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CD81 Controls Immunity to *Listeria* Infection through Rac-Dependent Inhibition of Proinflammatory Mediator Release and Activation of Cytotoxic T Cells

Gloria Martínez del Hoyo,* Marta Ramírez-Huesca,* Shoshana Levy,† Claude Boucheix,‡ Eric Rubinstein,§ María Minguito de la Escalera,§ Leticia González-Cintado,§ Carlos Ardáñ,§ Esteban Veiga,§ María Yáñez-Mó,*, and Francisco Sánchez-Madrid*,‖

Despite recent evidence on the involvement of CD81 in pathogen binding and Ag presentation by dendritic cells (DCs), the molecular mechanism of how CD81 regulates immunity during infection remains to be elucidated. To investigate the role of CD81 in the regulation of defense mechanisms against microbial infections, we have used the *Listeria monocytogenes* infection model to explore the impact of CD81 deficiency in the innate and adaptive immune response against this pathogenic bacteria. We show that CD81−/− mice are less susceptible than wild-type mice to systemic *Listeria* infection, which correlates with increased numbers of inflammatory monocytes and DCs in CD81−/− spleens, the main subsets controlling early bacterial burden. Additionally, our data reveal that CD81 inhibits Rac/STAT-1 activation, leading to a negative regulation of the production of TNF-α and NO by inflammatory DCs and the activation of cytotoxic T cells by splenic CD8α+ DCs. In conclusion, this study demonstrates that CD81–Rac interaction exerts an important regulatory role on the innate and adaptive immunity against bacterial infection and suggests a role for CD81 in the development of novel therapeutic targets during infectious diseases. *The Journal of Immunology,* 2015, 194: 000–000.

Tetraspanins comprise a large superfamily of cell surface proteins that have an exceptional ability to associate with each other and with other proteins, promoting the formation of supramolecular structures that regulate multiple biological functions, including the modulation of immunity and/or inflammatory processes (1, 2). In the immune system, tetraspanins expressed on APCs regulate Ag recognition through association with C-type lectin receptors and Fc receptors (3) and regulate Ag presentation through association with MHC class II (MHC-II) molecules, promoting an efficient naive T cell stimulation (4, 5). In this regard, several studies have emphasized the role of the tetraspan CD81 in promoting a potent costimulatory signal to T cells (6) and in controlling sustained T cell signaling and molecular organization of the immune synapse (7). Additionally, whereas lymphoid development in CD81−/− mice is normal (8–10), T cells from CD81−/− mice present a hyperproliferative phenotype after anti-CD3 stimulation and impaired development of Th2 responses (8, 11).

Tetraspanins participate in the regulation of defense against infection through their involvement in the binding and uptake of pathogens and the subsequent initiation of the immune response. CD81 is a ligand for hepatitis C virus in hepatocytes (12) and in T, B, and dendritic cells (DCs) (3), and CD81 and CD9 can modulate HIV-1 membrane fusion and viral clustering at the viral synapse (13). CD81 is also required for hepatocyte infection by *Plasmodium* (14). The tetraspanins CD9, CD63, and CD81 were recently shown to be recruited to the bacterial entry site after infection of epithelial cells by *Listeria* (15). However, the molecular mechanisms underlying the regulatory role of CD81 during bacterial infections remain to be elucidated.

*Listeria* is a Gram-positive bacteria that causes severe infection in immunocompromised individuals and is a widely used model to study innate and adaptive immunity to intracellular infection (16). During the first days of infection, professional phagocytic cells trap bacteria from target organs, enabling control of bacterial growth (17). After internalization by phagocytic cells, *Listeria* escapes from the phagosome into the cytoplasm, where it replicates (18). *Listeria* can spread from cell to cell without leaving the intracellular compartment, which is the main reason why a specific CD8+ T cell response to *Listeria* is crucial for the control of the infection (19). The early control of *Listeria* burden largely depends on the innate immune response occurring in the spleen, which relies on a specialized subset of monocyte-derived DCs called TNF-inducible NO synthase iNOS (iNOS)–producing DCs.
CD81 MEDIATES SUSCEPTIBILITY TO LISTERIA INFECTION

(Tip-DCs). Tip-DCs are characterized by their ability to produce TNF-α and the enzyme iNOS, which leads to the production of NO (20). Another splenic DC subset, namely CD8α+ conventional DCs (cDCs), is responsible for the final resolution of infection against *Listeria* through the cross-priming of bacterial-derived Ags to specific CD8+ T cells and the subsequent induction of the cytotoxic T cell response (21, 22). Interestingly, Ag cross-presentation in DCs requires endosomal alkalinization through reactive oxygen species (ROS) production resulting from NADPH oxidase (NOX) 2 recruitment, a process dependent on the small GTPase Rac (23–25). Additionally, a direct interaction between CD81 and Rac was revealed by proteomics analysis (26), suggesting that CD81 could modulate the cross-priming capacity of DCs.

DC activation after encounter with *Listeria* essentially relies on the activation of two signaling pathways: a TLR-dependent pathway, resulting from cell surface and endosomal bacterial sensing, which leads to the activation of a MyD88-dependent response; and a cytosolic pathway, triggered after *Listeria* escapes into the cytosol by secretory listeriolysin O, which activates the cytosolic sensor stimulator of IFN genes (STING) by nucleotides. STING activation leads to IFN regulatory factor (IRF)-3–dependent production of IFN-β (27). IFN-β in turn activates an autocrine loop after binding to its cognate receptor, the heterodimer formed by the type I IFN receptor (IFNAR)-1 and IFNAR-2 receptors, promoting subsequently the triggering of the IFNAR signaling pathway, which controls the transcription of IFN target genes essential for antiviral and antibacterial immunity (28).

To investigate the role of CD81 in the modulation of defense against bacterial infections, we have explored the impact of CD81 deficiency in the innate and adaptive immune response after *Listeria* infection in vivo. Our data reveal that CD81−/− mice are protected against *Listeria* infection and support the view that CD81 negatively regulates the defense mechanisms to this bacterial infection by the inhibition of Rac/STAT-1 activation that interferes with the production of proinflammatory mediators by DCs and the potential to cross-present bacterial Ags of DCs to cytotoxic T cells.

