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Expression of the self-marker CD47 on dendritic cells governs their trafficking to secondary lymphoid organs

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Dendritic cells (DCs) capture and process Ag in the periphery. Thus, traffic through lymphatic vessels is mandatory before DCs relocate to lymph nodes where they are dedicated to T-cell priming. Here, we show that the ubiquitous self-marker CD47 selectively regulates DC, but not T and B cell trafficking across lymphatic vessels and endothelial barriers *in vivo*. We find an altered skin DC migration and impaired T-cell priming in CD47-deficient mice at steady state and under inflammatory conditions. Competitive DC migration assays and active immunization with myeloid DCs demonstrate that CD47 expression is required on DCs but not on the endothelium for efficient DC trafficking and T-cell responses. This migratory defect correlates with the quasi-disappearance of splenic marginal zone DCs in nonmanipulated CD47-deficient mice. Nonetheless, CCR7 expression and CCL19-driven chemotaxis remain intact. Our data reveal that CD47 on DCs is a critical factor in controlling migration and efficient initiation of the immune response.

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Introduction

Dendritic cells (DCs), sentinels of the immune system, are unique in their capacity to coordinate innate and adaptive immune responses. Upon encounter with pathogens or danger signals, DCs adapt their chemokine receptors and migrate to the lymph nodes (LN) where they transfer their information to T cells and initiate T-cell differentiation and polarization (Palucka and Banchereau, 2002; Steinman *et al*, 2003). The trafficking of DCs through lymphatic vessels (LV) is an obligatory route for the accomplishment of their functions (Randolph *et al*, 2005a). In homeostatic and inflammatory conditions, DC mobilization is under the control of CCR7, although additional signals that include leukotrienes, prostaglandin E2 and CD38 usually found at sites of inflammation, appear to be required to sensitize CCR7 to its ligands (Randolph *et al*, 2005b). Activated DCs are first attracted to the connective tissue by CCL21 expressed by lymphatic endothelial cells until they enter the afferent lymph and produce CCL19, a chemokine that promotes their final maturation (Bachmann *et al*, 2006).

Yet, a large gap persists in our understanding of the regulation of DC interactions with the LV, their passage across the endothelial barrier and finally their exit and relocation to LN, where they encounter Ag-specific T cells. Another important issue relates to how DCs egress from the bloodstream to penetrate lymph nodes through high endothelial venules (HEV) (Randolph *et al*, 2005a). Adhesion (ICAM), junctional (JAMA-1) and extracellular matrix (SPARC) proteins expressed by the host environment or DCs themselves favor DC retention in the peripheral tissues (Xu *et al*, 2001; Cera *et al*, 2004; Sangaletti *et al*, 2005).

A link exists between CD47/signal regulatory proteins (SIRP)-α interactions and neutrophil transepithelial migration *in vitro* (Liu *et al*, 2002; Zen and Parkos, 2003). Neutrophil mobilization is delayed *in vivo* in CD47-deficient mice after intraperitoneal inoculation of *Escherichia coli* (Lindberg *et al*, 1996). The CD47 Ag (integrin-associated protein) is a highly hydrophobic and unusual pentaspanning transmembrane protein that is physically and functionally associated with αvβ3 integrin, the vitronectin receptor (Brown and Frazier, 2001). Ubiquitously expressed on hematopoietic and nonhematopoietic cells, CD47 serves both as a receptor for the extracellular matrix protein thrombospondin-1 (TSP) and as a ligand for the transmembrane SIRP-α and -γ (Gao *et al*, 1996; van Beek *et al*, 2005). CD47 is considered to be a marker of self in immune and nonimmune cells *in vivo*, as it delivers a negative signal to SIRP-α expressed on resident macrophages or DCs, thus inhibiting the clearance of intact hematopoietic cells (Oldenborg *et al*, 2000; Gardai *et al*, 2005). Interaction of CD47 with either SIRP-α or TSP molecules regulates several important biological phenomena, including cell–cell communication, macrophage multinucleation, neuronal survival, maintenance of lung homeostasis as well as DC maturation and their pro-inflammatory cytokine production (Cant and Ullrich, 2001; Latour *et al*, 2001).

Considering that interference with CD47/SIRP-α interactions by means of specific antibodies or respective fusion proteins favors Langerhans cell (LC) retention in the epidermis under inflammatory conditions (Fukunaga *et al*, 2004; Yu *et al*, 2006), we hypothesized that CD47 deficiency affects the mobilization of SIRP-α-expressing myeloid DCs of the immune system, that is, LC, dermal DCs and $CD11b^+CD11c^+$ cells, and thus regulates the T-cell priming that ensues (Edwards *et al*, 2003; Fukunaga *et al*, 2004). We investigated the impact of CD47 expressed in DCs or as part of the host

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environment on their trafficking *in vivo*. We report that the lack of CD47 curtails *in vivo* skin DC migration and the Ag-specific T-cell response under steady state and inflammatory conditions. We demonstrate that CD47 expression is dispensable on the endothelium but its expression on DCs themselves is essential for their passage across HEV and vascular sinusoids prior their relocation to the LN and the spleen, respectively. *In vivo*, the impaired DC migration is reflected by a profound defect in myeloid CD11b$^+$CD4$^+$ DCs of the splenic marginal zone. Thus, we propose CD47 molecule as a positive self-regulator on myeloid DCs for the control of their trafficking to secondary lymphoid organs.

