the local impairment of Th1 immunity, which is accompanied by skewed CD4⁺ T-cell immunity.

L. major-infected *Batf3*^{-/-} mice have defects in monocyte-derived DC and macrophage differentiation

Monocyte-derived DCs are crucial for the generation of Th1 immunity against *Leishmania* and, together with macrophages, mediate nitric oxide production [5, 21]. We therefore explored the possible impact of *Batf3*-dependent DCs on the differentiation of monocytes in the dermis. CD11b^{hi} CD64⁺ myeloid cells were analyzed for CCR2, Ly6C, and MHC class II expression, as reported [22] (Fig. 4A). CCR2⁺ Ly6C^{hi} MHC class II⁻ subset, corresponding to monocytes (P1, Fig. 4A) was increased in *Batf3*^{-/-} mice, both in frequency in the CD11b^{hi} CD64⁺ CCR2⁺ subset (Fig. 4B) and number of infiltrating cells with P1 phenotype per ear (Fig. 4C). Monocyte-derived DCs expressing MHC class II (P2 and P3) were reduced in frequency but not in numbers (Fig. 4). CCR2⁻ MHC class II⁺ macrophages (P4) were increased in frequency at the expense of a decrease in frequency of the CCR2⁻ MHC class II⁺ macrophages (P5), which were reduced in numbers after 3 weeks p.i. (Fig. 4). Thus, *Batf3* deficiency partially affects the differentiation of monocytes to monocyte-derived DCs and macrophages, but the impact is limited to overall numbers in the lesion.

We also determined IL-12 production by monocyte-derived DCs in skin dLNs. Only the MHC class II^{hi} subset of monocyte-derived DCs (P3) induced IL-12 production in response to *L. major* 2 weeks p.i. (Supporting Information Fig. 3A). The frequency of IL-12 producers in this subset was reduced in *Batf3*^{-/-} mice, but was compensated in numbers by an increase in infiltrates of this subset in the lesion. We also found reduced iNOS staining in dermal CD11c⁺ and CD11c⁻ subsets of Ly6C⁺ MHC class II⁺ myeloid cells from *Batf3*^{-/-} mice at 3 weeks p.i. (Supporting Information Fig. 3B). Collectively, these results show that there is a partial impairment of differentiation and function of dermal monocyte-derived DCs and macrophages that could be a consequence rather than the cause of the distorted immunity.

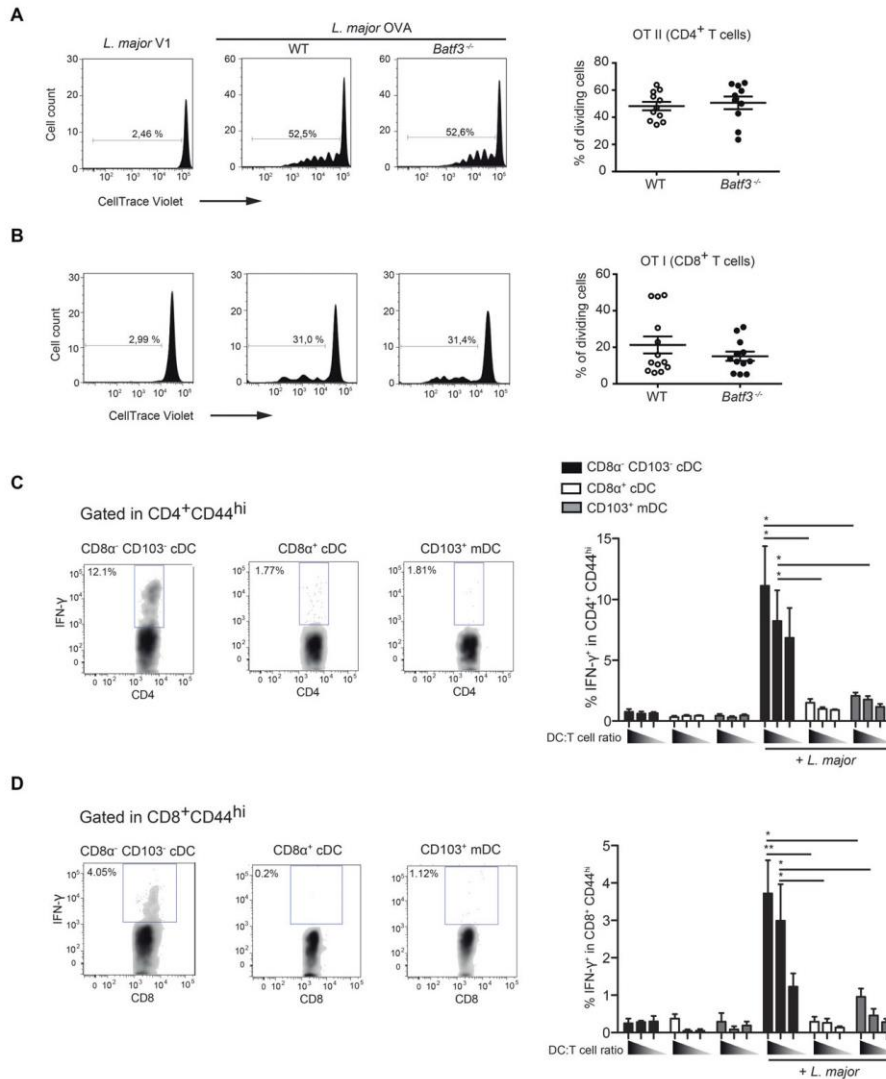


Figure 2. Priming of T-cell responses to *L. major* is mainly driven by *Batf3*-independent DCs. (A and B) WT and *Batf3*^{-/-} mice were transferred with (A) OTII (CD4⁺) or (B) OTI (CD8⁺) OVA-specific T cells labeled with cell violet and infected i.d. in the ear with 2×10^5 *L. major*-OVA parasites. Cell violet dilution was analyzed in the transferred cells present in the dLNs (A) 4 days or (B) 3 days p.i. Left: Representative plots of three independent experiments performed. Right: Data are shown as arithmetic mean \pm SEM of individual data ($n = 11$ –14 samples) and are data pooled from three independent experiments. (C and D) CD8 α ⁻ cDCs, CD8 α ⁺ cDCs, and CD103⁺ mDCs were purified from WT dLNs 2 weeks p.i. and cocultured with polyclonal T cells from *L. major* infected and healed WT mice in different DC:T-cell ratios (2:1; 1:1; 0.5:1). IFN- γ production by (C) CD4⁺ and (D) CD8⁺ T cells was analyzed 4 h later by intracellular staining. Left: Representative plots from three independent experiments performed. DCs were pooled from the dLN of ten mice. Right: Data are shown as arithmetic mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; unpaired ANOVA with Tukey post-hoc test.

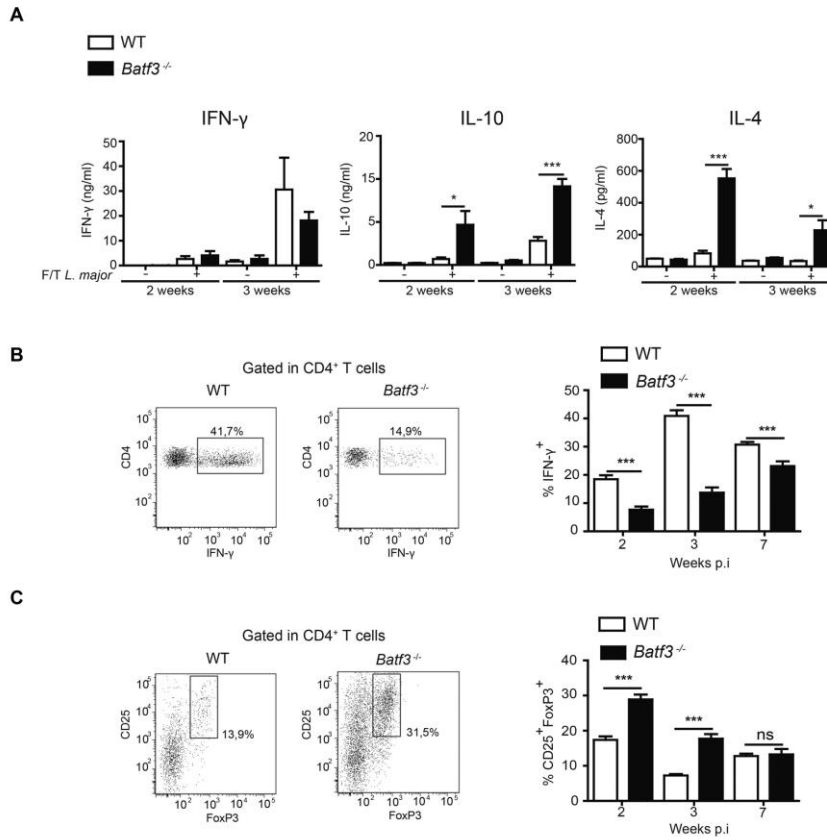


Figure 3. *Batf3* deficiency impairs local Th1 immunity and skews the adaptive response to *L. major*. (A) WT and *Batf3*^{-/-} mice were infected i.d. in the ear with 5×10^4 *L. major* parasites. dLN cells (2×10^6) obtained two and three weeks p.i. were restimulated with freeze-thawed (F/T) *L. major*, and IFN-γ, IL-10, and IL-4 were measured in the supernatant. (B, C) Ear cell suspensions obtained as in (A) 2, 3, and 7 weeks p.i. were restimulated with anti-CD3 and anti-CD28 and analyzed for (B) IFN-γ staining or (C) analyzed in steady state for FoxP3 expression. Representative plots of three independent experiments performed. (A–C) Data are shown as arithmetic mean + SEM ($n = 5$ mice (A) or 10 (B and C)) and are from a representative experiment of three performed. * $p < 0.05$; *** $p < 0.001$ unpaired two-tailed Student's *t* test.

Batf-3 dependent CD103⁺ DCs are major producers of IL-12 during *L. major* infection

We hypothesized that the local impairment in Th1 immunity could result from the loss of a T-cell differentiation signal from *Batf3*-dependent DCs. The analysis of skin dLNs from *Batf3*^{-/-} mice in the C57BL/6 background revealed normal development of CD8α⁺ cDCs and a partial but significant reduction in the CD103⁺ mDCs (Supporting Information Fig. 4A and B), compatible with previous results [11–13]. Through analysis of the ear skin, we further found a significant deficiency in

CD103⁺ dDC in the *Batf3*^{-/-} C57BL/6 mice (Supporting Information Fig. 4C). Similar results were obtained in an analysis 2 weeks after infection with *L. major* (Supporting Information Fig. 5A–C).

Batf3-dependent DCs may provide IL-12 for Th1 CD4⁺ T-cell differentiation [14, 16]. Examination of IL-12p40 and p35 expression in CD11c⁺ cells purified from LNs 2 weeks following infection with *L. major* revealed impaired expression of both genes in *Batf3*^{-/-} mice (Fig. 5A). This impaired IL-12 expression was also found in CD45⁺ cells locally infiltrating the ear 2 weeks after i.d. *L. major* infection (Fig. 5B). These results demonstrate that

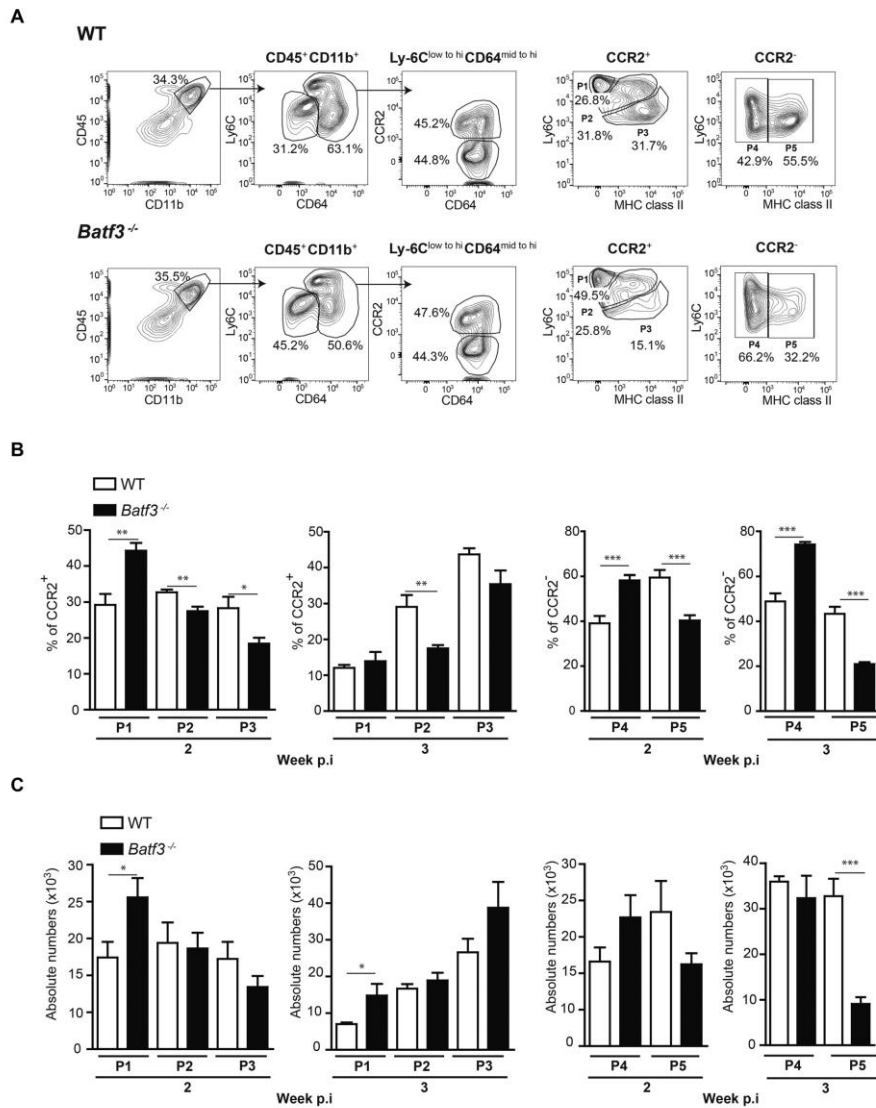


Figure 4. Batf3 deficiency partially affects differentiation of dermal monocyte-derived DCs and macrophages during *L. major* infection. (A) Monocyte differentiation to P1 dermal monocytes (CD11b⁺ CD64^{mid} CCR2⁺ Ly6C^{low} MHC-II⁻), monocyte-derived DCs (P2: CD11b⁺ CD64^{hi} CCR2⁺ Ly6C^{mid} MHC-II^{lo} and P3: CD11b⁺ CD64^{hi} CCR2⁺ Ly6C^{lo} MHC-II⁺) and dermal macrophages (P4: CD11b⁺ CD64^{hi} CCR2^{lo} MHC-II⁻ and P5: CD11b⁺ CD64^{hi} CCR2^{lo} Ly6C^{lo} MHC-II⁺) was tracked in ears of WT and Batf3^{-/-} mice 2 and 3 weeks p.i. with 5 × 10⁴ *L. major* parasites. (A) Representative plots and gating strategy are shown. (B) Right panels: Frequency of P1, P2, and P3 in the CD11b⁺ Ly6C^{lo-to-hi} CD64^{lo-to-hi} CCR2⁺ subset; Left panel: P4, and P5 frequency in the CD11b⁺ Ly6C^{lo-to-hi} CD64^{lo-to-hi} CCR2⁻ subset. (C) Absolute numbers of the populations in (B) per ear. (B, C) Data are shown as arithmetic mean + SEM (n = 10 samples) and are from one experiment representative of three performed. * p < 0.05; ** p < 0.01; *** p < 0.001 unpaired two-tailed Student's t test.

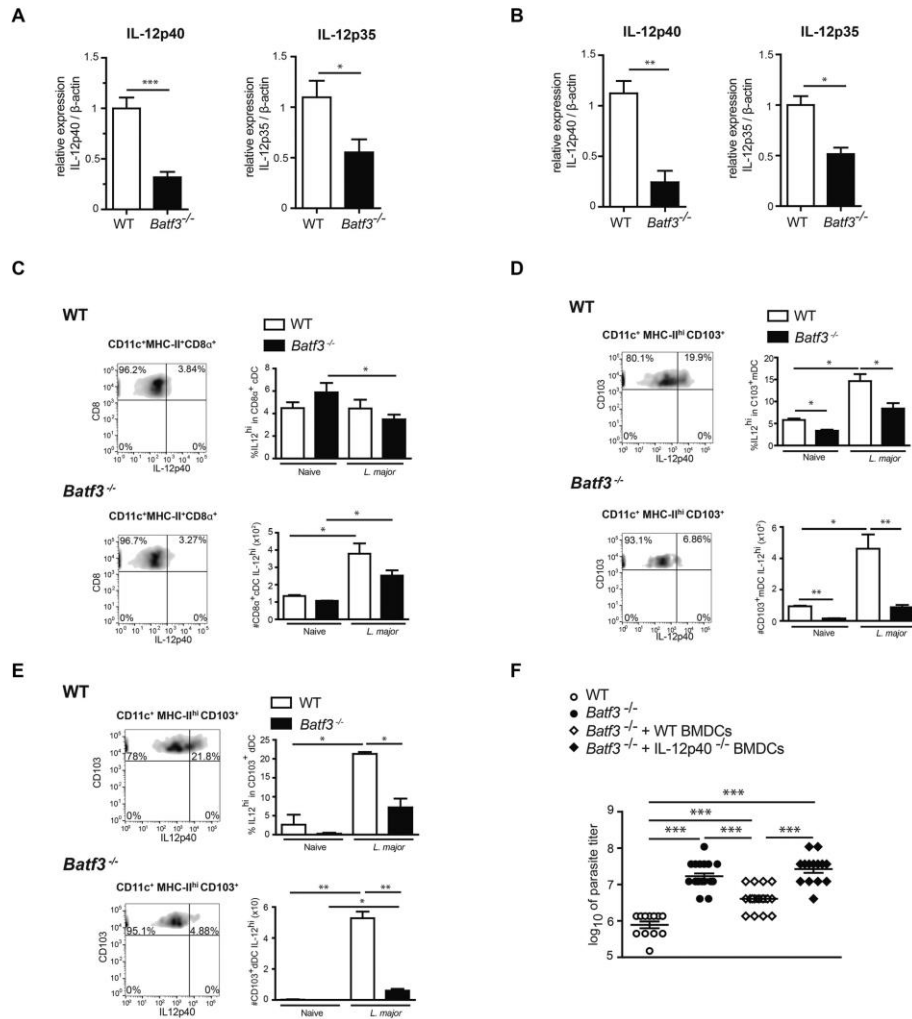


Figure 5. Batf3-dependent CD103⁺ DCs are major IL-12 producers. (A–E) WT and Batf3^{-/-} mice were infected with 5×10^4 *L. major* parasites i.d. in the ear and analyzed 2 weeks p.i. for IL-12p40 and IL-12p35 expression in (A) purified CD11c⁺ cells from the dLNs (B) CD45⁺ cells purified from the infected ears of WT and Batf3^{-/-} mice. RNA expression is standardized to the internal β -actin control and shown as fold induction to the WT average. (A and B) Data are shown as mean \pm SEM ($n = 6$ pooled samples analyzed in triplicate) from three independent experiments. (C–E) Five hours before sacrifice, mice were injected with Brefeldin A (250 μ g i.p.). (C and D) dLN cells were stained for CD11c, MHC-class-II, CD8 α , CD103, and intracellular IL-12p40. Plots show IL-12p40 staining in (C) CD8 α ⁺ CD11c⁺ MHC class II mid cDCs and (D) CD103⁺ CD11c⁺ MHC class II^{hi} DCs. (E) Ear dermal cells were extracted and stained for CD45, CD11c, MHC-class-II, CD103, and intracellular IL-12p40. Left: Plots showing IL-12p40 staining in CD11c⁺ MHC class II^{hi} CD103⁺ dermal DCs. Data in C–E are shown as arithmetic mean \pm SEM of frequency (upper panels) and absolute numbers (lower panels) in naive or infected mice ($n = 5$) and are from a representative experiment of three performed. (F) WT and Batf3^{-/-} mice were infected with 5×10^4 *L. major* parasites i.d. in the ear and CD24^{hi} cells from Flt3L BMDC cultures were transferred locally every 3 days starting at day 4 p.i. Ears and dLNs were analyzed for parasite load 3 weeks p.i. Individual data and arithmetic mean \pm SEM are shown for a representative experiment of two performed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ unpaired ANOVA with Tukey's post-hoc test.