### Materials and Methods

#### Mice

CD81−/− mice were described in Maeker and Levy (29). Experiments were performed using CD81−/− mice in a C57BL/6 genetic background (backcrossed >20 generations), and wild-type (WT) mice were littermates of sex and age matched. CD45.1 OT-I transgenic mice express a TCR specific for Listeria monocytogenes (HKLK), flagellin, polynosinic-polycytidylic acid (Invivo-Gen, San Diego, CA), or LPS from *Escherichia coli* (Sigma-Aldrich). TNF-α was analyzed with OptEIA ELISA kits (BD Biosciences, San Diego, CA) and IL-6 with the mouse ELISA Ready-SET-Go! kit from eBioscience (Affymetrix, San Diego, CA). NO was estimated from the nitrite concentration measured with a Griess reagent kit (Molecular Probes/Life Technologies, Thermo Fisher Scientific).

#### Measurement of reactive oxygen production

Production of ROS was assayed by luminol-ECL, using the L012-luminol derivative (Wako Chemicals, Neuss, Germany) and assessed for the gentamicin survival assay. BMDCs were seeded in 96-well luminometer plates (1 × 106 cells/well) in medium containing 500 μl of 10 mM L012 with or without HKLM (moi of 50:1) or PMA (250 ng/ml). Chemiluminescence was measured at 15-min intervals.

#### CD81 C-terminal peptide

To investigate the role of CD81 in the modulation of defense against bacterial infections, we have explored the impact of CD81 deficiency in the innate and adaptive immune responses after *Listeria* infection in vivo. Our data reveal that CD81−/− mice are protected against *Listeria* infection and support the view that CD81 negatively regulates the defense mechanisms to this bacterial infection by the inhibition of Rac/STAT-1 activation that interferes with the production of proinflammatory mediators by DCs and the potential to cross-present bacterial Ags of DCs to cytotoxic T cells.

CD81−/− mice were described in Maeker and Levy (29). Experiments were performed using CD81−/− mice in a C57BL/6 genetic background (backcrossed >20 generations), and wild-type (WT) mice were littermates of sex and age matched. CD45.1 OT-I transgenic mice express a TCR specific for *Listeria* monocytogenes (HKLK), flagellin, polynosinic-polycytidylic acid (Invivo-Gen, San Diego, CA), or LPS from *Escherichia coli* (Sigma-Aldrich). TNF-α was analyzed with OptEIA ELISA kits (BD Biosciences, San Diego, CA) and IL-6 with the mouse ELISA Ready-SET-Go! kit from eBioscience (Affymetrix, San Diego, CA). NO was estimated from the nitrite concentration measured with a Griess reagent kit (Molecular Probes/Life Technologies, Thermo Fisher Scientific).

#### Measurement of reactive oxygen production

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Analysis of Listeria-specific CD8+ T cell response in vivo

Spleens and lymph nodes were obtained from OT-1 CD45.1+ mice, and CD8+ T cells were isolated by negative selection in an autoMACS Pro separator (Miltenyi Biotec) according to the manufacturer’s instructions. Selected cells were >95% CD8a+Vo2+, and subsequently these cells were labeled with CellTrace Violet (Molecular Probes) and i.v. transferred into recipient WT and CD81−/− mice. One day later, these recipient mice were infected with Listeria-OVA (1 × 10⁷ CFUs per mouse). Three days later, splenocytes were obtained from infected mice to activate the differentiation of transferred OT-1 cells by flow cytometry. Additionally, splenocytes from infected mice were also cultured in 96-well plates (0.2 × 10⁶ cells/well) in the presence of 1 μg/ml OVA peptide 257–264 (SIINFEKL, purchased from GenScript, Piscataway,NJ), and 2 h later BD GolgiPlug (BD Biosciences) was added to block the secretion of cytokines overnight. Cells were washed and stained for detection of IFN-γ expression, after fixation and permeabilization using a BD Cytofix/Cytoperm kit (BD Biosciences), using an anti-mouse IFN-γ Ab (BD Biosciences). In parallel, endogenous OVA-specific CD8+ T cells were also analyzed in WT and CD81−/− mice infected with Listeria-OVA for 7 d. At this time point, splenocytes were obtained and Ag-specific CD8+ T cells were analyzed using SIINFEKL-H2b–PE tetramers, referred to as OVA tetramers (provided by Dr. D. Sancho, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain). Splenocytes were also cultured in the presence of OVA peptide 257–264 (1 μg/ml), and supernatants were collected to determine IFN-γ production by ELISA (mouse ELISA Ready-SET-Go! kit, eBioscience) after overnight culture; alternatively, splenocytes cultured with OVA peptide for 2 h were incubated with BD GolgiPlug, and intracellular IFN-γ was analyzed by flow cytometry.

Flow cytometry

Spleenic cell subsets in T/B cell–depleted fractions were analyzed by staining with FITC-conjugated anti-CD11b, allopurinol-conjugated anti-CD11c, biotin-conjugated anti-Ly-6C, biotin-conjugated anti-MHC-II, and biotin-conjugated anti-CD81, followed by incubation with PerCP-conjugated streptavidin, PE-conjugated anti-CD49b, and PE-conjugated anti-Ly-6G (all Abs from BD Biosciences). For the detection of intracellular iNOS, T/B cell–depleted splenocytes were fixed and permeabilized using a BD Cytofix/Cytoperm Kit (BD Biosciences) and stained with rabbit anti-iNOS (Cell Signaling Technology) followed by FITC-conjugated goat anti-rabbit Ab (Jackson Immunoresearch Laboratories, West Grove, PA). Serum and splenic cytokines were measured using a BD cytometric bead array mouse Th1/Th2/Th17 cytokine kit (BD Biosciences). BMDCs were analyzed after differentiation by incubation with PE-conjugated anti-CD11c, FITC-conjugated MHC-II, and allopurinol-conjugated Gr-1 (BD Biosciences). Spleenic DC-enriched cell fractions were stained for sorting with anti-CD11c–allophycocyanin, anti-CD11c–PE, anti-B220–FITC, and anti–CD11c–FITC (BD Biosciences). Live cells were identified by staining with DAPI (Molecular Probes). Flow cytometric analysis was performed on a FACSCanto II (BD Biosciences), and data were analyzed using FlowJo v8.8.5 (Tree Star) software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. A Student t test was used for individual comparisons of the mean between two groups. Differences were considered significant at p ≤ 0.05 and p ≤ 0.01. Data are shown in column bars representing the mean ± SD of at least three independent experiments unless otherwise indicated.

