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The alarmin Mrp8/14 as regulator of the adaptive immune response during allergic contact dermatitis

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Mrp8 and Mrp14 are endogenous alarmins amplifying inflammation via Toll-like receptor-4 (TLR-4) activation. Due to their pro-inflammatory properties, alarmins are supposed to enhance adaptive immunity via activation of dendritic cells (DCs). In contrast, analysing a model of allergic contact dermatitis (ACD) we observed a more severe disease outcome in Mrp8/14-deficient compared to wild-type mice. This unexpected phenotype was associated with an enhanced T-cell response due to an accelerated maturation of DCs in Mrp8/14-deficient mice. Accordingly, Mrp8, the active component of the heterocomplex, inhibits early DC maturation and antigen presentation in a TLR-4-dependent manner. Transfer of DCs purified from the local lymph nodes of sensitized Mrp8/14-deficient to wild-type mice determined the outcome of ACD. Our results link a pro-inflammatory role of the endogenous TLR-4 ligand Mrp8/14 to a regulatory function in adaptive immunity, which shows some similarities with the 'hygiene hypothesis' regarding continuous TLR-4 stimulation and decreased risk of allergy.

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Introduction

In industrialized countries, allergic contact dermatitis (ACD) represents one of the most common inflammatory skin diseases (Uter *et al*, 1998). The underlying mechanism is a contact hypersensitivity reaction depending on the generation of an allergen-specific T lymphocyte response (Martin *et al*, 2000; Bour *et al*, 2005; Vocanson *et al*, 2006). The resulting T-cell response is critically dependent on the state of differentiation and activation of dendritic cells (DCs) but the molecular mechanisms involved in the process of skin sensitization are still not fully understood (Banchereau and Steinman, 1998; Gutcher and Becher, 2007). Recently, so-called danger-associated molecular pattern proteins (DAMPs) or alarmins attracted attention. They represent endogenous danger signals inducing inflammatory responses after being released from activated or necrotic cells. Several receptors including the multiligand receptor for advanced glycation end products (RAGE), Toll-like receptor (TLR)-2, TLR-4, and TLR-9 appear to mediate effects of different DAMPs (Tian *et al*, 2007; Vogl *et al*, 2007; Zhang and Mosser, 2008; Ichikawa *et al*, 2011). Some DAMPs like High-mobility group nucleosome-binding protein 1 (HMGN1), High-mobility group box 1 protein (HMGB1), and eosinophil-derived neurotoxin are able to enhance antigen-specific immune responses via activation of DCs (Rovere-Querini *et al*, 2004; Yang *et al*, 2006, 2008, 2010, 2011). Moreover, a crucial role of TLR-4 has been shown recently in allergic contact hypersensitivity indicating that not only the environmental microbial flora but also endogenous ligands or metal ions as Ni^{2+} may contribute to the progress of the disease (Martin *et al*, 2008; Schmidt *et al*, 2010).

We recently demonstrated that Mrp8 (myeloid related protein 8, S100A8) and Mrp14 (S100A9) show typical characteristics of endogenous TLR-4 ligands and alarmins. Complexes of Mrp8 and Mrp14, also known as calprotectin, are released by activated phagocytes at sites of inflammation (Strupat *et al*, 2000; Foell *et al*, 2007; Korndörfer *et al*, 2007; Vogl *et al*, 2007; van Lent *et al*, 2008a; Ehrchen *et al*, 2009) and exhibit pro-inflammatory effects on endothelial cells, phagocytes, or lymphocytes (Viemann *et al*, 2005; Vogl *et al*, 2007; Frosch *et al*, 2009; Loser *et al*, 2010; Grevers *et al*, 2011). Mrp8 specifically interacts with TLR-4, thus representing an endogenous activator of this receptor (Vogl *et al*, 2007). In addition to TLR-4, RAGE has also been described as a receptor for Mrp8 and Mrp14 (Björk *et al*, 2009; Ichikawa *et al*, 2011). In animal models, we could show that Mrp8 and Mrp14 are able to promote arthritis (van Lent *et al*, 2008a, b) as well as the induction of autoreactive $CD8^+$ T cells in systemic autoimmunity in a TLR-4-dependent manner (Loser *et al*, 2010). In models of tumour development, $Mrp14^{-/-}$ mice show a shift between mature DCs and immature so-called myeloid-derived suppressor cells (MDSCs) (Cheng *et al*, 2008; Gabrilovich and Nagaraj, 2009).

In the present study, we demonstrate that Mrp8 and Mrp14 directly modulate adaptive immunity during allergic reactions. Remarkably, we found that mice lacking these proteins show a stronger inflammatory response during ACD. We deciphered a novel molecular mechanism by which prolonged exposure of myeloid progenitor cells to Mrp8 and Mrp14 blocks DC

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differentiation and antigen presentation resulting in a diminished T-cell response. Our data link the pro-inflammatory role of the danger molecule Mrp8/14 on innate immune mechanisms to a regulatory function in adaptive immunity, which may be an important mechanism to avoid tissue damage due to overwhelming immune responses.

Results

Mrp8 and Mrp14 activate bone marrow-derived DCs via TLR-4

To determine the effect of Mrp8 and Mrp14 on DC activation, we generated immature (i) bone marrow-derived DCs (BMDCs) from wild-type (wt) mice in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 6 days and incubated them thereafter for 48 h with Mrp8, Mrp14, or Mrp8/14 heterocomplex. Flow cytometric analyses revealed that the expression of activation markers (CD86 and MHC class II molecules, MHC-II) is upregulated on iBMDCs by Mrp8 and Mrp8/14 ($P<0.05$) but not by Mrp14 alone (Figure 1A). In addition, DCs stimulated by Mrp8 and Mrp8/14 induced allogenic T-cell proliferation to a significant greater extent than controls and comparable to LPS-treated BMDCs (Figure 1B and C). No induction of DC activation occurred in TLR-4$^{-/-}$ BMDCs whereas RAGE$^{-/-}$ BMDCs showed expression levels comparable to wt BMDCs (Figure 1D). These data indicate that DC activation by Mrp8 is mediated via TLR-4, while RAGE is not involved.

More severe disease outcome of ACD in Mrp14$^{-/-}$ mice

To investigate the biological relevance of the Mrp8-mediated effects on DC activation, we elicited ACD in C57BL/6 wt and Mrp14$^{-/-}$ mice as a model for a DC-mediated T-cell response. It is important to note that Mrp14$^{-/-}$ mice also lack Mrp8 on the protein level, probably due to high metabolic turnover of Mrp8 in the absence of its complex partner. Therefore, the Mrp14$^{-/-}$ strain represents a functional Mrp8/14 double knockout mouse (Hobbs *et al*, 2003; Manitz *et al*, 2003). Surprisingly, ear swelling during ACD was significantly more pronounced in Mrp14$^{-/-}$ mice in comparison to wt mice (Figure 2A). Accordingly, ear sections of Mrp14$^{-/-}$ mice showed an increased inflammatory infiltration by CD11b^{+} myeloid cells, Gr-1^{+} granulocytes, and F4/80^{+} macrophages (Figure 2B). No differences regarding the amounts of CD11b^{+}, Gr-1^{+}, and F4/80^{+} cells were found in untreated ear tissue of wt and Mrp14$^{-/-}$ mice (Supplementary Figure S1). As expected, ears of Mrp14$^{-/-}$ mice were completely negative for both, Mrp8 and Mrp14, whereas high levels of these proteins could be observed in wt mice upon challenge (Figure 2C). Our data clearly demonstrate an enhanced allergic response in Mrp14$^{-/-}$ mice compared to wt controls, which somehow contradicts our data regarding the activation of BMDCs by Mrp8 *in vitro*. This finding is not due to an anti-inflammatory effect of Mrp8 or Mrp14 since Mrp8/14$^{-/-}$ mice do not show higher inflammatory activity in a T cell-independent model of irritant contact dermatitis (ICD) (Figure 2D).

Mrp8 inhibits early differentiation of bone marrow progenitors to mature immunogenic BMDCs

In order to unravel the unexpected outcome of ACD in Mrp14$^{-/-}$ mice, we investigated the influence of the active component Mrp8 on early differentiation of bone marrow (BM) cells to iBMDCs (days 1–6 of GM-CSF and IL-4 treatment of BM cells) *in vitro*. Subsequently, cells were activated by LPS for another 48 h and the state of maturation was compared between Mrp8-pretreated [Mrp8/LPS] and untreated [-/LPS] BMDCs. In comparison to conventionally LPS-activated DCs [-/LPS], a significant reduction in expression of CD11c, CD86, and MHC-II could be observed in both mouse strains after pretreatment with Mrp8 (Figure 3A). Moreover, inhibition of BMDC differentiation by Mrp8 resulted in a markedly reduced capability of stimulating T cells (Figure 3B). These data indicate that Mrp8 impairs early BMDC differentiation.

With regard to our finding that TLR-4 is the relevant receptor for Mrp8-induced effects on activation of differentiated DCs (Figure 1D) we also investigated the influence of Mrp8 on early BMDC differentiation in TLR-4$^{-/-}$ BMDCs. In contrast to wt, no reduction of CD86 and MHC-II expression could be observed on TLR-4$^{-/-}$ BMDCs after differentiation in the presence of Mrp8 [Mrp8 (d0–d6)] (Figure 3C).

