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European Journal of Immunology

Tissue immunology and leukocyte trafficking

Basic

Research Article

Migration of murine intestinal dendritic cell subsets upon intrinsic and extrinsic TLR3 stimulation

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Initiation of adaptive immunity to particulate antigens in lymph nodes largely depends on their presentation by migratory dendritic cells (DCs). DC subsets differ in their capacity to induce specific types of immunity, allowing subset-specific DC-targeting to influence vaccination and therapy outcomes. Faithful drug design, however, requires exact understanding of subset-specific versus global activation mechanisms. cDC1, the subset of DCs that excel in supporting immunity toward viruses, intracellular bacteria, and tumors, express uniquely high levels of the pattern recognition receptor TLR3. Using various murine genetic models, we show here that both, the cDC1 and cDC2 subsets of cDCs are activated and migrate equally well in response to TLR3 stimulation in a cell extrinsic and TNF-α dependent manner, but that cDC1 show a unique requirement for type I interferon signaling. Our findings reveal common and differing pathways regulating DC subset migration, offering important insights for the design of DC-based vaccination and therapy approaches.

Keywords: Activation · Dendritic cells · TLR3 · Migration · Type I interferon

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

DCs are the major APCs in the body, which, upon migration to secondary lymphoid organs, initiate and shape naïve T-cell responses to peripherally acquired antigen. DCs are divided into two major subsets referred to as cDC1 and cDC2 [1]. In the intestine, migratory cDC1 are defined as XCR1$^+$CD103$^+$CD11b$^-$, while cDC2 can be divided into a major XCR1$^-$CD103$^+$CD11b$^+$ and a minor XCR1$^-$CD103$^-$CD11b$^+$ subset. Although both subsets present mucosa-derived antigen in the draining LNs, cDC1 and cDC2 differ in their capacity to induce specific immune responses [2]. While cDC1 are generally implicated in viral defense and cross-presentation of exogenous antigens to MHCI-restricted CD8$^+$

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T cells and MHCII-restricted $CD4^+$ T_H1 cells [3], cDC2 are highly effective at inducing T_H17 and T_H2 responses [4-6]. Specific targeting of DC subsets is thus of high relevance for DC-based strategies for vaccination and therapeutic approaches against different types of antigen.

Antigen-targeting to specific DC subsets using antibody-mediated delivery to differentially expressed surface receptors can indeed shape the resulting type of immunity [7]. One family of molecules expressed differentially by DC subsets is TLRs [8, 9], suggesting that differential engagement of DC subsets could also be achieved by using adjuvants specifically activating one subset but not the other. In support of this idea, the induction of fully functional cytotoxic $CD8^+$ T lymphocytes depends on simultaneous uptake of antigen together with cell-intrinsic stimulation of pattern recognition receptors expressed by the presenting DC (cis-activation), as shown in vivo in a mouse model for pulmonary DC activation [10]. TLR3 is an endosomal receptor that recognizes double-stranded RNA (dsRNA), a molecular pattern associated with viral infections [11, 12]. As several studies have demonstrated that TLR3 is preferentially expressed in cDC1 [8, 13-15] and promotes cross-presentation of antigen with high efficiency [16-18], targeting TLR3 is a promising strategy in cancer-immunotherapy and vaccination against viruses. A hallmark of DCs is to migrate to the draining LNs to present peripherally acquired antigen. In response to the TLR7-stimulating agent R848, plasmacytoid DC (pDC)-derived TNF-α drives cDC migration from the small intestinal lamina propria (SI-LP) to the mesenteric LNs (mLNs), while type I IFN regulates DC activation [19]. Subset-specific requirements were not assessed. Most TLR3 driven transcriptional changes in splenic DCs after stimulation with the dsRNA mimic polyinosinic:polycytidylic acid (poly(I:C)) result from secondary effects through the type I IFN receptor on cDCs [20]. This suggests that migration in response to poly(I:C) may also depend on type I IFN signaling. Here, we have analyzed in detail the major cellular and molecular players involved in the activation and migration of murine intestinal cDC subsets in response to poly(I:C) in vivo and provide novel insights regarding cis- and transregulation of these processes.

Results

Poly(I:C)-induced intestinal DC migration depends on TLR3 signaling

We first set out to analyze in detail the expression of TLR3 by immune cells of the spleen, mLNs, and SI-LP and confirmed that only cDC1 expressed high amounts of TLR3 in all organs (Fig. 1A, for a full-gating strategy employed throughout see Supporting information Fig. 1A). While macrophages also expressed low levels of TLR3, cDC2 were almost entirely negative and B and T cells showed no expression (Fig. 1A and Supporting information Fig. 1B). Importantly, stimulation with poly(I:C) did not change TLR3 expression across subsets (Fig. 1A). These results are consistent with in vitro data on the differential abilities of the cDC subsets to poly(I:C) stimulation [14] and therefore we hypothesized that poly(I:C) would drive migration of cDC1 preferentially in vivo. To test this, we quantified $CD103^+$ cDC1 and cDC2 in the mLNs after intraperitoneal injection of poly(I:C), based on the knowledge that CD103 expression by cDCs in the mLNs defines those which are derived from CCR7 dependent migration from the intestinal mucosa [21, 22]. Consistent with this idea, the numbers of both, migratory cDC1 and cDC2, increased after administration of poly(I:C), peaking at 12 h post injection and returning to steady-state levels after 24 h (Fig. 1B). Interestingly, cDC2 migrated almost as efficiently as cDC1, with only a small disadvantage being seen at early time points.

Although retinoic acid-inducible gene 1 (RIG-I)-like helicases that signal through mitochondrial antiviral-signaling protein (MAVS) can also sense poly(I:C) [23], DC migration of both subsets was completely abrogated in TLR3-deficient mice (Fig. 1C) and in mice deficient for the TLR3 adapter TRIF (TIR-domain-containing adapter-inducing IFN-β) (Supporting information Fig. 1C). As DC migration and activation, both crucial events for the induction of immunity, can be regulated independently [19, 24], we also measured the expression of the costimulatory molecule CD86 as a surrogate marker for DC activation. Again, activation of both DC subsets was also entirely depended on TLR3 and TRIF expression (Fig. 1D and Supporting information Fig. 1D), showing that poly(I:C) induces migration and activation of both, cDC1 and cDC2 in a strictly TLR3-dependent manner. Our findings are in accordance with previously published data showing that in vitro activation with poly(I:C) is abrogated in BM-derived DCs from TLR3-deficient mice [14]. However, as cDC2 themselves express virtually no TLR3, our data indicate that TLR3 stimulation might act in both, cell-intrinsic and -extrinsic, manners on cDCs in vivo.

Cell-intrinsic TLR3-sensing is dispensable for DC migration

Nonhematopoietic cells express TLR3 and support immune cell survival, maturation, and function. For example, TLR3 expression in intestinal epithelial cells is required for optimal clearance of rotavirus [25] and epithelial cells have previously been implicated in driving DC migration to the draining LNs during viral infection [26]. To determine whether TLR3-dependent sensing in nonhematopoietic cells could induce intestinal DC migration in response to poly(I:C), we reconstituted irradiated WT mice with TLR3-deficient BM and treated the mice with poly(I:C). The results showed that while DCs migrated well in response to poly(I:C) in WT recipients of WT BM, there were no significant increases in mLN DC numbers in recipients of TLR3-deficient BM after administration (Fig. 2A). Thus, TLR3 expression within the hematopoietic compartment is required to drive efficient DC migration in response to poly(I:C).