Batf3-dependent DCs are a key contributor for IL-12 production during *L. major* infection.

The specific Batf3-dependent DC subset that provides IL-12 may vary depending on the infection model [14, 16]. To document the Batf3-dependent DC subset contributing to IL-12 production, we performed intracellular staining of IL-12p40 in naive mice or 2 weeks following *L. major* infection (Fig. 5C–E). In the dLNs, *L. major* infection significantly induced IL-12p40 expression in CD103⁺ mDCs, but not in CD8 α ⁺ cDCs (Fig. 5C and D). In the absence of Batf3, not only were CD103⁺ mDCs significantly reduced (Supporting Information Figs. 4 and 5), but also their capacity to produce IL-12 in the remaining CD103⁺ mDCs in response to *L. major* infection was diminished (Fig. 5D). Dermal CD103⁺ DCs were also major Batf3-dependent IL-12 producers following *L. major* infection (Fig. 5E).

To test whether complementation with Batf3-dependent DCs could rescue the defective elimination of the parasite in *Batf3*^{-/-} mice, we generated Flt3L-BMDC cultures containing CD24^{hi} Batf3-dependent DCs [10, 23, 24]. Transfer of CD24^{hi} DCs could significantly revert the increased parasitemia observed in *Batf3*^{-/-} mice 3 weeks p.i. (Fig. 5F). Notably, transfer of IL-12p40^{-/-} CD24^{hi} DCs did not modify the *Batf3*^{-/-} phenotype (Fig. 5F). These results show that IL-12 produced by Batf3-dependent DCs is crucial for immunity against *L. major*.

Discussion

In this report, we have used the i.d. ear *L. major* infection model to explore the role of Batf3-dependent DCs in the generation of immunity against the parasite. We find that Batf3 deficiency results in exaggerated and unresolved pathology, with skewed T-cell differentiation in the dLNs and an impaired Th1-cell effector response at the infection site, leading to defective control of the parasite. During the preparation of this manuscript, Ashok et al. reported complementary findings, showing that *Batf3*^{-/-} mice exhibit enhanced susceptibility to *L. major* infection [17]. Taken together, the results of the two studies strongly suggest an important role for Batf3-dependent DCs in the generation of immunity against *L. major*. Our study further shows that Batf3 deficiency does not affect antigen presentation to T cells. Our results reveal impaired Th1 immunity at the infection site, with increased parasitemia and neutrophilia. We establish Batf3-dependent CD103⁺ DCs as key providers of IL-12 for maintenance of local Th1 immunity.

It has been suggested that the CD8 α ⁺ DC subset is likely to be the main mediator of the response, since they present *L. major* antigen more efficiently than CD103⁺ DCs [17]. We find that *L. major* antigen presentation to both CD4⁺ and CD8⁺ T cells is largely mediated by CD8 α ⁻ cDCs in the dLNs and is Batf3-independent, in agreement with previous results [6, 7]. We detect poor presentation of *L. major* antigens ex vivo by both the CD8 α ⁺ DC and the CD103⁺ DC subsets. Depletion of Langerin⁺ dermal DCs results in reduced priming of *L. major*-specific CD8⁺ T cells [9]. Depletion of CD103⁺ langerin⁺ dDCs is significant but incomplete in *Batf3*^{-/-}

C57BL/6 mice. It is conceivable that remaining CD103⁺ dDCs could be sufficient for mediating CD8⁺ T-cell priming in *Batf3*^{-/-} mice. It is also feasible that infected CD103⁺ dDCs could transport the pathogen to the dLNs [6] and transfer *L. major* antigens to an additional DC subset for presentation. In fact, cross-presentation is mostly TAP-dependent [25], whereas presentation of *Leishmania* antigens on MHC class I molecules is mainly TAP-independent [26]. Moreover, *Leishmania* inhibits cross-presentation [27]. Our data support the notion that susceptibility of *Batf3*^{-/-} mice to *L. major* infection is not caused by deficient antigen presentation.

CD8 α ⁺ cDCs are critical for producing IL-12 that drives the Th1 response to *Toxoplasma gondii* [14]. In the setting of *L. major* i.d. infection, we find a low and Batf3-independent contribution of CD8 α ⁺ cDC to IL-12 production. In addition to the different pathogen used, the distinct route of immunization could explain this apparent discrepancy: i.d. *L. major* versus i.p. *T. gondii*. In this regard, Th1 immunity in a cutaneous candidiasis model relies on CD103⁺ dDCs [16]. Notably, we find that CD103⁺ DCs both in the dLNs and the dermis are the major IL-12 producer after *L. major* i.d. infection. IL-12 impairment does not affect Th1 priming in the dLNs but may cause skewed polarization. This is consistent with IL-12 contributing to the clonal expansion, amplification, and phenotypic stabilisation of already-committed Th1 cells, while negatively regulating the Treg-cell pool [28, 29].

IL-12 is thus important not only for priming but also for the maintenance of Th1 immunity against *L. major* during infection [17, 30]. Local production of IL-12 by CD103⁺ dDCs may be critical for T-cell homing, migration, and local effector activity, especially when considering the concomitant accumulation of parasite-primed Treg cells [29, 31, 32]. Our proposed role of Batf3-dependent CD103⁺ DCs in maintenance rather than priming of local Th1 immunity would explain why depletion of cross-presenting DCs between days 17 and 19 p.i. transiently enhances susceptibility to infection [17]. We found that complementation by transfer of WT but not IL-12p40^{-/-} Batf3-dependent DCs significantly improved resistance to the infection. Batf3-dependent CD103⁺ DCs are thus essential mediators of IL-12 cytokine production that may contribute to maintenance of local Th1 CD4⁺ T-cell adaptive immunity against the parasite.

L. major infection recruits monocytes to the dermis that generate Th1-promoting dermal monocyte-derived DCs [5]. Our results show that Batf3-deficiency partially affects differentiation of monocytes to DCs or macrophages in the dermis following *L. major* infection, although this is compensated by increased infiltration of myeloid cells as a consequence of higher parasitemia. Rather than a direct effect of Batf3 deficiency, decreased IL-12 and a Th2 environment could affect monocyte differentiation and activation [21]. Reduced iNOS expression in monocyte-derived DCs and macrophages could also be a consequence of decreased IFN- γ production [33].

Our findings suggest that CD103⁺ Batf3-dependent DCs play a decisive role in providing IL-12 for generation of immunity against *L. major*. Batf3 deficiency is redundant for antigen presentation or even Th1-cell differentiation in the dLNs. IL-12 could however be essential for inhibition of Th2- and Treg-cell responses and for

generation and maintenance of local Th1 immunity against the parasite. This long-term role in maintenance, rather than priming, suggests that CD103⁺ DC could be potentially targeted throughout the infection for therapy.

Materials and methods

Mice

Mice were bred at the CNIC in specific pathogen-free conditions. *Batf3*^{-/-} mice backcrossed more than ten times to the C57BL/6 background (kindly provided by Dr. Kenneth M. Murphy, Washington University, St. Louis, MO, USA) were further backcrossed with C57BL/6 mice at the CNIC to establish WT and *Batf3*^{-/-} colonies from the heterozygotes. Animal studies were approved by the local ethics committee. All animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Leishmania parasites, preparation, inoculation, and quantification

In vivo experiments were carried out using different *L. major* lines. The *L. major* Friedlin strain FV1 (MHOM/IL/80/Friedlin) was generously provided by Dr. D. Sacks (NIH) [34]. *L. major* FV1 (MHOM/IL/80/Friedlin) parasites expressing ovalbumin (*Leishmania*-OVA) were kindly provided by Prof. Deborah Smith and Prof. Paul Kaye (University of York) [20]. For *Leishmania* challenge, parasites of the different lines were kept in a virulent state by passage in mice. Culture and differentiation of parasites was performed as described [35]. Mice were infected by i.d. injection of 1000 or 5×10^4 metacyclic *L. major* promastigotes into the dermis of both ears. Lesion size in the ear was determined with a digital calliper (Duratool) [34]. The limiting dilution assay was used to determine the number of parasites [35]. The parasite load was expressed as the number of parasites in the whole organ.

Generation and inoculation of mouse BMDCs

WT or IL-12p40^{-/-} BM cells (generously provided by Dr. Salomé LeibundGut-Landmann, Institute of Microbiology, ETH Zürich, Switzerland) were cultured in the presence of 150 ng/mL Flt3L (R&D Systems) and fresh medium was added supplemented with 20 ng/mL murine GM-CSF (Peprotech) as reported [23, 24]. Cells (1×10^5) were i.d. injected every 3 days into the ear, beginning 4 days after *L. major* i.d. infection with 5×10^4 parasites until 3 weeks p.i.

Cell purification, restimulation, ELISA, and RT-qPCR

Single-cell suspensions of LNs and ears were prepared by Liberase/DNAse digestion. At the indicated times after *L. major* infection, ears were recovered from naive or infected mice as described [35]. When further purification of CD4⁺ or CD8⁺ T cells was required, cell suspensions were negatively selected using a cocktail of biotin-conjugated antibodies (anti-CD11c, CD11b, B220, MHC-II, NK1.1) followed by separation with streptavidin-microbeads (Miltenyi Biotec). T cells were restimulated to induce cytokine production by incubation over plated anti-CD3 2C11 (10 µg/mL) and anti-CD28 (5 µg/mL, Bio × Cell). dLNs were restimulated with freeze-thawed *Leishmania*. ELISA kits for cytokines (IL-4, IFN-γ, and IL-10) were from BD Biosciences. DCs from LNs and ears were purified with anti-CD11c-microbeads (Miltenyi Biotec) or biotin-conjugated CD45 and streptavidin-microbeads (Miltenyi Biotec). For purification of DC subsets from dLNs, CD11c⁺ cells enriched using anti-CD11c microbeads were further sorted into CD8α⁺CD103⁻, CD8α⁻CD103⁺, and CD8α⁻CD103⁻ DCs in a FACSaria Sorter. RT-qPCR for *Gapdh*, *β-actin*, *Il12p40*, and *Il12p35* was performed using primers from Sigma Aldrich. Total RNA from cells was extracted with the RNeasy Plus Micro Kit (Qiagen, #74034). RNA was reverse transcribed to cDNA using random hexamers and High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, #4368814). Reverse transcription PCR was conducted in a C1000 Thermal Cycler (Bio-Rad). cDNA products were used for quantitative PCR, using the GoTaq[®] qPCR Master Mix (Promega, #A6001). PCR amplification was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystem, #4329001). All reactions were done in a 20 µL reaction in triplicate, following the manufacturer's protocol. Reverse transcription and PCR amplification of the housekeeping genes *Gapdh* and *β-actin* were performed to verify equal loading of RNA and cDNA. PCR primers used for SYBR Green assays were as follows: *β-actin* sense, (5') -GGCTGTATTCCCCTCCATCG - (3'), and *β-actin* antisense, (5') -CCAGTTGGTAACAATGCCATGT - (3'); *GADPH* sense, (5') -TGAAGCAGGCATCTGAGGG - (3'), and *GADPH* antisense, (5') -CAGGAAGTAGGTGAGGGCTTG - (3') IL-12p40 sense, (5') -GGAAGCACGGCAGCAGAATA - (3'); IL-12p40 antisense, (5') -AACTTGAGGGAGAAGTAGGAATGG - (3'), IL-12p35 sense, (5') -TACTAGAGAGACTTCTCCACAACAAGAG - (3'); IL-12p35 antisense, (5') -TCTGGTACATCTCAAGTCTCATAGA - (3'). Relative expression is defined as the arithmetic mean of triplicate 2^{-ΔCt} values relative to *β-actin* RNA. To compare data from different experiments, the biological replicate in each experiment was normalised as fold-induction compared to the average of the WT biological replicates.

Antibodies and flow cytometry

Samples for flow cytometry were stained in ice-cold PBS supplemented with 2 mM EDTA, 1% FCS and 0.2% sodium azide, with the appropriate antibody cocktails. Anti-mouse CD45, CD4, CD8α, CD11b, CD11c, CD103, CD24, Ly-6C, FoxP3, IL-4, and

I-A^b (MHC-II), antibodies conjugated to FITC, PE, PerCP-Cy5.5, V450, or Allophycocyanin were obtained from eBioscience. PE-conjugated CCR2 (R&D), Allophycocyanin-Cy7 CD45, and Allophycocyanin CD25 were from Tonbo Biosciences. PE-conjugated anti-mouse Ly-6G, Alexa Fluor 647-conjugated anti-mouse CD64-FITC-conjugated anti-mouse iNOS were from BD Biosciences. Allophycocyanin-anti-IFN- γ was from Miltenyi Biotec. Purified anti-Fc γ RIII/II (2.4G2) was used to block nonspecific antibody binding. Noncell-permeant Hoechst 33258 (0.1 μ M) was used as a counterstain to detect necrotic cells. Events were acquired using a FACSCanto or FACSDiva flow cytometer (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star). For the detection of intracellular iNOS, cells were fixed in 2% paraformaldehyde for 15 min and permeabilized in 0.3% saponin and 0.5% BSA in PBS for 20 min before staining, as described [36]. For intracellular IL-12p40 staining *in vivo*, mice were intraperitoneally inoculated with brefeldin A (250 μ g/mouse) [37] 2 weeks after *L. major* infection. Ear and ear-dLNs were recovered 5 h after brefeldin A injection. The cells obtained were fixed and permeabilized and were stained with PE-conjugated anti-IL-12p40 antibody (eBioscience). The cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences).

Statistical analysis

Statistical differences were analyzed with Prism software (GraphPad Software, Inc.). Comparisons of samples with a normal distribution (Shapiro-Wilk test for normality) were made using the unpaired two-tailed Student's *t* test for comparison of two groups or ANOVA with Tukey's Post-hoc test for comparison of more than two groups. Differences were considered significant at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Acknowledgments: We are grateful to members of the Immunobiology of Inflammation lab for discussions and Carlos Ardavin for critical reading of the manuscript. We thank the CNIC Cellomics and Comparative Medicine Units and the technicians and assistants in the Department of Vascular Biology and Inflammation for technical support. We thank Simon Bartlett (CNIC) and Kenneth McCreath for providing English editing. We are indebted to all the scientists who shared reagents with us. Work in the DS laboratory is funded by the CNIC and grants from the Spanish Ministry of Economy and Competitiveness (SAF-2013-42920-R) and the European Research Council (ERC Starting Independent Researcher Grant 2010, ERC-2010-StG 260414). D.S. is the recipient of a Ramón y Cajal fellowship (RYC-2009-04235) from the Spanish Ministry of Economy and Competitiveness. M.M.-L. is the recipient of a FPU fellowship (AP2010-5935) from the Spanish Ministry of Education.

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

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Abbreviations: **Batf3**: basic leucine zipper transcription factor, **ATF-like 3**: conventional DC **cDC**: conventional DC **dDC**: dermal DC **mDC**: migratory DC **dLN**: draining lymph node **i.d.**: intradermal **p.i.**: postinfection

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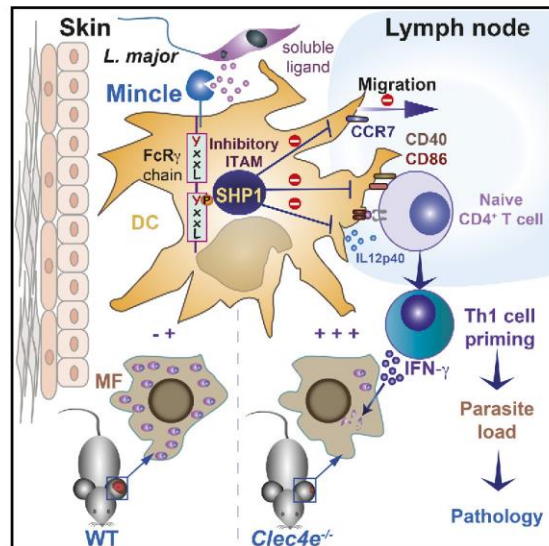
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Received: 13/3/2014
Revised: 24/8/2014
Accepted: 8/10/2014
Accepted article online: 14/10/2014

Immunity

Leishmania Uses Mincle to Target an Inhibitory ITAM Signaling Pathway in Dendritic Cells that Dampens Adaptive Immunity to Infection

Graphical Abstract



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In Brief

Iborra et al. show 90% reduced parasitemia during *Leishmania* infection in Mincle-deficient mice. These mice have an enhanced adaptive response through increased DC activation and migration. *Leishmania* releases a proteinaceous ligand for Mincle, triggering an inhibitory ITAM signaling axis that involves Mincle, FcR γ chain, and SHP1.

Highlights

- *Leishmania* releases a proteinaceous ligand that binds human and mouse Mincle
- *Clec4e*^{-/-} mice show enhanced immunity with reduced parasite burden and pathology
- *Leishmania* inhibits activation and migration of DCs in a Mincle-dependent manner
- *Leishmania* shifts Mincle to an inhibitory FcR γ /SHP1 axis in DCs



Iborra et al., 2016, Immunity 45, 788–801
October 18, 2016 © 2016 Elsevier Inc.
<http://dx.doi.org/10.1016/j.immuni.2016.09.012>

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Leishmania Uses Mincle to Target an Inhibitory ITAM Signaling Pathway in Dendritic Cells that Dampens Adaptive Immunity to Infection

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<http://dx.doi.org/10.1016/j.immuni.2016.09.012>

SUMMARY

C-type lectin receptors sense a diversity of endogenous and exogenous ligands that may trigger differential responses. Here, we have found that human and mouse Mincle bind to a ligand released by *Leishmania*, a eukaryote parasite that evades an effective immune response. Mincle-deficient mice had milder dermal pathology and a tenth of the parasite burden compared to wild-type mice after *Leishmania major* intradermal ear infection. Mincle deficiency enhanced adaptive immunity against the parasite, correlating with increased activation, migration, and priming by Mincle-deficient dendritic cells (DCs). *Leishmania* triggered a Mincle-dependent inhibitory axis characterized by SHP1 coupling to the FcR γ chain. Selective loss of SHP1 in CD11c⁺ cells phenocopies enhanced adaptive immunity to *Leishmania*. In conclusion, *Leishmania* shifts Mincle to an inhibitory ITAM (ITAMi) configuration that impairs DC activation. Thus, ITAMi can be exploited for immune evasion by a pathogen and may represent a paradigm for ITAM-coupled receptors sensing self and non-self.