To further test whether the dampened immune response in the presence of Mrp8 may be the result of a classical desensitization of TLR-4, we incubated iBMDCs with Mrp8 for 24 h prior to LPS activation [Mrp8 (d5–d6)]. No impaired expression of DC activation markers as well as no impaired stimulation of allogenic T-cell proliferation could be observed (Figure 3D and E). Hence, Mrp8 does not induce a classical desensitization of TLR-4.

Since Mrp8 impairs BMDC differentiation we asked whether this effect contributes to the enhanced ACD in Mrp14$^{-/-}$ mice. Comparing the maturation state of wt and Mrp14$^{-/-}$ iBMDCs after 6 days of differentiation (GM-CSF/IL-4), we found substantially higher expression levels of maturation markers (CD86 and MHC-II) expressed on BMDCs obtained from Mrp14$^{-/-}$ mice compared to controls (Figure 3F, left part). Additionally, LPS-activated Mrp14$^{-/-}$ BMDCs expressed maturation markers to a greater extent and induced a stronger allogenic T-cell proliferation than wt BMDCs (Figure 3F, right part and G) indicating an accelerated BMDC differentiation in Mrp14$^{-/-}$ mice. In addition, we analysed the physical contacts of C57BL/6 wt and Mrp14$^{-/-}$ BMDCs with allogenic BALB/c T cells. Therefore, time-lapse video microscopy of cells embedded in a 3-D collagen matrix was used. We found that the ability to establish contacts with T cells was improved in Mrp14$^{-/-}$ BMDCs compared to wt BMDCs (Figure 3H, left part). The embedded T cells were isolated from the collagen gels after 8 h and further analysed for the expression of the activation marker CD69. We observed a clearly higher CD69 expression on T cells incubated with Mrp14$^{-/-}$ BMDCs than with wt BMDCs (Figure 3H, right part). These data indicate that Mrp8 does not simply inhibit the expression of co-stimulatory molecules but induces a more complex modulation of DC differentiation. Furthermore, by quantifying serum concentrations in wt mice before and after sensitization we observed a strong increase in Mrp8/14 release (about ten-fold) 2 days after 2,4-dinitrofluorobenzene (DNFB) application (Figure 3I). These findings suggest that release of Mrp8/14 during sensitization may result in impaired DC differentiation and restricted antigen-presenting capacity.

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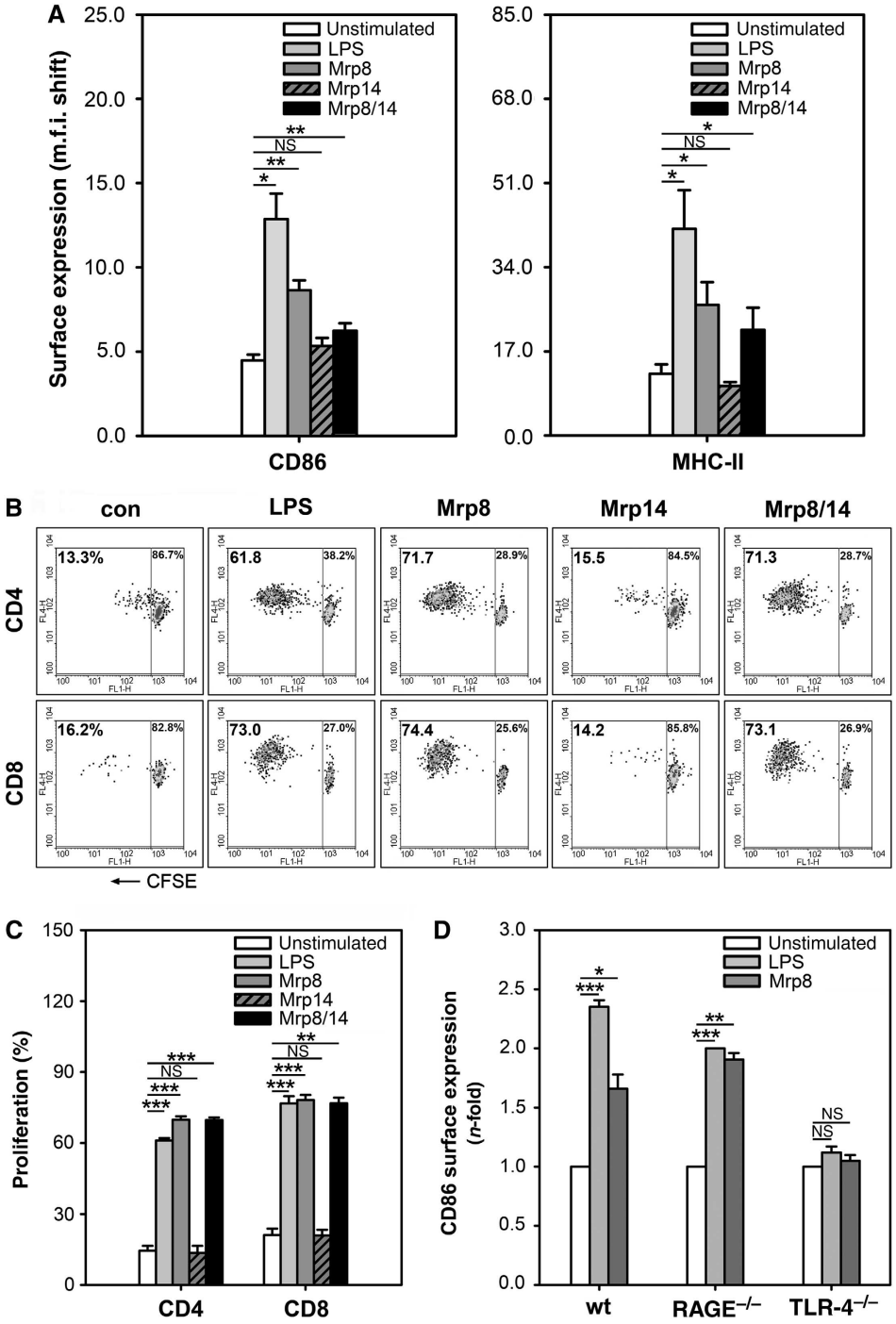

Figure 1 Mrp8/14-induced TLR-4-mediated activation of iBMDCs. (**A**) Expression of activation markers CD86 and MHC-II on *in vitro* generated iBMDCs from C57BL/6 mice. iBMDCs were activated for 48 h in the presence of Mrp8 or Mrp14 (8 μg/ml), Mrp8/14 (20 μg/ml), or LPS (300 ng/ml). Unstimulated iBMDCs served as control. M.f.i. shifts were determined by flow cytometry (three independent experiments; mean ± s.e.m.). (**B**, **C**) Proliferation of CFSE-labelled $CD4^+$ and $CD8^+$ allogenic spleen T cells from BALB/c mice after 5 days of culture with unstimulated, Mrp8-, Mrp14-, Mrp8/14-, or LPS-stimulated C57BL/6 BMDCs (DC:T-cell ratio was 1:5). Panel (**B**) shows results from one of three independent experiments which are summarized in (**C**) (mean ± s.e.m.). (**D**) Comparison of CD86 expression of unstimulated, LPS- (300 ng/ml) and Mrp8- (8 μg/ml) stimulated BMDCs from C57BL/6 wt, $RAGE^{-/-}$, and $TLR\text{-}4^{-/-}$ mice. Following 48 h of stimulation, m.f.i. shifts were measured by flow cytometry and normalized to unstimulated control cells (three independent experiments; mean ± s.e.m.). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and NS = not significant.

Enhanced DC activation leads to an amplified T-cell response in Mrp14$^{-/-}$ mice during ACD

Since DCs of $Mrp14^{-/-}$ mice showed an enhanced maturation phenotype, we examined Mrp-induced effects on DC-mediated T-cell proliferation during ACD *in vivo*. Forty-eight hours after challenge with DNFB, spleen T cells were isolated and incubated with either BMDCs obtained from naive mice, loaded with 100 μM dinitrobenzene-sulfonate (DNBS), the water soluble analogue of DNFB, or *in vivo*-primed epithelial cell adhesion molecule negative ($EpCAM^-$) dermal DCs

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(dDCs) isolated from ear-draining lymph nodes (LNs) 48 h after ear challenge. We found that both BMDCs and dDCs from Mrp14$^{-/-}$ mice induced a significantly higher T-cell proliferation than the corresponding wt cells (Figure 4A). Moreover, we observed an enhanced secretion of interferon-γ (IFN-γ) and IL-17 by Mrp14$^{-/-}$ T cells cultured with Mrp14$^{-/-}$ dDCs compared to their wt counterparts (Figure 4B).

