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Cutting Edge

Cutting Edge: Clec9A+ Dendritic Cells Mediate the Development of Experimental Cerebral Malaria

Lucia Piva,* Piotr Tetlak,* Carla Claser,† Klaus Karjalainen,* Laurent Renia,† and Christiane Ruedl*

Plasmodium infections trigger strong innate and acquired immune responses, which can lead to severe complications, including the most feared and often fatal cerebral malaria (CM). To begin to dissect the roles of different dendritic cell (DC) subsets in Plasmodium-induced pathology, we have generated a transgenic strain, Clec9A-diphtheria toxin receptor that allows us to ablate in vivo Clec9A+ DCs. Specifically, we have analyzed the in vivo contribution of this DC subset in an experimental CM model using Plasmodium berghei, and we provide strong evidence that the absence of this DC subset resulted in complete resistance to experimental CM. This was accompanied with dramatic reduction of brain CD8+ T cells, and those few cerebral CD8+ T cells present had a less activated phenotype, unlike their wildtype counterparts that expressed IFN-γ and especially granzyme B. This almost complete absence of local cellular responses was also associated with reduced parasite load in the brain. The Journal of Immunology, 2012, 189: 1128–1132.

The immune response during malaria infection has to be tightly controlled and regulated because an excessive and inappropriate immune activation can lead to severe pathology like cerebral malaria (CM), the most severe clinical manifestation of human P. falciparum infections. Mechanisms leading to severe malaria are still debated, but sequestration of parasitized erythrocytes in deep tissue microvasculature is thought to be an essential factor for CM to occur, which leads to obstruction and hypoxia of the surrounding brain parenchyma (1). However, even if tissue parasite burden in the brain appears to be a crucial indicator for the progress-fatality of the disease, there is growing evidence that systemic or local leukocytes and their secreted proinflammatory cytokines may be involved in CM pathogenesis (2, 3).

Several experimental CM (ECM) animal models have been established including the infection of mice with Plasmodium berghei ANKA (PbA) to investigate the still elusive cellular mechanisms underlying the pathogenesis of CM. In this model, sequestration of parasitized erythrocytes in the cerebral microvasculature, as well as intravascular accumulation of CD8+ T cells in the brain, clearly promotes the disease (3–5). In particular, CD8+ T cells contribute to the pathology of ECM most likely through perforin-dependent destruction of cerebral endothelial cells (6, 7), via granzyme B (GzmB) production (5), or via systemic and local secretion of proinflammatory cytokines such as IFN-γ or LT-α (3, 7, 8).

It is well established that dendritic cells (DCs) are not only the major APCs initiating malaria immunity, but also the APCs contributing to the pathogenesis of ECM. In fact, it was recently shown in vitro that PbA-expressed Ags are cross-presented by this DC subset, which subsequently can prime naive CD8+ T cell proliferation and CTL effector functions (11).

To investigate the contribution of the CD11c+CD8+ DC subset in the ECM immunopathology in vivo, we have generated a Clec9A-DTR transgenic mouse capable of ablating this subset on diphtheria toxin (DT) injection. Clec9A, also known as NK lectin group receptor-1 (DNGR-1), was selected because it is expressed at high levels on lymphoid CD11c+CD8+ DCs, on CD11c+CD103+ DCs and non-lymphoid organs, as well as in lower amounts on plasmacytoid DCs (pDCs) (12, 13). In this in vivo study, we demonstrate the direct role of CD11c+ Clec9A+ DCs in ECM development. In fact, ablation of this specific DC subset in PbA-infected mice resulted in complete protection from severe cerebral pathology. This resistance correlates with

*School of Biological Sciences, Nanyang Technological University, Singapore 637551; and Singapore Immunology Network, Agency for Science, Technology and Research, Singapore 138648

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Address correspondence and reprint requests to Prof. Christiane Ruedl, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551. E-mail address: Ruedl@ntu.edu.sg

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Abbreviations used in this article: BAC, bacterial artificial chromosome; CM, cerebral malaria; DC, dendritic cell; DT, diphtheria toxin; DTR, DT receptor; ECM, experimental CM; GzmB, granzyme B; PbA, Plasmodium berghei ANKA; PbA-GFP, PbA clone expressing GFP; pDC, plasmacytoid DC; RMCBS, rapid murine coma and behavior scale; WT, wildtype.