INTRODUCTION

C-type lectin receptors (CLRs) are equipped with the C-type lectin domain, a versatile structure for binding diverse ligands that allows sensing of self and non-self (Dambuzza and Brown, 2015; Sancho and Reis e Sousa, 2012). Eukaryote parasites,

such as *Leishmania*, are detected by CLRs, Toll-like receptors, and opsonizing antibodies via Fc receptors, which trigger a combination of activating and inhibitory pathways (Lefèvre et al., 2013; Woelbing et al., 2006). Mice infected intradermally with *Leishmania major* develop lesions similar to those seen in patients with localized cutaneous leishmaniasis (Belkaid et al., 2000). *L. major* is a poor inducer of dendritic cell (DC) activation and inhibits migration of DCs to draining lymph nodes (dLNs) (Ng et al., 2008; Ribeiro-Gomes et al., 2012), although DCs do eventually migrate and promote T helper 1 (Th1) cell immunity and macrophage microbicidal activity (León et al., 2007). The mechanisms by which *Leishmania* initially blunts DC activation and T cell priming remain ill defined. It has been argued that they may involve uptake of apoptotic infected neutrophils by DCs (Ribeiro-Gomes et al., 2012) or direct DC contact with parasite products (Srivastav et al., 2012). However, the receptor(s) mediating *L. major*-induced DC suppression have not been identified.

Mincle (macrophage-inducible C-type lectin, also known as Clec4e or Clec5f9) (Matsumoto et al., 1999) is weakly expressed in myeloid cells, including DCs, and is induced upon their activation in a macrophage C-type lectin (MCL, Clec4d, Clec5f8)-dependent fashion (Miyake et al., 2013; Yamasaki et al., 2008). Mincle was identified as an Fc receptor γ (FcR γ) chain-coupled CLR for endogenous SAP-130 that is exposed and released by dead cells (Yamasaki et al., 2008) but also recognizes glycolipids on the cell walls of bacteria and fungi, including trehalose-6, 6-dimycolate (TDM), and its synthetic analog trehalose-6, 6-dibehenate (TDB) (shikawa et al., 2009, 2013; Schoenen et al., 2010; Wells et al., 2008; Yamasaki et al., 2009). Binding of these ligands to Mincle triggers phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) tyrosine residues in the FcR γ chain by Src-family kinases, followed by the recruitment and activation of the kinase Syk, which is facilitated by the phosphatase SHP2 as a scaffold (Deng et al., 2015). Subsequently,

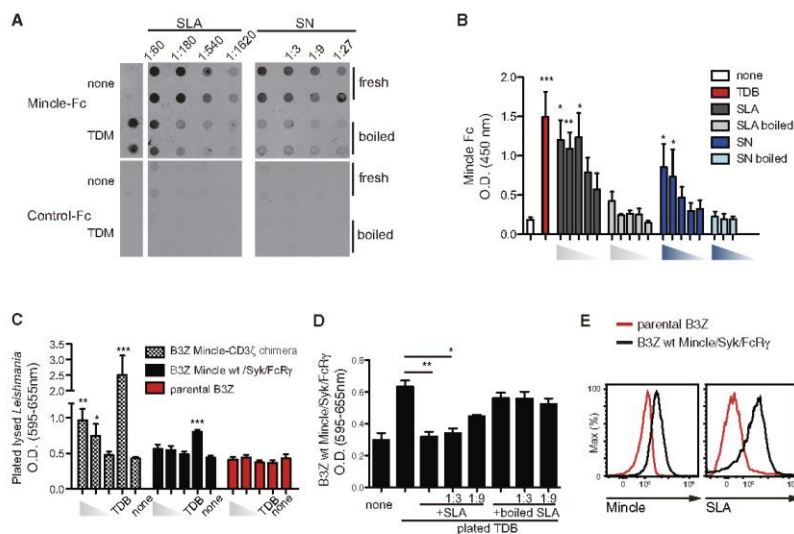


Figure 1. *Leishmania* Releases a Soluble Ligand for Mincle
 (A) Dot blots for Mincle-Fc (top) or control-Fc (bottom) with membranes spotted with fresh or boiled SLAs (left) or SNs (right) from the indicated dilutions of stationary cultured parasites. Culture medium (none) or TDM were used as controls.
 (B) ELISA with Mincle-Fc of different doses of SLA or supernatants (fresh or boiled) from *L. major* promastigotes and controls (none or TDB).
 (C) NFAT reporter activity in response to 10^6 , 10^5 , or 10^4 of plated lysed *Leishmania* or TDB in B3Z cells expressing human Mincle-CD3 ζ chimera, WT mouse Mincle receptor co-expressing Syk and FcR γ , or the parental cells.
 (D) NFAT reporter activity in B3Z cells expressing WT mouse Mincle receptor, FcR γ , and Syk and exposed to plated TDB in the presence of the indicated dilutions of fresh or boiled SLA.
 (E) Staining with anti-Mincle (left) and fluorochrome-labeled SLA (right) on control and Mincle-expressing B3Z cells.
 (A and E) Data are from one representative experiment of four (A) or three (E) performed.
 (B, C, and D) Bars show arithmetic mean + SEM corresponding to three independent experiments.
 * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (one way ANOVA with Bonferroni post hoc test).

Syk generates an activating signal mediated by the protein CARD9 that boosts immunity to infections and inflammation in response to bacterial adjuvants (Ishikawa et al., 2009; Schoenen et al., 2010; Shenderov et al., 2013; Sousa et al., 2011; Yamasaki et al., 2009). Classically considered an activating CLR, Mincle has recently been associated with dampening of immunity (Seifert et al., 2016; Wevers et al., 2014; Wüthrich et al., 2015), acting by repressing IL12-p35 transcription through a Syk-Akt-PKB-dependent pathway in response to *Fonsecaea* (Wevers et al., 2014).

Here, we have found that loss of Mincle resulted in reduced parasitemia and enhanced immunity to *L. major*, correlating with stronger DC activation, priming, and migration to dLN. *Leishmania* released a soluble, proteinaceous Mincle ligand and induced a Mincle-dependent inhibitory axis. This inhibitory axis involved transient Syk activation that mediated coupling of SHP1 to FcR γ chain and dampened DC activation. Recruitment of SHP1 to the ITAM and mediating inhibitory signaling toward heterologous receptors (inhibitory ITAM, ITAMI) have been described for Fc receptors binding monomeric immunoglobulins

(Aloulou et al., 2012; Ben Mkaddem et al., 2014; Hamerman et al., 2009; Pasquier et al., 2005), but not downstream of pattern-recognition receptors. Our results reveal the relevance of the ITAMi pathway activated via Mincle after detection of a pathogen and as a mechanism of immune evasion by *L. major*. This ligand-dependent dual sensing and activation of the ITAM domain may be a paradigm for other ITAM-coupled receptors that have to deal with diverse exogenous and endogenous ligands.

RESULTS

Leishmania Releases a Soluble Proteinaceous Ligand for Mincle

While screening for pathogens expressing Mincle ligands by dot blot, we found that the human Mincle ectodomain-Fc chimera (Mincle-Fc) specifically bound soluble *Leishmania major* extracts from freeze-thawed (F-T) promastigotes (Figure 1A, left). Mincle-Fc also bound to blotted supernatants from *L. major* promastigotes kept for 3 hr at 37°C to favor secretion (Figure 1A, right)

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and detected plated soluble *Leishmania* antigen (SLA) or supernatants (SN) by ELISA (Figure 1B); in contrast, control-Fc or macrophage C-type lectin (MCL)-Fc did not bind to blotted or plated *Leishmania* extracts (Figure S1A). Loss of binding upon boiling of the parasite preparations indicated that the ligand is heat sensitive (Figures 1A and 1B). Treatment of plated *Leishmania* extract with sodium periodate, which oxidizes glycans, did not affect binding of Mincle-Fc to the *Leishmania* extract, but it did inhibit the trehalose-dependent binding to TDM (Figure S1B).

To determine whether the ligand bound cellular Mincle, B3Z NFAT reporter cells (Karttunen et al., 1992) were transduced with a chimera comprising the extracellular human Mincle and intracellular CD3 ζ or, alternatively, with the wild-type (WT) mouse Mincle receptor co-transduced with the FcR γ chain and Syk. The CD3 ζ chimera responds to any multimeric ligand, whereas WT Mincle requires the Syk kinase transduction pathway to activate an NFAT reporter (Sancho et al., 2009). Plated *Leishmania* lysates triggered the Mincle-CD3 ζ reporter, but not the WT Mincle-FcR γ -Syk or the parental cell line (Figure 1C). SLA did not trigger the Mincle-CD3 ζ chimera or the WT Mincle (data not shown), suggesting a low valency of the soluble ligand. In contrast, SLA blocked the triggering of WT Mincle or CD3 ζ chimera by plated TDB in a dose-dependent and heat-sensitive manner (Figure 1D and data not shown). SLA-mediated blockade did not affect the triggering of Mincle by plated 1B6 anti-Mincle antibody, indicating specificity for a TDB-Mincle binding site (Figure S1C). In addition, fluorochrome-labeled SLA bound to Mincle-expressing B3Z cells, but not to the parental cell line (Figure 1E and data not shown).

Mincle-Fc also stained fixed and permeabilized *L. major* promastigotes, whereas Dectin-1-Fc did not (Figures 2A and S2A). Binding of Mincle-Fc to fixed and permeabilized *L. major* was specifically inhibited by preincubation of the ectodomain with 2F2 anti-Mincle or with soluble TDM (Figures 2B and S2B), but not with 1B6 anti-Mincle (data not shown). Moreover, treatment of fixed and permeabilized *Leishmania* promastigotes with proteinase K, trypsin, heat, or low pH, but not DNaseI, inhibited labeling by Mincle-Fc chimera, suggesting a proteinaceous nature of the ligand (Figure 2C). Notably, other *Leishmania* species were also specifically stained by Mincle-Fc (Figure S2C).

Confocal analysis of Mincle-Fc staining in fixed and permeabilized *L. major* promastigotes revealed an intracellular granular pattern, including the flagellar pocket close to the kinetoplast, a unique site for exocytosis (Figure 2D). Mincle-Fc also stained the parasitophorous vacuole containing *L. major* amastigotes after uptake of the parasite by cultured macrophages (Figure 2E) alongside the staining of the endogenous nuclear ligand for Mincle (Yamasaki et al., 2008). Dectin-1 Fc did not stain fixed and permeabilized promastigotes or amastigotes (Figures 2D and 2E) but did label endocytosed zymosan (Figure S2D). Thus, *Leishmania* produced a proteinaceous ligand(s) for Mincle that was detected in all tested *Leishmania* species and was present at both the promastigote and amastigote stages.

Mincle Is Expressed during *Leishmania* Infection

The typical route of *Leishmania* infection is a skin bite by a parasite-inoculated sandfly. We therefore analyzed Mincle expression in dermal cell types of WT and Mincle-deficient (*Clec4e*^{-/-})

mice after *L. major* infection. The pinnae of both ears were inoculated by intradermal (i.d.) injection of 1,000 *L. major* metacyclic promastigotes, and ear infiltrates were analyzed 24 hr later and compared with dermis taken from the ears of uninfected mice. Mincle expression by myeloid cells was modest in unchallenged dermis (Figures 3A and S3A) but was upregulated upon *L. major* infection in tissue macrophages, neutrophils, and monocyte-derived DCs (MoDCs) infiltrating the infection site (Figures 3A and 3B) and was maintained throughout the course of infection (Figure 3B). Mincle staining of myeloid cells was also observed in human skin samples and serial spleen sections from patients infected with *Leishmania infantum* (Figures S3B and S3C).

Mincle Deficiency Increases Resistance to Cutaneous Leishmaniasis

To determine the contribution of Mincle to the immune response against *L. major*, we monitored cutaneous disease during an 11-week period after ear inoculation with 1,000 *L. major* metacyclic promastigotes in WT or *Clec4e*^{-/-} mice. In the first 2 weeks after infection, the inflammatory pathology in *Clec4e*^{-/-} mice was similar to or greater than that in WT mice, but the response subsequently plateaued and there was no development of dermal lesions (Figure 3C). A similar pathology was provoked with inoculation of 5×10^4 parasites (Figures S3D and S3E). The dose was subsequently used to induce a robust adaptive response in the challenge region. Since the third week of infection, parasite loads in the ears and dLNs of *Clec4e*^{-/-} mice were 90% lower than those of their WT counterparts (Figures 3D and 3E). Real-time tracking of i.d. ear infection with *L. major* mCherry confirmed that better control of infection was in *Clec4e*^{-/-} mice, with significantly lower parasite load at all times analyzed (Figure 3F). Mincle-deficient mice thus controlled the infection earlier and more effectively than WT mice, leading to reduced pathology.

Mincle Deficiency Strengthens the Adaptive Response to *L. major*

Polyclonal effector CD4⁺ T cells producing interferon- γ (IFN- γ) (but not CD8⁺ T cells) were significantly more abundant in the ears of infected *Clec4e*^{-/-} mice at 3, 6, and 10 weeks post-infection (p.i.) (Figures 4A and S4A). CD4⁺ T cells present in dLNs from infected *Clec4e*^{-/-} mice showed augmented production of IFN- γ , but not IL-10, in response to SLA (Figures 4B and S4B). The strong Th1 effector CD4⁺ T cell response also correlated with higher anti-*Leishmania* IgG2a, but not IgG1 antibodies, in *Clec4e*^{-/-} mice (Figure S4C).

To investigate the mechanism of the enhanced adaptive response to *L. major* in the absence of Mincle, we analyzed early CD4⁺ T cell priming. As described (Pagán et al., 2013; Ribeiro-Gomes et al., 2012), infection with *L. major* expressing the model antigen ovalbumin (OVA) induced poor priming of OVA-specific CD4⁺ T cells (Figure 4C). Priming was boosted in *Clec4e*^{-/-} mice, with enhanced CD4⁺ T cell proliferation in vivo and IFN- γ production upon OVA restimulation ex vivo (Figures 4C, 4D, and S4D). The specificity of the Mincle-dependent decrease in CD4⁺ T cell priming for *L. major* was confirmed by identical effector responses in WT and *Clec4e*^{-/-} mice upon infection with OVA-expressing vaccinia virus (Figures 4C, 4D, and S4D). These data show that *Leishmania* targets Mincle to decrease priming of a CD4⁺ Th1 cell-type response against the parasite.

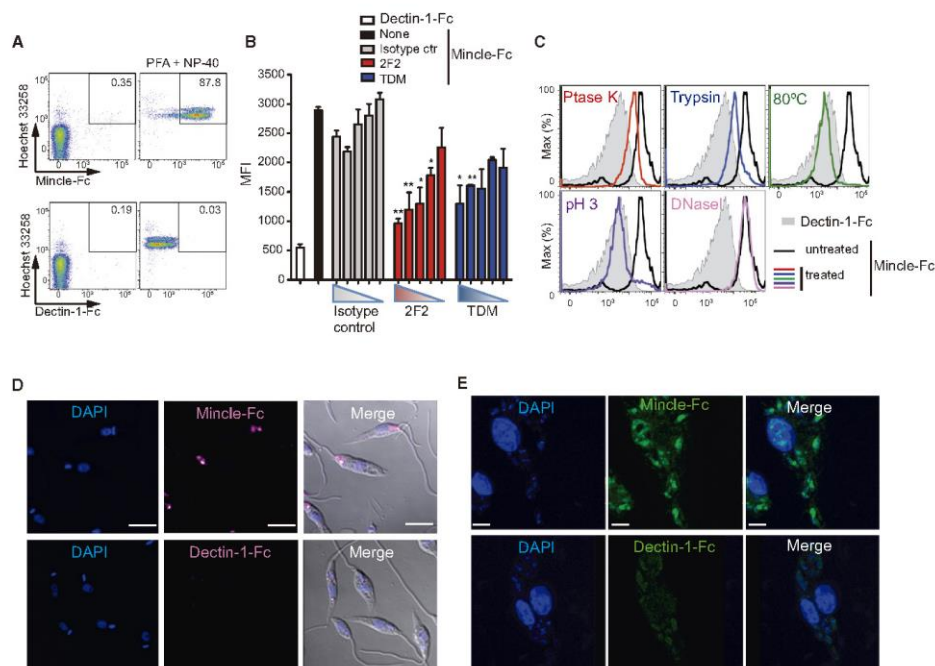


Figure 2. The *Leishmania* Ligand for Mincle Is Proteinaceous and Present at All Parasite Stages
 (A) Mincle-Fc and Dectin-1-Fc staining with Hoechst 33258 counterstaining in live *L. major* promastigotes or paraformaldehyde (PF)-fixed parasites permeabilized with NP-40.
 (B) Mean fluorescence intensity in fixed and permeabilized *L. major* promastigotes stained with Dectin-1-Fc (Control-Fc) or Mincle-Fc preincubated with titrated dilutions of anti-Mincle (clone 2F2), isotype control antibody (mouse IgM), or TDM (10 μ g/mL starting dose, 3-fold dilution). Bars show arithmetic mean + SEM corresponding to three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA with Bonferroni post hoc test).
 (C) Fixed and permeabilized *L. major* promastigotes were subjected to the indicated treatments (colors) or untreated (black) and stained with Mincle-Fc chimera. Gray histograms show Dectin-1-Fc staining.
 (D and E) Confocal images of Mincle-Fc and Dectin-1-Fc staining in fixed and permeabilized *Leishmania* promastigotes (D) and bone-marrow-derived macrophages preincubated with promastigotes (E). Nuclei are counterstained with DAPI. Scale bar: 5 μ m.
 (A and C-E) Plots and images are from single representative experiments of three performed.