As cDC1 uniformly expressed TLR3, we explored the role of this subset in sensing poly(I:C) for driving DC migration directly, by generating a mouse model that allows for cell-specific

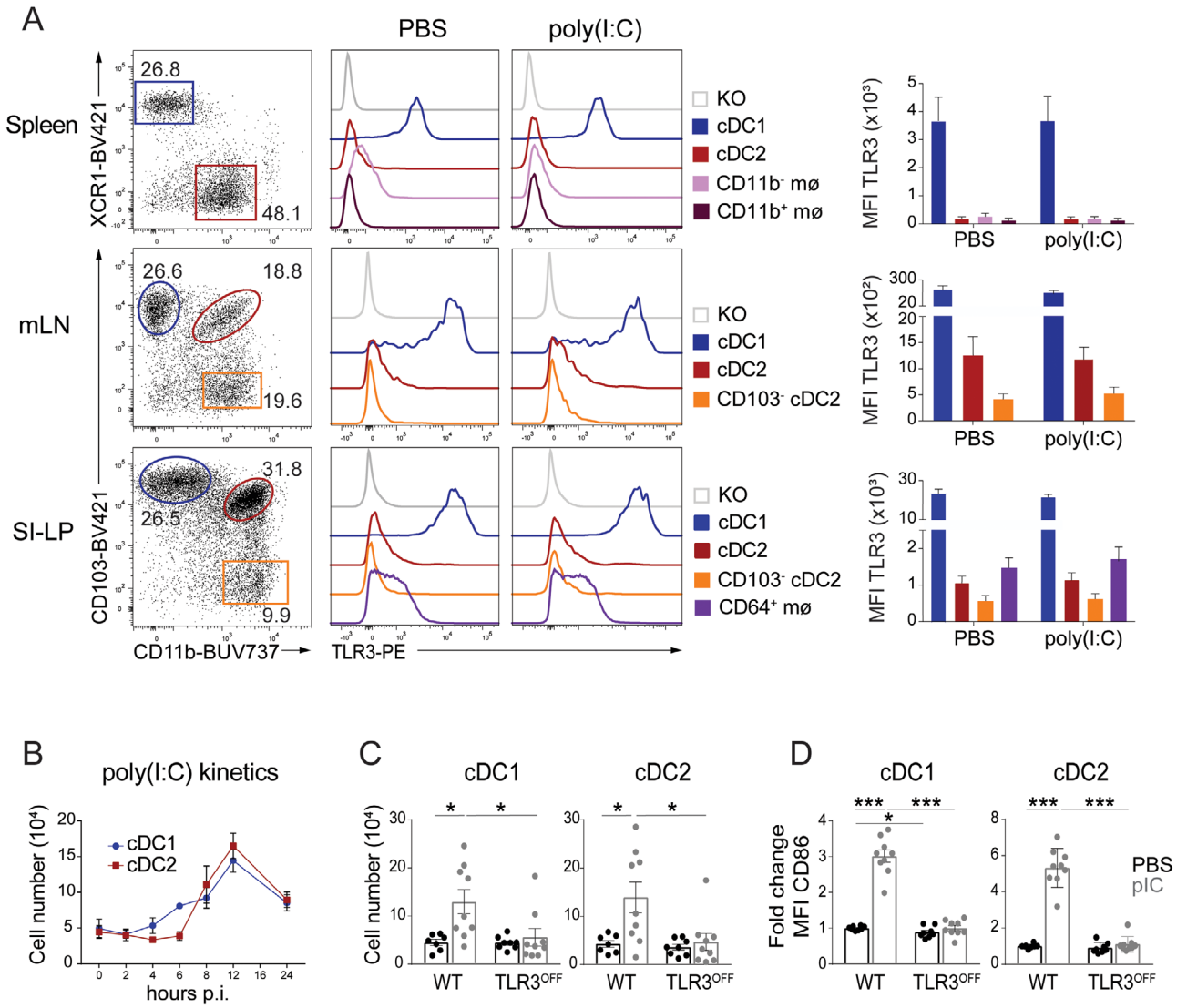

Figure 1. TLR3 expression by mononuclear phagocytes and migration of cDCs in response to poly(I:C). (A) Left: representative flow cytometry plots of spleen, mLN, and SI-LP DC subsets from C57BL/6 mice. All populations were gated on live, lineage (CD3, CD19, NK1.1) negative, single cells. DC in spleen and mLN were further pregated as $CD11c^+MHCII^+$ cells and in SI-LP as $CD11c^+MHCII^+CD64^-$ cells. Macrophages in spleen were further pregated as $CD11c^{int}$ and $CD11b^+$ or $CD11b^-$, while macrophages in SI-LP were further gated as $CD11c^+CD64^+$ cells. Histograms: intracellular TLR3 staining of the indicated DC and macrophage populations 12 h after i.p. injection of PBS or 100 μg poly(I:C) into WT mice and by bulk DC in resting $TLR3^{OFF}$ mice (KO; $TLR3^{LSL/LSL}$ mice not bred to any cre line). Right: quantification of TLR3 expression by DC subsets and macrophages in C57BL/6 mice 12 h after i.p. injection of PBS or poly(I:C). Data shown are means ± 1 sem pooled from two independent experiments with three mice per group per experiment. (B) Kinetics of intestinal cDC1 and cDC2 migration after i.p. injection of 100 μg poly(I:C). Data shown are mean numbers of mLN cDC1 (blue circles) and cDC2 (red squares) ± 1 sem pooled from two to four independent experiments with two to three mice per group per experiment. Differences between cDC1 and cDC2 are not significant. (C) Total numbers of cDC1 and cDC2 in the mLNs of WT and $TLR3^{OFF}$ mice 12 h after i.p. injection of PBS (black circles) or 100 μg poly(I:C) (gray circles). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with three mice per group per experiment. Two-way ANOVA, $^*p < 0.05$. (D) Activation of cDC subsets in mLNs of WT and $TLR3^{OFF}$ mice by poly(I:C). Results shown are fold change in CD86 expression 12 h after injection of PBS (black) or 100 μg poly(I:C) (gray) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from three independent experiments with three mice per group per experiment. Two-way ANOVA, $^*p < 0.05$, $^{***}p < 0.0005$.

re-expression of TLR3 from the endogenous locus in a TLR3 KO background. To this end, a floxed transcriptional termination cassette was inserted into the coding sequence of the TLR3 gene ($TLR3^{OFF}$), abolishing TLR3 expression. Expression of TLR3 by cDC1s could then be restored in cDC1.$TLR3^{ON}$ mice, in which the TLR3 stop codon was deleted using XCR1-driven cre recombinase (XCR1.cre [27]) (Fig. 2B and Supporting information Fig. 2A). Poly(I:C)-induced DC migration and activation of both, cDC1 and cDC2 occurred in cDC1.$TLR3^{ON}$ mice, but to a lesser extent compared to WT mice (Fig. 2C and D). As expected, DC migration was absent in $TLR3^{OFF}$ mice (Fig. 2C and D). These findings suggest that while cDC1-restricted TLR3 expression can drive