Results

CD81−/− mice are resistant to a lethal dose of Listeria

To investigate the contribution of CD81 to immunity against Listeria infection, we first analyzed the bacterial load in target organs at different time points after sublethal infection of WT and CD81−/− mice. We did not observe any significant differences between WT and CD81−/− mice using a low bacterial infection dose (10⁷ CFUs per mouse; Supplemental Fig. 1A). When we used a higher infection dose (2 × 10⁸ CFUs per mouse), bacterial load in the spleen was also similar in WT and CD81−/− mice at day 1 postinfection (Fig. 1A), and liver CFUs were below the detection limit of the assay (data not shown). However, analysis of bacterial burden at longer times revealed that CD81−/− mice showed significantly lower CFUs than did WT mice in both the spleen and the liver at 2, 3, and 4 d postinfection (Fig. 1A). These results show that CD81−/− mice are able to control bacterial growth more efficiently than are WT mice.

We next analyzed the survival of WT and CD81−/− mice after lethal Listeria infection. At a dose that caused 50% mortality in WT mice after 10 d of infection, ~75% of CD81−/− mice survived (Fig. 1B), showing that the low bacterial burden observed in CD81−/− mice correlates with reduced mortality.

Absence of CD81 allows effective differentiation and survival of inflammatory DCs after Listeria infection

To understand the protective effect conferred by the absence of CD81 during Listeria infection, we analyzed the recruitment and differentiation of cells that control the innate (monocytes, Tip-DCs, and neutrophils) and the adaptive (CD8α+ and CD8α− cDCs) immune responses. Analysis of WT and CD81−/− mice before infection did not reveal differences in the numbers or percentages of these subsets (Fig. 2A, day 0), indicating that the development of the different splenic subsets was not affected by CD81 deficiency. Between days 2 and 4 postinfection, both genotypes increased the numbers of inflammatory monocytes and Tip-DCs described upon Listeria infection (20). Importantly, in CD81−/− mice this increase was significantly more pronounced compared with WT mice (Fig. 2A). cDC numbers increased from day 0 to day 2 in CD81−/− mice and subsequently dropped to a similar level in both genotypes by day 4 postinfection (Fig. 2A); neutrophils displayed a comparable increase in number in WT and CD81−/− mice from day 0 to day 4 (Fig. 2A). Additionally, analysis of lymphocyte subpopulations revealed that T and B cell numbers were drastically reduced in the spleen of both WT and CD81−/− mice during the first days of Listeria infection (Supplemental Fig. 1B). Interestingly, the surface expression of CD81 was modulated by Listeria infection in the different splenic subsets analyzed of WT mice (Supplemental Fig. 1C). Whereas the expression of CD81 was downregulated in inflammatory monocytes, neutrophils, and cDCs, Tip-DCs increased CD81 expression levels during Listeria infection. To investigate how CD81 controls early bacterial replication during Listeria infection, we analyzed Tip-DC activation in WT and CD81−/− mice, because these cells have been claimed to be critical cells in limiting Listeria burden owing to the production of the microbicidal mediators TNF-α and NO (20). Tip-DC activation, assessed by intracellular iNOS expression, was increased in CD81−/− mice compared with WT mice after 3 d of Listeria infection (Fig. 2B). We next analyzed whether CD81 deficiency could also affect the production of inflammatory cytokines, as an early event of the innate immune response. We found that CD81−/− mice exhibited increased serum levels of TNF-α, but comparable levels of serum IL-6, than did WT mice after Listeria infection (Fig. 2C). Additionally, TNF-α production in the spleen of infected CD81−/− mice was also higher than WT mice production (Fig. 2C).

Therefore, our results indicate that Listeria infection in the absence of CD81 leads to efficient differentiation of inflammatory monocytes and Tip-DCs, whose numbers and activation increase notably during the first days after infection, correlating with the observed protective phenotype.

CD81 regulates Listeria-induced production of TNF-α and NO by infected DCs

Early protective immunity against Listeria relies on the production of proinflammatory cytokines, such as TNF-α and IL-6, mainly driven by TLR2-dependent signaling, as well as microbicidal mediators, such as NO, after STING-dependent signaling triggering the IFNAR autocrine pathway (27, 28). To investigate the role of CD81 in the production of microbicidal mediators in re-
response to Listeria, we compared the capacity of WT and CD81−/− BMDCs to produce proinflammatory cytokines after Listeria infection in vitro. According to the results obtained on cytokine production in serum and spleen (Fig. 2C), infected CD81−/− BMDCs produced higher levels of TNF-α than did WT BMDCs (Fig. 3A), whereas the levels of IL-6 were similar in the two genotypes (Fig. 3B). In parallel with the increased TNF-α production observed, CD81−/− BMDCs produced also higher levels of NO (Fig. 3C).

The higher TNF-α and NO production potential displayed by CD81−/− BMDCs compared with WT BMDCs could reflect a differential bacteria internalization capacity and susceptibility to infection leading to a stronger DC activation or, alternatively, that CD81 directly modulates Listeria-mediated DC activation and consequently the production of proinflammatory mediators. We first analyzed the capacity of BMDCs to internalize bacteria in vitro by the gentamicin survival assay (30, 31). These experiments revealed that WT and CD81−/− BMDCs had a comparable susceptibility to infection, as assessed by the number of CFUs at 2 h, indicating that both genotypes internalized Listeria with a similar efficiency (Fig. 3D). Accordingly, CD81−/− and WT BMDCs displayed a similar ability to internalize fluorescent beads, suggesting that CD81 does not interfere with the phagocytic capacity of DCs (Fig. 3F). Moreover, no differences were found in the capacity of CD81−/− and WT BMDCs to kill Listeria, as revealed by the similar decrease of CFUs observed between 4 and 6 h (Fig. 3D). Importantly, both genotypes displayed a comparable viability 9 h after in vitro infection (Fig. 3E). These data confirmed that the higher TNF-α and NO production capacity of CD81−/− BMDCs did not result from a higher susceptibility to infection.