Results

CD47 is required for DC entry into dermal lymphatic vessels

CD47 homophilic and/or CD47/SIRP-α interactions appear to be involved in leukocyte trafficking (Liu *et al*, 2004b; Rebres *et al*, 2005). Whether these interactions are also implicated in DC migration and which is the precise step of DC trafficking affected by CD47 deficiency remains to be elucidated. We thus examined the role of CD47 expression for LC emigration from the epidermis to the LN. We quantified the number MHC I-E$^+$ cells in freshly isolated epidermal sheets and first noticed a slight reduction in LC density (10%) ($P<0.01$) in CD47$^{-/-}$ skin (Figure 1A, top). After *in vitro* culture of the skin, the epidermis was separated from the dermis to examine LC morphology and distribution (Figure 1A, middle and bottom panels). LCs acquired a round shape in the epidermis of both mice. Whereas CD47$^{+/+}$ DCs accumulated along LV forming dermal cord structures, CD47$^{-/-}$ DCs were distributed evenly within the dermis. The absence of dermal cord formation in the CD47-deficient mice suggests that CD47 is required for DC entry into LV. We observed a significant decrease in the accumulation of skin-derived DCs in the CD47$^{-/-}$ LN (Figure 1B and C). As the CD11c$^+$IE^{high} subset may represent activated resident DCs, we examined CD40 expression and found a significant reduction in the proportion and absolute number of both dermal (CD11c^{low}CD40$^+$, I) and epidermal (CD11c^{high}CD40$^+$, II) skin-derived DCs. However, CCR7 expression on CD11c$^+$IE^{high} and CD11c$^+$CD40^{high} DCs was not significantly affected by the lack of CD47 expression. The proportion of resident CD8$^+$DEC205$^+$ DCs was comparable in both strains of mice, but the total DC number was significantly reduced in CD47-deficient mice. This reflected a decrease in CD11c^{low}B220 DCs (Supplementary Figure S1).

To further clarify the impairment of DC trafficking across the LV to the LN, we examined the impact of CD47 deficiency on the Ag-specific response of passively transferred DO11.10 TCR transgenic CD4$^+$ T cells following subcutaneous (s.c.) immunization with OVA protein in the absence of adjuvant (Figure 2A). T-cell proliferation, measured by CFSE dilution on day 2, was impaired in the ipsilateral LN in CD47-deficient compared to BALB/c mice. No T-cell priming was detected in the contralateral LN owing to specificity of the response (data not shown). The decreased T-cell priming was observed at 72 h whether mice were immunized with 1 or 10 μg OVA, as demonstrated by the percent of undivided cells. Note that a similar proportion of transferred naïve T cells were recovered in both strains of unmanipulated mice, suggesting that the CD47-deficient environment did not hamper their migration to LN (Figure 2C).

Altogether, the observed impairment of dermal cord formation, decreased accumulation of skin-derived DCs in the LNs and inefficient T-cell priming in CD47-deficient mice support the hypothesis of altered skin DC migration.

CD47/SIRP-α interactions are required for skin DC migration under inflammatory conditions

To examine how the absence of CD47 influences DC trafficking under inflammatory conditions, mice were immunized with OVA peptide in IFA 1 day after passive transfer of

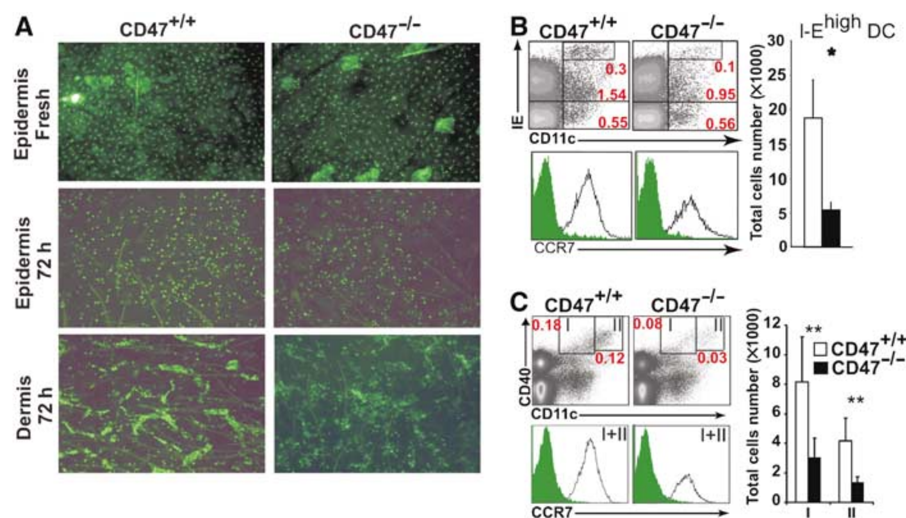

Figure 1 Effect of CD47 deficiency on the distribution and mobilization of LCs and skin DCs. (**A**) Epidermal sheets were prepared from untreated CD47$^{-/-}$ and CD47$^{+/+}$ mice and stained with MHC I-E mAb (top panel). After 72 h culture, the epidermis (middle panel) was separated from the dermis (bottom panel) and stained with I-E mAb. The data represent one of three individually analyzed mice. (**B**, **C**) LN cell suspensions were analyzed for the expression of CD11c, I-E and CD40. The proportion of CD11c$^+$I-E^{high} cells (B) and of each CD11c$^+$CD40$^+$ subset (I, dermal DCs and II, epidermal DCs) (C) is shown. CCR7 staining was examined for CD11c$^+$ I-E^{high} (B, lower panel) and skin-derived DCs (I + II) (C, lower panel). Dot plots are representative of five individually analyzed LN from separate mice. Bars represent absolute number (mean ± s.d., $n=5$ mice) of CD11c$^+$IE^{high} cells and CD11c$^+$CD40$^+$ DC subsets. *$P<0.05$, **$P<0.01$.

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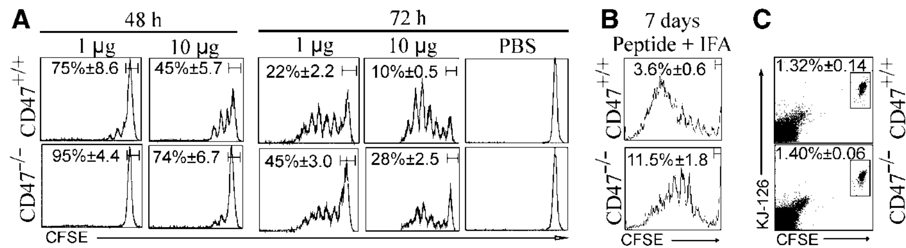

Figure 2 Impaired T-cell response in CD47$^{-/-}$ mice under homeostatic and inflammatory conditions. (**A**, **B**) One day after adoptive transfer of CFSE-labelled CD4$^+$ DO11.10 T cells into CD47$^{+/+}$ and CD47$^{-/-}$ mice, the recipients were immunized s.c. into the left footpad with 1 or 10 μg OVA protein (A) or 10 μg OVA peptide with IFA (B). PBS was injected into right footpad. Proliferation of CD4$^+$ T cells was measured by CFSE dilution in the draining LN after 48 or 72 h (A) and 7 days (B). CFSE profile (% of undivided cells, mean±s.e.m.) is representative of five individually analyzed LN from separate mice. (**C**) Proportion of CFSE-labelled CD4$^+$ DO11.10 T cells (KJ-126$^+$ cells) recovered from naive CD47$^{+/+}$ and CD47$^{-/-}$ mice after 24 h.

transgenic T cells and T-cell priming was examined at day 7 (Figure 2B). At this time point, T-cell proliferation continued to be decreased in CD47$^{-/-}$ hosts demonstrating that CD47 deficiency has a negative impact on the priming of T cells under inflammatory conditions. By using an OVA peptide, we also ascertained that impaired Ag processing did not account for the altered T-cell response.