To determine the relevance of enhanced priming in a context of vaccination, we transferred OVA-specific CD4⁺ T cells intravenously (i.v.) and subsequently injected 1×10^5 F-T *L. major*-OVA i.d. into the ear. Injection of dead parasites into *Clec4e*^{-/-} mice resulted in increased numbers of OVA-specific CD4⁺ T cells producing IFN- γ upon restimulation ex vivo (Figures 4E and S4E). We next analyzed whether Mincle deficiency also strengthens the function of the memory CD4⁺ T cell compartment. Vaccination with F-T *Leishmania* followed by *L. major* rechallenge 4 weeks later induced IFN- γ ⁺ CD4⁺ effector T cells in the ear of *Clec4e*^{-/-}, but not WT, mice (Figure 4F), thus generating a protective response with reduced parasitemia (Figure 4G). This Mincle-dependent vaccination deficiency using F-T *Leishmania* extracts in WT mice could be reverted by the use of CpG as adjuvant (Fig-

ures 4F and 4G), consistent with published findings (Walker et al., 1999). These results indicated that upon sensing *Leishmania*, Mincle inhibited the generation of effector and memory CD4⁺ T cells and impaired the adaptive response to *L. major*.

Mincle Absence Increases DC Activation and Migration to dLNs after *L. major* Infection

Given the increased adaptive response, we next investigated whether Mincle-deficient DCs had an enhanced ability to prime anti-*L. major* responses. DCs extracted from dLNs of *Clec4e*^{-/-} mice were better than WT at restimulating *L. major*-specific CD4⁺ T cells obtained from healed WT mice (Figure 5A). Early after *L. major* infection, CD40 expression on DCs in the ear was significantly upregulated in *Clec4e*^{-/-} mice compared with WT

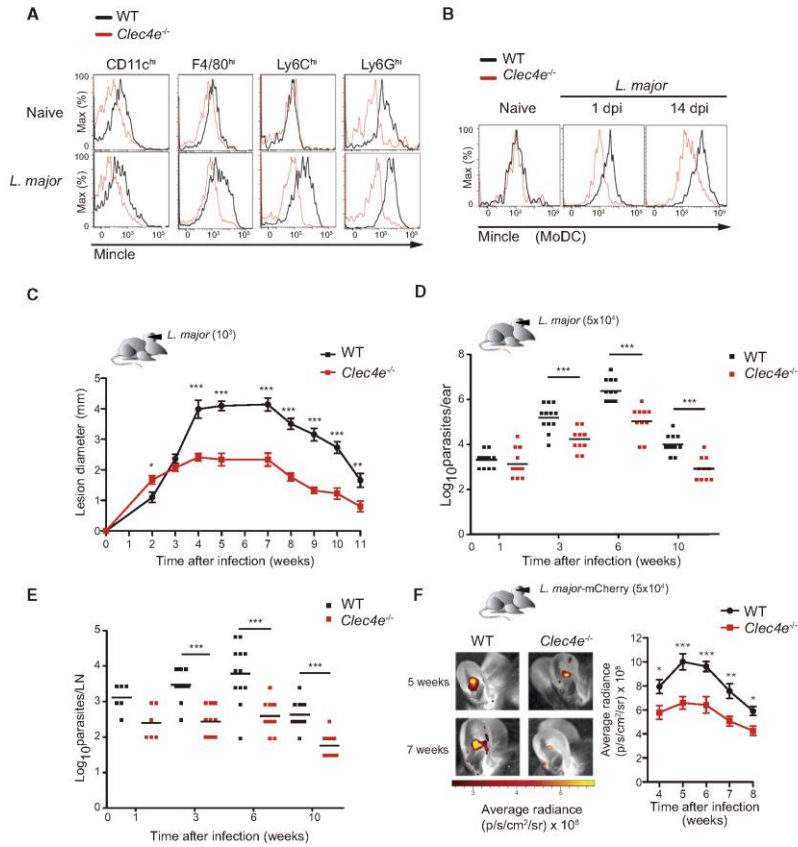


Figure 3. Mincle Deficiency Increases Resistance to Cutaneous Leishmaniasis

(A and B) Expression of Mincle on the indicated cell subsets from the ear of naive or *L. major*-infected WT or *Clec4e*^{-/-} mice 1 day p.i. (A) or on MoDC at the indicated time points after infection (B).

(A and B) Histograms depict representative data from three independent experiments (n=9).

(C) Time profiles of lesion diameter in the ear pinnae of WT and *Clec4e*^{-/-} mice infected i.d. with 1,000 *L. major* parasites. Data arithmetic means ± SEM from a representative experiment (n = 16) of three performed.

(D and E) Parasite load in the ear (D) and dLNs (E) of WT and *Clec4e*^{-/-} mice at the indicated times after i.d. infection in the ear with 5 × 10⁴ *L. major* parasites. Squares show individual data and horizontal bars show arithmetic means from a representative experiment of three performed.

(F) Left: In vivo imaging of mouse ears at the indicated times after i.d. inoculation with 5 × 10⁴ mCherry⁻ *L. major* metacyclic promastigotes. Right: Progression of fluorescence signal (pixel/second/cm²/sr) expressed as arithmetic mean ± SEM (n = 6).

(C-F) *p < 0.05; **p < 0.01; ***p < 0.001 (Student's *t* test at each time point).

mice (Figure S5A). Moreover, MoDCs infiltrating the dermis of Mincle-deficient mice also showed upregulation of the activation markers CD40 and CD86 and the chemokine receptor CCR7 at 20 hr and 14 days after infection (Figure 5B).

In addition, *L. major* infection decreased the numbers of migratory DCs in a Mincle-dependent manner (Figure 5C). The

effect of Mincle on the capacity of dermal DCs to migrate to the dLNs was further investigated in FITC skin sensitization assays. *L. major* infection inhibited migration of FITC⁺ CD11c⁺ DCs to dLNs in WT mice, but not in *Clec4e*^{-/-} mice (Figure 5D). Mincle-dependent inhibition of DC migration was maintained 2 weeks after infection (Figure S5B). These results suggest that

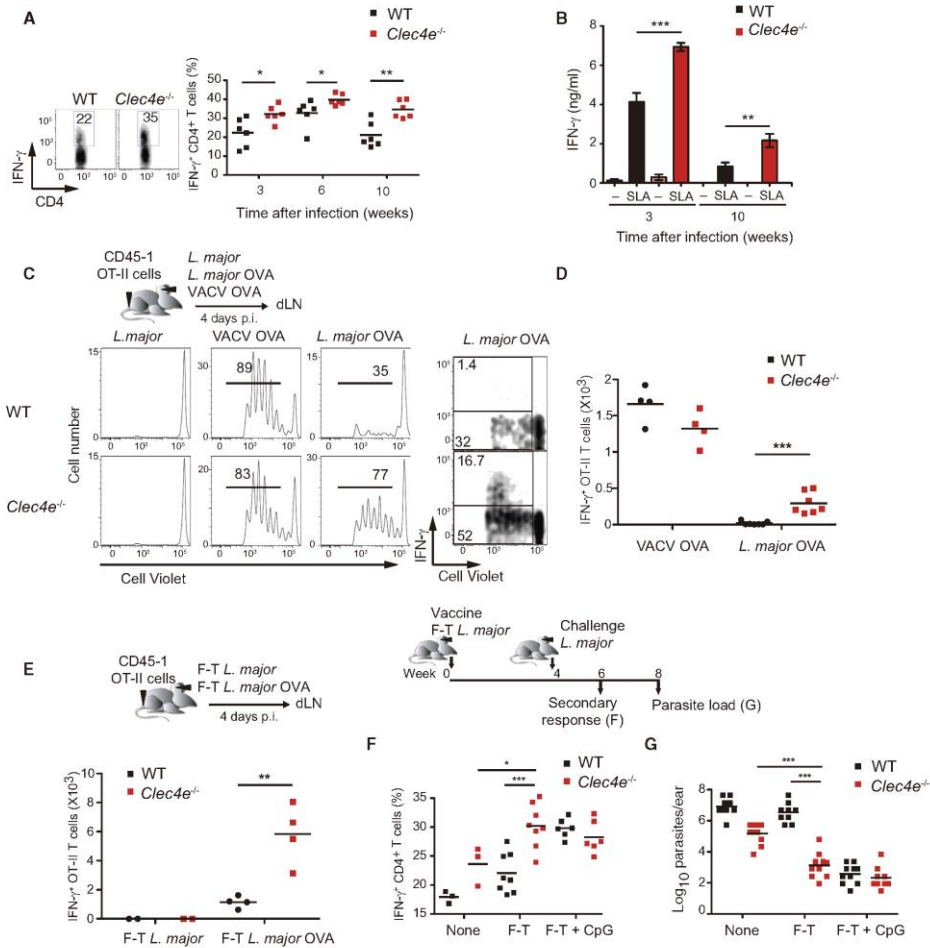


Figure 4. Increased Adaptive Response and Enhanced CD4 $^{+}$ T Cell Priming during *L. major* Infection in Mincle-Deficient Mice

(A and B) WT and $Clec4e^{-/-}$ mice were infected i.d. in the ear with 5×10^4 *L. major* parasites. (A) IFN- γ production in CD4 $^{+}$ T cells in response to polyclonal restimulation of ear infiltrates at the indicated times. Left: representative plots 3 weeks p.i. Right: individual data and arithmetic means. (B) IFN- γ in supernatants from dLN cells extracted at the indicated times and restimulated with SLA. Data are arithmetic means \pm SEM ($n = 6$) of one representative experiment of three performed.

(C and D) WT and $Clec4e^{-/-}$ mice were transferred with CD45.1 $^{+}$ OTII OVA-specific T cells labeled with Cell Violet and infected i.d. in the ear with 5×10^4 particles of either *L. major*, *L. major*-expressing OVA (*L. major*-OVA), or recombinant vaccinia virus expressing OVA (VACV-OVA). (C) Left: Representative histograms showing Cell Violet dilution in OTII cells in dLNs, 4 days p.i. Right: Representative plots of Cell Violet dilution and IFN- γ production following ex vivo restimulation with OVA peptide. (D) Quantification of IFN- γ $^{+}$ OT-II absolute numbers in the dLNs.

(E) Mice were vaccinated in the ear with 1×10^5 F-T parasites and transferred with OTII as in (C). Quantification of OTII cells that were IFN- γ $^{+}$ in the dLNs upon ex vivo restimulation with OVA peptide.

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Leishmania sensing by Mincle impaired DC activation in the infection site and subsequently limited their capacity to migrate to dLNs, contributing to the reduced priming to *L. major* in the presence of Mincle.

L. major Promotes a Mincle- and SHP1-Dependent Inhibitory Axis in DCs

To test whether increased DC activation in the absence of Mincle was intrinsic, we generated GM-CSF (granulocyte-macrophage colony-stimulating factor) bone-marrow-derived cells akin to DCs (GM-DCs) from WT and *Clec4e*^{-/-} mice (Figure S6A). Stimulation with F-T *L. major* induced increased expression of CD40, CD86, and CCR7 in Mincle-deficient CD11c⁺ GM-DCs (Figures 6A, 6B, S6B, and S6C), suggesting an intrinsic effect. As Syk is downstream Mincle (Yamasaki et al., 2008), we tested the absence of Syk in the CD11c compartment (CD11cΔSyk) (Iborra et al., 2012). GM-DCs from CD11cΔSyk mice showed impaired activation by F-T *L. major* (Figures 6B and S6C), suggesting the possible existence of an unidentified activating Syk-coupled DC receptor for *L. major* (Lefèvre et al., 2013).

MCL and Mincle are mutually regulated and act as heterodimers for binding to TDM (Kerscher et al., 2016; Lobato-Pascual et al., 2013; Miyake et al., 2013, 2015). Consistent with these reports, GM-DCs derived from *Clec4d*^{-/-} mice lacked expression of not only MCL, but also Mincle (Figure S6D). Mincle expression was rescued by transduction with WT MCL or MCL^{WAA} (Figure S6D), which contains a mutation in the calcium-binding motif of the C-type lectin domain (Miyake et al., 2015). The impaired expression of Mincle and MCL in *Clec4d*^{-/-} mice resulted in increased activation of DCs exposed to F-T *L. major* (Figure 6C). Reexpression of Mincle mediated by transduction of both MCL or MCL^{WAA} correlated with impaired DC activation by *L. major* (Figure 6C), suggesting that regulation of Mincle expression by MCL contributes to responses to *L. major*.

Infection with *Fonsecaea* triggers Akt-dependent repression of IL12p35 transcription (Wevers et al., 2014). In contrast, F-T *L. major* did not induce Akt activation in WT mice (Figure S6E). We hypothesized that DC activation by *L. major* might be antagonized by Mincle through the recruitment of SHP1 in an inhibitory ITAM (ITAMI) configuration (Aloulou et al., 2012; Ben Mkaddem et al., 2014; Hamerman et al., 2009; Pasquier et al., 2005). Consistent with this notion, treatment with the SHP1/2 phosphatase inhibitor NSC-87877 increased DC activation by *L. major* (Figure S6F), contrasting with the absence of an effect with the Akt inhibitor VIII. Notably, NSC-87877 did not further activate Mincle-deficient DCs in response to the parasite (Figure S6G), suggesting that Mincle and phosphatase activity act in the same pathway. Supporting this conclusion, the enhanced F-T *L. major*-mediated activation seen in Mincle-deficient mice was phenocopied in GM-DCs from mice lacking SHP1 in the CD11c compartment (CD11cΔSHP1) (Abram et al., 2013) (Figures 6D and S6H). F-T *L. major*-induced cytokine production was also higher in GM-DCs lacking Mincle or SHP1 (Figure 6E). Moreover, like *Clec4e*^{-/-} mice tested in parallel, CD11cΔSHP1

mice displayed lower ear and LN parasitemia in response to *L. major* infection (Figure 6F) and showed increased adaptive immunity (Figure 6G). Thus, our results suggested that Mincle inhibited DC activation through SHP1.

L. major Shifts Mincle to an Inhibitory ITAM Configuration that Suppresses Heterologous Receptors

Participation of Mincle and SHP1 in the same axis was further supported by Mincle-dependent phosphorylation of SHP1 (but not SHP2) in F-T *L. major*-stimulated GM-DCs (Figures 7A, S7A, and S7B). Pull-down of SHP1 in WT or FcRγ-chain-deficient GM-DCs revealed specific FcRγ-dependent association of SHP1 with Mincle (Figure 7B). Notably, treatment of GM-DCs with plated TDB induced FcRγ-dependent association of Mincle with Syk, but not with SHP1 (Figure S7C). Moreover, pull-down of Mincle from B3Z transfectants expressing tyrosine mutants in the FcRγ ITAM domain demonstrated that the membrane-distal tyrosine 76 was crucial for association of Mincle-FcRγ with SHP1, whereas tyrosine 65 was at least partially dispensable (Figure 7C), consistent with the ITAMI configuration (Ben Mkaddem et al., 2014).

We next tested the effect of the *L. major*-induced Mincle-dependent inhibitory axis on GM-DC activation promoted by lipopolysaccharide (LPS). F-T *L. major* dampened LPS-induced activation in GM-DCs, and this inhibition was dependent on Mincle and Syk (Figure 7D). The ITAMI configuration is dependent on transient activation of Syk (Ben Mkaddem et al., 2014). We found that Syk transiently associated with Mincle in GM-DCs stimulated with F-T *L. major* in a manner dependent on the FcRγ chain (Figure 7E). Notably, CD11cΔSyk DCs showed impaired SHP1 recruitment to Mincle (Figure 7F). These results suggest that *L. major* shifts Mincle to an ITAMI configuration that suppresses heterologous activating receptors, dampening DC activation and thus impairing the induction of adaptive immune responses.

DISCUSSION

Parasites that depend on an invertebrate vector for cyclical transmission have evolved mechanisms to delay or prevent sterilizing immunity in vertebrate hosts, thereby prolonging parasite availability to the vector (Yazdanbakhsh and Sacks, 2010). *Leishmania* parasites replicate silently in the skin for several weeks after inoculation (Belkaid et al., 2000), suggesting that they might actively dampen DC recognition or activation (Srivastav et al., 2012) and establish a functional immune privilege in the skin (Peters and Sacks, 2006). In this study, we have found that *L. major* parasites release a soluble ligand that binds Mincle, triggering an ITAMI signaling pathway that suppresses DC activation by heterologous activating receptors concomitantly sensing *L. major*. Mincle deficiency thus favored stronger DC activation in response to *L. major* infection, manifested in higher expression of costimulatory molecules, migration to dLNs, and priming of a Th1 cell response to parasite antigens. Increased Th1 cell-type

(F and G) WT and *Clec4e*^{-/-} mice were vaccinated i.d. in the ear with F-T *L. major* and challenged with live parasites in the same site 4 weeks later. (F) IFN-γ production in CD4⁺ effector T cells in the ear upon restimulation as in (A), assessed 2 weeks p.i. (G) Parasite load in the infected ears was evaluated 4 weeks p.i. (A, D-G) Individual data and arithmetic mean of a representative experiment of three performed. *p < 0.05; **p < 0.01; ***p < 0.001; (A-E) Student's t test; (F, G) one way ANOVA with Bonferroni post hoc test.