We next addressed whether the differential production of TNF-α and NO by WT and CD81−/− BMDCs reflected that CD81 interferes with Listeria-mediated DC activation. In this regard, in response to Listeria, the production of TNF-α and NO is controlled both by TLR signaling and by cytosolic STING-mediated signaling triggering the IFNAR autocrine pathway, whereas IL-6 production is not dependent on STING/IFNAR signaling. To determine whether CD81 differentially affects Listeria-induced signaling through the TLR2 versus STING/IFNAR-induced signaling, we analyzed the response of WT and CD81−/− BMDCs to HKLM, which activates the TLR2 signaling pathway but not the STING/IFNAR-dependent signaling. Our data revealed that HKLM induced

FIGURE 1. CD81 mediates susceptibility to lethal systemic Listeria infection. (A) Bacterial load in the spleen and the liver of WT and CD81−/− mice postinfection with 2 × 10^5 Listeria per mouse at the indicated time points (mean ± SD; n = 5 mice/condition). Data are representative of three independent experiments with similar results. *p < 0.05 (unpaired t test). (B) Survival of WT and CD81−/− mice postinfection with 5 × 10^5 Listeria per mouse (n = 10 mice/group). Data are representative of three independent experiments with similar results. *p < 0.05, log-rank test.
a significantly lower production of TNF-α and IL-6 than did live *Listeria*, and NO was not produced after HKLM stimulation (Fig. 3G). Therefore, the production of TNF-α, IL-6, and NO in response to HKLM was not affected in CD81^−/−^ BMDCs (Fig. 3G). Interestingly, similar results were obtained in response to the synthetic ligand Pam3Cys, which activates TLR2 signaling but not STING/IFNAR signaling (Fig. 3H), although Pam3Cys induced similar IL-6 levels than did live *Listeria* that were not affected by CD81 deficiency (Fig. 3H). Because BMDCs stimulated with HKLM or TLR2 ligands induced lower TNF-α production compared with BMDCs infected with live *Listeria*, we performed a dose-response curve to determine TNF-α production by *Listeria*-infected DCs at different doses to compare similar ranges of TNF-α production. We found that TNF-α production of *Listeria*-infected DCs were higher than HKLM- or Pam3Cys-stimulated DCs, even at the lower infection rate used (moi of 1; Supplemental Fig. 2A).
FIGURE 3. Production of proinflammatory cytokines and NO by WT and CD81−/− DCs after Listeria infection. WT and CD81−/− BMDCs were infected with Listeria and 16 h later TNF-α (A), IL-6 (B), and NO (C) were analyzed in noninfected or Listeria-infected DCs. Data are presented as the mean ± SD and represent the fold differences of TNF-α and NO production from WT BMDC production after Listeria stimulation and are also shown, and data are pooled from four independent experiments. *p < 0.05, unpaired t test. (D) Analysis of Listeria infection by gentamicin survival assay after incubation of WT and CD81−/− BMDCs with Listeria (moi of 10) for the indicated times. The chart shows the numbers of CFUs present in 5 × 10^4 DCs (mean ± SD). Data are pooled from four independent experiments. (E) WT and CD81−/− BMDCs were incubated with Listeria (moi of 10) for 9 h and DC viability was determined by staining with propidium iodide and 7-aminoactinomycin D. The chart shows the percentage of viable DCs (mean ± SD; representative data from three independent experiments). (F) Phagocytosis of fluorescent beads by DCs. WT and CD81−/− BMDCs were incubated with fluorescent microspheres, and phagocytized particles were analyzed by flow cytometry. Data are presented as the percentage of DCs with internalized beads (mean ± SD; representative data from four independent experiments). WT and CD81−/− BMDCs were stimulated with HKLM (G) or Pam3Cys (H) for 16 h, and TNF-α, IL-6, and NO were determined. Data are presented as the mean ± SD and are representative of three independent experiments with similar results.
TLR ligands flagellin (TLR5 ligand), polyinosinic-polycytidylic acid (TLR3 ligand), and LPS (TLR4 ligand) also induced similar levels of TNF-α, IL-6, and NO in WT and CD81<sup>−/−</sup> BMDCs (Supplemental Fig. 2B, 2C, and 2D, respectively). These results demonstrated that the observed differences in TNF-α and NO production were dependent on live <i>Listeria</i> and suggest that CD81 regulates TNF-α and NO production in <i>Listeria</i>-infected DCs by interfering with STING/IFNAR signaling. Therefore, it can be hypothesized that the higher survival and lower splenic bacterial load observed in CD81<sup>−/−</sup> mice is, at least in part, due to a higher production of TNF-α and NO by splenic inflammatory Tip-DCs.

To further investigate the involvement of CD81 in the STING/IFNAR signaling in response to <i>Listeria</i>, we analyzed the expression of IFN-β by <i>Listeria</i>-infected BMDCs. <i>Listeria</i> triggers a STING-driven cytosolic signaling pathway after bacterial nucleotide sensing in DCs that leads to the production of IFN-β (27). Our results showed that IFN-β mRNA was expressed by BMDCs at 3 h of <i>Listeria</i> infection and this expression was increased at 5 h postinfection (Supplemental Fig. 3). However, comparable levels of IFN-β were observed in infected WT and CD81<sup>−/−</sup> BMDCs (Supplemental Fig. 3). Our results show that CD81 does not affect the STING-mediated production of IFN-β in response to <i>Listeria</i> infection, but these results are consistent with the possibility that CD81 interferes with the activation of the autocrine IFNAR signaling pathway, which controls the transcription of IFN-regulated genes essential for antiviral and antibacterial immunity (28).