Cutaneous sensitization with FITC is an inflammatory stimulus commonly used to induce a robust migration of skin DCs, along with other immune cells, into draining LN (Figure 3A). However, LN cellularity as well as T and B cell number remained unchanged in CD47-deficient mice after FITC painting (Figure 3A). The absence of immune cell recruitment to the LN is paralleled by a significant decrease in the proportion and accumulation of DCs carrying FITC (Figure 3B and C). The early (i.e. 24 h) and late (i.e. 72 h) defects in DC migration delineated a decrease in the accumulation of dermal DCs and LCs, respectively (Kissenpfennig *et al*, 2005). From 24 to 72 h after FITC painting, we observed a 3.1- and 5.3-fold increase in the recovery of CD47$^{-/-}$ and CD47$^{+/+}$IE$^+$FITC$^+$ DCs, respectively, in the LN. These data suggest that the defect in migration is more pronounced for LCs than dermal CD47$^{-/-}$ DCs.

To examine the contribution of CD47 to SIRP-α interactions in BALB/c mice, intradermal injections of CD47-Fc or control fusion protein were performed 1 h before FITC application. CD47-Fc treatment significantly reduced the accumulation of epidermal (72 h) and dermal (24 h) IE$^+$FITC$^+$DCs in BALB/c mice (Figure 3C, data not shown). The results were expressed as a ratio of IE$^+$FITC$^+$DCs/Total IE$^+$DCs in the LN to eliminate independent variability owing to differences in LN cellularity in the two strains of mice. In contrast to BALB/c mice, pretreatment with CD47-Fc did not further inhibit DC migration at any time point in CD47-deficient mice (Figure 3C). These data demonstrate that CD47-Fc probably disrupted CD47/SIRP-α interactions between DCs and their environment, rather than delivering a negative signal via SIRP-α ligation on DCs and/or the endothelium.

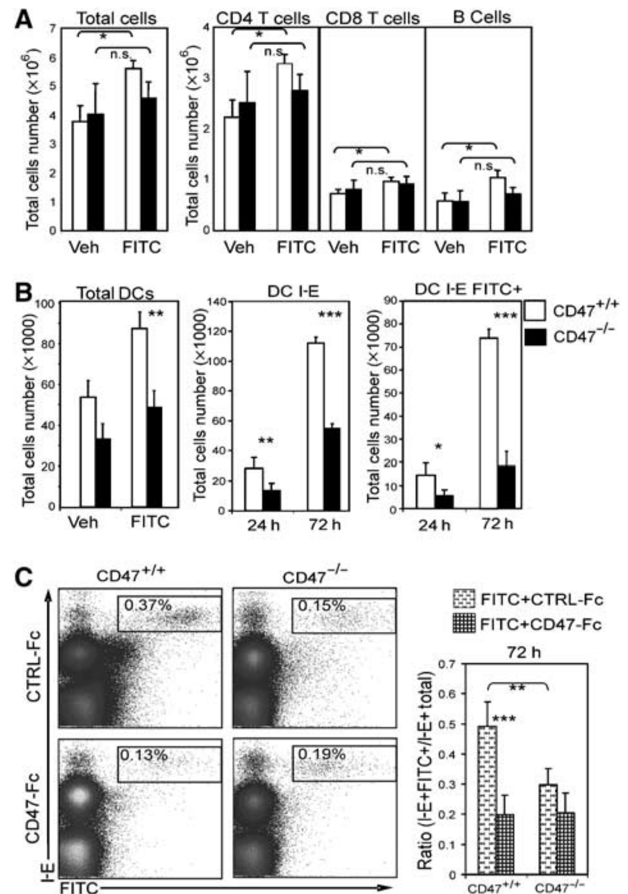

Figure 3 CD47/SIRP-α requirement for skin DC migration under inflammatory conditions. LN cell recovery 24 h after FITC painting is shown for various cell subsets (**A**). Relative number of total DCs (24 h), MHC-I-E$^+$ and MHC-I-E$^+$ carrying FITC (**B**). Proportion of MHC-I-E$^+$ FITC$^+$ DCs in LN (72 h) of mice injected intradermally with CD47-Fc or CTRL-Fc. Sixty minutes before FITC challenge (**C**). For each panel, data are representative of at least five individually analyzed LN from separate mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Decreased trafficking of CD47$^{-/-}$ DCs across lymphatic vessels

In vitro transepithelial migration of leukocytes requires CD47 to be expressed on the epithelium side and CD47 overexpression enhances this passage (Liu *et al*, 2004a; Rebres *et al*, 2005). We thus examined whether a similar requirement applied to *in vivo* transendothelial DC migration. To investigate the importance of CD47 as part of the host environment for DC trafficking, OVA peptide-pulsed bone-marrow-derived (BM) CD47$^{+/+}$ DCs were administered s.c. to CD47$^{+/+}$ and CD47$^{-/-}$ mice in which CFSE transgenic T cells had been adoptively transferred (Figure 4A). The T-cell response was comparable in the two types of mice and directly correlated with the number of injected DCs (Figure 4A, top and middle