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the decrease of serum IFN-γ and GzmB expressing CD8+ T cells, and accumulation of parasitized erythrocytes in the brain.

**Materials and Methods**

**Trangenic DTR mouse strains, parasite, and infection**

Clec9A-DTR and Siglec-H–DTR transgenic lines were generated via BALB/c ES cells (generous gift of Dr. Birgit Ledermann, Novartis Pharma AG, Basel, Switzerland) transfected with recombined bacterial artificial chromosome (BAC) clones (Siglec-H: RP24-265E12; Clec9A: RP23-394L7; BACPAC Resources, Children’s Hospital Oakland) carrying insertions of human DTR sequence with its pA site in the initiation codons replacing the first coding exons of Clec9A or Siglec-H genes. Recombineering was performed using RED/ET recombination kits following the instructions of the manufacturer (Gene Bridges, Heidelberg, Germany). PI-Sce 1 linearized BACs were electro-porated in ES cells, and those ES cell clones containing intact BAC sequences, that is, both vector ends and middle modification verified by PCR, were selected for further transgenesis. Heterozygous mice were used to monitor the ablation efficacy on DT injection.

To obtain ECM-susceptible mouse strain, we generated F1 mice by crossing C57B6/J (B6) mice with wildtype (WT) BALB/c (control), homozygous Clec9A-, and Siglec-H–DTR, respectively (F1 BALB/c/B6, F1 Clec9A-DTR/ B6, and F1 Siglec-H–DTR/B6). For simplicity, these strains are referred in the text as WT, Clec9A, and Siglec-H, respectively.

Groups of 6- to 7-wk-old females/males were treated twice with 10 ng/g DT before infection and every 3–4 d during the infection period for a constant ablation of the targeted DC subsets (Supplemental Fig. 1B, right panel). DT injection completely eliminated CD11chighCD8+ DCs (but not CD11cintCD8+ DCs), whereas pDCs (CD11cintSiglec-H+) known to express lower levels of Clec9A were only partially depleted (~50%; Fig. 1A, lower panel; Fig. 1B, right panel). However, similar injections of DT in heterozygous Siglec-H–DTR mice led to 100% ablation of the CD11cintSiglec-H+ pDCs population without affecting the conventional CD11cintCD8+ DCs (Fig. 1A, lower panel; Fig. 1B, right panel).

**Preparation and analysis of tissue mononuclear cells**

Spleen single-cell suspension was obtained as described in Ruedl et al. (16). For brain-sequestered leukocyte analysis, mice were perfused intracardially with PBS, brains were digested with collagenase, and single-cell suspension was obtained by homogenization through a needle followed by a 33% Percoll gradient. For quantification of accumulation of parasitized erythrocyte in the brain, brains were minced and homogenized after perfusion. After Percoll centrifugation, the cells were contaminated with Hoechst and anti-CD45.2.

For intracellular cytokine staining, cells were incubated in brefeldin A for 3 h at 37°C before proceeding with cell surface staining, fixation/ permeabilization, and subsequent intracellular staining.

**Brain histology**

Brains dissected from infected and uninfected mice were fixed in 4% phosphate-buffered paraformaldehyde for 48 h, dehydrated, and embedded in paraffin. Five-micrometer sections were stained with H&E and analyzed by light microscopy.

**Statistical analysis**

Comparison between two groups was performed using the Student t test or Mann–Whitney U test, depending on whether data followed a normal distribution. Survival curves were analyzed using the Mantel–Cox logrank test. Statistical significance was accepted at p < 0.05. Data were analyzed with GraphPad Prism software.

**Results and Discussion**

**CD11cintCD8+ DCs can be efficiently ablated in Clec9A-DTR mice**

We selected Clec9A for our target locus because it is expressed on CD11cintCD8+ DCs (Supplemental Fig. 1A) (12). Because Clec9A is also weakly expressed on pDCs (12), we have generated, in addition, Siglec-H–DTR transgenic mice using a similar BAC recombineering strategy. This mouse strain allows us to ablate only pDCs, and hence it provides us an extra “specificity” control for this study.