<i>CD81 downregulates IFNAR signaling by controlling Rac activation and STAT-1 phosphorylation in <i>Listeria</i>-infected DCs</i>

IFN triggers an autocrine loop after engagement of its cognate receptor IFNAR promoting the activation of STAT-1, a central mediator of IFN responses that is crucial for IFN-induced target genes, such as the production of NO (32). Based on the reported role of Rac1 in the regulation of STAT-1 activation during IFN signaling (33) and on the described ability of CD81 to regulate Rac activity (26), we investigated whether CD81 could interfere with IFNAR signaling by regulating the activation of STAT-1.

For this purpose, we first analyzed the activation of the small GTPase Rac after <i>Listeria</i> infection of WT and CD81<sup>−/−</sup> BMDCs. We assessed Rac activation in <i>Listeria</i>-infected BMDCs by pull-down of the active (GTP-bound) form of Rac with GST-PAK-Cdc42/Rac interactive binding domain. CD81<sup>−/−</sup> BMDCs contained higher levels of active Rac than did WT cells 30 min after <i>Listeria</i> infection, and these levels were maintained almost constant in CD81<sup>−/−</sup> DCs at 45 min postinfection (Fig. 4A). These results suggest that CD81 negatively regulates Rac activation in <i>Listeria</i>-infected DCs.

We next analyzed whether the higher levels of active Rac displayed by CD81<sup>−/−</sup> BMDCs after <i>Listeria</i> infection correlated with a stronger STAT-1 activation. In this regard, NO production by DCs is driven by IFN-β-mediated triggering of the IFNAR autocrine pathway (34). Thus, we investigated whether CD81 could interfere with IFNAR signaling by regulating the activation of STAT-1 in BMDCs after <i>Listeria</i> infection. Phosphorylation of STAT-1 was evident in DCs 3 h after in vitro infection with <i>Listeria</i>, and it was enhanced in CD81<sup>−/−</sup> DCs compared with WT DCs (Fig. 4B). These data support that CD81 exerts a negative regulation on the production of NO and other proinflammatory mediators such as TNF-α in <i>Listeria</i>-infected DCs by interfering with IFNAR signaling through the inhibition of the Rac/STAT-1 activation pathway.

To demonstrate that the mechanism responsible for the inhibition of NO and proinflammatory cytokine production by CD81 in <i>Listeria</i>-infected DCs involves a downregulation of Rac activity resulting from direct CD81–Rac interaction, we performed in vitro infection assays in the presence of a cell-permeable peptide corresponding to the C-terminal sequence of CD81. This CD81 peptide prevents Rac–CD81 association, leading to an increase in Rac activation (26). For this purpose, we incubated WT DCs with fluorescently labeled CD81 peptide or with a control peptide resulting from a scrambled combination of the same eight amino acids. These peptides were internalized by DCs and 1 h after incubation, 100% of the DCs were fluorescently labeled. The mean fluorescence intensity of the cells increased up to 4 h and declined thereafter, although 100% of the cells retained detectable fluorescence after 20 h.
Once we confirmed that DCs internalized CD81 or scrambled peptides, we assessed the capacity of *Listeria*-infected DCs to produce TNF-α and NO after the treatment with the peptides. As shown in Fig. 5B, WT DCs treated with the CD81 peptide but not with the control peptide increased the production of TNF-α to levels comparable to those displayed by CD81−/− DCs. Likewise, CD81 peptide-treated WT DCs induced a significantly higher production of NO than did those treated with control peptide (Fig. 5C). Interestingly, the production of TNF-α and NO was not affected in *Listeria*-infected CD81−/− DCs treated with the CD81 peptide. These data show that inhibition of Rac–CD81 interaction results in a significantly higher production of TNF-α and NO by *Listeria*-infected WT DCs, further supporting that CD81 exerts a negative regulatory role during the DC-mediated anti-*Listeria* immune response through the inhibition of Rac activity.

In *vivo* priming of effector CD8+ T cells is more efficient in CD81−/− than in WT mice in response to systemic *Listeria* infection

DCs are highly efficient in Ag cross-presentation mainly due to phagosomal alkalinization, which is mainly regulated by Rac2-mediated recruitment of NOX2 to the endosomal compartment. The recruitment of NOX2 to the phagosomes leads to the generation of ROS in CD8α+ cDCs, which are also dependent on Rac2 (25). Our data revealing that CD81 inhibits Rac activation through direct association prompted us to investigate whether CD81 deficiency interferes with the production of ROS in DCs during *Listeria* infection. For this purpose, ROS production was assessed by WT and CD81−/− BMDCs stimulated with HKLM, because live *Listeria* presents inhibitory mechanisms of ROS production, or PMA, during a 180-min period using a chemiluminiscence assay. CD81−/− BMDCs induced higher ROS levels than did WT BMDCs after stimulation with either HKLM or PMA (Fig. 6A), suggesting that CD81 could exert a negative effect on the microbicidal potential of BMDCs through the regulation of Rac-mediated ROS production.