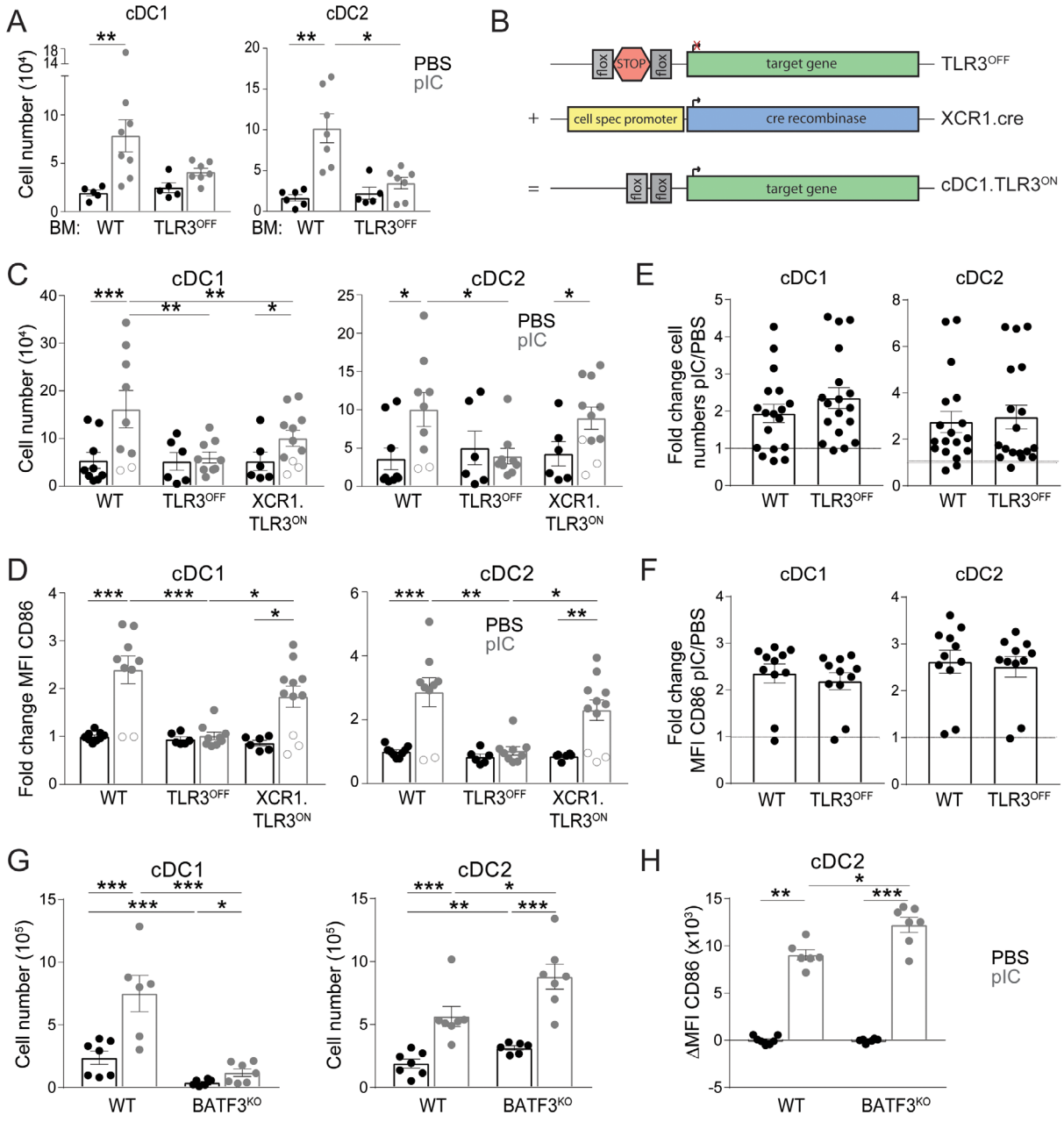

Figure 2. Cellular requirements for TLR3-mediated DC migration in response to poly(I:C). (A) Total numbers of cDC1 and cDC2 in the mLNs of WT recipients reconstituted for 6–8 weeks with either WT or TLR3^{OFF} BM 12 h after i.p. injection of PBS (black circles) or 100 μg poly(I:C) (gray circles). Data shown are mean numbers of cells ± 1 sem from one representative experiment out of two with three to eight mice per group per experiment. Mann–Whitney U test, $^{*}p < 0.05$, $^{**}p < 0.005$. (B) Schematic diagram of generation of cell-type specific TLR3^{ON} mice in which TLR3^{OFF}, which were created by crossing mice harboring a floxed STOP codon in the endogenous TLR3 locus to mice expressing cre under the control of a cell-type specific promoter (e.g. XCR1 for cDC1). Cre-mediated deletion of the STOP codon drives re-expression of the endogenous TLR3. (C) Total numbers of cDC1 and cDC2 in the mLNs of WT, TLR3^{OFF}, and XCR1.TLR3^{ON} mice 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with three to four mice per group per experiment. Two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. Open circles were used to mark those poly(I:C) injected mice that also did not show upregulation of CD86 (panel D); these were not excluded from statistics. (D) Activation of cDC subsets in mLNs of WT, TLR3^{OFF}, and XCR1.TLR3^{ON} mice by poly(I:C). Results shown are fold change in CD86 expression 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from three independent experiments with three to four mice per group per experiment. Two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. (E) Fold change of total number of cDC1 and cDC2 in the mLNs 12 h after i.p. injection of 100 μg poly(I:C) versus PBS derived from the indicated BM in 50:50 WT:TLR3^{OFF} mixed BM chimeras. Data shown are means ± 1 sem pooled from three independent experiments with four to seven mice per group per experiment. Two-way ANOVA, not significant. (F) Activation of cDC1 and cDC2 in mLNs of 50:50 WT:TLR3^{OFF} mixed BM chimeras by poly(I:C). Results shown are fold change in CD86 expression 12 h after i.p. injection of 100 μg poly(I:C) versus PBS and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from two out of three representative independent experiments with four to seven mice per group per experiment. Two-way ANOVA, not significant. (G) Total number of cDC1 and cDC2 cells in BATF3^{KO} mice 12 after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray). Data shown

poly(I:C)-induced DC migration, other TLR3-expressing cells contribute to optimal DC migration in response to poly(I:C). Of note, careful analysis of XCR1-driven re-expression of TLR3 revealed that re-expression of TLR3 also occurred in approximately 20% of $CD64^+CD11b^+XCR1^-$ macrophages in the intestine, but not in splenic macrophages (Supporting information Fig. 2A). This phenomenon is not specific for the TLR3 locus, as XCR1.cre could also drive YFP expression by some intestinal macrophages when crossed to ROSA-STOP-YFP (data not shown). We therefore examined whether off-target re-expression of TLR3 by intestinal macrophages might account for the restored DC migration in $cDC1.TLR3^{ON}$ mice. However, migration of both, cDC1 and cDC2, was entirely normal after administration of poly(I:C) to CCR2-deficient mice that lack most of the monocyte-derived intestinal macrophages [28] (Supporting information Fig. 2B). Finally, we could not detect any migration or activation of either DC subset if TLR3 expression was restricted to intestinal epithelial cells of $TLR3^{OFF}$ mice using villin-cre ($villin.TLR3^{ON}$, Supporting information Fig. 2C). Together, these data suggest that cDC1-specific TLR3 expression can drive DC migration in response to poly(I:C), although a contributory role for a residual population of CCR2-independent, TLR3 expressing intestinal macrophages in $cDC1.TLR3^{ON}$ mice is likely.