Pleasingly, heterozygous Clec9A-DTR mice in BALB/c genetic background ablated efficiently the splenic target cells as shown by flow cytometry and absolute cell counts (Fig. 1A, upper panel; Fig. 1B, left panel). DT injection completely eliminated CD11cintCD8+ DCs (but not CD11chighCD8+ DCs), whereas pDCs (CD11cintSiglec-H+) known to express lower levels of Clec9A were only partially depleted (~50%; Fig. 1A, lower panel; Fig. 1B, right panel). However, similar injections of DT in heterozygous Siglec-H–DTR mice led to 100% ablation of the CD11cintSiglec-H+ pDCs population without affecting the conventional CD11chighDC8+ DCs (Fig. 1A, lower panel; Fig. 1B, right panel).

**Clec9A+ DCs control susceptibility of mice to ECM**

To investigate the role of Clec9A+ DCs in ECM, we first monitored the ablation efficiency in the F1 mouse strains (see Materials and Methods). After having confirmed the effective ablation of the targeted DC subsets (Supplemental Fig. 1B, 1C), 10 ng/g DT was injected on days −1 and −2, and mice were subsequently infected with PbA-GFP. Survival, neurologic symptoms, as well as parasitemia were monitored and scored over a period of 20 d. Unlike most control mice, which developed clear signs of ECM and started to die between days 7 and 10 postinfection (~75%), DT-treated Clec9A-DTR mice showed no visible clinical symptoms of ECM by the
applied RMCBS scoring system, and none of them died during the ECM critical period (Fig. 2A, 2B). No significant differences in parasitemia were observed during the first 12 d postinfection in surviving mice (Fig. 2C). Although control brains showed, as expected, intravascular accumulation of parasitized erythrocytes and leukocytes and large areas of hemorrhage, brains collected from Clec9A-depleted mice did not show any of these severe signs (Fig. 2D). To exclude the contribution of Clec9Alow pDCs in ECM, we infected also Siglec-H–DTR mice. Unlike the Clec9A-DTR, Siglec-H–DTR mice developed ECM and the course of the disease was comparable with WT mice (Fig. 2A–C, triangles). These results indicate that only depletion of CD11chighClec9Ahigh DCs and not of the CD11cintClec9Alow pDCs leads to ECM resistance.

Because proinflammatory cytokines such as IFN-γ are essential for ECM to occur, we next investigated whether CD11chighClec9A+ DCs regulate its levels (Fig. 2E). Serum obtained at days 4 and 6 postinfection from Cleac9A-depleted mice showed significantly reduced levels of IFN-γ, suggesting that CD11chighClec9A+ DCs regulate systemic IFN-γ levels, one of the essential immune mediators in ECM. To dissect further the cellular mechanisms involved in ECM, we next focused only on Clec9A-DTR mice that are clearly protected from ECM.

Reduced splenic T cell activation in absence of Clec9A+ DCs after PbA infection

During ECM, parasite-specific T cells are primed by DCs in the spleen and are found in the brain at the onset of the neurologic symptoms (9, 17). To investigate whether there is a difference in splenic T cell priming in the absence of Clec9A+ DCs during PbA infection, we analyzed the expression of CD69 on CD4+ and CD8+ T cells at day 4 postinfection in WT and Clec9A DTR mice. As shown previously (18), the fraction of spleen CD69+ T cells is gradually increasing during PbA infection, reaching a peak at day 4. Similarly, we found 17 and 25% of WT CD4+ and CD8+ T cells being CD69+ after 4 d of PbA infection (Fig. 3A, middle panel). However, in the absence of Clec9A+ DCs, CD69-bearing CD4+ and CD8+ T cells were significantly lower or comparable with T cells obtained from uninfected mice (Fig. 3A, 3B), suggesting a direct involvement of this particular DC subset in activating splenic T cell responses.