Presentation of *Listeria*-derived Ags during *Listeria* infection has been claimed to rely essentially on the CD8α+ cDC subset. Splenic CD8α+ cDCs are the first infected subset in the spleen during the early phases of *Listeria* infection (35). *Listeria*-infected CD8α+ cDCs in the spleen are essential not only for CD8+ T cell generation but also for *Listeria* expansion and dissemination within the host.
CellTrace Violet–labeled CD45.1+ OT-I cells were adoptively transferred into WT or CD81−/− mice. (A) WT and CD81−/− BMDCs were stimulated with PMA or heat-killed Listeria and ROS were assayed by luminol-ECL. Data are presented as mean ± SD and are representative of two independent experiments with similar results. (B) WT and CD81−/− mice were infected with Listeria and 15 h later CD8α+ and CD8α− cDCs were sorted and the purified populations were Triton X-100 lysed. The dot plot shows the identification of the two subsets of CD8α+ and CD8α− cDC, defined on the basis of CD8α expression after gating on CD11c+ cells. The charts show the numbers of live intracellular Listeria present in 5 × 105 cells after lysis and dilution plating on BH agar (mean ± SD of four mice per condition). Data are representative of two independent experiments with similar results. (C) CellTrace Violet–labeled CD45.1+ OT-I cells were adoptively transferred into WT or CD81−/− mice. After 24 h, mice were infected with Listeria-OVA and activation of transferred OT-I cells was analyzed in infected spleens. Graphs show OT-I absolute cell numbers among total splenocytes and cell proliferation determined by loss of CellTrace Violet, assessing the number of OT-I cells that had divided at 72 h after activation of transferred OT-I cells was analyzed in infected spleens. Graphs show OT-I absolute cell numbers among total splenocytes and cell proliferation determined by loss of CellTrace Violet, assessing the number of OT-I cells that had divided at 72 h after Listeria-OVA infection. (D) CD25 expression levels in OT-I cells are shown. (E) Absolute numbers of IFN-γ+ cells in OT-I cells after OVA peptide restimulation of splenocytes from Listeria-OVA–infected WT or CD81−/− mice. (F) WT or CD81−/− mice were infected with Listeria-OVA and 7 d later mice were analyzed for the presence of endogenous Listeria-OVA–specific CD8α+ T cell in spleens. Dot plots show the proportion of tetramer+CD44+ cells, and graph shows absolute cell numbers of Listeria-OVA–specific CD8α+ T cells in infected WT or CD81−/− mice. (G) Splenocytes from infected WT or CD81−/− mice were restimulated in the presence of OVA peptide, and IFN-γ production was analyzed by flow cytometry, assessing the number of IFN-γ+ cells in CD8α+ T cells or by ELISA determining IFN-γ production in cell supernatants. Data are presented as mean ± SD (five mice per condition, three independent experiments with similar results). *p < 0.05, **p < 0.01, unpaired t test.

FIGURE 6. Susceptibility of splenic CD8α+ cDCs to in vivo infection and induction of T cell responses in WT and CD81−/− mice. (A) WT and CD81−/− BMDCs were stimulated with PMA or heat-killed Listeria and ROS were assayed by luminol-ECL. Data are presented as mean ± SD and are representative of two independent experiments with similar results. (B) WT and CD81−/− mice were infected with Listeria and 15 h later CD8α+ and CD8α− cDCs were sorted and the purified populations were Triton X-100 lysed. The dot plot shows the identification of the two subsets of CD8α+ and CD8α− cDC, defined on the basis of CD8α expression after gating on CD11c+ cells. The charts show the numbers of live intracellular Listeria present in 5 × 105 cells after lysis and dilution plating on BH agar (mean ± SD of four mice per condition). Data are representative of two independent experiments with similar results. (C) CellTrace Violet–labeled CD45.1+ OT-I cells were adoptively transferred into WT or CD81−/− mice. After 24 h, mice were infected with Listeria-OVA and activation of transferred OT-I cells was analyzed in infected spleens. Graphs show OT-I absolute cell numbers among total splenocytes and cell proliferation determined by loss of CellTrace Violet, assessing the number of OT-I cells that had divided at 72 h after Listeria-OVA infection. (D) CD25 expression levels in OT-I cells are shown. (E) Absolute numbers of IFN-γ+ cells in OT-I cells after OVA peptide restimulation of splenocytes from Listeria-OVA–infected WT or CD81−/− mice. (F) WT or CD81−/− mice were infected with Listeria-OVA and 7 d later mice were analyzed for the presence of endogenous Listeria-OVA–specific CD8α+ T cell in spleens. Dot plots show the proportion of tetramer+CD44+ cells, and graph shows absolute cell numbers of Listeria-OVA–specific CD8α+ T cells in infected WT or CD81−/− mice. (G) Splenocytes from infected WT or CD81−/− mice were restimulated in the presence of OVA peptide, and IFN-γ production was analyzed by flow cytometry, assessing the number of IFN-γ+ cells in CD8α+ T cells or by ELISA determining IFN-γ production in cell supernatants. Data are presented as mean ± SD (five mice per condition, three independent experiments with similar results). *p < 0.05, **p < 0.01, unpaired t test.

enabling the establishment of protective T cell responses (36). We therefore analyzed whether infection of splenic CD8α+ cDCs in vivo was affected by CD81 deficiency in the early postinfection period. We sorted CD11c+CD8α+ and CD11c−CD8α− splenic DC subsets 15 h after systemic Listeria infection of CD81−/− and WT mice and determined the number of viable Listeria recoverable from these cells. Consistent with previous reports, our results revealed that infection was readily detectable in CD8α+ cDCs at 15 h postinfection before bacteria spread to CD11c−CD8α+ or CD11c−CD8α− Tip-DCs (Fig. 6B). Interestingly, CD8α− cDCs isolated from spleens of infected CD81−/− mice presented significantly more CFUs than did WT CD8α− cDCs at 15 h postinfection in vivo, indicating a higher infection rate (Fig. 6B) and providing an additional explanation for the more efficient immune response against Listeria observed in CD81−/− mice.

Induction of Listeria-specific effector CD8α+ T cells is crucial for bacterial clearance and the induction of protective immunity. Thus, we analyzed whether Rac–CD81 interaction could affect the capacity of DCs to activate cytotoxic T cells. For this purpose, we used OVA-expressing Listeria (Listeria-OVA) to assess the activation of Listeria-specific effector T cells during infection. WT and CD81−/− mice were adoptively transferred with OVA-specific transgenic OT-I CD8α+ T cells. Twenty-four hours later, mice were systemically infected with Listeria-OVA. In this system, splenic CD8α+ cDCs are the main DC subset involved in the presentation of Listeria-derived Ag to CD8α+ T cells. Proliferation and activation of OT-I cells transferred to WT or CD81−/− mice were analyzed at 72 h postinfection (Fig. 6C–E). The expansion of OT-I cells in the spleen was higher in CD81−/− than in WT mice, as assessed by the analysis of their absolute cell number at 72 h postinfection.
CD81 mediates susceptibility to Listeria infection