Next, T cells of DNFB-challenged Mrp14$^{-/-}$ mice were incubated with either wt or Mrp14$^{-/-}$ DCs. We found that DNBS-loaded BMDCs as well as dDCs from DNFB-sensitized Mrp14$^{-/-}$ mice induced a stronger T-cell response compared to wt DCs (Figure 4C, left part). Similar results were observed when using wt T cells in proliferation assays (Figure 4C, right part) indicating that the T-cell source does not affect proliferation rates. Of note, T cells do not express Mrp8/14, therefore T-cell proliferation rates cannot be influenced by missing intracellular Mrp8/14. These findings demonstrate that the more pronounced T-cell response in Mrp14$^{-/-}$ mice is a consequence of higher stimulatory capacities of Mrp14$^{-/-}$ DCs as compared to wt controls.

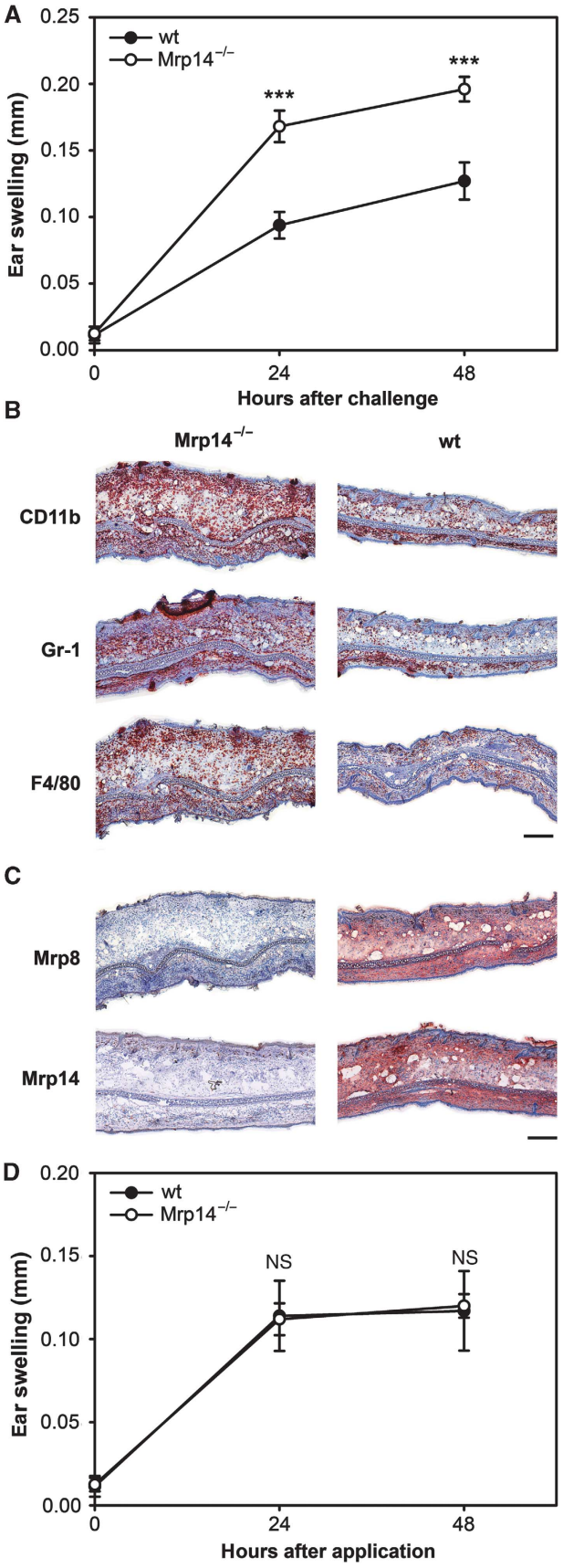

Figure 2 (**A–C**) DNFB-induced ACD response in ears of C57BL/6 wt and Mrp14$^{-/-}$ mice. (**A**) Ear swelling of wt and Mrp14$^{-/-}$ mice after elicitation of ACD. Mice were sensitized by painting 0.4% DNFB in acetone/olive oil on the shaved abdomen. After 6 days, the right ears were challenged with 0.4% DNFB in acetone/olive oil. The left ears were treated with acetone/olive oil only and served as controls. Ear thickness was measured 24 and 48 h after challenge. Swelling was determined by calculating the difference between the control ear and the challenged ear (four independent experiments with each 8–15 mice per group; mean ± s.e.m.) (***$P<0.001$). (**B**, **C**) Histological analyses of ear sections from DNFB-treated wt and Mrp14$^{-/-}$ mice 48 h after challenge. Sections were stained for (**B**) immigrated immune cell (CD11b^{+} myeloid cells, Gr-1^{+} granulocytes, F4/80^{+} macrophages) and (**C**) expression of Mrp8 and Mrp14. (**B**, **C**) scale bar, 250 μm. (**D**) Croton oil-induced ICD response in ears of C57BL/6 wt and Mrp14$^{-/-}$ mice. Mice were painted with 10 μl 3% croton oil in acetone/olive oil (4:1) onto the right ears. The left ears served as controls and were treated with acetone/olive oil only. Ear thickness was measured 24 and 48 h after the treatment. Ear swelling was determined by calculating the difference between the untreated ear and the treated ear (three independent experiments with five mice per group each; mean ± s.e.m.).

Reduced numbers of MDSCs partially contribute to the enhanced DC-mediated T-cell response of Mrp14$^{-/-}$ mice during ACD

Blocking the differentiation of mature professional antigen-presenting cells (APCs) may not only result in a decrease of mature DCs but could also induce a shift to more immature myeloid cell populations with known suppressive effects on adaptive immunity, like CD11b^{+}Ly-6C^{+} so-called monocytic MDSCs which are known to be associated with autoimmunity, inflammation, and cancer (Cheng *et al*, 2008; Gabrilovich and Nagaraj, 2009). Therefore, we quantified MDSC fractions in ear-draining LNs 48 h after elicitation of ACD. We observed decreased numbers of CD11b^{+}Ly-6C^{+} 'double positive' MDSCs in allergic Mrp14$^{-/-}$ mice compared to wt controls (Figure 5A, $P=0.04$). We next investigated whether the quantitative difference of MDSCs affects the T-cell response during ACD. We isolated dDCs from ear-draining LNs of DNFB-challenged wt and Mrp14$^{-/-}$ mice and removed MDSCs by positive selection (Ly-6C). The MDSC-depleted dDC fractions were used for co-culture experiments with spleen-derived T cells of the same animals. We found that in the absence of MDSCs the wt T-cell response increases moderately but still significantly ($P=0.017$), whereas the increase in Mrp14$^{-/-}$ T-cell proliferation was less pronounced, as expected (Figure 5B and C)

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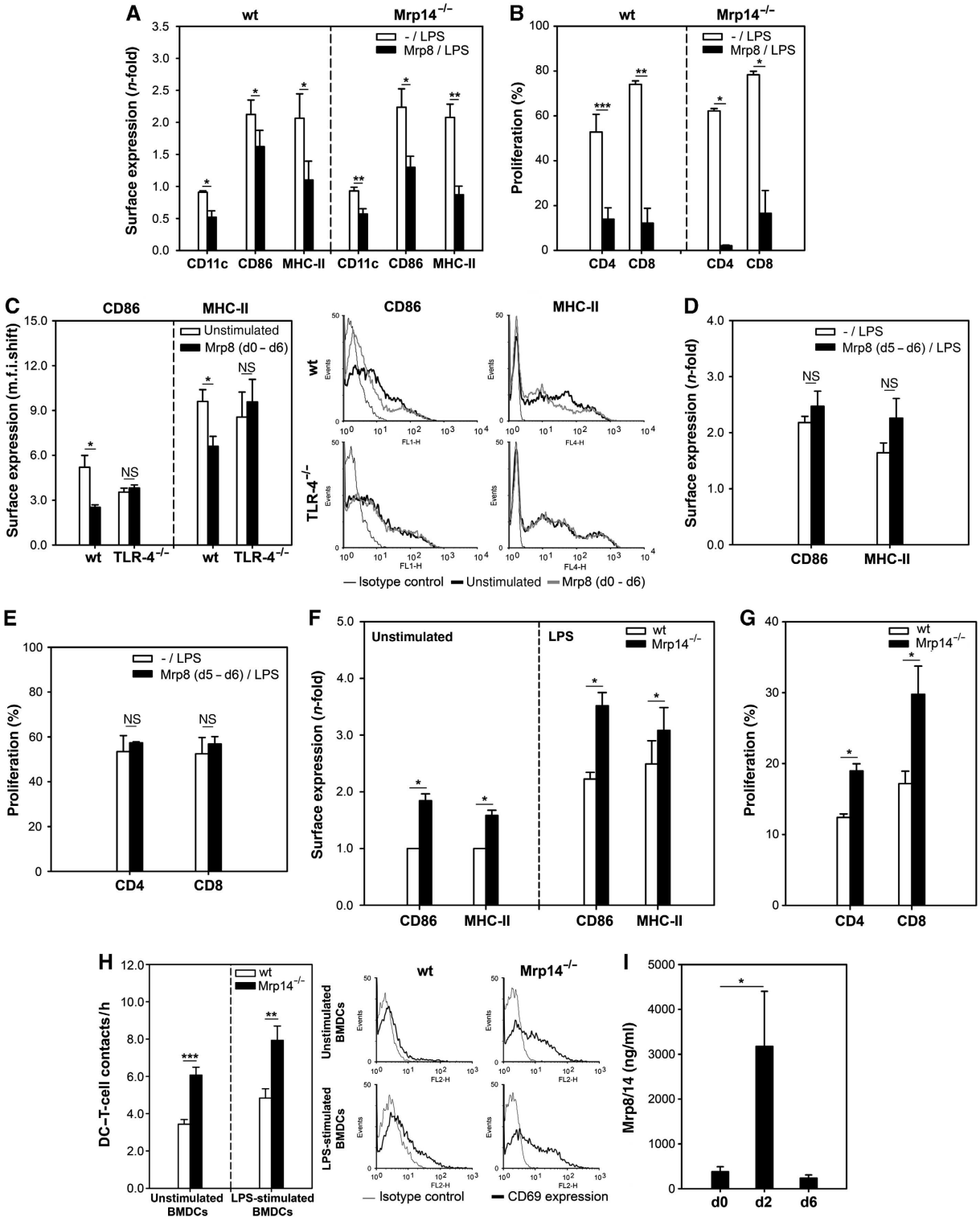
indicating that the reduced number of MDSCs have some impact on the enhanced T-cell response in Mrp14$^{-/-}$ mice. However, the different proliferation rates of wt and Mrp14$^{-/-}$ T cells in the absence of MDSCs again confirm an enhanced antigen-presenting capacity of Mrp14$^{-/-}$ DCs compared to wt controls. The T-cell proliferation data are in accordance with the increased secretion of the pro-inflammatory cytokine IL-17 in the absence of MDSCs by wt T cells as well as by Mrp14$^{-/-}$ T cells (Figure 5D). Taken together, these data indicate that Mrp8 induces a shift from mature, immunogenic DCs to rather immature myeloid cells, which finally results in a minor but still significant inhibitory effect on adaptive immunity.