The fact that expression of TLR3 restricted to XCR1-expressing cDC1 can drive the migration and activation of cDC2 indicates a cell extrinsic effect of poly(I:C) on cDC2. To test whether cell extrinsic effects on cDC1 are equally sufficient, we generated mixed-BM chimeras in which WT recipients on a CD45.1/.2 congenic background were reconstituted with a 50:50 mix of $CD45.1^+$ WT and $CD45.2^+$ TLR3-deficient BM. Under these conditions, administration of poly(I:C) induced the activation and migration of TLR3-deficient cDC1 and cDC2 to the same extent as their WT counterparts in the same hosts (Fig. 2E and F), indicating that both, cDC1 and cDC2 can respond to TLR3 stimulation in a cell extrinsic manner. This is presumably driven by the TLR3-competent BM-derived cells of WT origin present in the mixed chimeras. Interestingly, cDC1 themselves do not appear to play an essential role in this process, as complete deficiency of cDC1 in $BATF3^{KO}$ mice [29] did not abrogate the activation and migration of cDC2 in response to poly(I:C), showing that hematopoietic cells other than cDC1 can also contribute (Fig. 2G and H). Macrophages are a potential candidate for this role, as they express and respond to TLR3 stimulation [30] and we attempted to explore their involvement by generating macrophage-specific $TLR3^{ON}$ mice using LysM.cre [31] to delete the TLR3 stop codon in $TLR3^{OFF}$ mice. However, this approach was unsuccessful, as TLR3 was re-expressed by approximately 50% of cDC1 of $LysM.TLR3^{ON}$ mice and thus the role of macrophages in responding to TLR3 in vivo requires further investigation (Supporting information Fig. 2D).

DC migration in response to poly(I:C) is independent of MyD88, but requires TNF- receptor signaling

The cell extrinsic effect of TLR3 on DC migration in response to poly(I:C) suggests that inflammatory mediators produced following TLR3 signaling on $TLR3^+$ target cells might play a key role in this process. We therefore measured the expression of cytokines that have been implicated in DC activation and migration by qPCR analysis of whole SI tissue samples at different times after administration of poly(I:C). This showed increased levels of mRNA for TNF-α, IL-1β, IFN-α, IFN-β, and IFN-λ after 2 and 4 h after poly(I:C) injection (Fig. 3A).

Steady-state migration of intestinal DCs depends on MyD88 signaling through NFκB [22, 32] and although TLR3 signaling itself does not require MyD88, the IL-1 receptor signals through MyD88 [33]. However, the activation and migration of both, cDC1 and cDC2 occurred normally in poly(I:C)-treated $MyD88^{KO}$ mice (Fig. 3B and C). Although TNF receptor 1 (TNFR1) signaling is not important for intestinal DC migration in the steady state, it is required for DCs to migrate in response to R848 [22]. TNFR1 signaling is important for the induction of the $CD8^+$ T cell response toward mouse hepatitis virus, and expression on DCs alone is sufficient to confer protection [34]. As we found TNF-α to be upregulated in the intestine after injection with poly(I:C), we examined its role in poly(I:C)-induced DC migration. Indeed, there were no significant increases in migration of either cDC1 or cDC2 in response to poly(I:C) in either $TNFR1^{KO}$ mice or mice treated in vivo with a blocking anti-TNFR1 antibody, while there was minimal DC activation in poly(I:C)-treated $TNFR1^{KO}$ mice (Fig. 3D–F). These results are consistent with previous studies of the effect of mast cell derived TNF-α on skin DCs [35, 36] and show that TNFR1 signaling is a crucial secondary signal that mediates the extrinsic response of DCs to TLR3-sensing in vivo.

DC subsets differ in type I IFN signaling requirements in response to poly(I:C)

In addition to elevated expression of TNF-α and IL-1β, type I IFNs were significantly upregulated in the intestine after poly(I:C) injection (Fig. 3A). Previous studies have shown a prominent role for type I IFN in the activation and maturation of splenic DCs in response to poly(I:C), acting via the type I IFN receptor on DCs [20]. Conversely, the TNF-α dependent migration of intestinal DCs in response to R848 does not require type I IFN signaling but it is rather needed for their activation [19]. We therefore tested directly the role of type I IFN in the activation and migration of intestinal DCs in response to poly(I:C).

are mean numbers of cells ± 1 sem pooled from two representative experiment out of three with two to four mice per group. Two-way ANOVA, $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. (H) Activation of cDC2 in mLNs of $BATF3^{KO}$ mice by poly(I:C). Results are shown as MFI of CD86 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray) in WT and $BATF3^{KO}$ mice. Data shown are delta MFIs over the mean of all untreated WT CD86 MFI values ± 1 sem pooled from two representative experiment out of three with two to four mice per group per experiment. Two-way ANOVA, $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