(Total 10) Correspondingly, the number of cells in proliferation was higher in the spleen of CD81−/− mice when analyzed by the number of cells that had undergone three, four, and five divisions at 72 h postinfection (Fig. 6C). Additionally, activation, assessed by CD25 surface expression (Fig. 6D) and intracellular expression of IFN-γ (Fig. 6E) in OT-1 cells, was significantly higher in CD81−/− than in WT mice. In parallel, we also analyzed the activation of endogenous OVA-specific CD8+ T cells postinfection of WT and CD81−/− mice with Listeria-OVA. According to our data on the activation of adoptively transferred transgenic OT-1 cells, the expansion of endogenous OVA-specific effector CD8+ T cells was higher in CD81−/− than in WT mice as assessed by staining with OVA tetramers (Fig. 6F). Likewise, these CD8+ T cells produce higher levels of IFN-γ (Fig. 6G), demonstrating that OVA-specific CD8+ T cells were functionally more active in CD81−/− than in WT mice. Taken together, our results reveal that CD81 exerts a negative regulation on the immune response against Listeria through the inhibition of Rac/STAT-1 activation that leads to a reduction of the production of proinflammatory mediators and the activation of effector CD8+ T cells.

Discussion

In this study, we report that CD81 regulates the anti-Listeria immune response by limiting the differentiation and recruitment of inflammatory monocytes and Tip-DCs to infected spleens and ultimately inhibiting the induction of a protective anti-Listeria T cell response. The synthesis by DCs of TNF-α and NO, critical microbicidal mediators in the response to Listeria infection, was more efficient in the absence of CD81. This functional effect involves the direct association of CD81 C-term cytoplasmic domain with Rac that is crucial for the initial control of bacterial replication. Recent studies have demonstrated that failed recruitment of Tip-DCs reduces host survival after Listeria infection (20) in a process that depends on cDCs and NK cells (37). Furthermore, recruitment of Tip-DCs to gut-associated tissues has also been described during infection with Salmonella typhimurium (38) and is critical for the control of Toxoplasma gondii infection (39).

Our results show that CD81 regulates Rac activity in DCs after Listeria infection, but this activity does not impair internalization of bacteria by CD81−/− DCs. In DCs, phagocytosis requires a dynamic regulation of the actin cytoskeleton, and Rac is involved in this function (40, 41). Moreover, tetraspanins exhibit a remarkable capacity to form functional complexes with microbial receptors in the host cell membrane during infection. Recent data have demonstrated the recruitment of tetraspanins CD9, CD63, and CD81 to the Listeria entry site during in vitro infection of nonphagocytic target cells, highlighting the role of CD81 in this step. However, CD81 does not affect the recruitment of Met, the Listeria receptor in epithelial cells, to the bacterial entry site, suggesting that CD81 acts as a membrane organizer of signaling events at Listeria entry sites (15).

Tetraspanin microdomains facilitate the clustering of membrane receptors and signaling molecules for the regulation of signal transduction events. Listeria is detected by multiple signaling pathways during infection (27). TLR engagement by Listeria induces a MyD88-dependent pathway leading to the synthesis of proinflammatory cytokines. Additionally, Listeria is detected by cytosolic sensors that trigger the production of type I IFN through the STING/IRF3-dependent pathway. Our results show that CD81 does not affect the STING-dependent production of type I IFN in Listeria-infected DCs, but is able to negatively regulate the IFN-induced Rac/STAT-1 signaling through the triggering of the IFNAR autocrine loop. It has been claimed that Rac is involved in the activation of STATs in response to a variety of inflammatory mediators. Rac deficiency leads to attenuation of IFN-γ-induced inflammatory responses, with cells expressing a Rac dominant-negative mutant presenting reduced expression of several IFN-γ-responsive genes such as MCP-1, ICAM-1, and phosphorylated STAT-1 and STAT-3 (33). Conditional STAT-1 ablation reveals the importance of IFN signaling for immunity to Listeria infection (42), although the effect is cell type–dependent (42, 43). Type I IFN is important for host defense against viral and bacterial infections, although the outcome of the IFN response highly depends on the timing of production, the cellular context, and type of pathogen (44). In this sense, it has been shown that low levels of type I IFN are required at an early stage during Listeria infection for the resolution of the infection (45). Type I IFN promotes DC activation, increasing the expression of costimulatory molecules, IL-12 secretion, and their potential to activate T cells (46). Conversely, type I IFN can also exert detrimental effects during Listeria infection. The main mechanism described to this harmful role relies on the induction of apoptosis, particularly of lymphocytes as well as macrophages (47, 48). It has been shown, using IFN-β reporter mice, that Tip-DCs are an important source of IFN-β during Listeria infection (49) as are CD11b+ DCs (50). This finding might suggest that type I IFN production can act as a mechanism of self-regulation by immune cells, which could be subverted by Listeria for its own profit. However, whether Tip-DCs as well as CD11b+ DCs are targets of type I IFN–induced cell death remains unclear (44). The IFN-β–dependent autocrine loop, initiated by interaction of IFN-β with IFNAR receptors, activates the transcription factor STAT-1 through IRF1, regulating the induction of inducible NO synthase and NO production (34). TNF-α activates an IFN-β–dependent autocrine loop, leading to sustained expression of STAT-1–dependent type I IFN (32). The high levels of TNF-α and NO produced by CD81−/− BMDCs after Listeria infection seem to be specific for live Listeria, and notably signaling through other TLRs was unaffected in CD81−/− DCs. Other studies have described the role played by tetraspanins in preventing signaling downstream of pathogen receptors. For example, CD37 regulates antifungal immunity by stabilizing the C-type lectin receptor dectin-1 on the cell surface of APCs and controlling dectin-1–mediated IL-6 production in response to fungal infection (51). Moreover, CD9 acts as a negative regulator of CD14TLR4 complex formation and signaling in macrophages, as shown by the increased macrophage infiltration and TNF-α production in LPS-treated CD9−/− mice (52). Additionally, CD9 associates with ADAM17 and negatively regulates the shedding activity of ADAM17 against its substrate TNF-α (53).