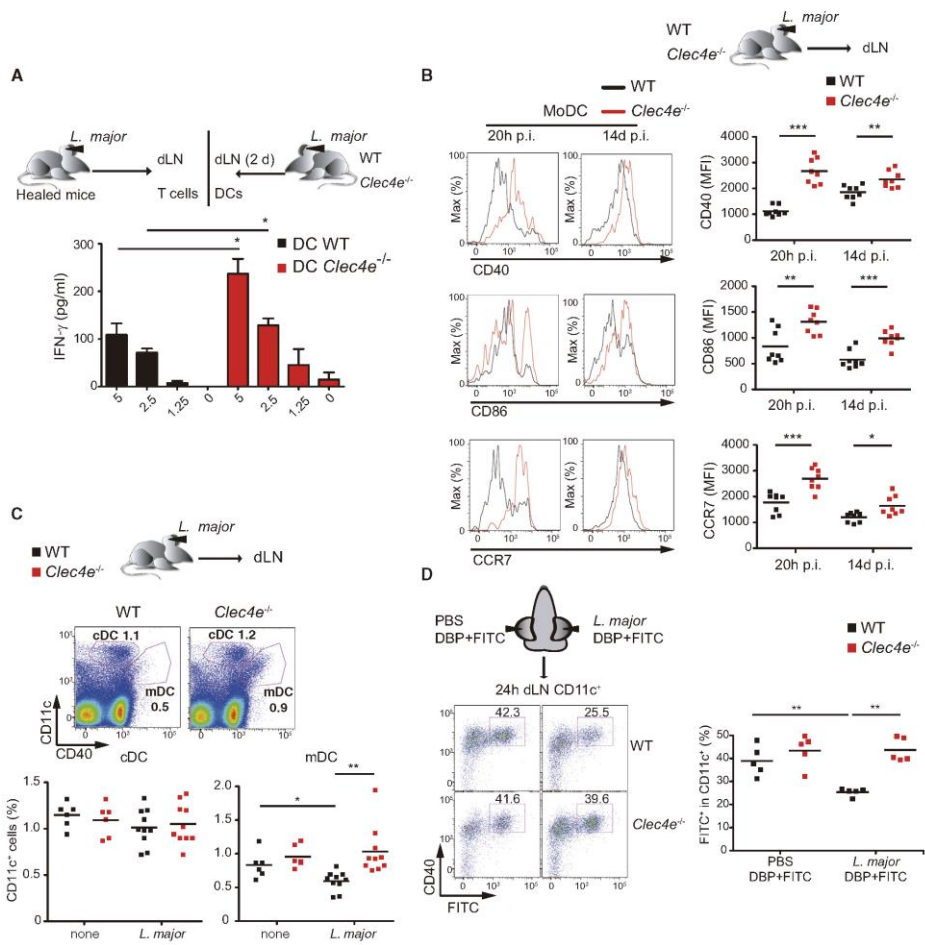
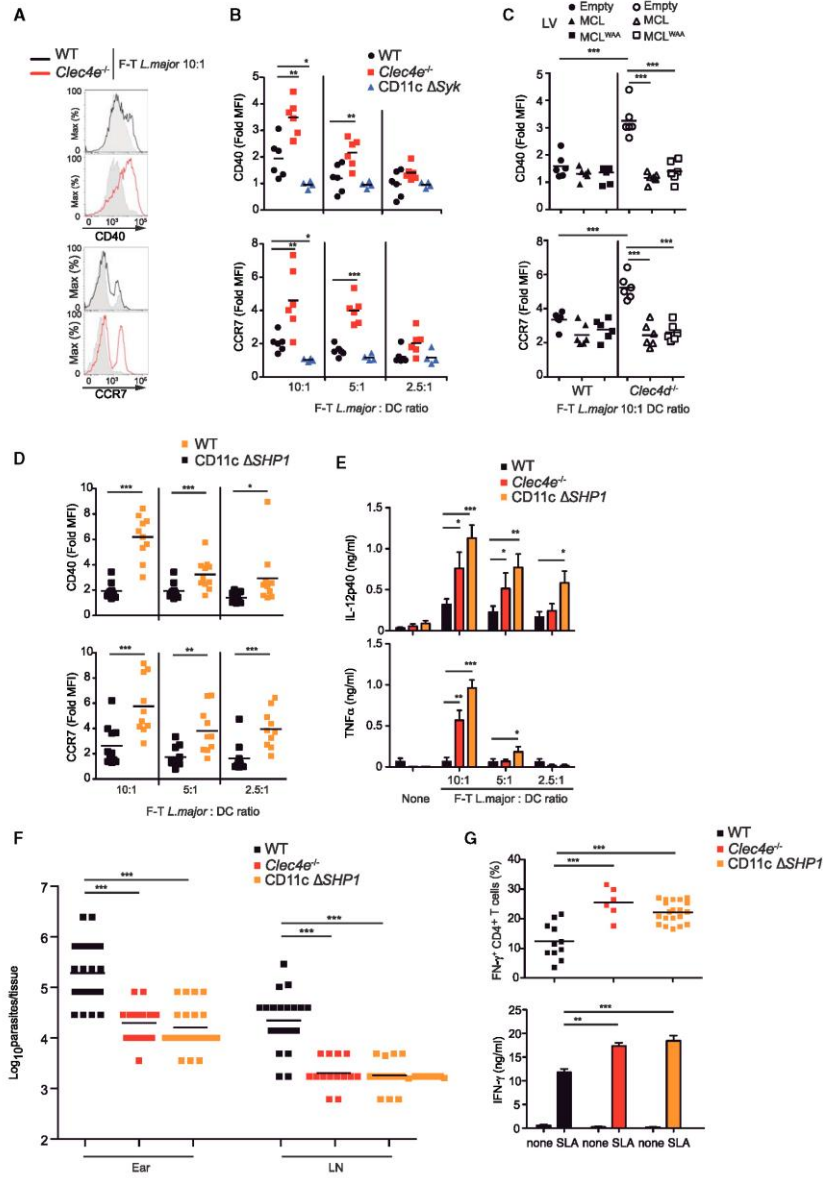


Figure 5. Enhanced DC Activation and Migration to dLNs after *L. major* Infection in Mincle-Deficient Mice
 (A) IFN- γ in supernatants of T cells from healed *L. major*-infected mice after co-culture for 3 days with CD11c⁺ cells recovered from the dLNs of WT and *Clec4e*^{-/-} mice 2 days p.i. Data are arithmetic means + SEM from two independent experiments (n = 6).
 (B) Left panels: Representative histograms of CD40, CD86, and CCR7 staining in MoDCs (CD11b⁺Ly6C⁺CD11c⁺MHCII⁺ gated cells) from ears of infected mice. Right panels: Mean fluorescence intensity (MFI) of CD40, CD86, and CCR7 expression on MoDCs.
 (C) Representative dot plots (top) and frequencies (bottom) of CD11c⁺- and CD40-positive cells in dLNs from uninfected mice or 24 hr after *L. major* infection in the ear.
 (D) Ears of mice inoculated with PBS in the left ear and *L. major* parasites (10⁵) in the right ear were FITC painted, and dLNs were harvested 24 hr later. Left: Representative plots of dLN cells gated for CD11c and stained with anti-CD40 and FITC. Right: frequencies of FITC⁺ CD11c⁺ dLN cells.
 (B-D) Individual data and arithmetic means corresponding to a representative experiment of two (B) or three (C and D) performed.
 (A-D) *p < 0.05; **p < 0.01; ***p < 0.001 (Student's t test).



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immunity correlated with reduced parasite load and pathology in Mincle-deficient mice. These results reveal how the ITAMi pathway can be targeted by a pathogen as a mechanism to evade immune surveillance and illustrate a SHP1-based inhibitory pathway in an ITAM-coupled CLR.

Mincle is a FcR γ -Syk-coupled CLR (Kerscher et al., 2013; Sancho and Reis e Sousa, 2012, 2013) with a well-established role in inducing inflammation and host immunity in response to glycolipid ligands in the cell wall of bacteria and fungi (Ishikawa et al., 2009, 2013; Schoenen et al., 2010; Shenderov et al., 2013; Sousa et al., 2011; Wells et al., 2008; Yamasaki et al., 2009). However, recent reports point to an additional, negative role for Mincle in the control of immunity (Seifert et al., 2016; Wevers et al., 2014; Wüthrich et al., 2015). Mincle detection of *Fonsecaea* involves an Akt-dependent pathway that selectively impairs IL12p35 transcription (Wevers et al., 2014). Therefore, the finding that Mincle sensing of *Leishmania* induces global DC inhibition through a SHP1-dependent and Akt-independent pathway was highly unexpected.

Engagement of FcR γ chain-coupled receptors by low-affinity or avidity ligands may cause hypophosphorylation of ITAM domains and result in recruitment of SHP1, a configuration termed inhibitory ITAM (ITAMi) (Aloulou et al., 2012; Ben Mkaddem et al., 2014; Hamerman et al., 2009; Pasquier et al., 2005). Our results provide an example of a functional ITAMi coupled to a pattern-recognition receptor and support the potential physiological relevance of this signaling module (Aloulou et al., 2012; Blank et al., 2009; Pasquier et al., 2005). Transient Syk activation is required for the ITAMi configuration (Ben Mkaddem et al., 2014). We found transient Syk association with Mincle following F-T *L. major* stimulation, and we showed that Syk is indeed required for SHP1 recruitment to Mincle. However, we found that the overall response of CD11c Δ Syk DCs to F-T *L. major* was impaired, likely because Syk was required for intracellular signaling pathways by other pattern-recognition receptors that mediate activating signals to the parasite. Consistent with the ITAMi configuration, SHP1 associated through the membrane distal tyrosine 76 (Ben Mkaddem et al., 2014), which was crucial for association of Mincle-FcR γ to SHP1. Notably, soluble *Leishmania* extract inhibited DC activation upon LPS challenge in a Mincle-dependent manner, showing the potential of this FcR γ /SHP1 axis to interfere with diverse activating pathways through heterologous receptors, all these features defining the ITAMi pathway.

Given that the *Leishmania* ligand was soluble, avidity for Mincle could be reduced (Iborra and Sancho, 2015). In contrast to *Leishmania* ligand, we did not find SHP1 associated with Mincle when GM-DCs were treated with plated TDB. Together with Mincle, the CLR MCL binds to and is essential for TDM adjuvant potential (Furukawa et al., 2013; Miyake et al., 2013). Mincle, MCL, and FcR γ form a heteromeric complex that facilitates signaling (Lobato-Pascual et al., 2013). In addition, MCL and Mincle mutually regulate their expression (Kerscher et al., 2016; Miyake et al., 2013, 2015). Here, we have found that control of Mincle expression by MCL is required for dampening DC activation in response to *L. major*. Our results do not support a direct role for MCL in the recognition of *L. major* by Mincle, since MCL-Fc did not bind to *Leishmania* extract and its inhibitory effect on DCs was maintained with a MCL^{WAA} mutant in the lectin domain that allows Mincle expression (Miyake et al., 2015), although the possibility that MCL could contribute directly cannot be completely ruled out. Our data show that the *L. major* ligand triggers SHP-1 phosphorylation via Mincle in B3Z cells in the absence of MCL. It is therefore feasible that the signal triggered by the binding of *L. major* ligand to Mincle (in homo or heteromeric configuration) could be weaker than that triggered by the binding of TDM-coated structures to the Mincle-MCL heteromer, and this weaker signaling may favor the ITAMi configuration.

The presence of a ligand for Mincle may contribute to the low effectiveness of candidate vaccines based on whole-killed *Leishmania* or attenuated parasites (Duthie et al., 2012). Our results indicate that blocking Mincle or SHP1 during a vaccination setting may improve vaccine efficiency by allowing Th1 responses to be induced. Moreover, our findings suggest that Mincle can couple to an activating ITAM or to an ITAMi configuration depending on the nature of the ligand, an idea that could apply to other ITAM-coupled CLRs with a diverse ligand range or that can heterodimerize with multiple receptors (Iborra and Sancho, 2015).

EXPERIMENTAL PROCEDURES

Mice

Mouse colonies were bred at the CNIC under specific pathogen-free conditions. Colonies included C57BL/6; *Clec4e*^{-/-} (B6.Cg-Clec4e^{tm1.1C9}) backcrossed more than 10 times to C57BL/6J-CrI (kindly provided by Scripps Research Institute, through R. Ashman and C. Wells, Griffiths University) (Wells et al., 2008); CD11c Δ Syk (Iborra et al., 2012); *Fcer1g*^{-/-} (B6.129P2-*Fcer1g*^{tm1Fw/J}) from

Figure 6. Mincle and SHP1 Inhibit DC Activation by Freeze-Thawed *L. major*

(A) Histogram overlays for CD40 and CCR7 in CD11c⁺ GM-DCs from WT and *Clec4e*^{-/-} mice left untreated (gray histograms) or treated with F-T *L. major*. Data are representative of three independent experiments (n = 6).
(B–D) Fold induction of MFI for CD40 and CCR7 upon F-T *L. major* treatment of (B) GM-DCs obtained from WT, *Clec4e*^{-/-}, and CD11c Δ Syk mice; (C) WT and *Clec4e*^{-/-} GM-DCs transduced with empty vector, MCL, or MCL^{WAA}; and (D) GM-DCs from WT and CD11c Δ SHP1 mice.
(E) IL-12p40 and TNF α in culture supernatants 20 hr after exposure of WT, *Clec4e*^{-/-}, and CD11c Δ SHP1 GM-DCs to different doses of F-T *L. major*. Data are arithmetic means \pm SEM of three independent experiments.
(F and G) WT, *Clec4e*^{-/-}, and CD11c Δ SHP1 mice inoculated with 5×10^6 *L. major* parasites i.d. in the ear were sacrificed 3 weeks after infection. (F) Parasite load in the infected ear and dLNs. (G) Top: intracellular IFN- γ in CD4⁺ T cells after polyclonal restimulation of ear infiltrates. Bottom: IFN- γ in supernatants after SLA restimulation of 2×10^5 dLN cells. Data are arithmetic means \pm SEM of three independent experiments.
(B, D, F, and G) Individual data and arithmetic mean corresponding to one representative experiment of three performed; (C) Pooled data from three independent experiments (n = 6).
(B–G) *p < 0.05; **p < 0.01; ***p < 0.001.
(B, E–G) One-way ANOVA with Bonferroni post-hoc test.
(C and D) Student's t test.

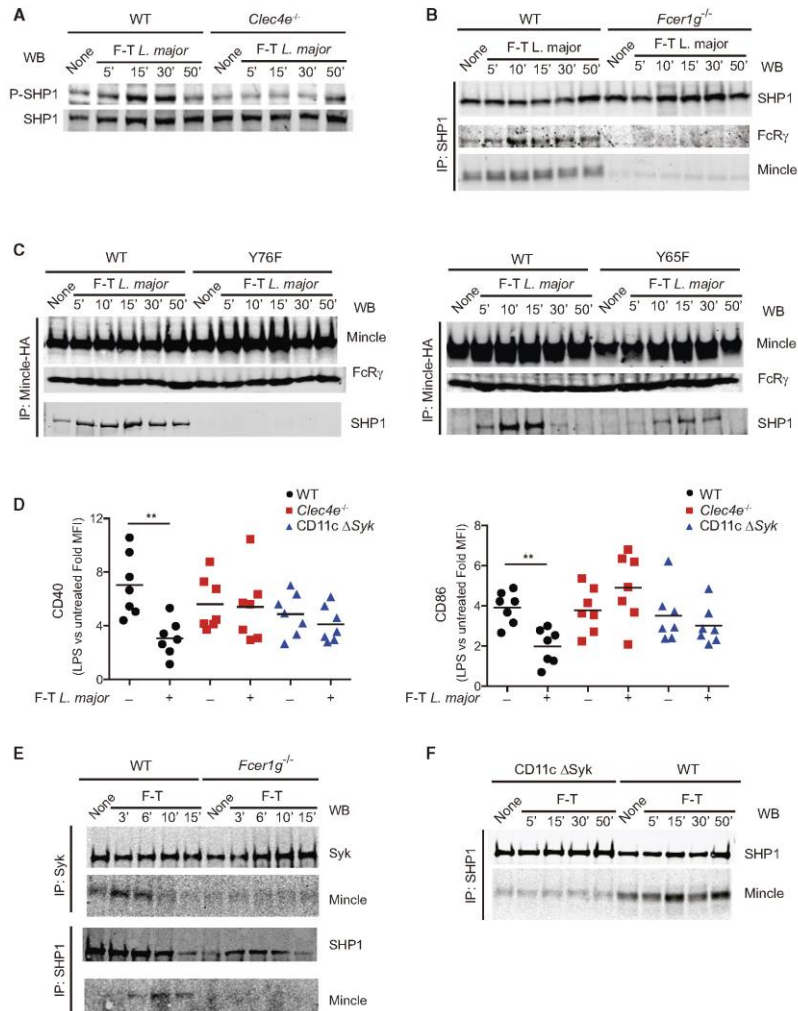


Figure 7. *L. major* Promotes a Mincle/FcR γ /SHP1 Axis that Impairs DC Activation
 (A) Western blot (WB) for P-SHP1 and total SHP1 in WT and *Clec4e*^{-/-} GM-DCs lysed at the indicated times of stimulation with F-T *L. major*.
 (B) SHP1 immunoprecipitation and WB for SHP1, FcR γ , and Mincle in WT and *Fcer1g*^{-/-} GM-DCs lysed at the indicated times of stimulation with F-T *L. major*.
 (C) Mincle immunoprecipitation in B3Z cells transduced with mouse Mincle, Syk, and either WT FcR γ chain or the Y76F (left) or Y65F (right) mutants. WB for Mincle, FcR γ , and SHP1.
 (D) Fold induction of MFI for CD40 and CCR7 upon LPS stimulation (200 ng/mL) of F-T *L. major*-pretreated for 30 min (+) or non-pretreated (-) GM-DCs from WT, *Clec4e*^{-/-}, and CD11c Δ Syk mice. **p < 0.01; Student's t test comparing F-T *L. major* pretreatment and no pretreatment within each genotype.

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The Jackson Laboratory (Takai et al., 1994); CD11cΔSHP1 (Abram et al., 2013); and OT-II CD4⁺ TCR transgenic mice in C57BL/6 background (B6.Cg-Tg(TcrαTcrβ)425Cbn/J) and mated with B6/SJL expressing CD45.1 isoform to facilitate cell tracking. Animal studies were approved by the local ethics committee. All animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Leishmania Parasite Preparation, Inoculation, and Quantitation

For *Leishmania* challenge, parasites of different lines were cultured and kept in a virulent state as described (Martínez-López et al., 2015). Mice were infected by i.d. inoculation of 1,000 or 5 × 10⁶ metacyclic *L. major* promastigotes into the dermis of both ears (Martínez-López et al., 2015). Lesion size in the ear and number of viable parasites was determined as described (Martínez-López et al., 2015). The parasite load is expressed as the number of parasites in the whole organ.

Parasite Preparation of Protein Extracts and Binding to Mincle-Fc Chimera

For preparation of soluble *Leishmania* extract, also known as SLA, ~10⁸ promastigotes were harvested and washed twice in PBS. After three cycles of freezing and thawing, the suspension was centrifuged at 13,000 × g for 20 min at 4°C, and supernatant containing SLA was collected and stored at -80°C. Protein concentration was estimated by the Bradford method.

F-T *L. major* parasites were prepared by three cycles of freezing and thawing of 10⁸ stationary parasites in complete RPMI medium or PBS. Fixed and permeabilized *Leishmania* parasites were prepared by fixing 10⁸ parasites with 0.5 mL of 4% paraformaldehyde and immediate addition of 0.5 mL 1% NP-40. After incubation for 10 min at room temperature, parasites were extensively washed with PBS. To obtain culture supernatants, stationary promastigotes were washed three times in phosphate buffer saline (PBS), resuspended at 5 × 10⁸ parasites/mL in serum free DMEM, and incubated for 3 hr at 37°C. Culture supernatants were collected by two steps of centrifugation, first at 1,500 × g for 5 min at 4°C, followed by a second step at 2,500 × g for 10 min at 4°C. Protein concentration was estimated by the Bradford method. For dot-blot determination of Mincle ligands in *Leishmania* extracts, protein samples were applied to 0.2 μm membranes (BioRad) using a vacuum dot blot apparatus (BioRad). To load different protein amounts in each dot, protein samples were serially diluted in PBS (1:3). Similarly, for ELISA, high-binding plates were loaded with protein samples serially diluted in PBS (1:3). Plates were incubated for 24 hr at 4°C. Later, membranes and plates were washed with PBS and incubated with blocking solution (2% defatted milk in PBS) for 120 min at room temperature, followed by incubation with Mincle-Fc chimera or control Fc (2 μg/mL) for 2 hr. Membranes and plates were then incubated with anti-human IgG (Fc gamma-specific) conjugated to biotin. Membranes were imaged with the LI-COR Odyssey infrared imaging system.