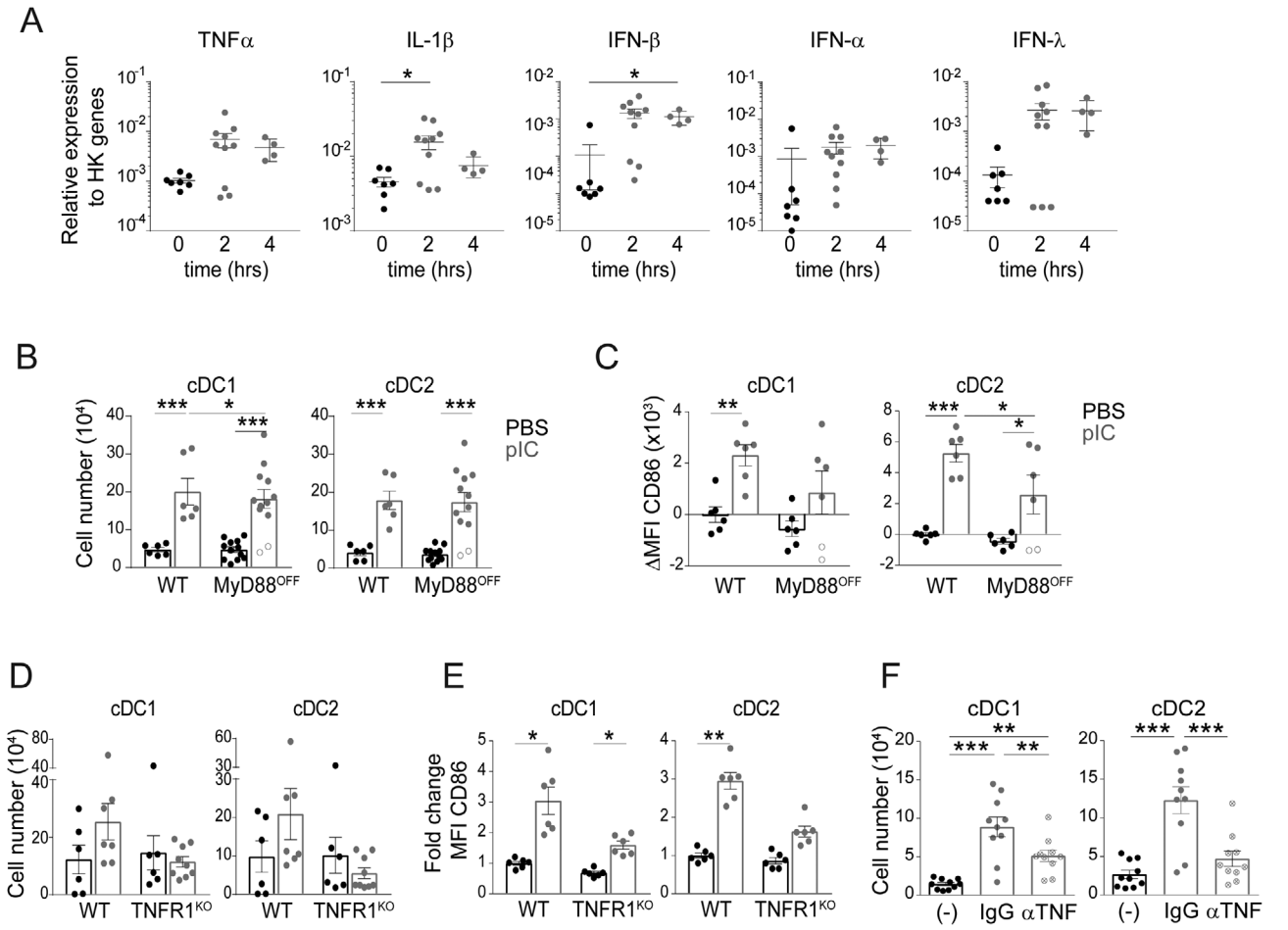

Figure 3. Role of cytokines and MyD88 in the DC response to poly(I:C). (A) Expression of cytokine mRNA in total SI-LP of WT C57BL/6 mice at indicated times after i.p. injection of 100 μg poly(I:C). Each point represents the mean of qPCR triplicates for every gene as assessed by RT-qPCR and measured relative to mean of three housekeeping genes (Reep5, β-actin, GAPDH). Data shown are means ± 1 sem pooled from three independent experiments with two to four mice per group per experiment. Two-way ANOVA, $^{*}p < 0.05$. (B) Total number of cDC1 and cDC2 in the mLNs of WT and MyD88^{OFF} (equals full KO) 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray). Data shown are mean numbers of cells ± 1 sem pooled from four independent experiments with three to four mice per group per experiment (only two experiments included WT controls). Two-way ANOVA, $^{*}p < 0.05$, $^{***}p < 0.0005$. Open circles were used to mark those poly(I:C) injected mice that also did not show upregulation of CD86 (panel C); these were not excluded from statistics. (C) Activation of cDC subsets in mLNs of WT and MyD88^{OFF} mice by poly(I:C). Results shown are delta MFI of CD86 expression 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray) over the mean of all untreated WT CD86 MFI values. Data shown are means ± 1 sem pooled from two independent experiments with three to four mice per group per experiment. Two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. (D) Total number of cDC1 and cDC2 in the mLNs of TNFR1^{KO} mice 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with one to three mice per group per experiment. Two-way ANOVA, not significant. (E) Activation of cDC subsets in mLNs of WT and TNFR1^{KO} mice by poly(I:C). Results shown are fold change in CD86 expression 12 h after injection of PBS (black) or 100 μg poly(I:C) (gray) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from three independent experiments with one to three mice per group per experiment. Two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.005$. (F) Total number of cDC1 and cDC2 in the mLNs of C57BL/6 mice pretreated with TNF-α antibody-blocking and 12 h after i.p. injection of 100 μg poly(I:C). Control mice were treated with the isotype antibody (IgG). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with two to four mice per group per experiment. Two-way ANOVA, $^{**}p < 0.005$, $^{***}p < 0.0005$.

Type I IFN receptor-deficient mice (IFNAR^{KO}) showed defective migration of both, cDC1 and cDC2 in response to poly(I:C) (Fig. 4A) and similar results were found in mice lacking IFNAR in all CD11c-expressing cells (CD11c.IFNAR^{KO}), although cDC2 showed some residual migration upon deletion of IFNAR in these mice (Fig. 4B). The activation of both DC subsets as assessed by CD86 expression was also greatly diminished in CD11c.IFNAR^{KO} mice (Fig. 4C). Conversely, while cDC1-specific deletion of the IFNAR in XCR1.IFNAR1^{KO} mice abrogated the poly(I:C)-induced migration and activation of cDC1, this had no effect on either parameter in cDC2 (Fig. 4B and C). In the human system, langerin (CD207) is expressed in Langerhans cells and intestinal cDC2 [37]. Cre expression under the control of the human langerin promoter in transgenic mice efficiently targets intestinal cDC2 [37]. We

therefore bred floxed IFNAR mice to CD207.cre mice to analyze the role of IFNAR specifically on cDC2. We found that deletion of IFNAR in cDC2 had no effect on the migration or activation of either DC subset, apart from a small decrease in CD86 upregulation by cDC2 (Fig. 4B and C). These data suggest that type I IFN signaling in cDC2 is not required for their migration to the mLNs in response to poly(I:C).

Since we observed that TLR3-signaling was not required cell intrinsically (Fig. 2E), we next checked whether this was also the case for type I IFN signaling. Mixed BM chimeras using a 50:50 combination of WT and XCR1.IFNAR1^{KO} BM showed that the requirement for type I IFN signaling in cDC1 migration was cell intrinsic (Fig. 4D), as XCR1.IFNAR^{KO}-derived cDC1 migrated less than WT-derived cDC1 in the mouse in response to poly(I:C). As expected, poly(I:C)-induced migration of cDC2 remained equal regardless of the donor background in these chimeras (Fig. 4D).

We next set out to test whether differential requirements for type I IFN signaling in the two DC subsets was specific to stimulation through TLR3-engagement, potentially caused by the specific high expression of TLR3 in cDC1 (Fig. 1A). Mirroring our findings using poly(I:C), we however observed a similar defect in cDC1 migration in XCR1.IFNAR1^{KO} mice treated orally with R848, the ligand for TLR7 expressed mostly by pDCs and cDC2, indicating a need for type I IFN signaling in cDC1 regardless of whether stimulation occurred in a direct (poly(I:C)) or indirect (R848) manner (Fig. 4E). cDC2 migration was also induced by R848 in huCD207.IFNAR1^{KO} mice lacking type I IFN signaling specifically on cDC2, although this was somewhat reduced in comparison with that in WT mice (Fig. 4F). Hence, the impact of cDC2-specific IFNAR deficiency on the migration of this subset is minor in response to both, poly(I:C) and R848.