Rac plays a well-established role in the development and function of CD8α+ cDCs (23). Despite the critical role of CD8α+ cDCs in initiating T cell–mediated immunity, they are also a required entry point for productive Listeria infection (21, 35). Thus, Batf3−/− mice, which lack CD8α+ cDCs, exhibit an enhanced resistance to Listeria (21). Our results show that the efficient early infection of CD8α+ cDCs in CD81−/− mice increases survival. Unlike other phagocytes, such as neutrophils, macrophages, and CD8α− cDCs, CD8α− cDCs are highly efficient at Ag cross-presentation, due in part to their delayed phagosomal acidification and their limited...
proteolytic activity in endosomes. This efficient cross-presentation relies on Rac, because in the absence of Rac, CD80+ cDCs behave like other phagocytes in terms of phagosomal acidification and cross-presentation (23, 25). Contrasting observations that Rac activity is like other phagocytes in terms of phagosomal acidification and cross-presentation (23, 25). Contrasting observations that Rac activity is like other phagocytes in terms of phagosomal acidification and cross-presentation (23, 25). Contrasting observations that Rac activity is like other phagocytes in terms of phagosomal acidification and cross-presentation (23, 25).

...CD8+ T cell responses in vivo (55).

...are severely compromised in their ability to generate Listeria infection in vivo. Cross-presentation of exogenous Ags has been recognized as a major mechanism of induction of CD8+ T cell responses against virus and tumor Ags; however, the contribution of this mechanism on T cell immunity to bacteria in vivo is not well established. In this sense, it has been recently shown that DC cross-presentation is essential for immune responses to Listeria, because mice presenting a defective cross-presentation capacity are severely compromised in their ability to generate Listeria-specific CD8+ T cell responses in vivo (55).

In summary, our data reveal that CD81 plays a regulatory role during systemic Listeria infection by controlling different aspects of immune cell invasion through the modulation of downstream signaling pathways and the regulation of the presentation activity of DCs, which lead to the T cell–protective immunity. Our results suggest that tetraspansins could play a prominent role as novel therapeutic targets in the treatment of infectious diseases.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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In summary, our data reveal that CD81 plays a regulatory role during systemic Listeria infection by controlling different aspects of immune cell invasion through the modulation of downstream signaling pathways and the regulation of the presentation activity of DCs, which lead to the T cell–protective immunity. Our results suggest that tetraspansins could play a prominent role as novel therapeutic targets in the treatment of infectious diseases.

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Supplemental Figure 1. Analysis of leukocyte recruitment in spleens of WT and CD81-/- mice after *Listeria* infection.

(A) Bacterial load in spleens of WT and CD81-/- mice after infection with $10^3$ *Listeria* per mouse at the indicated time points (mean ± SD; of five mice per condition). Data are representative of two independent experiments with similar results. (B) Bar charts
represent absolute cell numbers of CD4+ T cells, CD8+ T cells and B cells from non-infected (day 0) and *Listeria*-infected WT and CD81-/- after day 1, 3 and 4 (mean ± SD of four mice per condition, three independent experiments with similar results). (C) Surface expression of CD81 in monocytes, Tip-DCs, conventional DCs and neutrophils after immunomagnetic depletion of T and B cells, at days 0, 2, 3 and 4 post-infection with 2x10^4 *Listeria* per mouse (mean ± SD of four mice per condition). Data are representative of three independent experiments with similar results.
Supplemental Figure 2. Production of pro-inflammatory cytokines and NO by wild-type and CD81-/- DCs stimulated with TLR ligands.
Wild-type and CD81−/− BMDCs were stimulated with live *Listeria* (A), flagellin (B), poly I:C (C) or LPS (D) for 16 h, and TNFα, IL-6 and NO were measured in cell supernatants by ELISA. Data are presented as the mean ± SD and are representative of 3 independent experiments with similar results.
Supplemental Figure 3. Expression of IFNβ mRNA in DCs after infection with

*Listeria in vitro.*

BMDCs from WT or CD81-/− mice, non-infected or infected with *Listeria* (moi = 10) for the indicated times, were analyzed for the expression of mRNA for IFNβ by real-time PCR, normalized to β-actin. Data are expressed as induction fold relative to non-infected control BMDCs (mean ± SD of triplicates, two independent experiments with similar results). RNA from BMDCs was extracted using the High Pure RNA Isolation kit (Roche, Mannheim, Germany). RNA was retro-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Real-time PCR
was performed using a FluoCycle SYBR Green mix (EuroClone, Milano, Italy) on an
ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Primer sequences (5’- 3’):

\textbf{IFNβ:}
\textit{IFNβ} for: TCAGAATGAGTGGGTTG
\textit{IFNβ} rev: GACCTTCAAATGCA

\textbf{β-actin:}
\textit{β-actin-for: GGCTGTATTTCCCCTCCATCG}
\textit{β-actin-rev: CCAGTTGGTAACAATGCCATGT}
Supplemental Figure 4. Model of the regulatory role of CD81 during the immune response against *Listeria*.

*Listeria* is initially recognized by TLR2 at the cell membrane and internalized into the phagosome. The TLR2/MyD88 signaling pathway induces the synthesis of inflammatory cytokines. *Listeria* is able to escape from the phagosome to the cytosol, inducing a signaling pathway driven mainly by the adaptor molecule STING upon activation by bacteria-derived cyclic di-nucleotides. This pathway triggers the synthesis of type I IFN, which induces an autocrine signaling pathway through the IFNAR receptor that involves STAT1-induced synthesis of NO. The absence of CD81 from the
cell membrane increases the activation of the GTPase Rac, which increases STAT1 phosphorylation and consequently the production of NO and other microbicidal mediators. The increased activation of Rac also affects ROS production, which contributes to the elimination of bacteria, and increases the capacity of DCs to cross-present antigens to specific CD8+ T cells. The combined effect of the increased levels of microbial mediators, elevated ROS production, and more efficient antigen cross presentation leads to a stronger immune response and more effective control of infection.