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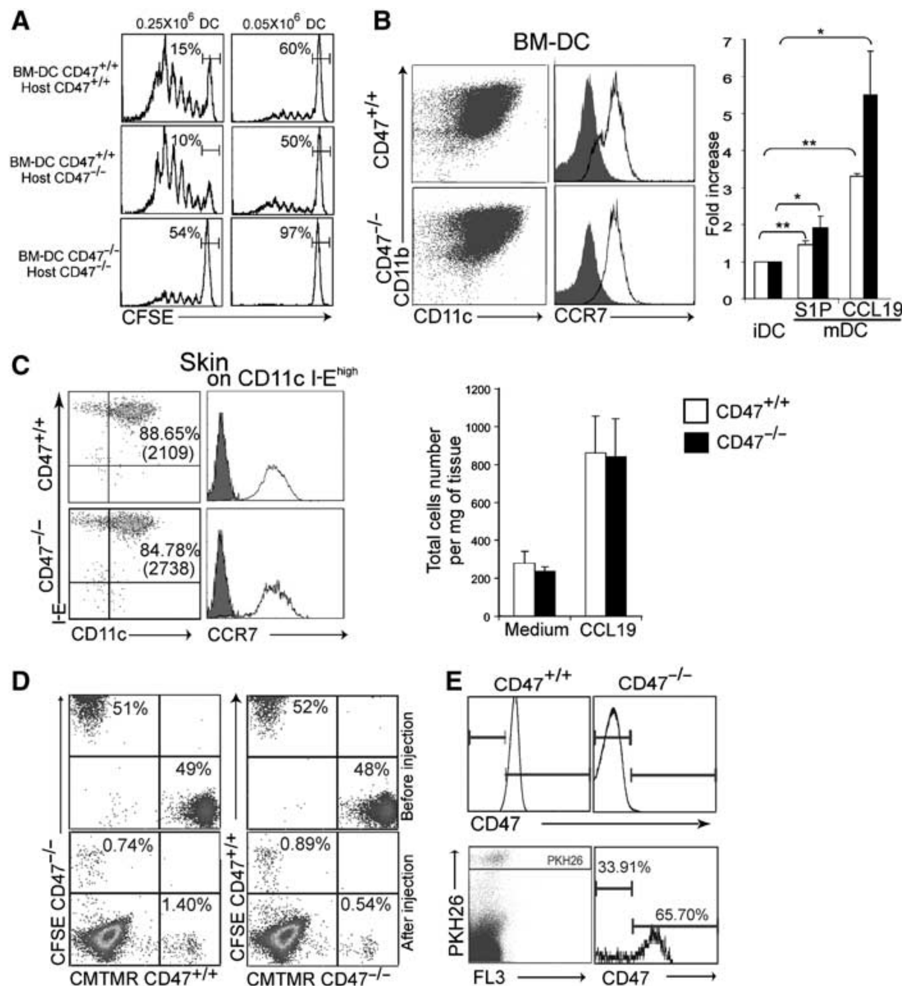

Figure 4 Decreased CD47$^{-/-}$ DC trafficking across lymphatic vessels. (**A**) 0.25 and 0.05×10^6 CD47$^{+/+}$ and CD47$^{-/-}$ LPS-activated and OVA-pulsed BM-DCs were injected s.c. into the footpad 24 h after adoptive transfer of CFSE-labelled transgenic CD4$^+$ T cells. Proliferation of CD4$^+$ T cells in the draining LN was analyzed after 72 h. Data are representative of four individually analyzed mice. (**B**) Activated BM-DCs were analyzed for CD11b and CCR7 expression and their *in vitro* migration toward CCL19 and S1P in transwell chambers. Dot plots and histograms represent one of four BM-DC preparations. Bars represent fold increase DC migration compared to immature DCs (mean±s.d., $n=5$ mice). (**C**) Ears from four to ten mice were pooled to determine the proportion (MFI, in parenthesis) of I-E$^+$ cells emigrated into the tissue culture medium after 72 h. CCR7 staining is shown for CD11c$^+$I-E^{high} DCs. Bars represent the number of IE$^+$ cells/mg of tissue cultured in the absence or presence of CCL19 (mean±s.d., $n=3$ pools of mice). (**D**) For the competitive migration assay, activated CD47$^{+/+}$ and CD47$^{-/-}$ BM-DCs were stained with CFSE or CMTMR and vice versa, and mixed at a 1:1 ratio before s.c. injection into mice. Top panel; a mixture of cells before injection. Bottom panel; cells were isolated from LN on day 2 and retraced (**E**) CD47$^{+/+}$ and CD47$^{-/-}$ activated BM-DC were stained with PKH-26 and injected s.c. in CD47$^{-/-}$ footpad, then traced by staining with anti-CD47 antibody after gating on PKH-26-positive cells. Upper panel shows negative and positive CD47 staining of a BALB/c LN. For panels D and E, data are representative of at least five individually analyzed mice. *$P<0.05$, **$P<0.01$.

panels). Indeed, a five-fold reduction in the number of BM-CD47$^{+/+}$ DCs (0.05 versus 0.25×10^6) resulted in an equivalent decrease in T-cell response. These results indicated that the absence of CD47 in the host did not influence the efficiency of CD47$^{+/+}$ DC migration and the quality of the ensuing T-cell response. The next series of experiments were then exclusively performed in CD47-deficient hosts, a mandatory setting considering that injected CD47$^{-/-}$ cells were readily eliminated from BALB/c mice as a result of the lack of an inhibitory signal delivered through SIRP-α (Gardai *et al*, 2005). When compared to CD47$^{+/+}$ BM-DCs, CD47$^{-/-}$ BM-DCs yielded a strong decrease in the magnitude of the T-cell response in CD47$^{-/-}$ mice. In fact, the number of undivided T cells was comparable for CD47$^{-/-}$ mice immunized with either 0.25×10^6 CD47$^{-/-}$ or 0.05×10^6 CD47$^{+/+}$ BM-DCs. Therefore, inefficient T-cell priming *in vivo* is observed only when CD47 is absent on the DC and not in the endothelium or in the microenvironment.

As CD47$^{+/+}$ DCs primed naive transgenic T cells *in vitro* as efficiently as CD47$^{-/-}$ DCs (Supplementary Figure S2), the altered T-cell priming observed *in vivo* could result from an impaired DC response to chemokines or transmigration through the endothelial barrier. It was thus essential to ascertain the *in vitro* migratory capacity of these two types of DCs to chemo-attractants. We found that CD47$^{+/+}$ and CD47$^{-/-}$ DCs displayed equivalent amounts of CCR7 and chemotaxis to CCL19 (Figure 4B). Sphingosine-1-phosphate (S1P) also controls egress of lymphocytes from the LN and is a CCR7-independent mediator of mature DC migration (Lan *et al*, 2005). We found no difference in the *in vitro* migratory potential of the two types of DCs toward S1P. Similarly, CD47$^{+/+}$ and CD47$^{-/-}$ DCs isolated from *ex vivo* skin

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explants migrated efficiently toward CCL19. CCR7 expression and cell recovery of CD11c^{+}IE^{high} cells were comparable in both mice (Figure 4C).