Ablation of Clec9A+ DCs decreases dramatically the numbers of brain CD8+ T cells after PbA infection

ECM is characterized by an influx of leukocytes and, in particular, CD8+ T cells in the brain when mice display distinct neurologic signs (5, 7, 17). Because the Clec9A DTR mice are ECM resistant, we monitored cerebral accumulation of different leukocytes such as NK, NKT, CD4+ T, and CD8+ T cells in WT and Clec9A-DTR mice at day 7 postinfection. Interestingly, DT-treated Clec9A-DTR mice showed significantly decreased cerebral accumulation especially of CD8+ T cells but also CD4+ T cells and NKT cells, whereas the absolute numbers of NK cells were comparable between the two groups (Fig. 4A).

In ECM, the majority of brain-recruited CD8+ T cells show an activated phenotype, secrete IFN-γ, and express high levels of GzmB. Therefore, we measured the production of both IFN-γ and GzmB by CD8+ T cells present in the brain of PbA WT and Clec9A-infected mice. Seven days postinfection, a large portion of CD8+ T cells obtained from WT mice...
secreted IFN-γ and strongly expressed GzmB. In comparison, those few CD8+ T cells detectable in ablated mice showed lower IFN-γ and GzmB levels (Fig. 4B). Clearly, the almost total absence of cerebral CD8+ T cells and the poor activation status of those few that were present can explain the ECM resistance in Clec9A-ablated mice.

Decreased brain parasite load in the absence of CD11chighClec9A+ DCs

Although CD8+ T cells are necessary for ECM pathology, they are per se not sufficient to induce pathology unless parasitized erythrocytes are sequestered in brain microvasculature (3, 4). It is clear that an interdependent relationship between brain parasite burden and cerebral effector GzmB-producing CD8+ T cells, as well as IFN-γ, is the prerequisite for ECM occurrence (3–5). Therefore, we measured the cerebral parasite burden in perfused brains of PbA-GFP–infected WT mice and in Clec9A-DTR transgenic mice. Interestingly, flow cytometry analysis showed a greater proportion of mature (HoechsthighGFPhigh) but not young (HoechstlowGFP+) parasitized erythrocytes in the brains of infected WT (Fig. 4C, 4D). This indicated that sequestration of mature parasitized erythrocytes is reduced in the absence of CD11chighClec9A+ DCs. The lower numbers of parasitized erythrocytes in the brains of PbA-infected Clec9A DTR mice are most likely due to the reduced numbers of infiltrating cerebral IFN-γ–producing CD8+ T cells because parasite sequestration in the brain is dependent on CD8+ T cells and IFN-γ (3, 19).

Our in vivo results are consistent with previously obtained in vitro data suggesting that CD11chighCD8+ DCs are crucial cross-presenting cells in stimulating CD8+ T cells on malaria infection (11). CD11chighClec9A+ DCs are anatomically optimally located together with marginal zone macrophages in the marginal zone of the spleen (20), a location that allows an efficient uptake of the parasitized erythrocytes from the blood. However, different from macrophages, which have a specific function to digest and clear parasitized erythrocytes (21), CD11chighClec9A+ DCs possess the unique capacity to cross prime efficiently CD8+ T cell responses, which can lead to a severe acute cerebral pathology.

The identification of a human counterpart, the CD141+ DC subset with similar cross-priming characteristics to those of the mouse CD11chighClec9A+ DCs (22, 23), may suggest a potential role of this cell type during human malaria infections. As this particular DC subset was recently suggested as an attractive clinical target to enhance CTL cross-priming in tumor therapy (24), this could also be considered as a possible target in the development of immunotherapies to prevent severe malaria.

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Disclosures
The authors have no financial conflicts of interest.

References
Suppl. Fig. 1.

In vivo ablation of DC subsets (A) Representative dot plots showing the co-expression of Clec9A and CD8 on CD11c^{high} DCs and its ablation in DT treated Clec9A-DTR mice. (B) Representative dot plots showing the expression of CD11b and CD8 on CD11c^{high} DCs in DT treated F1 BALB/c/B6, F1 Clec9A-DTR/B6 and F1 Siglec-H-DTR/B6. (C) Representative dot plots showing the expression of CD11c and Siglec-H in DT treated F1 BALB/c/B6, F1 Clec9A-DTR/B6 and F1 Siglec-H-DTR/B6.
Suppl. Fig. 1