Generation and Assay of B3Z Cell Lines Expressing Mincle and FcRγ Chain Mutants

B3Z cells (kindly provided by N. Shastri, University of California) express a β-gal reporter for nuclear factor of activated T cells (NFAT) (Karttunen et al., 1992). B3Z cells were transduced with retroviruses expressing FcRγ chain, Syk, and mouse Mincle. FcRγ chain ITAM tyrosine 65 and 76 phenylalanine mutants were generated using the QuickChange lightning site-directed mutagenesis kit (Agilent). Binding of ligands can be detected by NFAT reporter activation and induction of β-gal activity. B3Z cells were plated in 96-well plates and incubated with plated TDB or anti-Mincle (1B6) in the presence or absence of *Leishmania* extract. Lysed parasites used in B3Z assays were opsonized with fresh serum from infected Balb/c mice for 2 hr at RT and washed twice with cold PBS. Before B3Z cell plating, promastigotes were seeded on plates coated with 50 μg/mL poly-L-Lysine (Sigma) for 30 min at 37°C.

After overnight culture, cells were washed in PBS, and LacZ activity was measured by lysis in CPRG (Roche)-containing buffer. Four hours later, O.D. 595 nm was measured relative to O.D. 655 nm used as a reference.

Adoptive Transfer and Antigen Presentation Studies In Vitro

For adoptive transfer experiments, CD4⁺ T cells were purified from pooled spleens and lymph nodes of OT-II CD4⁺ TCR transgenic mice by negative selection (Miltenyi Biotec). Purified CD4⁺ T cells were incubated at 5 × 10⁶ cells/mL in PBS with 0.5 μM CellTrace Violet (Invitrogen) for 10 min at 37°C. The reaction was stopped with 5% FCS PBS. CellTrace Violet-labeled purified CD4⁺ OT-II T cells (2–5 × 10⁵) were transferred just after challenge in the ear dermis either with 5 × 10⁴ metacyclic promastigotes of *Leishmania*-OVA, rVACV-OVA (kindly provided by J. Yewdell, NIAID) or dead *Leishmania* OVA (1 × 10⁵). Four days after adoptive transfer, the dLNs were removed and LN cell suspensions were prepared and seeded in the presence of 10 μM I-A^b-restricted OVA peptide (323-339) and brefeldin A. LN cells were stained and analyzed by intracellular flow cytometry. In some experiments, T cells were purified from retroaxillary LNs of infected and healed mice and co-cultured with DCs enriched from dLNs of mice infected 48 hr before. IFN-γ release was determined in culture supernatants 72 hr later.

Statistical Analysis

The statistical analysis was performed using Prism software (GraphPad Software, Inc). Statistical significance for comparison between two sample groups with a normal distribution (Shapiro-Wilk test for normality) was determined by unpaired two-tailed Student's *t* test. Comparisons of more than two groups were made by one-way ANOVA and Bonferroni post-hoc test. Differences were considered significant at *p* < 0.05 (* *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2016.09.012>.

AUTHOR CONTRIBUTIONS

S.I., M.M.-L., F.J.C., C.D.F., H.M.J., R.C.-G., Y.C.-M., and D.S. did the experiments; C.L.A., C.A.L., D.M., B.K., S.Y., M.J.R., R.M.R., and M.S. provided essential reagents. S.I. and D.S. conceived and designed experiments, analyzed data, and wrote the manuscript. All of the authors discussed the results and the manuscript.

ACKNOWLEDGMENTS

We are grateful to C. Reis e Sousa, C. Ardavin, A. Corbí, A. Hidalgo, and members of the D.S. laboratory for discussions and critical reading of the manuscript. We thank the CNIC facilities, personnel, and S. Bartlett for editorial assistance. We are indebted to G. Brown, J. Willment, A. Corbí, J. Yewdell, N. Shastri, C. Wells, R. Ashman, H. Miyoshi, RIKEN BRC, and the Scripps Research Institute for providing reagents. S.I. is funded by grant SAF2015-74561-JIN. Work in the D.S. laboratory is funded by the CNIC and grants from the Spanish Ministry of Economy and Competitiveness (MINECO, SAF-2013-42920R), the European Commission (635122-PROCROP H2020), and the European Research Council (ERC-2010-SIG 260414). The CNIC is supported by the MINECO and the Pro-CNIC Foundation and is a Severo Ochoa Center of Excellence (MINECO award SEV-2015-0505). M.J.R. and B.K. are employees of MedImmune and shareholders in the parent company AstraZeneca.

(E) Syk (upper blots) and SHP1 (lower blots) immunoprecipitation from F-T *L. major*-treated WT and *Fcer1g*^{-/-} GM-DCs and WB for Syk and Mincle (upper) or SHP1 and Mincle (lower).

(F) SHP1 immunoprecipitation from F-T *L. major*-treated WT and CD11cΔSyk GM-DCs and WB for SHP1 and Mincle.

(A–F) Western Blots are from single representative experiments of at least three performed.

Received: July 25, 2015
 Revised: June 17, 2016
 Accepted: August 4, 2016
 Published: October 11, 2016

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Leishmania Hijacks Myeloid Cells for Immune Escape

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Protozoan parasites of the *Leishmania* genus are the causative agents of leishmaniasis, a group of neglected tropical diseases whose clinical manifestations vary depending on the infectious *Leishmania* species but also on host factors. Recognition of the parasite by host myeloid immune cells is a key to trigger an effective *Leishmania*-specific immunity. However, the parasite is able to persist in host myeloid cells by evading, delaying and manipulating host immunity in order to escape host resistance and ensure its transmission. Neutrophils are first in infiltrating infection sites and could act either favoring or protecting against infection, depending on factors such as the genetic background of the host or the parasite species. Macrophages are the main host cells where the parasites grow and divide. However, macrophages are also the main effector population involved in parasite clearance. Parasite elimination by macrophages requires the priming and development of an effector Th1 adaptive immunity driven by specific subtypes of dendritic cells. Herein, we will provide a comprehensive outline of how myeloid cells regulate innate and adaptive immunity against *Leishmania*, and the mechanisms used by the parasites to promote their evasion and sabotage. Understanding the interactions between *Leishmania* and the host myeloid cells may lead to the development of new therapeutic approaches and improved vaccination to leishmaniases, an important worldwide health problem in which current therapeutic or preventive approaches are limited.

Keywords: myeloid cell, *Leishmania*, immune escape, neutrophils, macrophages, dendritic cells

OPEN ACCESS

Edited by:

Celio Geraldo Freire-de-Lima,
Universidade Federal do Rio
de Janeiro, Brazil

Reviewed by:

Fabienne Tacchini-Cottier,
Université de Lausanne, Switzerland
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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Microbiology

Received: 10 February 2018

Accepted: 17 April 2018

Published: 07 May 2018

Citation:

Martínez-López M, Soto M, Iborra S
and Sancho D (2018) *Leishmania*
Hijacks Myeloid Cells for Immune
Escape. *Front. Microbiol.* 9:883.
doi: 10.3389/fmicb.2018.00883

INTRODUCTION

The trypanosomatid protozoa *Leishmania* spp. belonging to the order kinetoplastida are the causative agents of leishmaniases, whose clinical manifestations can range from cutaneous, mucocutaneous or diffuse cutaneous forms to visceral forms, depending on both the parasite species and the host's immune response (Pace, 2014). *Leishmania* is a digenetic parasite, whose life cycle involves two hosts, the insect vector and a vertebrate host. *Leishmania* parasites are transmitted to the vertebrate host by the bite of infected female sandflies belonging to the genera *Phlebotomus* and *Lutzomyia* (Akhoundi et al., 2016). Inside the sandflies the extracellular flagellated, motile form, called procyclic promastigotes generate the infective, non-dividing metacyclic promastigotes, which are inoculated into the host during blood feeding. Once there, *Leishmania* become into the aflagellate intracellular form, called amastigotes, that undergo

replication within host cells, mainly phagocytes such as macrophages. The transmission cycle is complete when infected phagocytes are taken up during a sandfly blood meal, and amastigotes then convert into promastigotes in the sandfly midgut. As a successful parasite, *Leishmania* has developed strategies to evade host immune mechanisms in order to survive within the host. The ability of *Leishmania* to maintain a chronic infectious state within its host depends largely on its immune evasion potential (Geiger et al., 2016). We will review how myeloid cells drive innate and adaptive immunity against *Leishmania* and how the parasites escape host resistance.

INNATE AND ADAPTIVE IMMUNITY AGAINST *Leishmania*

The generation of protective immunity against *Leishmania* requires the cooperation between the innate and adaptive host immune cells. Clearance of *Leishmania* parasites that promotes healing requires IFN- γ -producing effector cells, mainly CD4⁺ T helper 1 (Th1). IFN- γ production by NK cells (Bajenoff et al., 2006) and type 1 CD8⁺ T cells (Belkaid et al., 2002b) also correlates with protection against *L. major* in mice, whereas CD8⁺ T cells play an important role in controlling visceral leishmaniasis (Stäger and Rafati, 2012). However, cytotoxic T lymphocytes (CTLs) play a detrimental role during infection with other *Leishmania* species, such as *L. braziliensis* (Novais and Scott, 2015). IFN- γ signaling in infected macrophages promotes expression of inducible nitric oxide (NO) synthase (iNOS, NOS2) and NO production that, together with reactive oxygen species (ROS) generated during phagocytosis, are essential to kill intracellular parasites (Bogdan et al., 1990; Green et al., 1990). However, *L. amazonensis* are resistant to IFN- γ -mediated killing, and parasite control during the early stages of infection in mice is independent of this cytokine (Kima and Soong, 2013). Besides IFN- γ , other inflammatory cytokines, such as TNF, can activate the infected macrophages in an autocrine manner to produce NO (Bronte and Zanovello, 2005). On the contrary, CD4⁺ T helper 2 (Th2)-related cytokines, such as IL-4, IL-13, IL-10, and antibody production are associated with alternative activated macrophages (Gordon, 2003), which favors parasite survival inside the macrophages (Kropf et al., 2005), and a non-healing phenotype (Scott et al., 1988; Heinzel et al., 1989; Chatelain et al., 1992; Sacks and Noben-Trauth, 2002).

Although macrophages are the primary host cell for *Leishmania* parasites, monocytes, dendritic cells (DCs) and neutrophils can be infected and contribute differentially to the immune response and the outcome of the infection. Acting as a bridge between innate and adaptive immune system, DCs have a prominent role for the development of immune response against the parasite. *Leishmania* infection of DCs results in IL-12 production (Marovich et al., 2000), an essential cytokine for the polarization of naïve T cells toward Th1 subset and subsequent IFN- γ production to control the infection (Heinzel et al., 1993; Sypek et al., 1993; von Stebut et al., 1998). DCs derived from inflammatory monocytes (moDCs) and the migratory

CD103⁺ DCs are the main source of IL-12 upon *Leishmania* infection (Leon et al., 2007; Martínez-López et al., 2015).

Leishmania infection resolution generates a long-lasting immunity to reinfection mediated primarily by a population of short-lived *Leishmania*-specific effector CD4⁺ T cells maintained by low number of parasites that persist after resolution (Peters et al., 2014). Apart from this, *Leishmania*-specific effector memory T (TEM) cells and central memory T (TCM) cells are detected upon *Leishmania* infection (Zaph et al., 2004; Colpitts et al., 2009). Only TCM cells can proliferate, differentiate into effector T cells, and migrate to the lesion site, protecting the host against the infection (Zaph et al., 2004). In addition, CD4⁺ T resident memory cells (TRM) have been identified at sites distant from the primary lesion in *L. major* immune mice and increase the ability of circulating effector cells to mediate protection against the infection (Glennie et al., 2015). After resolution of infection, there are also CD8⁺ T cells, which can contribute to host protection after reinfection or vaccination (Gurunathan et al., 1997; Muller et al., 1997; Rhee et al., 2002; Colmenares et al., 2003; Jayakumar et al., 2011). Understanding how parasites subvert the host innate immune response could help to target these mechanisms in future vaccine strategies that promote more effective and longer-term protection in leishmaniasis mediated by these TCM and TRM cells.

Leishmania TARGETING NEUTROPHILS AS FIRST LINE OF DEFENSE

Neutrophils are recruited early after *Leishmania* infection in response to several factors derived from the host, the sand fly, or the parasite itself. These cells contribute to kill the invading pathogens by formation of neutrophil extracellular traps (NETs) or by a potent oxidative burst generation and granule-derived toxic compound secretion in the surrounding environment or into the phagosome (Kolaczowska and Kubes, 2013). Neutrophils from visceral leishmaniasis patients are highly activated and degranulated (Yizengaw et al., 2016). In addition, CCL3 secreted by neutrophils also attracts monocytes to the site of infection in an experimental model of *L. major* infection (Charmoy et al., 2010b). However, depending on the parasite species and the host, neutrophils can contribute to parasite elimination, or, conversely, favor immune escape by the parasite (Carlsen et al., 2015; de Menezes et al., 2016; Hurrell et al., 2016). Notably, the parasite itself can cause a delay in neutrophil apoptosis that allows parasite replication within these cells (see Regli et al., 2017 for a recent review on how *Leishmania* parasites are able to survive into these myeloid cells).

NETs in *Leishmania* Infection

Neutrophils release NETs composed by granule proteins together with chromatin that form extracellular fibers that can kill microorganisms (Brinkmann et al., 2004). This process can be ROS-dependent and concludes with the death of the neutrophil by a process named NETosis (Fuchs et al., 2007; Kirchner et al., 2012). In addition, there is a ROS-independent early NETosis

(Pilszczek et al., 2010). *Leishmania* promastigotes induce both types of NET formation in human and mouse neutrophils both *in vitro* and *in vivo* (Figure 1A) (Rochaël et al., 2015; Regli et al., 2017). Depending on the species, some promastigotes are resistant to NET-mediated killing as described for *L. mexicana* in mice and *L. donovani* in humans, while others are susceptible as demonstrated for *L. amazonensis* in response to human neutrophils, and this is dependent on lipophosphoglycan (LPG), a glycolipid molecule abundantly found in the surface of the promastigote forms (Guimaraes-Costa et al., 2009; Gabriel et al., 2010; Hurrell et al., 2015). Moreover, *Leishmania* can escape from NET-mediated killing, through the expression of nucleases, as well as by the presence of endonuclease (Lundep) in the vector's saliva, allowing parasites to survive (Figure 1B), as occurred in the interaction between *L. infantum* and *L. major* and human neutrophils (Chagas et al., 2014; Guimaraes-Costa et al., 2014). On the other hand, NETs formation in response to *Leishmania* can interfere with the generation of adaptive immunity, given that NETs isolated from human neutrophils activated by *L. amazonensis* promastigotes are able to inhibit monocyte-derived DCs differentiation and function, thus favoring parasite survival (Barrientos et al., 2014; Guimaraes-Costa et al., 2017).

Inhibition of Neutrophil-Mediated Oxidative Burst

Leishmania promastigote has evolved to survive within neutrophils following phagocytosis. Promastigotes from some *Leishmania* species like *L. donovani* or *L. major* express particular molecules, like LPG, that inhibit phagosome maturation (Gueirard et al., 2008; Mollinedo et al., 2010). In addition, others molecules from *L. donovani*, like tartrate-resistant acid phosphatase (ACP) and *Leishmania* chemotactic factor (LCF) inhibit the respiratory burst, (Figure 1C) (Remaley et al., 1984; Al Tuwaijri et al., 1990; Wenzel and Van Zandbergen, 2009). LCF from some *Leishmania* species, including *L. major*, *L. aethiopica*, and *L. donovani*, shares features of both LTB₄ and LXA₄ mediators. Similar to LTB₄, LCF contributes to increased neutrophil recruitment *in vitro* either directly or by inducing IL-8 secretion by neutrophils (Figure 1D). Similar to LXA₄, LCF from the cited species, increases parasite engulfment and survival within neutrophils *in vitro* through the inhibition of oxidative burst, mediating its effects via the LXA₄ receptor (ALX/FPRL-1) (Figure 1E) (van Zandbergen et al., 2002). In line with this, *L. major*-infected neutrophils release an increased amount of LTB₄, whereas LXA₄ production is reduced, contributing to the initial establishment of the infection (Plagge and Laskay, 2017). On the other hand, the presence of apoptotic cells at the site of infection could contribute to the parasite evasion of the oxidative-mediated killing, since apoptotic cells promote *L. major* survival within neutrophils by downregulating ROS production (Figure 1F) (Salei et al., 2017).

Extending Neutrophil Lifespan

Neutrophils could be considered non-suitable host cells for intracellular parasites due to their short lifespan. Notwithstanding, *Leishmania* infection increases the survival of

neutrophils both *in vitro* and *in vivo* (Aga et al., 2002). *L. major* increases neutrophil lifespan by activation of ERK1/2 and induction of anti-apoptotic proteins Bcl-2 and Bfl-1 (Figure 1G) (Sarkar et al., 2013). However, the neutrophil response to *Leishmania* infection may depend on its location, since *L. major* delays mouse peritoneal and human blood-derived neutrophil apoptosis (Aga et al., 2002; Charmoy et al., 2010a), but this apoptosis delay is not observed in infected mouse dermal neutrophils (Ribeiro-Gomes et al., 2012).

MACROPHAGES AS KEY HOSTS AND EFFECTORS AGAINST *Leishmania*

Leishmania infects macrophages directly after being released from neutrophils (Peters et al., 2008) or following the phagocytosis of apoptotic neutrophils containing intact parasites. The later mechanism mediates a silent transmission of *Leishmania* promastigotes to macrophages and couples to the triggering of an anti-inflammatory response associated to uptake of apoptotic cells, with TGF- β secretion, which favors the survival and division of parasites within the macrophages in a model of transmission so that called "Trojan Horse" (Figure 1H) (Laskay et al., 2003, 2008; John and Hunter, 2006). MIP-1 β secretion by infected neutrophils favors attraction of macrophages to the site of the infection (van Zandbergen et al., 2004). This process requires *Leishmania*-mediated neutrophil apoptosis, which does not occur in all species (Hurrell et al., 2015). In addition, *in vivo* imaging revealed that parasites can also escape dying neutrophils to infect macrophages, which was termed the "Trojan rabbit" strategy (Ritter et al., 2009). Once inside macrophages, *Leishmania* amastigotes differentiate and multiply, which requires manipulating macrophage function to escape ROS generation and the action of lysosomal enzymes and the acidic milieu of the phagolysosome. In addition, parasites modulate the cytokine repertoire secreted by the infected macrophages and their ability to act as antigen presenting cells, in order to avoid a proper generation of the adaptive immune response (de Menezes et al., 2016).