Taken together, these data strongly support the concept of an altered passage of CD47$^{-/-}$ DCs through LV rather than a defect in chemotaxis toward CCL19. The following experiments were designed to directly and concomitantly trace the *in vivo* migration of CD47$^{+/+}$ and CD47$^{-/-}$ DCs in CD47-deficient hosts (Figure 4D). Each BM-DC population was labelled with CFSE or CMTMR, respectively, and *vice versa* before s.c. injection at a 1:1 ratio (top panel). On day 2, DCs were found in the draining LN in the proportion of one CD47$^{-/-}$ to two CD47$^{+/+}$ DCs (bottom panel). As a complementary approach to visualize the differential DC migration, both types of DCs were labelled with the same red fluorescent vital dye (i.e. PKH-26), coinjected at 1:1 ratio and retraced in the LN by CD47 staining (Figure 4E). On day 2, the recovery of CD11c^{+}CD47^{+}cells gated on PKH-26 positive cells was 66% $\pm$4.1 (mean$\pm$s.d. of four experiments). These two competitive *in vivo* migration assays performed in a CD47-deficient host demonstrate that CD47 must be expressed on the DC to allow for efficient lymphatic transendothelial trafficking, but is dispensable on the endothelium itself.

The selective altered CD47$^{-/-}$ DC migration is correlated with a depletion of marginal zone DCs in the spleen

As CD47 expression on DCs was required to gain entry into the LV and prime T cells in the LN, we hypothesized that it was also critical for DCs to exit the bloodstream and travel across the endothelial barriers to enter the spleen. BM-DCs were administered intravenously (i.v.) into mice passively transferred with transgenic T cells 1 day before and the T-cell response was examined at day 3. As depicted in Figure 5A, CD47 expression was dispensable on the endothelium for migration into the spleen as CD47$^{+/+}$ BM-DCs efficiently primed T cells in both strains of mice. In contrast, i.v. injection of CD47$^{-/-}$ DCs resulted in impaired T-cell priming in CD47$^{-/-}$mice. Note that no T-cell response occurred when CD47$^{-/-}$ DCs were injected in BALB/c mice because of their elimination. *In vivo* competitive migration assays in CD47-deficient hosts further showed that the absence of CD47 on DCs led to a decrease in their recovery from the spleen. However, we demonstrated that the altered entry into the spleen was rather selective for CD47$^{-/-}$ DCs, as we observed a comparable recovery of CD47$^{-/-}$ and CD47$^{+/+}$ T and B cells (Figure 5B).

The faulty *in vivo* migration of the *in vitro* generated CD47$^{-/-}$ BM-DCs was taken as a strong argument in favor of their inefficient trafficking through vascular sinusoids in the spleen. In support of this concept, we next demonstrated in unmanipulated CD47-deficient mice, a drastic reduction in the proportion and the accumulation of splenic CD11b^{high}CD11c^{high} myeloid DCs that correlated with a significant increase of immediate DC precursors in the blood (Figure 6A). In fact, the absolute number of CD11c^{high} DCs was significantly diminished; more specifically, the CD4^{+}CD11c^{high} subset was virtually absent in CD47-deficient mice, whereas the number of CD8^{+}CD11c^{high} and plasmacytoid DCs (120G8 positive cells) remained unchanged as it was weighed down by a 30% decrease in the cellularity of the spleen (Figure 6B and C). By immunohistological techniques, we observed that the marginal zone was almost devoid of CD11c staining, the precise location of the CD4^{+}CD11c^{high} subset. The number of DCs in T-cell areas, mostly comprised of CD8^{+}CD11c^{high} was similar in both strains of mice (Figure 6D). Of interest, the CD4^{+} DC subset expressed the lowest quantity of CD47 in BALB/c mice suggesting that the regulation of CD47 expression may be critical for the localization of this DC subset (Supplementary Figure S3).

At this point, one may speculate that there is a selective developmental defect of the CD4^{+} DC subset in CD47$^{-/-}$ mice. Because BM chimera experiments are not feasible in CD47$^{-/-}$ mice (Blazar *et al*, 2001), we relied on *in vitro* and *in vivo* DC differentiation experiments to disprove such a hypothesis. No defect was observed during BM-DC differentiation *in vitro* with GM-CSF (Figure 4 and data not shown) and the analysis of DC subsets after *in vivo* administration of Fms-like tyrosine kinase 3 ligand (Flt3-L), known to promote the differentiation and the expansion of both CD4^{+} DC and CD8^{+} DC subsets, showed that the number of progenitors and total DCs recovered in the BM and blood was comparable in Flt3-L-treated CD47$^{+/+}$ and CD47$^{-/-}$ mice. However, the reduction in CD4^{+} DCs was maintained in the spleen of Flt3-L-treated CD47 mutants (Supplementary Figure S4).

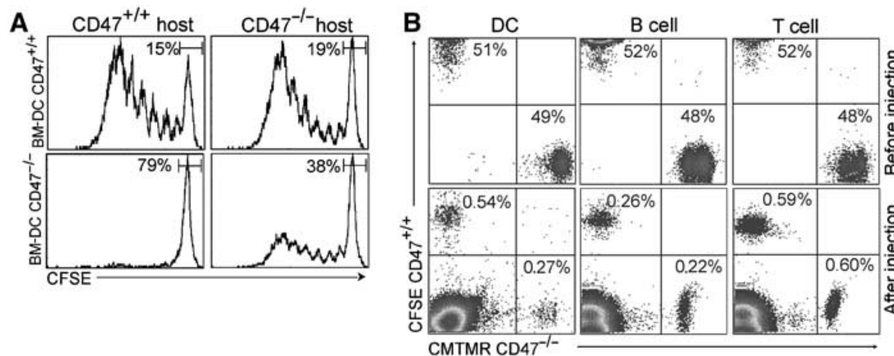

Figure 5 Selective altered CD47$^{-/-}$ DC transendothelial migration. (**A**) 0.5×10^6 CD47$^{+/+}$ and CD47$^{-/-}$ LPS-activated and OVA-pulsed BM-DCs were injected i.v. 24 h after adoptive transfer of CFSE-labelled transgenic CD4^{+} T cells. Proliferation of CD4^{+} T cells in the spleen was analyzed after 72 h. (**B**) For the competitive migration assay, activated CD47$^{+/+}$ and CD47$^{-/-}$ BM-DC, and unfractionated B and T cells were stained with CFSE or CMTMR and mixed at 1:1 ratio before i.v. injection into mice. Top panel: a mixture of the cells before injection. Bottom panel: cells were isolated from the spleen and retraced at day 1 postinjection. In each panel, data represent one of four individually analyzed spleens.

