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Differential Intrasplenic Migration of Dendritic Cell Subsets Tailors Adaptive Immunity

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SUMMARY

Evidence suggests that distinct splenic dendritic cell (DC) subsets activate either CD4+ or CD8+ T cells in vivo. This bias has been partially ascribed to differential antigen presentation; however, all DC subsets can activate both T cell lineages in vitro. Therefore, we tested whether the organization of DC and T cell subsets in the spleen dictated this preference. We discovered that CD4+ and CD8+ T cells segregated within splenic T cell zones prior to immunization. After intravenous immunization, the two major conventional DC populations, distinguished by 33D1 and XCR1 staining, migrated into separate regions of the T cell zone: 33D1+ DCs migrated into the CD4+ T cell area, whereas XCR1+ DCs migrated into the CD8+ T cell area. Thus, the post-immunization location of each DC subset correlated with the T cell line-age it preferentially primes. Preventing this co-localization selectively impaired either CD4+ or CD8+ T cell immunity to blood-borne antigens.

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AUTHOR CONTRIBUTIONS

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SUPPLEMENTAL INFORMATION

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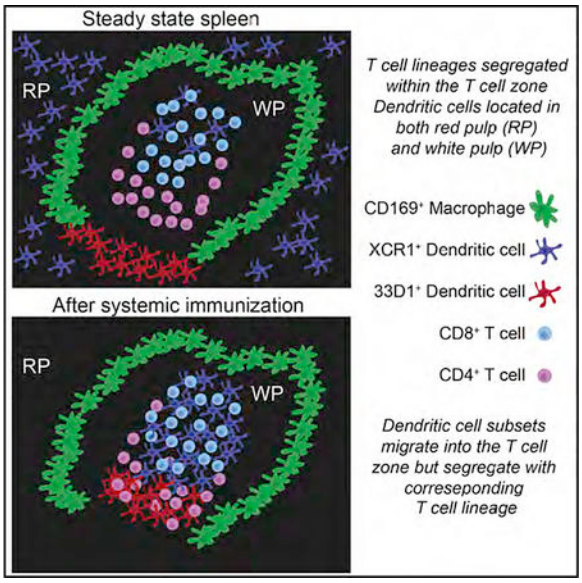
Graphical Abstract



In Brief

Calabro et al. demonstrate that, upon immunization, dendritic cell subsets in the spleen migrate into non-overlapping zones that correspond to regions enriched for CD4+ or CD8+ T cells. This differential migration results in the selective induction of either CD4+ or CD8+ T cell responses.

INTRODUCTION

Activation of naive T lymphocytes is the first step in the induction of most adaptive immune responses, such as those to vaccines or pathogens. Given that this key step dictates a metabolically costly and potentially deleterious cascade of cellular events, it is not surprising that a coordinated series of checkpoints exist to regulate naive T cell priming. One crucial checkpoint is antigen presentation. This is accomplished primarily by mature dendritic cells (DCs) not only because they express the requisite stimulatory signals to activate naive T cells, but also because, after antigen capture from tissues and maturation by an innate immune stimulus, they efficiently migrate via lymphatics to draining lymph nodes (LNs) (Itano and Jenkins, 2003); circulation of naive T cells is restricted to such secondary lymphoid organs.

For blood-borne antigens, this entire process occurs in the spleen, which, unlike all other secondary lymphoid structures, does not contain afferent lymphatics (Bronte and Pittet, 2013). The spleen filters the blood of aging red blood cells (RBCs), as well as foreign antigens or pathogens that have gained access to the bloodstream. It is divided by function and structure into red pulp (RP) and white pulp (WP); between these two regions is the marginal zone (MZ) in mice or the perifollicular zone in humans (Mebius and Kraal, 2005). Most lymphocytes are located in the WP and reside in distinct zones, such as the T cell zone, where T lymphocytes are concentrated. The WP is where adaptive immune responses are generated to blood-borne antigens.