As well as type I IFN, poly(I:C) also induces cDC1 to express IFN-λ in a manner that requires IFNAR on splenic DCs [38]. IFN-λ is a type III IFN that drives thymic stromal lymphopoietin expression by M cells in response to nasal vaccination with an influenza vaccine, which in turn drives cDC1 migration from the respiratory tract to the mediastinal lymph nodes [26]. We therefore tested whether IFN-λ was required for the poly(I:C)-induced migration of DCs, using mice deficient for IL28R, the receptor for IFN-λ. However, the activation and migration of both, cDC1 and cDC2 in response to poly(I:C) were normal in these animals (Fig. 4G and H).

Taken together, type I IFN but not type III IFN signaling plays a global role in the migration of cDC1 in response to TLR stimulation but has little or any effect on cDC2 migration in response to either TLR3 or TLR7 stimulation.

Discussion

DCs are uniquely capable of inducing adaptive immunity upon first encounter of the antigen, making them formidable targets for vaccination strategies. Yet, their subset specific activation to different adjuvants and the cellular requirements for their migration from the periphery to inductive sites are incompletely understood. Here, we show that DC migration from the intestines to the draining LNs induced by poly(I:C) injection critically depends on TLR3-signaling despite the well-defined role of other pattern recognition receptors in sensing dsRNA [39]. Given the major differences in their TLR3-expression patterns and the dependence on TLR3 for migration, it is surprising that both, cDC1 and cDC2 migrate equally well in response to poly(I:C). We demonstrate that the hematopoietic compartment is responsible for TLR3-dependent migration and activation of DCs, but these processes can occur in a cell extrinsic manner, with cDC1-derived signals not being essential, despite the high levels of TLR3 expression by these cells. Residual intestinal macrophages might provide the signals required for migration and activation, given that they also express some TLR3. Unfortunately, we are not aware of a clean system allowing for complete deletion of macrophages. Our findings of exogenous triggers sufficing for full DC migration from the SI LP to the mLN is in accordance with a study from Yrlid et al., demonstrating that R848-stimulation (a potent agonist of TLR7) leads to robust DC migration [19]. TLR7 is prominently expressed in pDCs, and pDCs were necessary to drive migration. However, a bystander cell type sensing the trigger driving migration does not suffice in all cases: TLR5-signaling specifically induces cDC2 migration as a consequence of specific expression of TLR5 only in cDC2 [40]. This suggests different mechanisms of cell migration induction in response to different triggers.

Our data further reveal a previously unappreciated differential role for type I IFN in cDC migration from the intestine to the mLNs. While IFNAR signaling drives maturation of both major subsets of migratory DCs, only cDC1 critically depend on direct type I IFN signals for migration in response to poly(I:C), while IFNAR expression in mature intestinal cDC2 was dispensable for their migration. These findings suggest that total deficiency of IFNAR (KO) or the global, earlier absence of IFNAR in DCs before peripheral maturation (CD11c.cre) broadly affects DCs, but that the mechanisms and requirements inducing peripheral DC migration are subset specific. Although the migration and upregulation of CD86 in both, cDC1 and cDC2 were entirely TLR3-dependent, this could occur in a cell extrinsic manner. Consistent with previous reports in other models [19, 20, 41], we found that TNF-α and type I IFN signaling played important roles as secondary mediators in TLR3-mediated intestinal DC migration and activation. Subset-specific differences were not assessed in previous studies and likely account for prior reporting of a major role for type I IFN only in activation, but not migration of intestinal DCs [19]. Using an elegant BM chimera approach, type I IFN signaling downstream of TLR3 activation has previously been shown to induce most of the poly(I:C)-induced transcriptional changes in splenic DCs [20, 40]. In light of our findings suggesting that type I IFN has a much stronger effect on cDC1 than on cDC2, it would be interesting to revisit some of these published findings, which were based on the assessment of bulk DCs.

Of note, the ability of DCs to induce proliferation by naïve CD4$^+$ T cells also does not require cell intrinsic expression of pattern recognition receptors by DCs, whereas the functional polarization of T cells depends on direct sensing of the PAMP by the presenting