Antigen-presenting capability of DCs is crucial for the enhanced ACD outcome in Mrp14$^{-/-}$ mice in vivo

Finally, we aimed to analyse whether the Mrp14$^{-/-}$ phenotype during ACD can be induced in wt mice by transferring



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Mrp14$^{-/-}$ DCs. dDCs from ear-draining LNs of DNFB-challenged wt and Mrp14$^{-/-}$ mice 48 h after elicitation of ACD were transferred intravenously into two identical groups of DNFB-sensitized wt mice. Subsequently, ear swelling was measured 24 and 48 h after challenge with DNFB (Figure 6). After transferring Mrp14$^{-/-}$ DCs to wt animals we observed an increase in ear swelling comparable to Mrp14$^{-/-}$ mice in ACD. The ear swelling of wt mice was not affected by transfer of wt dDCs. To make sure that these differences were not due to differences in cellular homing of the injected dDCs from wt and Mrp14$^{-/-}$ mice, isolated DNFB-primed dDCs were stained with cell tracker orange (CTO) and injected into sensitized wt mice prior to challenge. After 48 h, the ear-draining LNs were analysed for the number of immigrated CTO-positive dDCs. Identical numbers of CTO-positive cells were found in LNs of mice injected with wt and Mrp14$^{-/-}$ DCs (Supplementary Figure S2A). In a second independent approach to test whether cellular homing of wt and Mrp14$^{-/-}$ dDCs is identical FITC was applied to the ears of wt and Mrp14$^{-/-}$ mice. After 18 h, the draining LNs were isolated and analysed for FITC-positive DCs migrated from the skin to regional LNs. No differences in the number of FITC-positive immigrated cells could be observed in wt and Mrp14$^{-/-}$ mice (Supplementary Figure S2B). Thus, the observed differences in wt and Mrp14$^{-/-}$ mice are not due to differences in cellular homing of DCs. Taken together, these

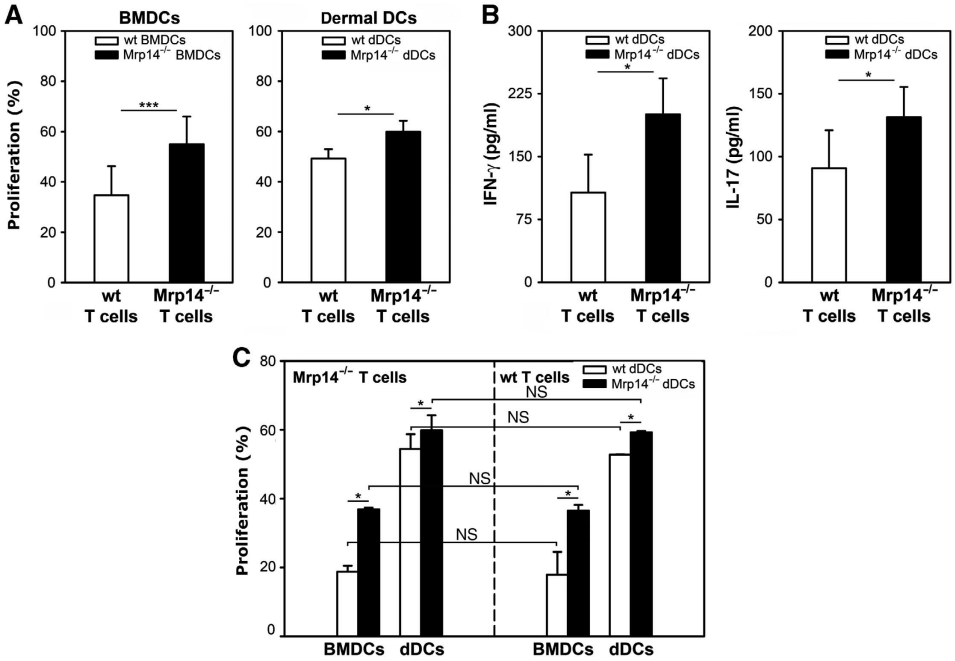

Figure 4 Increased DC-mediated T-cell response in Mrp14$^{-/-}$ mice during ACD. (**A**) Proliferation of wt and Mrp14$^{-/-}$ T cells during ACD. Wt and Mrp14$^{-/-}$ spleen T cells isolated from DNFB-challenged C57BL/6 mice were cultured with DNBS-loaded wt or Mrp14$^{-/-}$ BMDCs of naive C57BL/6 mice (left) or *in vivo*-primed EpCAM-negative dDCs from ear-draining LNs of the allergic wt or Mrp14$^{-/-}$ C57BL/6 mice (right) (DC:T-cell ratio was 1:5). Proliferation was measured after 5 days of co-culture (four independent experiments; mean ± s.e.m.). (**B**) Expression of pro-inflammatory cytokines IFN-γ and IL-17 by spleen T cells of DNFB-challenged wt and Mrp14$^{-/-}$ C57BL/6 mice in response to stimulation with wt or Mrp14$^{-/-}$ DNFB-primed EpCAM-negative dDCs from the same ACD mice, respectively (three independent experiments; mean ± s.e.m.). (**C**) Proliferation of Mrp14$^{-/-}$ T cells (left) or wt T cells (right) from spleens of DNFB-challenged C57BL/6 mice co-cultured with either DNBS-charged BMDCs of naive C57BL/6 mice or *in vivo*-primed EpCAM-negative dDCs from ear-draining LNs of the allergic wt or Mrp14$^{-/-}$ C57BL/6 mice. T-cell proliferation was measured after 5 days of co-culture (three independent experiments; mean ± s.e.m.). $^{*}P<0.05$, $^{***}P<0.001$, and NS, not significant.

Figure 3 Extracellular Mrp8 impairs DC differentiation. (**A**) C57BL/6 wt and Mrp14$^{-/-}$BMDCs generated in the presence of 8 μg/ml Mrp8 [Mrp8/LPS] were stimulated with 300 ng/ml LPS for 48 h on day 6. CD11c, CD86, and MHC-II surface expression was compared to conventionally LPS-stimulated BMDCs [-/LPS] (four independent experiments; mean ± s.e.m.). (**B**) Proliferation of allogenic T cells (BALB/c) induced by C57BL/6 BMDCs generated as described in (**A**) (DC:T cells, 1:5) (four independent experiments; mean ± s.e.m.). (**C**) CD86 and MHC-II expression on C57BL/6 wt and TLR-4$^{-/-}$ BMDCs generated in the absence or presence of 8 μg/ml Mrp8 [Mrp8 (d0–d6)]. The right part shows one of three independent experiments that are summarized in the left part (mean ± s.e.m.). (**D**) Phenotype of C57BL/6 wt BMDCs stimulated with 8 μg/ml Mrp8 on day 5 for 24 h [Mrp8 (d5–d6/LPS)]. On day 6, 300 ng/ml LPS was added for further 48 h. CD86 and MHC-II expression was compared to conventionally LPS-stimulated BMDCs [-/LPS] (three independent experiments; mean ± s.e.m.). (**E**) Proliferation of allogenic T cells (BALB/c) induced by C57BL/6 wt BMDCs generated as described in (**D**) (DC:T cells, 1:5) (three independent experiments; mean ± s.e.m.). (**F**) Accelerated BMDC differentiation due to the lack of Mrp8/14. CD86 and MHC-II expression on wt and Mrp14$^{-/-}$ BMDCs stimulated for 48 h with LPS (300 ng/ml) (right) or left unstimulated (left). M.f.i. shifts were normalized to unstimulated wt cells (four independent experiments; mean ± s.e.m.). (**G**) Proliferation of allogenic spleen T cells (BALB/c) induced by C57BL/6 wt or Mrp14$^{-/-}$ BMDCs (DC:T cells, 1:5) (four independent experiments; mean ± s.e.m.). (**H**) Improved ability of Mrp14$^{-/-}$ BMDCs to contact T cells. Allogenic T cells (BALB/c) were embedded with unstimulated or LPS-stimulated wt and Mrp14$^{-/-}$ BMDCs (C57BL/6) in 3-D collagen gels. Interactions were monitored by time-lapse microscopy. Left part: Number of DC–T-cell contacts per hour (two independent experiments; 40 DCs evaluated per condition). Right part: Embedded T cells were analysed for CD69 surface expression. (**I**) Mrp8/14 serum levels of DNFB-sensitized wt mice. Sera of 10 mice were analysed (two independent experiments, five mice each, mean ± s.e.m.) $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and NS, not significant.