Interfering Phagosome Maturation in Macrophages

Leishmania parasites delay phagosome formation and maturation, as shown by hindered expression of late endosomal markers LAMP-1 and Rab7, as occurred with *L. donovani* (Figure 2A) (Scianimanico et al., 1999; Seguin and Descoteaux, 2016). In addition, some *Leishmania* (such as *L. amazonensis* and *L. mexicana*) promote the formation of large parasitophorous vacuoles by the lysosomal trafficking protein to dilute the leishmanicidal effect of NO (Wilson et al., 2008). Several factors from the parasite participate in this evasion strategy, depending on the different *Leishmania* species. *L. donovani* LPG prevents the assembly of the NADPH oxidase complex (Figure 2B) (Lodge et al., 2006), excludes the proton-ATPase from the phagosome (Vinet et al., 2009), and reduces its fusion with the endosome (Desjardins and Descoteaux, 1997; Scianimanico et al., 1999), what has also been demonstrated for *L. major* LPG

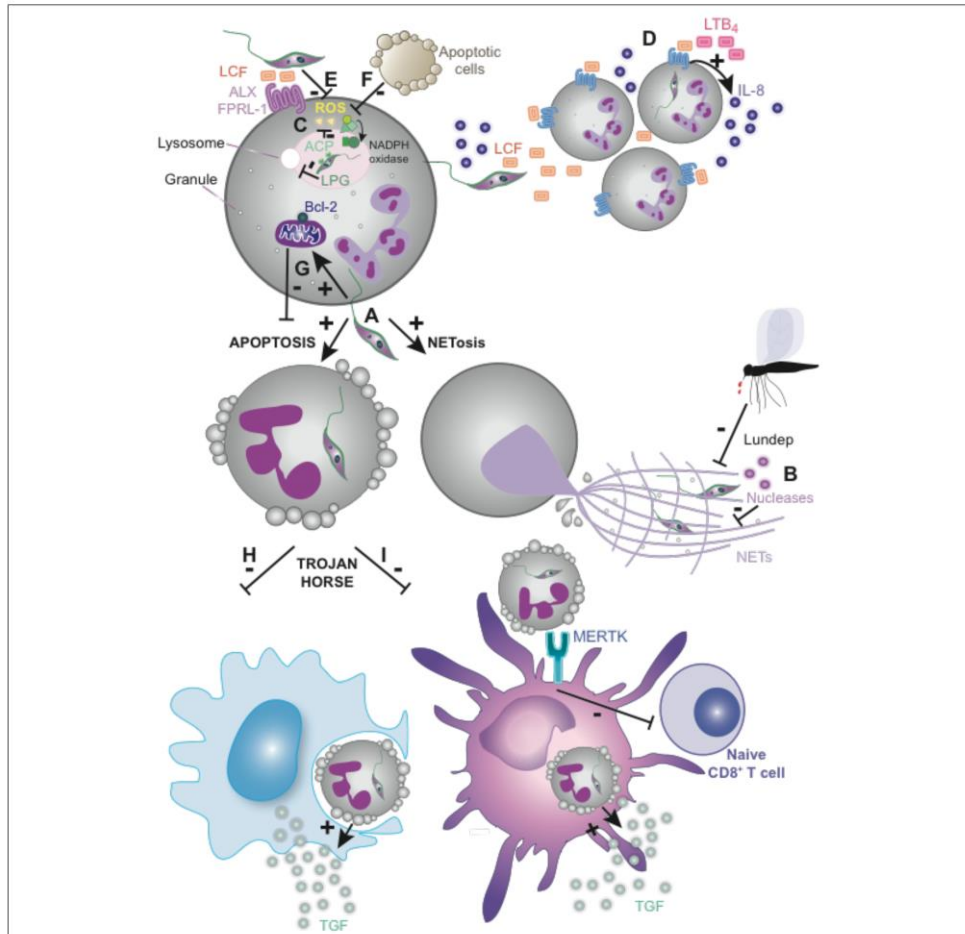
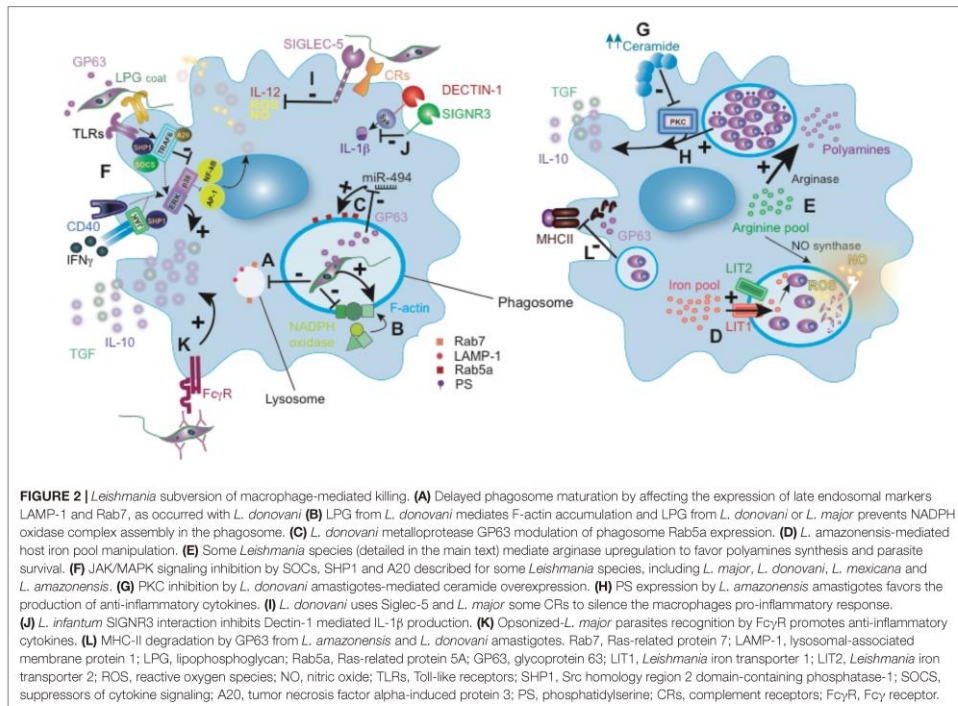


FIGURE 1 | Some *Leishmania* species evade neutrophils and target them for silent transmission. **(A)** *L. major* and *L. amazonensis* promastigotes induce apoptosis and/or NETosis in neutrophils. **(B)** NET-mediated killing inhibition by *L. infantum* and *L. major* nucleases and vector's endonuclease (Lundep). **(C)** LPG from *L. donovani* or *L. major* contributes to evade phagosome maturation while ACP and LCF from *L. donovani* inhibit the respiratory burst. **(D)** Recruitment of neutrophils to the site of the infection is mediated by LTB₄ and might be induced by LCF from several *Leishmania* species, including *L. major*, *L. donovani*, and *L. aethiopia*. **(E)** LCF from the mentioned species inhibits the oxidative burst via the LXA₄ receptor (ALX/FPRL-1) *in vitro*. **(F)** Apoptotic cell-mediated ROS downregulation promoting *L. major* survival. **(G)** *L. major* extends neutrophils lifespan by induction of anti-apoptotic protein Bcl-2 within other mechanisms (see the main text for more detailed information). **(H,I)** Silent transmission of *Leishmania* or "Trojan Horse" hypothesis. **(H)** Phagocytosis of apoptotic neutrophils containing intact parasites promotes an anti-inflammatory response by macrophages. **(I)** The engagement of MERTK on DCs by infected apoptotic neutrophils, upon infection with *L. major* Friedlin V1 strain, inhibits CD8⁺ T-cell priming. LPG, lipophosphoglycan; ACP, tartrate-resistant acid phosphatase; LCF, *Leishmania* chemotactic factor; LTB₄, leukotriene B₄; LXA₄, lipoxin A₄; ROS, reactive oxygen species; Bcl-2, B-cell lymphoma 2; MERTK, receptor tyrosine kinase Mer.

(Dermine et al., 2000). Moreover, *L. donovani* LPG promotes the accumulation of periphagosomal F-actin (Holm et al., 2001) (Winberg et al., 2009), avoiding in this manner the

phagosome acidification and favoring the parasite intracellular survival (Figure 2B). The *L. donovani* metalloprotease GP63 (a surface and secreted glycoprotein of 63 kDa) downregulates



miR-494, which induces Rab5a expression in the phagosome to prevent lysosome fusion (Figure 2C) (Verma et al., 2017). Moreover, the cysteine peptidase B from *L. mexicana* regulates GP63 expression, thus indirectly influencing phagosome formation (Casgrain et al., 2016). *Leishmania* can also exploit host sphingolipids and lipid droplets as energy source and to neutralize the acidic environment inside the phagolysosome (Ali et al., 2012; Rabhi et al., 2016).

To proliferate intracellularly, *Leishmania* amastigotes require iron and arginine-derived polyamines, essential nutrients for their survival within the macrophage phagolysosome (Iniesta et al., 2001; Huynh and Andrews, 2008). *L. amazonensis* compensates the host iron efflux pump by the activation of its own iron transporters, LIT1 and LIT2, providing their mitochondria with iron to generate ROS and regulate the differentiation of virulent *Leishmania* amastigotes (Figure 2D) (Huynh et al., 2006; Mitra et al., 2013; Mitra et al., 2016; Mitra et al., 2017). During infection, the macrophage arginine pool is utilized to produce metabolites (NO and polyamines) for the host defense and its suppression, respectively. *Leishmania* infection up-regulates arginase activity in host macrophages, which favors polyamine synthesis and subverts NO synthase-dependent killing by competing for arginine (Gaur et al., 2007; Reguera et al., 2009;

Rogers et al., 2009; Badirzadeh et al., 2017). In fact, the arginase encoded by the parasite can influence macrophage responses (Boitz et al., 2017). Polyamines contribute to proliferation and to the synthesis of anti-oxidants, such as trypanothione, which neutralize ROS and enable *L. donovani* amastigote survival inside macrophage phagolysosomes (Figure 2E) (Colotti and Ilari, 2011; Goldman-Pinkovich et al., 2016). However, this mechanism seems to be not essential for the survival of *L. donovani* amastigotes (Boitz et al., 2017), but it is relevant during the promastigote stage.

Modulating Macrophage Microbicide Response

Once inside the macrophage, *Leishmania* modulates the pattern of cytokine secretion and inhibits the generation of NO and ROS to increase its survival inside the host. *L. major* promastigotes inhibit IL-12 while promoting IL-10 and TGF- β production from infected host macrophages (Figure 2F) (Reiner et al., 1994; Carrera et al., 1996). Most of these immunosuppressive actions depend on *Leishmania*-mediated host protein tyrosine phosphatases (PTPs) or phosphatidylinositol-3 kinase (PI3K) recruitment, which leads to inhibition of JAK/STAT or MAPK signaling pathways, thus transforming

macrophages to anti-inflammatory state as described for *L. donovani* and *L. amazonensis* (Nandan and Reiner, 1995; Blanchette et al., 1999; Nandan et al., 1999; Forget et al., 2006; Ruhland and Kim, 2009; Calegari-Silva et al., 2015).

Toll-like receptors (TLRs) expressed on innate immune cells are critical for *Leishmania* recognition, which determines the outcome of the infection (Faria et al., 2012). As a strategy to modulate the TLR response, depending on the species, *Leishmania* recruits suppressors of the cytokine signaling family proteins, SOCS-1 and SOCS-3, activates host de-ubiquitinating enzyme A20 or the Src homology 2 domain phosphotyrosine phosphatase 1 (SHP-1) (Figure 2F) (de Veer et al., 2003; Lapara and Kelly, 2010; Shweash et al., 2011; Srivastav et al., 2012, 2015). Moreover, *L. donovani* suppresses p38 phosphorylation while activates ERK1/2, resulting in inhibition of TLR2 and TLR4-stimulated IL-12 and increase in IL-10 production (Chandra and Naik, 2008). Similarly, the activation of the ERK and MAPK pathway in response to IgG-opsonized *L. amazonensis* boosts IL-10 production (Yang et al., 2007). Several virulence factors derived from the parasite have been implicated in this process; LPG inhibits protein kinase C (Descoteaux and Turco, 1999), and stimulates ERK (Severn et al., 1992; Proudfoot et al., 1996; Feng et al., 1999; Prive and Descoteaux, 2000; Delgado-Dominguez et al., 2010), favoring downregulation of iNOS and IL-12 production. *L. donovani* amastigotes similarly inhibit IL-12 production despite lacking LPG on their surface, indicating that other parasite-derived molecules are involved in PKC activity inhibition (Olivier et al., 1992). For example, altering macrophage intracellular ceramide homeostasis by *L. donovani* results in impaired PKC signaling (Figure 2G) (Ghosh et al., 2001).

Similar to other *Leishmania* factors, like the elongation factor-1alpha (EF-1alpha) and fructose-1,6-bisphosphate aldolase described in *L. donovani* (Nandan et al., 2002, 2007), *L. major* GP63 can also promote the activation and recruitment of host PTPs, like SHP-1, which suppresses several kinase pathways, inhibiting several microbicidal macrophage functions (Gomez et al., 2009) (Figure 2F). Moreover, GP63 can inhibit macrophages inflammatory response through mTOR signaling pathway, which regulates the IL-12/IL-10 axis (Jaramillo et al., 2011; Cheekatla et al., 2012). GP63 also mediates proteolysis of some macrophage transcription factors, like AP-1 and NF- κ B, and adaptor molecules, such as Dok family proteins (Gregory et al., 2008; Contreras et al., 2010; Alvarez de Celis et al., 2015). Using the same mechanism, GP63 also modulates protein tyrosine kinases and PKC activity (Chawla and Vishwakarma, 2003; Halle et al., 2009). In addition, the *L. mexicana* cysteine peptidase B promotes SHP-1 function in the macrophage, inhibiting NF- κ B signaling and consequently IL-12 and NO production (Cameron et al., 2004; Abu-Dayyeh et al., 2008, 2010). In addition, the glycosylphosphatidylinositol structure common for different *Leishmania* surface molecules (including LPG and GP63) inhibits TNF expression and dampens macrophage response to infection (Tachado et al., 1997). The kinetoplastid membrane protein-11 is another pathogenicity factor expressed in different *Leishmania* spp., including *L. amazonensis* amastigote stage (Matos et al., 2010) that increases IL-10 production and arginase activity while reduces NO production by macrophages

(Lacerda et al., 2012). At this stage, *L. amazonensis* amastigotes can expose phosphatidylserine analogs, promoting TGF- β and IL-10 and inhibiting NO synthesis (Figure 2H) (Wanderley et al., 2006).

Leishmania can also target macrophage membrane-bound receptors to subvert the inflammatory response. Sialic acids in the parasite surface bind to Siglecs receptors on macrophages to dampen the immune response. Sialic acids recognition by Siglec-5 reduces levels of ROS, NO generation and promotes a Th2-prone cytokine response to *L. donovani* (Figure 2I) (Roy and Mandal, 2016). The C-type lectin receptor (CLR) SIGNR3 is targeted by *L. infantum* to inhibit Dectin-1-mediated IL-1 β secretion, favoring parasite survival (Figure 2J) (Lefevre et al., 2013). Mannose receptor (MR) expressed by dermal macrophages is targeted by a non-healing strain of *L. major*. These cells are permissive for parasite growth even in a Th1-immune environment, affecting the severity of cutaneous disease (Lee et al., 2018). In addition, the engagement of complement receptors (CRs) type 1 and type 3 by *L. major* inhibits respiratory burst and IL-12 production (Da Silva et al., 1989; Ricardo-Carter et al., 2013) (Figure 2I). Moreover, the engagement of the Fc receptor (Fc γ R) by opsonized-parasites promotes IL-10 and TGF- β production by *L. major* infected macrophages (Figure 2K) (Padigel and Farrell, 2005). *L. amazonensis* and *L. major* can also induce the expression of CD200 in macrophages, mediating iNOS inhibition and promoting the virulence of the parasites (Cortez et al., 2011). Additional mechanisms like secretion of exosomes or microRNA-mediated post-transcriptional regulation of inflammatory immune response genes have been described for *L. donovani* and *L. amazonensis*, which can modulate cytokine and NO generation by macrophages (Silverman et al., 2010; Muxel et al., 2017; Tiwari et al., 2017).

Modulating Macrophage Antigen Presentation and Costimulatory Signals

Another way used by *Leishmania* to perpetuate its presence inside the host is suppressing T cell-mediated immune responses by inhibiting presentation of *Leishmania* antigens in major histocompatibility complex (MHC) and dampening costimulatory signals provided by macrophages. Infection of macrophages affects their membrane lipid rafts fluidity and the disposition of MHC class II (MHC-II) molecules, leading to defective antigen presentation and T cell priming (Courret et al., 1999; Chakraborty et al., 2005; Roy et al., 2016). In addition, cysteine proteases from *L. amazonensis* and *L. donovani* amastigotes contribute to this process by degrading MHC-II molecules (Figure 2L) (De Souza Leao et al., 1995; Antoine et al., 1999). *L. donovani*-infected macrophages exhibit decreased expression of the co-stimulatory molecule B7-1 (Saha et al., 1995).

Increasing Macrophage Survival

Leishmania has evolved several mechanisms to extend the survival of the infected macrophages. Programmed death-1 receptor (PD-1), which mediates T-cell exhaustion, is negatively modulated by *L. donovani* to avoid macrophage apoptosis (Roy et al., 2017). In addition, *L. donovani* triggers AKT

activation of the anti-apoptotic β -catenin, inhibiting the pro-apoptotic transcriptional regulator FOXO-1 (Gupta et al., 2016). *L. donovani* also prevents mitochondria-dependent apoptosis by inducing anti-apoptotic protein MCL-1 (Giri et al., 2016). Some factors encoded by the parasite, like the orthologs of the cytokine macrophage migration inhibitory factor (MIF), are involved in blocking macrophage apoptosis and prevent clearance of internalized parasites upon *L. major* infection (Holowka et al., 2016).