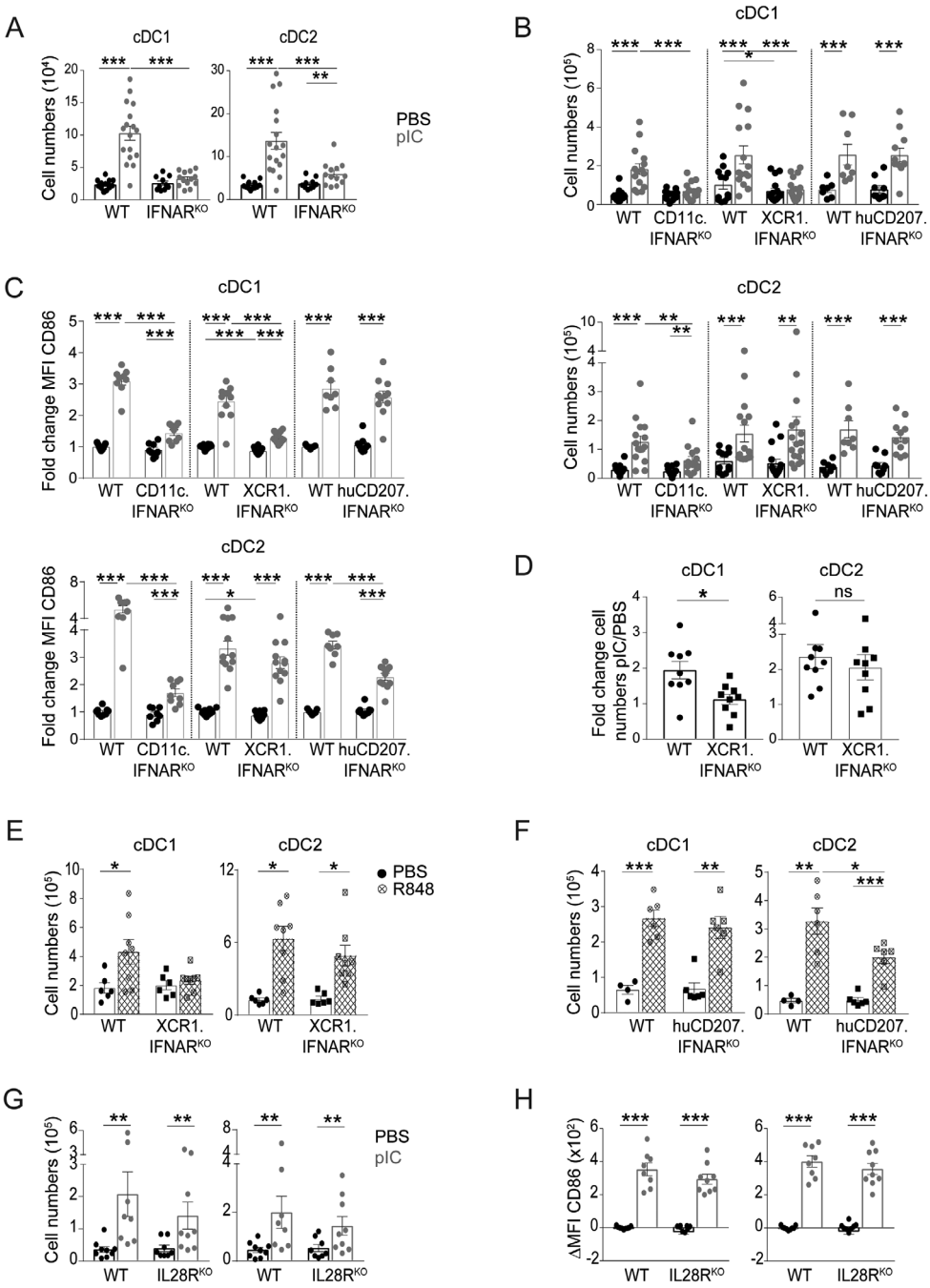

Figure 4. Type I IFN signaling in migration and activation of DC subsets. (A) Total number of cDC1 and cDC2 in the mLNs of WT and IFNAR^{KO} mice 12 h after i.p. injections of PBS (black) or 100 μg poly(I:C) (gray). Data shown are mean numbers of cells ± 1 sem pooled from four independent experiments with three to five mice per group per experiment. Two-way ANOVA, $^{**}p < 0.005$, $^{***}p < 0.0005$. (B) Total number of cDC1 (top) and cDC2 (bottom) cells in the mLNs of WT, CD11c.IFNAR^{KO} (lack IFNAR in CD11c expressing cells), XCR1.IFNAR^{KO} (lack IFNAR in cDC1), and huCD207.IFNAR^{KO} (lack IFNAR in cDC2) mice 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray). Data shown are mean numbers of cells ± 1 sem pooled from five independent experiments with two to three mice per group for WT versus CD11c.IFNAR^{KO}, five independent experiments with three to five mice per group per experiment for XCR1.IFNAR^{KO}, and three independent experiments three to five mice per group per experiment for huCD207.IFNAR^{KO}. Two-way ANOVA between littermates, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. (C) Activation of cDC1 (top) and cDC2 (bottom) in the mLNs of WT, CD11c.IFNAR^{KO}, XCR1.IFNAR^{KO}, and huCD207.IFNAR^{KO} mice by poly(I:C). Results shown are fold change in CD86 expression 12 h after i.p. injection of 100 μg poly(I:C) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from three out of five independent experiments with two to three mice per group per experiment for WT versus CD11c.IFNAR^{KO}; four out of five independent experiments with three to five mice per group per experiment for XCR1.IFNAR^{KO}, and three independent experiments with three to five mice per group per experiment for huCD207.IFNAR^{KO}. Two-way ANOVA between littermates, $^{*}p < 0.05$, $^{***}p < 0.0005$. (D) Fold change of total number of cDC1 and cDC2 in the mLNs 12 h after i.p. injection of 100 μg poly(I:C) versus PBS derived from the indicated BM in 50:50 WT:XCR1.IFNAR^{KO} mixed BM chimeras. Data shown are means ± 1 sem pooled from two independent experiments with three to nine mice per group per experiment. Two-way ANOVA, $^{*}p < 0.05$. (E) Total number of cDC1 and cDC2 in the mLNs of XCR1.IFNAR^{KO} mice

DC [10, 42]. Our data showing that the migration and upregulation of costimulatory molecules by DCs in response to TLR stimuli in vivo can result from transactivation of DCs therefore suggest that differences in migration and activation capacities may not be linked to the polarization of T cell responses. Specifically, the strong signals required for the activation of cytotoxic T cell activation were shown to depend on direct activation of the presenting DC [10]. Particularly in the context of intestinal immunity, it is intriguing to speculate that transactivation of DCs may in fact facilitate the maintenance of immune homeostasis. Indeed, a recent study by Anandasabapathy et al. described the immunomodulatory bias of migratory versus resident DCs [43]. Here, the authors used primarily TLR4-ligation in a vaccine approach to show that migratory DCs from the skin were not required for, but instead modulated the efficacy of a CD205-targeted protein-based vaccination formula. Future studies using different adjuvants matched with assessment of DC subset specific priming capacities will be necessary to fully understand context-dependent immune induction. Our data suggest that the level of type I IFN induction by the chosen adjuvants may affect the type of induced immunity due to a specific requirement in cDC1, uniquely capable of cross-presenting antigen to $CD8^+$ T cells.

Taken together, our findings indicate that TLR-based adjuvants and targeting need to be examined individually for their impact on specific DC subsets if their effects on the immune system are to be understood in depth. Future research aiming at better understanding the role of potently migrating and activating transactivated DC subsets, as well as the signals responsible, will be critical for the design of selective immune interventions in vaccination and therapy.

Material and methods

Mice

All animal were housed under specific pathogen-free conditions at the Danish Technical University (Denmark), Lund University (Sweden), or Monash University (Australia). The experiments were performed under the appropriate national licenses and guidelines for animal care. Both male and female mice were used between 8 and 16 weeks of age as no obvious age differences were detected. CD11c.cre mice (B6.Cg-Tg(Itgax-cre)1-1Reiz/J [44]) allow floxed gene deletion in CD11c-expressing cells, huCD207.cre mice drive floxed gene deletion in Langerhans cells and intestinal cDC2 [37], XCR1.cre mice permit to specifically delete floxed genes in cDC1 [27], villin.cre (B6.Cg-Tg(Vil-cre)997Gum/J) mice excise floxed genes in intestinal epithelial cells [45], and Rosa26-STOP-YFP mice allow tracking of cre specificity (B6.129 × 1-Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$/J). We used "switch-on" mutants carrying a floxed stop cassette in the endogenous locus prior to the gene of interest, allowing for re-expression of the targeted gene in the presence of cre for MyD88 [46], TLR3 and TRIF (unpublished, manuscript in preparation) (both generated at TU Munich, Germany). To generate $TLR3^{OFF}$ mice, an intron gene trap cassette [46] was placed between exons 3 and 4 of the Tlr3 gene. Flow cytometry analysis of BM-derived macrophages confirmed that $TLR3^{OFF}$ cells did not express detectable amounts of TLR3. Moreover, $TLR3^{OFF}$ macrophages did not produce IL-6 in response to poly(I:C) stimulation but produced normal levels of IL-6 when stimulated with CpG-DNA (unpublished, manuscript in preparation). IFNAR floxed mice were obtained from U. Kalinke [47]. $BATF3^{KO}$ (B6.129S(C)-Batf3^{tm1Kmm}/J) were maintained at DTU, $TNFR1^{KO}$ (crossed out from TNFR1/2^{KO} [48]), $IFNAR^{KO}$ [49], and $CCR2^{KO}$ (B6.129S4-Ccr2^{tm1Ifc}/J) at Lund University and $IL28RA^{KO}$ [50] (kindly provided by Sean Doyle, Zymogenetics/BMS) at Monash University. All mice were on the C57BL/6J background (B6.SJL-Ptprc^{a}Pepc^b/BoyJ for CD45.1 BM donors) and littermates were used as controls.

In vivo treatments

Mice were injected with PBS or 100 μg poly(I:C) (Sigma-Aldrich) in PBS intraperitoneally (i.p.) and mLNs and SI LP were collected 12–14 h later if not indicated otherwise. For TLR7 stimulation, 20 μg of R848 (Invivogen) in PBS was given orally. αTNF-α (XT3.11, BioXcell) was blocked with 0.5 mg on day 1 and 0.5 mg at the time of stimulation.