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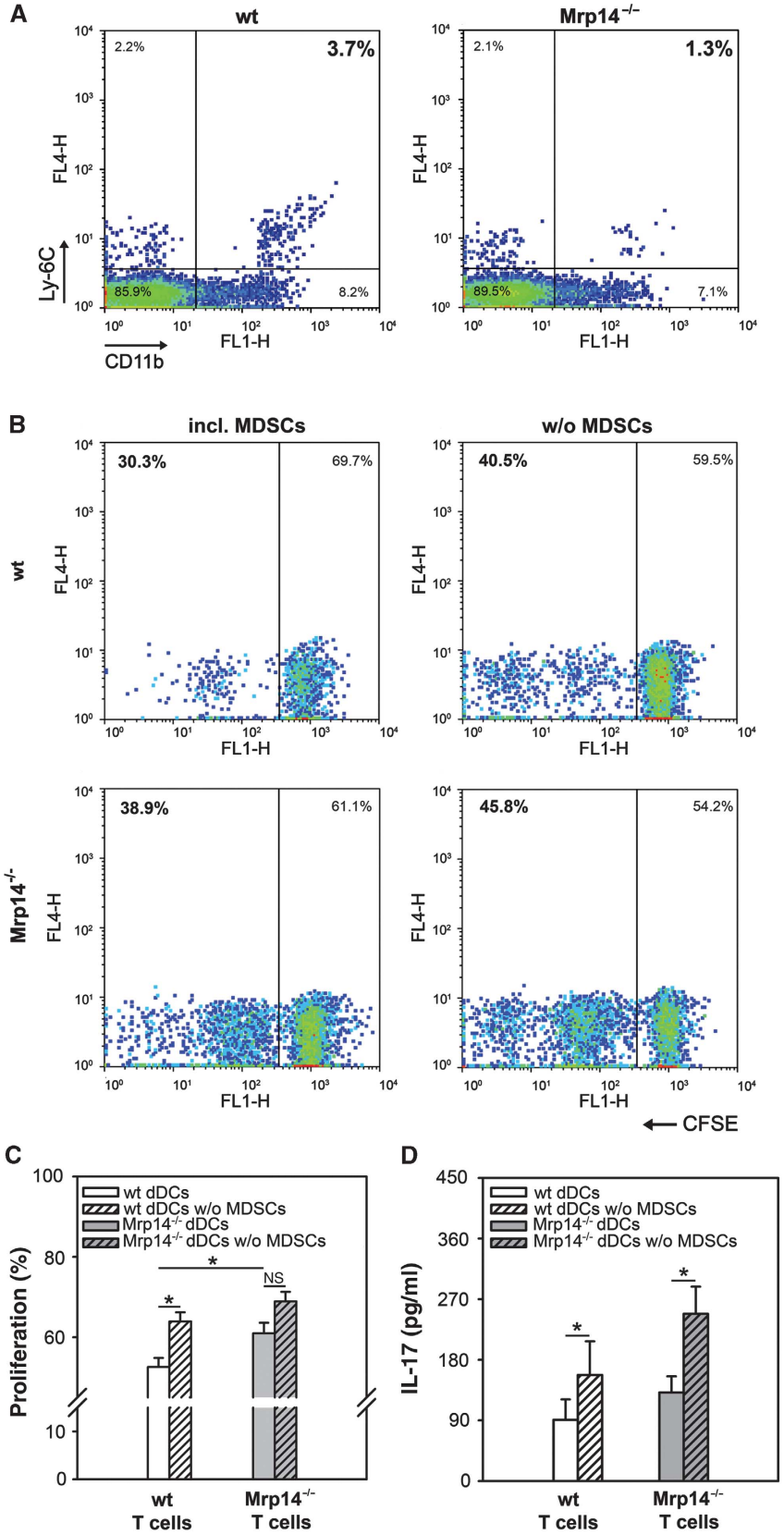

Figure 5 Increased T-cell response during ACD is partly dependent on the number of MDSCs. (**A**) Quantitative analysis of $CD11b^{+}Ly\text{-}6C^{+}$ MDSCs in LN suspensions from wt and $Mrp14^{-/-}$ mice 48 h after ACD elicitation with DNFB ($^{*}P = 0.04$). Results from one of four independent experiments are shown. (**B**, **C**) Proliferation of T cells from allergic wt or $Mrp14^{-/-}$ mice (C57BL/6) induced by MDSC-depleted EpCAM-negative dDC fractions (**B**, right and **C** white or grey hatched bars) in comparison to untreated EpCAM-negative dDCs (**B**, left and **C** white or grey bars) from the same DNFB-challenged wt and $Mrp14^{-/-}$ mice. Proliferation was determined by CFSE dilution (four independent experiments). $^{*}P < 0.05$ and NS, not significant. (**D**) Release of the pro-inflammatory cytokine IL-17 by spleen T cells from DNFB-treated wt and $Mrp14^{-/-}$ mice (C57BL/6) in response to MDSC-depleted EpCAM-negative dDCs or untreated EpCAM-negative dDCs of the same mice (three independent experiments; mean ± s.e.m.). $^{*}P < 0.05$.

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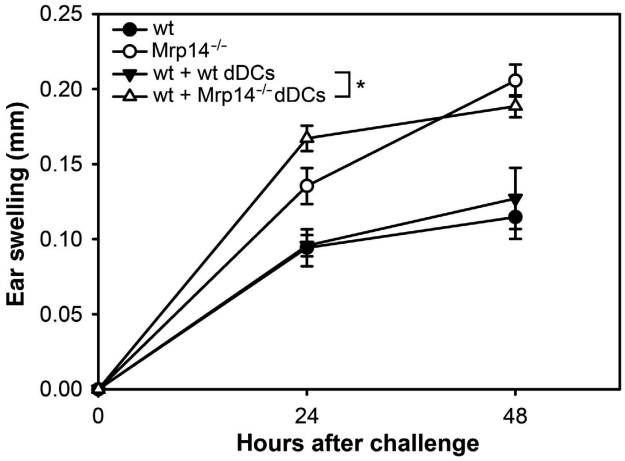

Figure 6 Wt mice develop an increased ear swelling after adoptive transfer of DNFB-primed Mrp14$^{-/-}$ dDCs. EpCAM-negative dDCs were isolated from wt and Mrp14$^{-/-}$ mice 48 h after challenge with DNFB. Subsequently, two identical groups of sensitized wt mice were injected intravenously with these freshly isolated wt or Mrp14$^{-/-}$ dDCs (1.6×10^6 dDCs per mouse) immediately before challenge with 0.4% DNFB. Ear swelling of both groups was evaluated 24 and 48 h after challenge. Data were compared to ear swelling of DNFB-challenged wt and Mrp14$^{-/-}$ mice. Data are from five mice per group (mean ± s.e.m.). $^*P < 0.05$.

results clearly illustrate that DCs are mainly responsible for the more pronounced inflammatory response of Mrp14$^{-/-}$ mice during ACD and show that altered DC migration rates could not account for the observed effects.