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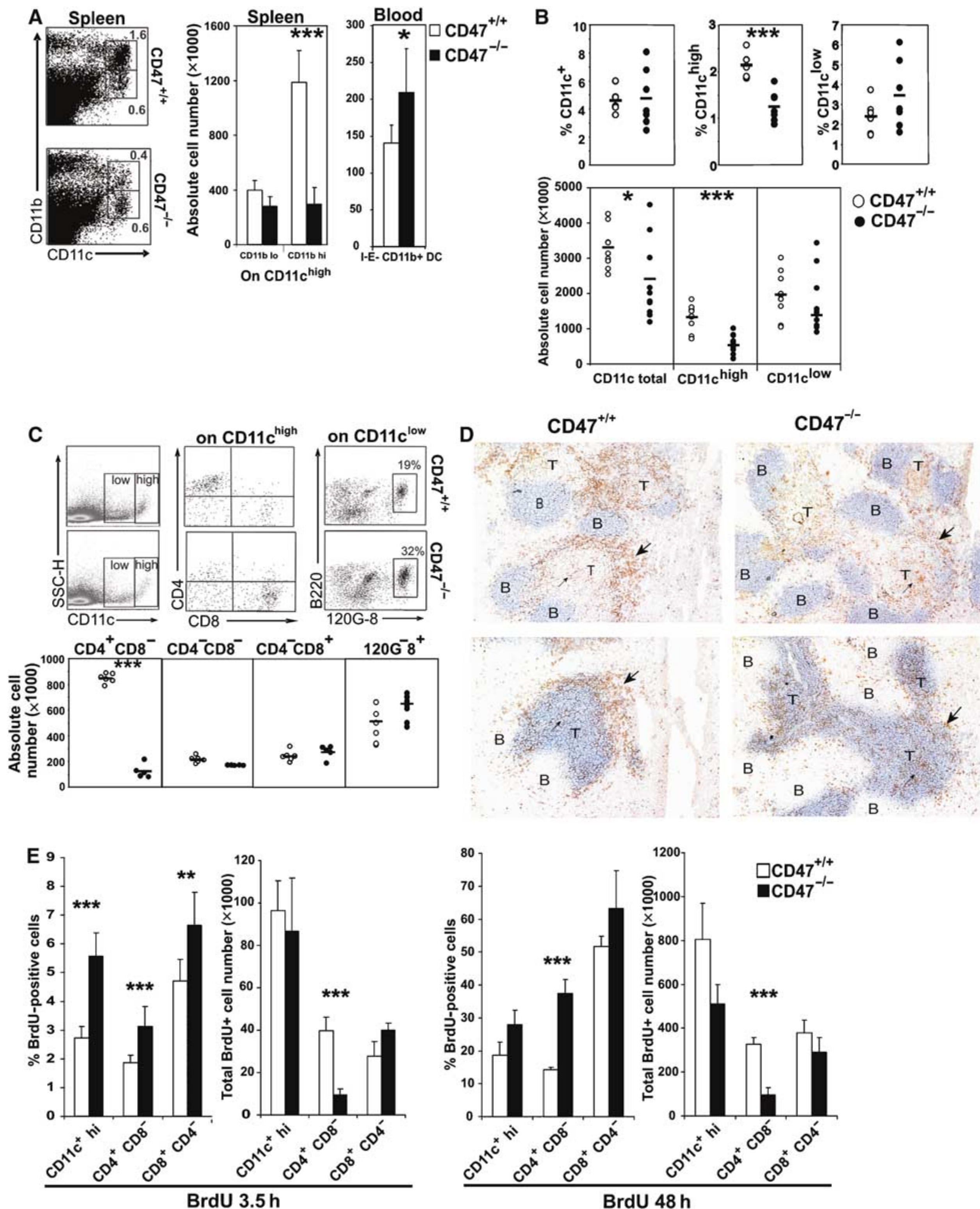

Figure 6 Depletion of myeloid $CD4^+CD8^-$ $CD11c^{high}$ DC subset in the spleen of CD47-deficient mice. (**A**) Proportion and absolute number of $CD11c^+CD11b^+$ DCs in spleens (left bar graph; mean±s.d., $n=4$ mice) and immediate DC precursors ($CD11c^+CD11b^+I\text{-}E^-$ DCs) in blood (right bar graph; mean±s.d., $n=5$ mice). (**B–C**) Proportion and absolute number of total DCs, $CD11c^{high}$, $CD11c^{low}$ and the different DC subsets per spleen. (**D**) Serial sections from frozen spleens were stained with CD11c (brown) and B220 (blue, top) or Thy1 (blue, bottom). Large arrowhead points to marginal zone DCs whereas narrow arrowhead points to DCs in the T-cell area. Data are representative of three individually analyzed spleens. (**E**) Proportion and absolute number of BrDU-positive $CD11c^{high}$, $CD4^+$ and $CD8^+$ DCs in mice treated with BrdU for 3.5 and 48 h. Bars represent mean±s.d. of six (3.5 h) and four (48 h) individually analyzed mice. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Recent studies provided evidence that the pool of splenic myeloid DCs is maintained by a combination of continuous extravasation from the blood of DC precursors and the local proliferation of immediate DC precursors in the spleen (Kabashima *et al*, 2005). Here, we show a similar or increased proportion of $BrdU^+$ cells among $CD11c^{high}$ DCs, as well as

the CD4$^+$ and CD8$^+$ subsets, in the two strains of mice 3.5 or 48 h after BrdU administration (Figure 6E and Supplementary Figure S5). However, the absolute number of proliferating DC precursors that had previously differentiated into the CD4$^+$ subset was significantly reduced in the CD47$^{-/-}$ spleen mice. Thus, an additional mechanism underlying the quasi-disappearance in CD4$^+$CD11c^{high} DCs in CD47-deficient mice may be through an altered migration into the spleen of the low-frequency immediate DC precursors that otherwise proliferated efficiently.