DENDRITIC CELLS COMMANDING IMMUNITY AGAINST *Leishmania*

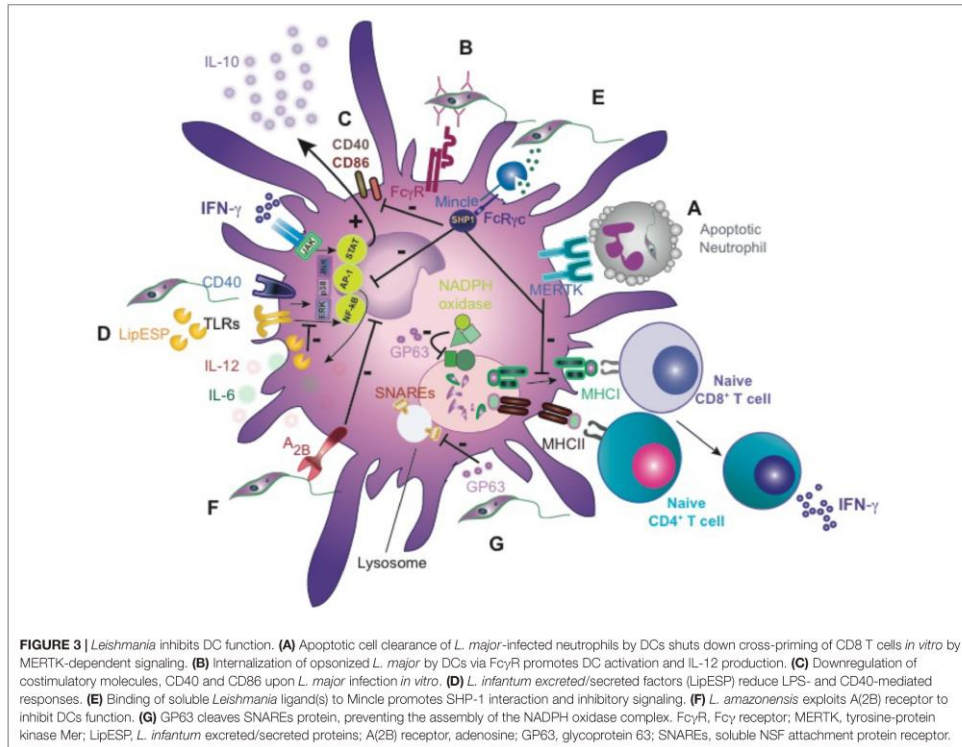
Dendritic cells play a unique role in the immune system as antigen presenting cells that promote and sustain adaptive immunity while contribute at the same time to the induction of tolerance to self-antigens. DCs uptake and process *Leishmania* parasites or their antigens and subsequently migrate to lymph nodes (LNs) to prime T cells. *Leishmania* sensing by DCs triggers IL-12p70 production in both human and mouse DCs, a key cytokine to prime and maintain Th1 responses that ultimately lead to the control of the parasite (Gorak et al., 1998; von Stebut et al., 1998; Marovich et al., 2000; Leon et al., 2007). In order to escape, *Leishmania* parasites target DC activation either being silent or even inhibiting DC activation, motility and migration to draining LNs (Ponte-Sucre et al., 2001; Jebbari et al., 2002; De Trez et al., 2004; Revest et al., 2008; Sanabria et al., 2008; Figueiredo et al., 2012; Hermida et al., 2014; Iborra et al., 2016; von Stebut, 2017). DC-*Leishmania* interaction can vary depending on the different DC subset involved, as they are equipped with different pattern recognition receptors. In addition, several *Leishmania* species and different strains might be endowed with different pathogen associated molecular patterns and/or immune evasion strategies. Moreover, the interaction of the parasite and DCs can be direct or indirect, through other infected cells, and even the sandfly saliva may also modulate DCs function.

L. major inoculation induces a huge infiltration of neutrophils that phagocytose the majority of parasites but fails to kill them, although this is not the case for other *Leishmania* species (Regli et al., 2017). DCs reaching the inflammation site would thus mainly encounter apoptotic neutrophils harboring intracellular parasites. The capture of infected neutrophils by DCs in the skin acts as a key mechanism to inhibit their functions, delaying the development of adaptive immunity (Figures 1I, 3A) (Ribeiro-Gomes et al., 2012). In fact, treatment of mice with two neutrophil-depleting antibodies, the GR-1-specific antibody RB6-8C5, which recognizes an epitope shared by Ly6G and Ly6C, and the Ly6G-specific antibody, 1A8, just before infection augments DCs maturation in the skin and the priming of *L. major* specific CD4⁺ T cells *in vivo*, which correlates with faster parasite clearance (Peters et al., 2008; Ribeiro-Gomes et al., 2012). Moreover, uptake of infected neutrophils inhibits DC maturation and their subsequent function as cross-priming DC *in vivo* (Ribeiro-Gomes et al., 2015). Upon *L. major* infection (Friedlin strain FV1), the engagement of the receptor tyrosine kinase Mer

(MERTK) on the DCs phagocytosing apoptotic neutrophils led to the impaired capacity for CD8⁺ T-cell priming *in vitro*. MERTK acted as a tolerogenic receptor in resting macrophages and in the absence of inflammation (Figures 1I, 3A) (Zagorska et al., 2014). Interestingly, the related protozoan parasite *Toxoplasma gondii* does not elicit this inhibitory response to the same extent (Ribeiro-Gomes et al., 2015). In addition, the parasites co-evolved a strategy where the virulent inoculum comprises viable and dying promastigotes, which expose phospholipids analogs to phosphatidylserine (Weingartner et al., 2012), a prototypical apoptotic eat-me signal promoting phagocytosis in a "silent" way. Thus, DCs can also engulf free extracellular *Leishmania* promastigotes (Ng et al., 2008).

Leishmania Modulates DC Maturation and Migration

Upon recognition of pathogen-derived molecules, DCs migrate to lymphoid tissues and undergo a process of "maturation" that enhances antigen processing and presentation, expression of costimulatory molecules and cytokine secretion, governing the fate of adaptive immunity. Several *in vivo* studies demonstrate the importance of fully activated migratory DCs (CD86^{high}, CD40^{high}, CCR7⁺, and IL-12⁺) in activation of NK cells and in the generation of protective Th1 responses against *Leishmania* parasites (Soong, 2008). Therefore, incomplete and delayed DC maturation could favor the establishment and amplification of *Leishmania* infection before the onset of immune responses. Of note, in an experimental model mimicking natural infection (low number *L. major* metacyclic promastigotes challenged in the ear dermis) a silent phase was observed with parasite replication in the absence of an inflammatory response. In this model, IL-12⁺ DCs were not detected until week 4 post-infection, peaking at week 6 and preceding full development of T cell-associated IFN- γ release (Belkaid et al., 2000). *L. major* internalization by DCs is facilitated by IgG via Fc γ RI and Fc γ RIII, and engagement of these receptors is required for development of Th1 dependent immunity (Figure 3B) (Woelbing et al., 2006). However, it is unknown when B-cell priming against *Leishmania* occurs, and whether natural IgGs can opsonize *Leishmania* and promote DC engulfment. The existence of a "silent phase" suggests that *Leishmania* is able to modulate DC maturation, motility or migration. In fact, upon *in vitro* infection with high doses of *L. major* promastigotes, DCs did not exhibit upregulation of MHC class I/II, costimulatory molecules, such as CD40, CD86, as well as release of proinflammatory cytokines (Figure 3C) (von Stebut et al., 1998). Similarly, the presence of live *L. amazonensis* parasites during human DC differentiation *in vitro* decreased CD80 expression and IL-6 secretion (Favali et al., 2007). Mouse bone marrow-derived DCs (BMDCs) infected with *L. amazonensis*, *L. braziliensis*, *L. major*, or *L. infantum* metacyclic promastigotes showed decreased MHC-II and CD86 expression, and exhibited an impaired ability to induce T-cell proliferation (Neves et al., 2010; Figueiredo et al., 2012). In addition, some *L. infantum* excreted/secreted proteins (LipESP) reduced the ability of human DCs to respond *in vitro* to LPS, inhibiting maturation and IL-12p70 production (Figure 3D)



(Markikou-Ouni et al., 2015). Exosomes from *L. donovani* failed to prime monocyte-derived human DCs to drive the differentiation of naive CD4 T cells into IFN- γ -producing Th1 cells *in vitro*. Interestingly, vesicles from *L. donovani* deficient in HSP100, which exhibit a distinct protein cargo, have more proinflammatory phenotype in human DCs *in vitro* (Silverman et al., 2010).

The outcome of the DC interaction with the parasite depends on the *Leishmania* species and the developmental stage. DC maturation is not observed upon *L. major* promastigote infection, but can be induced by *L. major* amastigotes *in vitro* (von Stebut et al., 1998). In contrast, DCs infected with *L. mexicana* amastigotes do not show detectable levels of IL-12, or any other signs of activation (Bennett et al., 2001). Likewise, *L. amazonensis* amastigotes failed to induce CD40-dependent IL-12 *in vitro* production in DCs (Figure 3D) (Qi et al., 2001; Boggiatto et al., 2009). Notably, DCs infected with *L. amazonensis* promastigotes displayed a “semi-activation” phenotype, produced relatively low levels of IL-12, and preferentially induced pathogenic CD4⁺ T cells (Xin et al., 2007). *L. amazonensis* amastigote-infected DCs were less mature

and with lower antigen presenting capacity *in vitro* compared with promastigote-infected DCs (Xin et al., 2008). In contrast to parasite extract stimulation or infection, internalization of antibody-opsonized *L. amazonensis* promastigotes or amastigotes induces DC maturation, as shown by the overexpression of costimulatory, adhesion and MHC-II (Prina et al., 2004).

The mechanisms that *Leishmania* uses to sabotage DCs are still not fully defined. *L. mexicana* infection of the DC line DC2.4, inhibits the MAPK-signaling cascade, decreasing antigen-presentation capacity and IL-12 secretion (Figure 3D) (Contreras et al., 2014). This inhibition is mediated by the activation of PTPs. *Leishmania* can be detected by different PRRs, such as TLRs, CLRs and opsonizing antibodies via Fc receptors, which trigger activating and/or inhibitory signals (Woelbing et al., 2006; Lefevre et al., 2013). Mincle (Clec4e) mediates dampening of DC activation and migration following sensing of a ligand released by *Leishmania* (Iborra et al., 2016). Mincle couples to the Fc receptor γ (FcR γ) chain that bears immunoreceptor tyrosine-based activation motif (ITAM). Upon canonical signaling through Mincle, tyrosine residues in the FcR γ

chain are phosphorylated by Src-family kinases, followed by the recruitment and activation of the kinase Syk, which generates an activating signal that boosts inflammation (Sancho and Reis e Sousa, 2012, 2013). Notably, upon recognition of *Leishmania* ligand, Mincle shifts to an inhibitory ITAM configuration that recruits SHP-1 and dampens DC activation and migration induced by heterologous receptors sensing activating signals from *Leishmania* (Figure 3E) (Iborra et al., 2016). Thus, we observed a more robust IFN- γ -producing-CD4⁺ T cell response, milder dermal pathology and 10-fold reduction of the parasite burden compared to wild-type mice. Selective loss of SHP1 in CD11c⁺ cells phenocopies enhanced adaptive immunity to *Leishmania* (Iborra et al., 2016). Another way to dampen DC activation related to purinergic signaling has been demonstrated in DCs infected with *L. amazonensis*. Whereas extracellular ATP induces inflammation, adenosine is an important anti-inflammatory mediator. In the presence of MRS1754, a highly selective A(2B) adenosine receptor antagonist, DCs exhibit an increased expression of MHC-II, CD86 and CD40, enhancing their ability to induce T-cell proliferation. In conclusion, A(2B) receptor activation may be used by *Leishmania* to inhibit DC function and evade the immune response (Figure 3F) (Figueiredo et al., 2012).

Leishmania may also subvert adaptive immunity by interfering with DC migration. In the steady state, dermal DCs are highly motile, continuously crawling through the interstitial space. Intradermal delivery of *L. major* immobilizes dermal DCs (Ng et al., 2008). Products secreted by *L. major* promastigotes inhibit the motility of DCs by up to 93%, in a dose-dependent and reversible manner (Jebbari et al., 2002). Co-incubation with *Leishmania in vitro*, changes the migratory pattern of DCs when they are adoptively transferred to mice (Hermida et al., 2014). Similarly, we described that *Leishmania* inhibits DCs migration via Mincle (Iborra et al., 2016). DCs from mice with chronic *L. donovani* infection fail to migrate from the marginal zone to the periarteriolar region of the spleen. However, DCs eventually migrate and promote Th1 cell immunity and macrophage microbicidal activity (Leon et al., 2007).

Different DC subsets can coordinate the mounting of anti-*Leishmania* response. *L. major* infection recruits monocytes to the dermis that generate Th1-promoting dermal monocyte-derived DCs (Leon et al., 2007). In addition, cDC1s (Batf3-dependent DCs) are essential for the control of *L. major* (Ashok et al., 2014; Martínez-López et al., 2015). Although this DC subset does not seem essential for Th1 or CTL priming (Martínez-López et al., 2015), probably because cDC1 are resistant to infection (Henri et al., 2002), they excel in IL-12 production, which is crucial for maintenance of local Th1 immunity against *L. major* infection (Martínez-López et al., 2015).

Interfering With CD8⁺ T Cell Cross-Priming

CD8⁺ T lymphocytes are components of the adaptive immune response that play an important role in protection against intracellular pathogens. The role of CD8⁺ T cells in the primary control of *Leishmania* is still controversial, given the different

results obtained in different infection models. CD8⁺ T cells contribute to parasite control in visceral leishmaniasis (Stäger and Rafati, 2012), probably by recruiting inflammatory cells and maintaining granulomas. CD8⁺ T cells also contribute to parasite clearance against low doses of *L. major* (Belkaid et al., 2002b), where they also contribute to the cutaneous pathology associated to the infection, and even exacerbate disease (Novais and Scott, 2015). However, *L. donovani* induces defective antigen-specific CD8⁺ T cell responses, with a very limited clonal expansion (Joshi et al., 2009), compared with viral infections or following injection of irradiated *Plasmodium* (Sano et al., 2001). In fact, visceral leishmaniasis patients do not show CD8⁺ T cell effector responses (Gautam et al., 2014).

Limited and poor *Leishmania* antigen-processing and presentation into MHC Class I, could be one potential explanation. Processing of *Leishmania* antigens occurs in a TAP-independent, intraphagosomal pathway that is less efficient and requires higher amounts of secreted antigen than the endoplasmic reticulum-based, TAP-dependent cross-presentation pathway (Bertholet et al., 2006). In addition to other mechanisms discussed above, the major surface metalloprotease of *Leishmania* GP63 cleaves a subset of SNAREs, including VAMP8. The inactivation of VAMP8 prevents the assembly of the NADPH oxidase complex (NOX2), which is critical to limit the acidification in these cross-presentation compartments (Figure 3G) (Matheoud et al., 2013; Matte et al., 2016). The inhibition of acidification is critical to prevent the complete and premature destruction of MHC class I epitopes by the protease activity (Savina et al., 2006). As a consequence, the cross-presentation of *Leishmania* antigens on MHC class I molecules is actively inhibited by the parasite. CD8⁺ T cells undergo a second round of activation, become dysfunctional, and ultimately die from exhaustion during infection (Joshi et al., 2009). Given that high and constant antigenic stimulation causes CD8⁺ T cell “exhaustion” during chronic viral infections (Mueller and Ahmed, 2009), we could speculate that *Leishmania* antigens might be available for cross-presentation from other sources, like death infected macrophages.

FIGHTING BACK IMMUNE EVASION BY VACCINATION

Inoculation of live virulent *L. major* parasites causing auto-curing cutaneous leishmaniasis lesions, a procedure known as leishmanization (Khamesipour et al., 2005) is the only efficient vaccine that induces immunity in human subjects (Saljoughian et al., 2014; Mendonca, 2016). Resistance to reinfection with *L. major* in mice has been linked to the induction of parasite persistence by CD4⁺CD25⁺ regulatory T cells secreting IL-10 (Belkaid et al., 2001, 2002a). The presence of small number of parasites in macrophages and DCs after primary challenge (Mandell and Beverley, 2017) preserves the concomitant immunity necessary to induce long-lasting defense (Sacks, 2014), consisting of migrating IFN- γ -producing effector T cells to the site of reinfection (Uzonna et al., 2001; Peters et al., 2014; Romano et al., 2015), and CD4⁺ resident memory T cells

in the infected skin (Glennie et al., 2015) that can further recruit effector T cells and inflammatory monocytes to the infected dermal site (Glennie et al., 2017).

Generation of long-lasting cellular immunity is the main objective of vaccines based on parasite proteins or extracts. Immunotherapy using DC-based vaccination is an emerging potent approach for harnessing the potential of a patient's own immune system to induce protection. DCs can be pulsed with parasite extracts alone (Ahuja et al., 1999; Carrion et al., 2008; Majumder et al., 2012; Masic et al., 2012), combined with adjuvants such as CpG-ODN (Carrion et al., 2008; Agallou et al., 2011, 2012; Majumder et al., 2012; Masic et al., 2012) or peptidoglycan (ligand of the TLR-2) (Jawed et al., 2016) or in DCs engineered to secrete IL-12 (Ahuja et al., 1999). These different treatments boost their immunogenicity in murine models (Ahuja et al., 1999; Majumder et al., 2012; Jawed et al., 2016), dampening IL-10 responses associated to parasite infection (Schwarz et al., 2013), and decreasing the tissue damage induced by the inflammatory response after infective challenge in vaccinated animals (Masic et al., 2012). Due to the high cost of these procedures, an alternative to the use of DCs primed with recombinant parasite proteins in humans will be to target *Leishmania* proteins to DCs by constructing recombinant chimeras, such as recombinant antibodies recognizing DC-specific receptors and containing leishmanial proteins. Using this strategy, antigen-specific CD4⁺ T cells producing IFN- γ , IL-2, and TNF were found in vaccinated mice (Matos et al., 2013).

CONCLUDING REMARKS

Myeloid cells, including neutrophils, monocytes, macrophages and DCs, orchestrate the generation of protective innate and adaptive immunity against *Leishmania*. Neutrophils are the first line of defense and generate an inflammatory response that restrains the parasite but, at the same time, and for some *Leishmania* species, neutrophils may act as carriers that facilitate silent infection of macrophages (Laskay et al., 2003, 2008; John and Hunter, 2008). Once within the macrophage, and depending on the *Leishmania* species, parasites delay phagosome formation and maturation, preventing phagosome acidification and action of proteases, while securing the nutrients needed for their survival. Moreover, the parasites modulate the pattern of cytokine secretion and inhibit the generation of NO and ROS, while

extending the survival of the infected macrophages. Similarly, *L. major*-infected neutrophils are silently phagocytosed by DCs in the skin and inhibit DC maturation and migration, delaying the development of adaptive immunity (Ribeiro-Gomes et al., 2012, 2015; Peters et al., 2014). Both monocyte-derived DCs and cDC1s are essential for the generation of Th1 immunity resulting in the control of *L. major* (Leon et al., 2007; Ashok et al., 2014; Martínez-López et al., 2015). *Leishmania* acts at different levels to inhibit DCs, including dampening the MAPK pathway, decreasing antigen presentation capacity, IL-12 secretion and migration, being this inhibition mediated by the activation of PTPs (Contreras et al., 2014; Iborra et al., 2016). Understanding which DC populations are key to trigger and achieve immunity to *Leishmania* and how parasites inhibit their activation and migration will help to improve a rational design of vaccines aimed to counteract parasite virulence factors, along with the use of the most adequate adjuvants.

AUTHOR CONTRIBUTIONS

MM-L, MS, SI, and DS conceived and wrote the manuscript. MM-L did the figures.

FUNDING

SI is funded by grant SAF2015-74561-JIN from the Spanish Ministry of Economy, Industry and Competitiveness (MINECO) and FEDER (European Fund for Regional Development). MM-L received a FPU fellowship (AP2010-5935) from the Spanish Ministry of Education. Work in the DS laboratory is funded by the CNIC and grant SAF2016-79040-R from MINECO, Agencia Estatal de Investigación and FEDER; B2017/BMD-3733 Immunothercan-CM from Comunidad de Madrid; RD16/0015/0018-REEM from FIS-Instituto de Salud Carlos III, MINECO and FEDER; Acteria Foundation; Constantes y vitales prize (Atresmedia); La Marató de TV3 Foundation (201723); the European Commission (635122-PROCROP H2020) and the European Research Council (ERC-2016-Consolidator Grant 725091). The CNIC is supported by the MINECO and the Pro-CNIC Foundation, and is a Severo Ochoa Center of Excellence (MINECO award SEV-2015-0505).

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Conflict of Interest Statement: 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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