Discussion

In the present study, we demonstrate that loss of Mrp8/14 leads to accelerated DC differentiation. This effect results in increased T-cell activation, a mechanism promoting the initiation and intensity of allergen-specific immune responses. Previously, we have shown that Mrp8/14 plays an important activating role in innate immunity. Extracellular Mrp8/14 amplifies phagocyte activation via binding to TLR-4 (Rammes *et al*, 1997; Vogl *et al*, 2007; Frosch *et al*, 2009). Stimulation of this receptor is known to induce maturation of iBMDCs, which is prerequisite for effective antigen presentation to T lymphocytes (Rescigno *et al*, 1999). Accordingly, we found that Mrp8/14 promotes the activation of iBMDCs in a TLR-4-dependent manner inducing the expression of MHC and co-stimulatory molecules. Mrp8 is the active component of the Mrp8/14 complex inducing iBMDC activation. The role of TLR-4 in the pathogenesis of ACD is complex. The most common contact allergen Ni^{2+} has been shown to trigger pro-inflammatory signalling via direct interaction with TLR-4 (Schmidt *et al*, 2010). However, mice deficient in TLR-4 develop normal responses in contact hypersensitivity. On the other hand, several organic contact allergens indirectly activate TLR-4 via release of endogenous ligands like breakdown products of hyaluronic acid and simultaneous absence of IL-12 and TLR-4. This prevents development of ACD indicating that beside TLR activation additional mechanisms are necessary for effective sensitization (Martin *et al*, 2008; Kaplan *et al*, 2012). These results are in line with earlier reports demonstrating stimulatory effects of other DAMPs, for example, HMGB1 (Rovere-Querini *et al*, 2004; Yang *et al*, 2006, 2010), HMGN1 (Yang *et al*, 2011), or uric acid (Shi *et al*, 2003; Martinon *et al*, 2006) on DCs leading to the general conclusion that alarmins are capable to activate APCs (Yang *et al*, 2009; Nace *et al*, 2012). However, we obtained the unexpected result that Mrp14$^{-/-}$ mice, that represent functional 'double knockout' mice for Mrp8 and Mrp14 (Hobbs *et al*, 2003; Manitz *et al*, 2003; Vogl *et al*, 2007), show a more severe inflammatory response in ACD compared to wt mice, which is not due to a general anti-inflammatory function but rather specific for antigen-driven ACD. T cell-independent inflammation in ICD is not amplified in the absence of these proteins. The Mrp14$^{-/-}$ phenotype during ACD relies on the suppression of early differentiation of myeloid progenitors to (BM)DCs resulting in a diminished capacity of T-cell stimulation. Our data clearly show that the maturation state of DCs is the critical factor for the more severe course of ACD, rather than an intrinsic abnormality of T-cell function in Mrp14$^{-/-}$ mice. Thus, the alarmin Mrp8 has ambivalent effects on DC functions depending on the time and the duration of stimulation *in vivo*.

We show for the first time for members of the alarmin family that Mrp8 and Mrp14 are released during the sensitization phase of ACD. Since the functional state of DCs may depend on the local microenvironment, we analysed whether lack of Mrp8 and Mrp14 influences the antigen-presenting capacity of phagocytes at the site of inflammation in contrast to earlier reports focusing on spleen-derived DCs (Cheng *et al*, 2008). In accordance to our *in vitro* studies, we found a higher ratio of mature DCs to immature MDSCs in local ear-draining LNs of Mrp14$^{-/-}$ mice compared to wt mice during ACD *in vivo*.

We could confirm the biological relevance of changes in this local cell composition since transfer of DNFB-primed myeloid cells obtained from local ear-draining LNs of Mrp14$^{-/-}$ mice to wt mice increased the immune response to DNFB comparable to Mrp14$^{-/-}$ mice without cell transfer. In addition, the effect of these transfer experiments confirms the more potent antigen-presenting capacity of Mrp14$^{-/-}$ DCs compared to wt controls and cannot be explained by the lower number of MDSCs in Mrp14$^{-/-}$ mice as described earlier in a model of tumour immunology (Cheng *et al*, 2008).

The regulatory function of DCs in inflammatory processes is dichotomous, including induction of specific immune responses as well as maintenance of self-tolerance depending on the expression of stimulatory or regulatory co-factors (Hume, 2008; Geissmann *et al*, 2010; Nace *et al*, 2012). Mononuclear phagocytes represent a continuum of mature APCs to immature suppressors of T-cell activation (Geissmann *et al*, 2010). Furthermore, resolution of inflammation is a crucial step in maintaining tissue homeostasis. Particularly, permanently occurring micro-traumatization and subclinical infections lead to the release of autoantigens, which are then presented by APCs. Therefore, elicitation of an adaptive immune response needs to be strictly controlled (Sallusto *et al*, 1999) to avoid allergic or autoimmune reactions and subsequent tissue damage. During development of an allergic reaction in otherwise healthy mice blocking of early differentiation of myeloid progenitors to i(BM)DCs by Mrp8 obviously hampers induction of an antigen-specific immune response.

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Despite the phenotype presented here for ACD, our data may also have impact for a wider field of DC biology. Intestinal mononuclear phagocytes, including DCs, are crucial for maintaining intestinal homeostasis allowing tolerance against the high number of commensal microorganisms, which is generally of mutual benefit (Sansonetti and Medzhitov, 2009; Varol *et al*, 2010). Maintenance of this homeostatic immune tolerance may involve active downregulation of immune responses by the host during contact with commensal bacteria to a state of 'physiological inflammation' (Sansonetti and Medzhitov, 2009). This is assured via readily killing of invading bacteria by macrophages, which do not elicit an immunological response, ensuring that the systemic immune system is essentially ignorant of these microorganisms (Macpherson and Harris, 2004). In this context, Mrp8 and Mrp14 may be reasonable candidates for maintaining 'physiological inflammation' since both proteins are highly upregulated in the intestine during infection or tissue damage (Foell *et al*, 2009), promote effector mechanisms of innate immunity, and show a moderate antimicrobial activity (Vogl *et al*, 2007; Ehrchen *et al*, 2009). On the other hand, Mrp8 and Mrp14 may limit induction of adaptive immune responses against harmless commensal microorganisms by blocking the development of pro-inflammatory DCs. Moreover, our finding of an endogenous TLR-4 activator suppressing ACD shows an interesting parallel to the so-called 'hygiene hypothesis'. Albeit short-term activation of TLR-4 by microbial products triggers innate immune mechanisms, this theory claims that continuous stimulation of TLRs by microbial products, especially LPS-mediated stimulation of TLR-4, is one major molecular mechanism preventing manifestation of allergic diseases (Macpherson and Harris, 2004; Okada *et al*, 2010; Ege *et al*, 2011). Our data point to the possibility that continuous exposure of APC progenitors to endogenous TLR-4 ligands may be involved in this process as well.

Breaking these control mechanisms on the other hand may be a possible cause promoting autoimmune disease. In an experimental model of autoimmunity driven by epidermal expression of CD40 ligand, we have previously shown that continuous pathologic co-stimulation of DCs obviously overruns inhibitory effects of Mrp8 on early DC differentiation (Loser *et al*, 2010). In this model, TLR-4-dependent inflammatory effects of Mrp8 on mature DCs dominate and promote the inflammatory process. Thus, the function of Mrp8, or potentially also of other alarmins, is strictly dependent on the time and duration of expression. Identification of triggers breaking those innate mechanisms of immunological self-control may be of relevance for future therapeutic strategies, since expression of Mrp8 and Mrp14 is prominently upregulated in a couple of clinically relevant autoimmune diseases like rheumatoid arthritis, vasculitis, systemic lupus erythematosus, or Crohn's disease (Foell and Roth, 2004; Ehrchen *et al*, 2009).

Taken together, we describe a new mechanism of immune regulation mediated by Mrp8, which has not been shown for another alarmin so far. Our data show for the first time that a member of this protein family is an important factor in orchestrating coordinated immune reactions. On the one hand, it allows elicitation of an appropriate innate inflammatory response by acting as a classical DAMP protein, but on the other hand it prevents hyper-activation of the adaptive immune system most likely in order to avoid tissue damage due to overwhelming and inadequate immune responses.

Materials and methods

Mice

Adult C57BL/6 and BALB/c mice were obtained from Janvier (France) and Harlan (Germany) laboratories. Mrp14$^{-/-}$ (Manitz *et al*, 2003) and RAGE$^{-/-}$ mice were generated by targeted gene disruption in our laboratory and backcrossed to C57BL/6 for at least 10 generations. TLR-4$^{-/-}$ mice were provided by P van Lent (Department of Rheumatology, Radboud University Medical Centre, Nijmegen, The Netherlands) or CJ Kirschning (Institute of Medical Microbiology, University of Duisburg-Essen, Essen, Germany). We performed all experiments according to institutional regulations (licence 8.87-50.10.37.09.244, District Government and District Veterinary Office, Münster, Germany).

Purification of Mrp8, Mrp14, and Mrp8/14

Mrp8, Mrp14, and Mrp8/14 were purified as described earlier (Vogl *et al*, 2007). All preparations revealed a purity of >98%. Endotoxin contaminations were excluded by limulus amebocyte lysate (LAL) assay or were below 2 pg LPS/μg protein and addition of polymyxin B did not affect the activities of the Mrp proteins.

Reagents

DNFB, DNBS, 3-amino-9-ethylcarbazol (AEC), LPS from *E. coli* 055:B5, periodic acid, albumin from bovine serum (BSA), FITC, polymyxin B, and croton oil were obtained from Sigma-Aldrich (Munich, Germany). LAL assay was from BioWhittaker (Walkersville, USA). RPMI-1640, L-Glutamine, Gentamycin, non-essential amino acids (NEAA), and PBS Dulbecco were from Biochrom (Berlin, Germany). Sodium azide (NaN_3), hydrogen peroxide (H_2O_2), and Mayer's hemalum solution were from Merck (Darmstadt, Germany). Normal goat serum (NGS) and 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE) were obtained from Invitrogen (Darmstadt, Germany). FlowCytomix Multiple Analyte Detection System was purchased from eBioscience (Frankfurt, Germany) and fetal bovine serum (FBS) from Biowest (Renningen, Germany). CTO was obtained from Molecular Probes (Darmstadt, Germany).