Cell isolation

Isolation of mLN and splenic DCs was performed by digesting the tissue with collagenase IV (0.5 mg/mL, Sigma–Aldrich) and DNase I (12.5 μg/mL) diluted in R10 media (RPMI 1640 + 10% FCS) for 40 min at room temperature. Remaining tissue was mashed and filtered through 70 μm cell strainer with R10. For spleens, RBCs were lysed using RBC lysing buffer, containing ammonium chloride, potassium bicarbonate, EDTA, and MiliQ water. The SI LP cell isolation was performed as described previously [51].

12 h after oral gavage of PBS (black) or 20 μg R848 (diamond grid). Data shown are mean numbers of cells ± 1 sem pooled from two independent experiments with three to five mice per group per experiment. Two-way ANOVA, $^*p < 0.05$. (F) Total number of cDC1 and cDC2 in the mLNs of huCD207.IFNAR^{KO} mice 12 h after oral gavage of PBS (black) or 20 μg R848 (diamond grid). Data shown are mean numbers of cells ± 1 sem pooled from two independent experiments with three to five mice per group per experiment. Two-way ANOVA, $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. (G) Total number of cDC1 and cDC2 in the mLNs of IL28R^{KO} mice 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with three mice per group per experiment. Two-way ANOVA, $^{**}p < 0.005$. (H) Activation of cDC1 and cDC2 in the mLNs of IL28R^{KO} mice by poly(I:C). Results shown are delta MFI of CD86 expression 12 h after i.p. injection of PBS (black) or 100 μg poly(I:C) (gray) over the mean of all untreated WT CD86 MFI values. Data shown are means ± 1 sem pooled from three independent experiments with three mice per group per experiment. Two-way ANOVA, $^{***}p < 0.0005$.

Flow cytometer

Ca/Mg-containing PBS with 2% FCS was used as buffer during the entire staining procedure. Nonspecific binding was blocked with rat antimouse CD16/CD32 Fc block (2.4G2, BD Biosciences) for 20 min at 4°C. Dead cells identified as propidium iodide$^+$ (Sigma–Aldrich) or by Aqua LIVE/DEAD Fixable Dead Cell Staining Kit (Life Technologies) and cell aggregates (identified on FSC-A versus FSC-H scatterplots) were excluded from analyses. DCs were identified by using the following antibodies: α-CD3 (145-2C11), α-CD19 (eBio1D3), α-NK1.1 (PK136), α-B220 (RA3-6B2), α-CD64 (X54-5/7.1), α-CD103 (M290), α-CD11b (M1/70), α-CD11c (HL3), α-CD8a (53-6.7), α-CD86 (GL1), α-MHC-II I (IA/I-E) (M5/114.15.2), α-CD45.1 (A20), α-CD45.2 (104), α-IFNAR (MAR1-5A3), α-XCR1 (ZET), α-SiglecH (551), and α-TLR3 (11F8). Intracellular staining was performed using the FoxP3 Fixation/Permeabilization Kit (eBioscience) according to the manufacturer's instructions. Data were acquired on a FACS Aria II or LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star). Experiments were designed in line with the recently published guidelines for flow cytometry [52].

Adoptive transfers

BM chimeras were generated by intravenous injection of BM (5×10^6) cells into irradiated (9 Gy) recipients. Analysis of BM chimeras was performed 6–8 weeks after cell transfer. In all mixed BM chimeras, WT cells were identified by CD45.1 expression.

Real-time PCR

Total RNA was isolated from SI using the RNeasy kit (QIAGEN). cDNA was generated using iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad), using SsoFast™EvaGreen® Supermix (Bio-Rad). The expression of all genes was normalized to the mean of β-actin, Reep5, and GAPDH. Primer sequences are as follows: IL-1β (GACAGTGATGAGAATGACCTGTT, TGGAAGGTCCACGGGAAAGACA), TNF-α (TGTCTACTGAACTTCGGGGTGA, TCTTTGAGATCCATGCCGTTG), IFN-α (TGCAATGACCTCCATCAGCA, TTCCTGGGTCAGAGGAGGTTC), IFN-β (CTGGAGCAGCTGAATGGAAAG, CTCCGTCATCTCCATAGGGAT), IFN-λ (GTTCAAGTCTCTGTCCCCAAAA, GTGGGAACTGCACCTCATGT), Reep5 (GCCATCGAGAGTCCCAACAA, AGCATCTCAGCCCCATTAGC), β-actin (CCGGGACCTGACAGACTA, GTTTCATGGATGCCACAGGAT), GAPDH (CCTGCACCACCAACTGCTTA, TCATACTTGGCAGGTTTCTCCA). Undetectable values were calculated based on the highest possible Cq + 1 (= 41 cyles).

Statistical analysis

Statistics were performed using two-way ANOVA considering treatment and experiment as factors for the analysis. Wherever indicated in the figure legends, Mann–Whitney U test was applied to compare two groups (e.g. different treatments [n = 2] within the same genotype), and Kruskal–Wallis test was applied to compare more than two groups (e.g. different genotypes [n = 3] within the same treatment). Statistical significance was estimated by using R Studio.

R Scripts:

- Two-way ANOVA: aov(value ~ genotype + day, data = Data)
  - Post-Hoc test Tukey: TukeyHSD(Data_anova2, which = "genotype")
- Mann–Whitney U test: wilcox.test(value ~ genotype, data = Data, exact = FALSE)

Genotype accounts for the analysis of different genotypes within the same treatment. Using treatment instead of genotype allows for analysis of different treatments within a genotype. Data refer to the data to be analyzed.

Acknowledgments: We thank Prof. Dan Kaplan for sharing huCD207-cre mice and Prof. Ulrich Kalinke for sharing IFNAR^{flox} mice. We thank the Lahl, Agace, Bekiaris, and Johansson-Lindbom laboratories for many fruitful discussions, Matthias Schiemann and Immanuel Andrä for flow cytometry support, Katrine Fog Thomsen for technical support, and Allan Mowat for editing of the manuscript. KL was supported by a Vetenskapsrådet Young Investigator Award (2014-3595), the Ragnar Söderberg Foundation Fellowship in Medicine, a Lundbeck Foundation Research Fellowship, the Åke Wiberg Foundation, the Carl Trygger Foundation, and the Crafoord Foundation. JH was supported by a MARIE CURIE Fellowship as part of the EU-funded project HC Ørsted Postdoc. AGL received travel support from the Niels Bohr Foundation. VB and WA were supported by a project grant from Vetenskapsrådet (Dnr. 521-2012-2637). The flow cytometer at Lund University was purchased with funding from the Lundberg Foundation.

Author contributions: AGL, VB, JH, WA, and KL conceived and designed the project; AGL, VB, JH, MO'K, WA, and KL designed experiments; AGL, VB, KM-L, JH, KGM, JN, IU, KK, and KL performed experiments and analyzed the data; BM and BH provided essential tools; AGL and KL wrote the manuscript; WA and KL supervised the study and acquired funding; all authors reviewed the manuscript.

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

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Abbreviations: **mLNs**: mesenteric LNs · **pDC**: plasmacytoid DC · **SI LP**: small intestinal lamina propria

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The peer review history for this article is available at https://publons.com/publon/10.1002/eji.201948497

Received: 3/12/2019
Revised: 27/3/2020
Accepted: 8/5/2020
Accepted article online: 8/5/2020