Collectively, the present results revealed that CD47 expression on DCs positively regulates myeloid DC trafficking and as such may control the pool of marginal zone DCs in the spleen.

Discussion

At peripheral sites, DCs require direct recognition of pathogens to appreciate the quality of the danger and initiate their migration toward the LN, where they are dedicated to T-cell priming. Yet, the integrated signals that lead to DC migration and the elicitation of a protective immune response versus tolerance are not completely identified.

Earlier *in vitro* studies indicated that through the interaction with its counter-receptor SIRP-α on neutrophils or monocytes, CD47 on the epithelium or endothelium controls leukocyte migration (Liu *et al*, 2002; de Vries *et al*, 2002; Zen and Parkos, 2003). Here, we hypothesized that CD47 expression governs epidermal and dermal skin transendothelial DC trafficking to the LN. In the absence of CD47, there was a decrease in the accumulation of skin-derived DCs in the draining LN under both homeostatic and inflammatory conditions. We have established that CD47 expression on DCs, rather than on the endothelium, is essential for DCs to gain entry into the afferent lymph and for their relocation to the LN, where stimulation of T cell occurs. We propose that after skin DCs have penetrated through the extracellular matrix network into the dermis, their entrance into LV is positively controlled by CD47.

Although it is not yet clear whether CD47/CD47 or CD47/SIRP-α interactions predominantly regulate LC retention in the epidermis, LC emigration is impaired in SIRP-α mutants lacking its intracytoplasmic domain (Fukunaga *et al*, 2004). In addition, phosphorylated SIRP-α expression on a melanoma cell line positively controls its migration (Motegi *et al*, 2003). In contrast, SIRP-α ligation by CD47-Fc fusion protein impairs skin DC migration in BALB/c mice and promotes SIRP-α tyrosine phosphorylation and SHP-1 recruitment under inflammatory conditions (Okazawa *et al*, 2005). Obviously, in CD47-deficient mice, SIRP-α is bound by CD47 neither *in trans* nor *in cis* and the predominant effect appeared to be impaired myeloid cell migration. Here, we report that the proportion of CD11c$^+$ DCs carrying FITC in the LN of mutant mice was not further altered by treatment with CD47-Fc fusion protein. These data suggest that CD47-Fc interrupted a positive interaction between CD47/SIRP-α rather than delivering a negative signal to the DCs via SIRP-α. Alternatively, the SIRP-α signalling pathway may not be functional in the absence of CD47 coexpression.

Inflammatory stimuli delivered by contact elicitation or active immunization with OVA peptide in the presence of adjuvant induces less efficient skin DC migration in CD47-deficient mice when compared to BALB/c mice. The general defect in skin DC migration is reminiscent of the phenotype observed in CCR7-deficient mice (Ohl *et al*, 2004). The latter is much more severe, as there is a near complete absence of naïve T and B cells in the LN, precluding DC/T-cell interactions (Forster *et al*, 1999). In contrast, T- and B-cell migration were entirely normal in CD47-deficient mice. Moreover, we show that the absence of CD47 did neither modulate CCR7 expression nor the chemotaxis toward CCL19. During the revision of this paper, Hagnerud *et al* (2006) similarly reported on the *in vivo* altered DC migration in CD47$^{-/-}$C57BL/6 mice with no modulation of CCR7 expression. In contrast to our data, these authors observed a defect in DC chemotaxis *in vitro*. As the function of CD47 is partly mediated through interactions with SIRP-α, this apparent controversy may be attributed to the polymorphism in SIRP-α in the two strains of mice (Sano *et al*, 1999).

We further showed that i.v. injected CD47$^{-/-}$ BM-DCs poorly primed T cells in the spleen, owing to their inefficient migration across endothelial barriers. Owing to the observed parallel between the cellular distribution of SIRP-α expression (i.e. neutrophils, LCs and dermal DCs) and the defect in cell migration of the corresponding cells in CD47$^{-/-}$ mice, we postulated that the absence of CD47 commonly affects the mobilization of SIRP-α-expressing cells. This prediction fits with our *in vivo* findings showing a selective depletion of the CD4$^+$CD11b$^+$CD11c^{high} marginal zone DC subset in the spleen. In support of our hypothesis, SIRP-α is not expressed on CD8$^+$ resident DCs, and their numbers were unaltered in the CD47-deficient spleen and LN.

Other mice carrying mutations for various transcription factors display a deficiency in CD4$^+$ DC numbers. Indeed, a reduction in the CD4$^+$ DC subset was demonstrated in Rel-B, PU.1, TRAF-6, IRF-2 and IRF-4-deficient mice as a result of a developmental defect in their DCs (Wu *et al*, 1998; Guerriero *et al*, 2000; Kobayashi *et al*, 2003; Ichikawa *et al*, 2004; Suzuki *et al*, 2004). Specifically, PU.1, Rel-B and IRF-4-deficient mice all show defects in BM-DC differentiation *in vitro*, and in contrast to CD47 deficiency, they show no evidence of altered DC migration. Hence, the decrease in splenic CD4$^+$ DCs observed in CD47$^{-/-}$ mice is most likely linked to a defect in cellular migration rather than differentiation.

Nonetheless, we cannot rule out a DC developmental problem in CD47$^{-/-}$ mice, although the following observations argued against such a defect. First, Flt3-L administration yielded a similar recovery of progenitors and total DCs in the BM and blood in CD47$^{+/+}$ and CD47$^{-/-}$ mice. Second, the proportion of CD11b$^+$I-E$^-$CD11c$^+$DC precursors was increased in the CD47$^{-/-}$ blood. Third, intrasplenic DC proliferation, a pathway to maintain DC homeostasis, was not decreased in the mutant mice. Yet, the absolute number of CD4$^+$ DCs was significantly reduced after BrdU or Flt3-L administration. We thus propose that a lack of CD47 leads to a defective migration of immediate DC precursors into the spleen, which results in the quasi-disappearance of marginal zone DCs. Naik *et al* (2006) recently identified such a low frequency DC precursor (0.05% total spleen cells), that is, CD11c^{int}, CD43^{int}, CD4$^-$CD8$^-$, MHC classII$^-$ SIRP-α^{int} DC, corroborating our hypothesis of a general defect in the mobilization of SIRP-α$^+$ cells in the absence of CD47.