Monoclonal anti-mouse antibodies

Purified CD4 (H129.19), CD8a (53-6.7), CD19 (ID3), CD11b (M1/70), CD16/CD32 (2.4G2), CD45R/B220 (RA3-6B2), Ly-6G/Ly-6C (RB6-8C5), TER-119 (TER-119), CD49b (DX5), as well as biotin-conjugated CD24 (M1/69) and CD11c (HL3) Abs were purchased from Becton-Dickson (Heidelberg, Germany). Purified F4/80 (CI:A3-1) Ab was obtained from AbD Serotec (Düsseldorf, Germany). Purified EpCAM (G8.8) and biotin-conjugated-γδ TCR (GL-3), as well as CD86 FITC (GL1), MHC class II FITC (M5/114), CD11c APC (N418), F4/80 FITC (BM8), CD4 APC (RM4-5), and CD8a APC (53-6.7) Abs were from eBioscience. CD69 PE (310106) Ab was from R&D Systems (Wiesbaden, Germany). Purified Ly-6C (HK1.4) and CD11b FITC (M1/70) Abs were obtained from BioLegend (Fell, Germany) and Ly-6C APC (1G7.G10) Ab from Miltenyi Biotech (Bergisch Gladbach, Germany). Peroxidase-conjugated affin pure F'(ab)$_2$ fragment goat anti rabbit IgG (H+L) was purchased from Dianova (Hamburg, Germany). Rabbit antisera against recombinant murine Mrp8 and Mrp14 were produced in our laboratory as described previously (van Lent *et al*, 2008a).

Allergic contact dermatitis

At day zero, C57BL/6 wt and Mrp14$^{-/-}$ mice were sensitized by painting 100 μl of 0.4% DNFB in acetone/olive oil (4:1) onto the shaved abdominal skin. Six days later, mice were challenged by applying 10 μl 0.4% DNFB in acetone/olive oil on the dorsal and ventral surface of the right ears, respectively. The left ears served as controls and were treated with acetone/olive oil only. Ear thickness was measured 24 and 48 h after challenge using a digital calliper (Roth, Karlsruhe, Germany). Ear swelling was determined by calculating the difference between the unchallenged ear (left) and the challenged ear (right).

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Irritant contact dermatitis

C57BL/6 wt and Mrp14$^{-/-}$ mice were painted with 10 μl 3% croton oil in acetone/olive oil (4:1) onto the right ears. The left ears served as controls and were treated with acetone/olive oil only. Ear thickness was measured 24 and 48 h after the treatment using a digital calliper. Ear swelling was determined by calculating the difference between the unchallenged ear and the challenged ear.

DC isolation from ear-draining LNs

For isolation of immigrated skin-derived DCs from ear-draining LNs, mice were sacrificed 48 h after challenge. Ear-draining LNs were extracted and single cell suspensions were produced. Cells were washed in cold PBS containing 1% FBS. For depletion of lymphocytes and erythrocytes, cells were incubated with rat anti-mouse CD4, CD8a, CD19, and TER-119 mAbs (0.5 μg/1 $\times 10^6$ cells, respectively) and magnetic labelled goat anti-rat IgG microbeads (Miltenyi Biotech). After washing with cold PBS/FBS, antibody-coated cells were removed on MACS columns (Miltenyi Biotech). To further enrich dDCs, the cells were depleted of EpCAM-positive epidermal Langerhans cells as described above, using a rat anti-mouse EpCAM mAb. In some experiments, EpCAM-negative dDC fractions were additionally depleted of MDSCs by positive selection using an anti-mouse Ly-6C mAb. In all experiments, enriched DC fractions were checked for macrophage contamination by CD11c-F4/80 double staining using flow cytometry. The amount of F4/80 single positive macrophages was always $<7\%$.

Measurement of Mrp8/14 concentrations

Mrp8/14 concentrations were determined in sera of sensitized wt mice by ELISA as described previously (Vogl *et al*, 2007).

Flow cytometry

Immunofluorescence staining was performed after washing the cells with PBS/1% FBS. In all experiments, 1–2 $\times 10^5$ cells were incubated for 20 min at 4°C with optimal dilution of fluorescently labelled mAbs in PBS/FBS. After washing, the cells were analysed by flow cytometry (FACS Calibur and CellQuestPro software; Becton-Dickson, Heidelberg, Germany). Mean fluorescence intensity (m.f.i.) shifts were calculated by dividing the geometric mean fluorescence of cells stained with antigen-specific mAb by the geometric mean fluorescence for the same cells stained with isotype control Ab.

Analysis of cell–cell interactions by time-lapse microscopy

Interactions of C57BL/6 wt and Mrp14$^{-/-}$ BMDCs with allogenic BALB/c T cells were analysed using 3-D collagen gels. DCs and T cells (ratio 1:10) were embedded in bovine collagen type I at a total cell number of 11 $\times 10^6$ cells/ml. Interactions were monitored for 8 h by time-lapse microscopy using an Olympus BX61 microscope (Olympus, Hamburg, Germany) with an UAPO lens (20 ×) and a FView camera with CellP software (Olympus, Münster, Germany).

Spleen T-cell purification and CFSE labelling

For purification of T cells, spleen cell suspensions were passed through a nylon wool matrix column. Further enrichment of T cells was achieved by depletion of monocytes, macrophages, granulocytes, B cells, erythrocytes, γδT cells, platelets, and DCs using a cocktail of rat-CD11b, rat-CD19, rat-CD16/CD32, rat-CD45R/B220, rat-Ly-6G/Ly-6C, rat-CD49b, rat-TER-119, rat-biotin-CD24, hamster-biotin-γδ TCR, and hamster-biotin-CD11c mAbs. Antibody-coated cells were removed using anti-rat IgG- and anti-biotin coated magnetic beads according to manufacturer's instructions (Miltenyi Biotech). For T-cell proliferation experiments, T cells were stained with CFSE (Invitrogen) for 4 min at 37°C. Following washing in PBS/1% FBS, cells were resuspended in complete medium (RPMI 1640 containing 1% NEAA, 1% L-Glutamine, 10% FBS, and 10 μg/ml Gentamycin) at 1 $\times 10^6$/ml.

T-cell proliferation assay

CFSE-labelled T cells (1 $\times 10^5$) were co-cultured with DCs (2 $\times 10^4$) in 200 μl complete medium in 96-well round-bottomed plates (Greiner bio-one, Frickenhausen, Germany) for 5 days (DC:T-cell ratio was 1:5). T-cell proliferation was assessed by CFSE dilution on a FACS Calibur. Supernatants were collected for detection of cytokines.

Measurement of cytokine production

Cell culture supernatants were subjected to cytokine analysis (IFN-γ, IL-17) by FlowCytomix Multiple Analyte Detection System (eBioscience) according to manufacturer's protocol.

In vitro generation of BMDCs

BM cells were isolated from murine femur and tibia. Cells were cultured in complete medium supplemented with 100 U/ml rmIL-4 and 100 U/ml rmGM-CSF (Immunotools, Friesoythe, Germany) for 6 days at 37°C and 5% CO_2. On days 3 and 6, half of the supernatant was replaced with fresh cytokine-containing medium. On day 6, immature DCs were cultured for further 48 h in the presence of different stimulants as indicated in the figures. On day 8, mature DCs were harvested. In some experiments, BM cells were cultured in the presence of 8 μg/ml Mrp8 throughout the course of the complete experiment.

Immunohistochemistry of ear sections

Tissue sections from DNFB-treated and untreated ears were fixed in acetone. For blockage of endogenous peroxidase, slides were incubated in PBS/NaN_3/H_2O_2. To avoid unspecific binding, Fc receptors were blocked by incubating sections in PBS/1% BSA including 50% NGS. Afterwards, slides were treated with appropriate dilution of primary antibody or isotype control. After washing, the samples were incubated with a horseradish peroxidase-conjugated secondary antibody for 45 min. To visualize peroxidase activity, AEC was used as chromogen. To completely destroy endogenous peroxidase, slides were incubated in 14.5 mM periodic acid. Counterstaining of tissue sections was performed with Mayer's hemalum solution. Images were acquired by using an upright microscope (Axioskop, Zeiss, Jena, Germany) and imported into Photoshop version 9.0 (Adobe Systems) for production of the final figures.

Statistical analysis

Statistical significance of the data was calculated using Student's *t*-test for paired samples. Probability (*P*-value) of <0.05 was considered to be significant.

Supplementary data

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

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Author contributions: TV, JR, and CS supervised the project. TV, JR, and BP designed experiments and wrote the paper. BP, JA, and MA performed experiments. BP and TV analysed data. MW, BP, KL, and VK performed microscopy experiments. PvL and DF performed experiments with TLR-4$^{-/-}$ and RAGE$^{-/-}$ mice, respectively.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Material and Methods

Homing of CTO-stained dDCs

EpCAM negative dDCs were isolated from wt and Mrp14 -/- mice 48h after challenge with DNFB and stained with cell tracker orange (CTO; 5µM; Molecular Probes, Darmstadt, Germany). $9x10^6$ of the stained wt and Mrp14 -/- cells were injected in the tail veins of two identical groups of DNFB-sensitized wt mice prior to challenge. After 48h the immigration of CTO positive cells into ear-draining LNs was determined by flow cytometry.