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Taken together, we provide evidence that the CD47 molecule expressed on myeloid DCs controls their ability to efficiently traffic across lymphatic and endothelial vessels, seed in secondary lymphoid organs and participate in T-cell priming. Thus, regulating CD47 expression and as such DC migration may have an impact on undesired T-cell responses in autoimmune diseases and organ transplantation.

Materials and methods

Mice

A 16–18th-generation BALB/c backcross of CD47-deficient mice was a generous gift from Dr Oldenborg. BALB/c mice were purchased from Charles River Labs. Eight- to 12-week-old mice were used in all experimental protocols as approved by the Canadian Council on Animal Care and maintained under SPF conditions.

Preparation of epidermal sheets and skin explant cultures

The dorsal halves of ear explants were incubated in PBS with 20 mM EDTA for 2 h. The epidermal and dermal fractions were separated using fine forceps. The tissues were stained with FITC-anti-I-E mAb (14.4.4S3). In some experiments, ear explants were floated on tissue culture medium with or without 100 ng/ml of CCL19 (R&D System) for 72 h the before dermis was separated from the epidermis and stained. The images were acquired on a microscope equipped with a mercury lamp, monochrome filter, Kodak camera, and with the Metamorph software. Emigrated cells in the culture supernatant were counted by flow cytometry using latex beads for calibration (Beckman Coulter) and stained for CD11c, I-E and CCR7 expression. The data were acquired on a FACSCalibur and analyzed using Cellquest Software.

Preparation of BM-DCs

BM-DCs were prepared as described previously (Inaba *et al*, 1992), with slight modifications. BM cell suspensions were obtained from femurs and tibias and left to adhere *in vitro* for 6 h in serum-free RPMI at 5×10^6 cells/ml in six-well plates. GM-CSF 5 ng/ml (Peprotech) was added for 10–14 days to 1×10^7 nonadherent cells/3 ml to induce DC differentiation. The culture medium was renewed every 3 days. Cell purity was >98% CD11c$^+$ cells. In some experiments, BM-DCs were activated overnight with 1 μg/ml of LPS (*E. coli*, Sigma) and 1 μg/ml OVA peptide (323–339, Peptide International).

In vitro migration assay

3×10^5 activated or nonactivated BM-DCs in RPMI were placed on the upper compartment of 5-μm pore size Transwell plates (ChemoTx, Neuro Probe). CCL19 measuring 100 ng/ml or 1 μM of S1P (R&D system) in RPMI was added to the lower chamber. After 3 h of incubation at 37°C, cells that had transmigrated to the bottom compartment were collected and counted by flow cytometry using latex beads for calibration (Beckman Coulter).

Passive transfer experiments

BALB/c and CD47-deficient mice were injected i.v. with 5×10^6 CFSE-labelled CD4$^+$ T cells from DO11.10 mice. One day later, the mice were immunized s.c in the right footpad with 1 or 10 μg of OVA protein dissolved in 20 μl of PBS or 10 μg of OVA peptide emulsified in IFA. The left footpad received 20 μl of PBS. The draining LN were extracted 2, 3 or 7 days after the immunization. In some experiments, mice were immunized with activated OVA-pulsed BM-DCs s.c. or i.v. and the T-cell response was examined in the LN or the spleen after 72 h.

Competitive migration assays

2×10^6 (s.c.) or 4×10^6 (i.v.) activated CD47$^{+/+}$ and CD47$^{-/-}$ BM-DCs were stained with two different colors, CFSE (Sigma) or CMTMR (Molecular Probes), respectively, and injected into CD47$^{-/-}$ hosts. Twenty-four hours later, DCs were retraced in draining LN or spleen by flow cytometry. For some experiments, activated CD47$^{+/+}$ and CD47$^{-/-}$ BM-DCs were stained with PKH26 (Sigma), injected s.c. and retraced by CD47 staining.

Spleen and lymph node staining

Antibodies were purchased from BD, except for anti-I-E (14.4.4S3) and anti-CD11c (N418), purified in our laboratory, and an antibody that specifically recognizes pDCs, 120G-8 (Asselin-Paturel *et al*, 2003), which was kindly provided by Dr G Trinchieri. Spleens were injected with 1 ml liberase at 0.4 mg/ml and minced in 2 ml (Roche). Axillary, brachial and inguinal LN were minced in 3 ml liberase. The tissues were incubated at 37°C for 15 min and passed through a 70-μm nylon cell strainer. Spleen cells were then treated with NH_4Cl for red blood cell lysis.

FITC sensitization

Mice were shaved and 100 μl of a 1% FITC (sigma) solution or vehicle alone (1:1 v/v acetone and dibutylphthalate) was painted on the abdomen. At 24 or 72 h after painting, the inguinal LN were stained for DCs, T and B cells, and analyzed by flow cytometry. In CD47-Fc experiments, mice received 50 μg of CD47-Fc or CTRL-Fc fusion proteins intradermally before FITC sensitization.

Spleen immunohistochemistry

Frozen spleen sections (8 μm) were fixed on slides in acetone for 20 min at −20°C, blocked with Universal Blocking reagent (DAKO, Mississauga, Ontario) and stained with biotin anti-CD11c and either FITC-anti-B220 or anti-Thy1. The biotin-coupled antibody was revealed using Vectastain ABC kit (Vector Labs, Burlingame, CA), followed by DAB, and the FITC-antibodies using alkaline phosphatase-coupled anti-FITC (Roche, Indianapolis, IN) followed by Vector Blue.

BrdU staining

BrdU (2 mg/mouse) was injected intraperitoneally and added to the drinking water (0.8 mg/ml). After 3.5 or 48 h, spleens were harvested and stained for different DC subsets. The cells were then fixed, permeabilized (BD Cytofix/cytoperm) and treated with DNase I (Sigma) for 1 h at 37°C before staining with anti-BrdU-FITC.

Statistical analysis

Student' *t*-test; $***P<0.001$, $**P<0.01$, $*P<0.05$.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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