FITC application

Ears of naïve C57BL/6 wt and Mrp14 -/- mice were painted with 30µl 0.5% FITC in an acetone/dibutylphthalate mixture (Sigma-Aldrich, Munich, Germany). After 18h ear-draining LNs were analyzed for immigrated FITC positive cells by flow cytometry.

Supplementary Figure legends

Figure S1. Histological analyses of wt and Mrp14 -/- ear sections from control ears, treated with acetone/olive oil only. Sections were stained for immune cells ($CD11b^+$ myeloid cells, $Gr\text{-}1^+$ granulocytes, $F4/80^+$ macrophages) as well as for Mrp8 and Mrp14. Scale bar: 250µm.

Figure S2. Identical cellular homing capacities of wt and Mrp14 -/- dDCs. **(A)** EpCAM negative dDCs were isolated from wt and Mrp14 -/- mice 48h after challenge with DNFB and stained with CTO. Subsequently, two identical groups of DNFB-sensitized wt mice were injected intravenously with these CTO-stained wt or Mrp14 -/- dDCs ($9x10^6$ dDCs per mouse) immediately before challenge with 0.4% DNFB. After 48h the immigration of CTO positive dDCs into ear-draining LNs was determined by flow cytometry. **(B)** Ears of naïve C57BL/6 wt and Mrp14 -/- mice were painted with 30µl 0.5% FITC. After 18h ear-draining LNs were analyzed for immigrated FITC positive cells by flow cytometry.

Figure S1

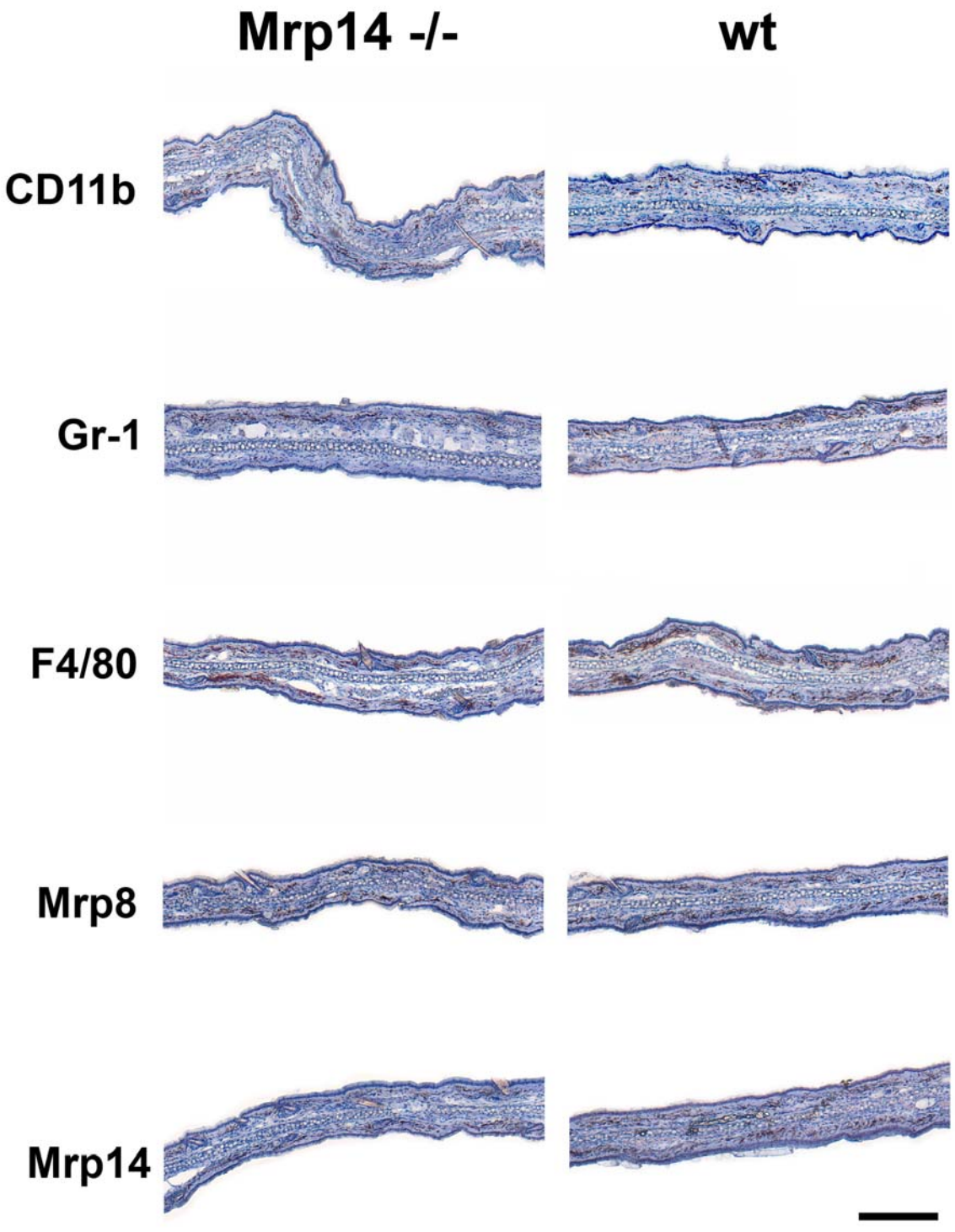
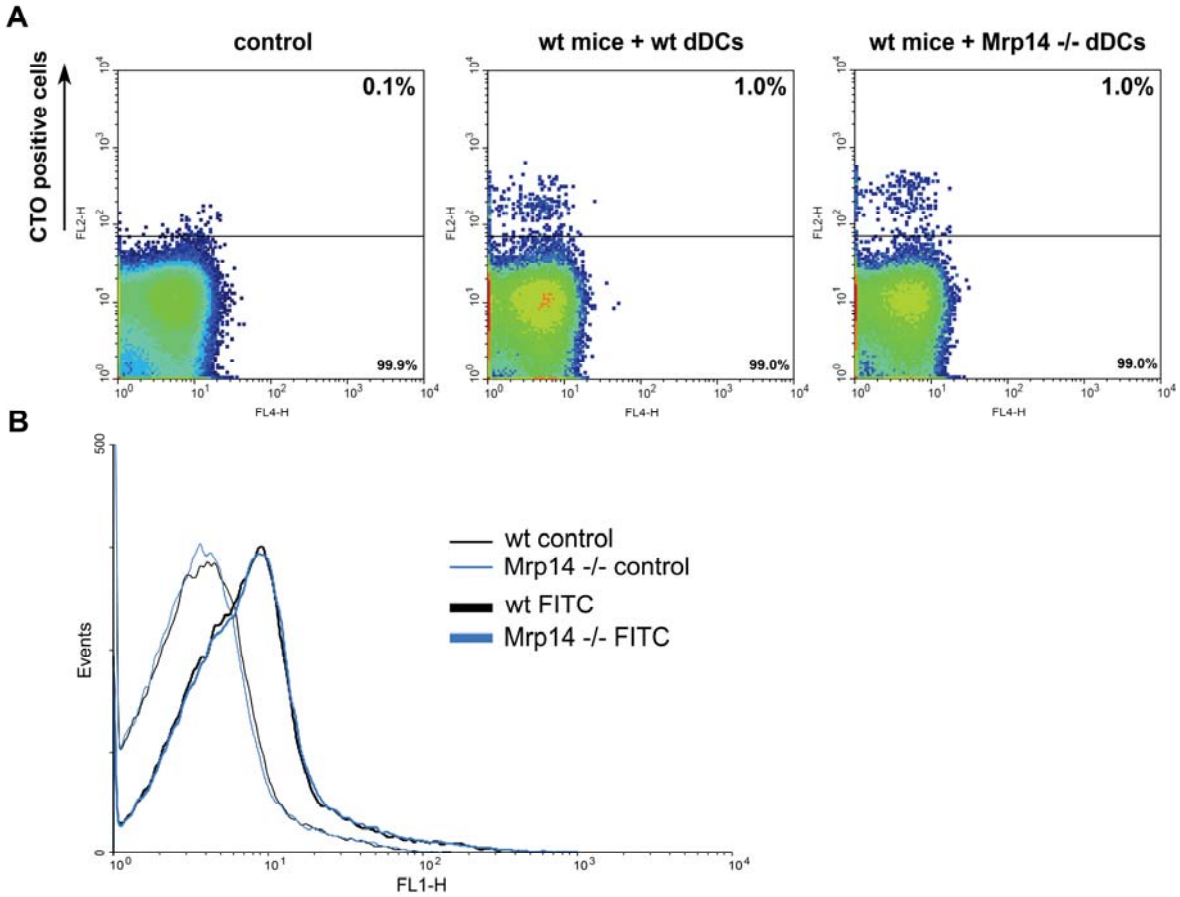


Figure S2


A
control
wt mice + wt dDCs
wt mice + Mrp14 -/- dDCs
CTO positive cells
0.1%
99.9%
1.0%
99.0%
1.0%
99.0%
FL2-H
FL4-H
B
Events
FL1-H
wt control
Mrp14 -/- control
wt FITC
Mrp14 -/- FITC