DCs are the primary cells in the spleen that prime T cells to antigens encountered in the blood (Meredith et al., 2012). Although the migration of tissue DCs to draining LNs is known to be a crucial step in the induction of T cell responses, it is not clear that the same holds true within the spleen (Czeloth et al., 2005; Ohl et al., 2004). The presence of CD8+ DCs in the T cell zone at steady state in both humans and mice (Idoyaga et al., 2009; Pack et al., 2008) raises the possibility that antigen transport via DC migration might not be necessary, unlike in other sites in the body, because the unique architecture of the spleen juxtaposes the antigen-exposed tissue (e.g., the MZ) with the lymphoid compartment (e.g., the WP) (Bronte and Pittet, 2013; Khanna et al., 2007). Indeed, the role of the primary DC homing receptor to LNs, CCR7, in DC movement within the spleen is debated (Czeloth et al., 2005; Gunn et al., 1999; Ritter et al., 2004; Yi and Cyster, 2013). However, the same kinds of innate stimuli that induce tissue DCs to migrate to LNs are also stimuli of DC migration within the spleen (Balázs et al., 2002; De Smedt et al., 1996; De Trez et al., 2005; Idoyaga et al., 2009; Reis e Sousa and Germain, 1999). If this relocalization is not necessary for adaptive immunity, then how is a threshold created to prevent T cell activation to innocuous or self-antigens in the blood? We aimed to characterize how particular splenic DCs migrate following immunization and how migration impacts the activation of each T cell lineage.

In the mouse spleen, DCs are divided into plasmacytoid DCs (pDCs), conventional DCs (cDCs), and monocyte-derived DCs such as TNFa-iNOS-producing (TIP) DCs (Serbina et al., 2003). cDCs are the primary cells that activate naive T cells and can be further divided into two main subsets based on transcription factor usage, surface marker expression, and the ability to prime CD4+ versus CD8+ T cells (Guilliams et al., 2014; Meredith et al., 2012; Satpathy et al., 2012; Segura and Amigorena, 2013). The *Irf4*-dependent cDC subset efficiently processes antigen for major histocompatibility complex class II (MHCII) and preferentially primes CD4+ T cells; almost all are marked by 33D1, SIRPa, and CD11b in the spleen, with variable expression of CD4 and ESAM (Dudziak et al., 2007; Pooley et al., 2001; Suzuki et al., 2004). In contrast, *Batf3*-dependent XCR1+CD8αα+ cDCs readily cross-present phagocytosed antigen on MHC class I (MHCI) to CD8+ T cells (den Haan et al., 2000; Hildner et al., 2008; Pooley et al., 2001; Schnorrer et al., 2006; Schulz and Reis e Sousa, 2002). This subset is also dependent on the transcription factor IRF8 and includes DCs that express DEC205 or CD103 (Hildner et al., 2008; Qiu et al., 2009).

Depending on the study, the preferential activation of CD4+ versus CD8+ T cells by the two cDC subsets is determined by differential antigen acquisition, cytokine elaboration, or the ability to present a given antigen on MHCI versus MHCII (Dudziak et al., 2007; Maldonado-López et al., 1999; Pulendran et al., 1999; Schulz and Reis e Sousa, 2002). These functional distinctions are not strict, in that both DC subsets can activate, albeit with different efficiency, CD4+ and CD8+ T cells when placed in vitro. Therefore, an absolute presence versus absence of MHCI or -II machinery on different DCs does not exist (Kamphorst et al., 2010; Maldonado-López et al., 1999; Pulendran et al., 1999; Schnorrer et al., 2006; Schulz and Reis e Sousa, 2002). For example, mice lacking CD11b+ splenic DCs secondary to the deletion of the Notch2 receptor have defective CD4+ T cell activation in vivo, but when DCs from these same mice were used to stimulate CD4+ T cells in vitro, no apparent defect was observed (Lewis et al., 2011). Therefore, although ample evidence

suggests that 33D1+ DCs preferentially prime CD4+ T cells, whereas XCR1+ DCs preferentially prime CD8+ T cells in vivo, why this preference exists is not clear, given that, in vitro, they are functionally similar. Since this preference has been primarily established in vivo, we hypothesized that the organization of DC and T cell subsets in the spleen determines this preference.

We found that CD4+ and CD8+ T cells preferentially occupy different areas of the T cell zone at steady state. Furthermore, we found that DC migration within the spleen is triggered by multiple forms of intravenous (i.v.) immunization, including RBC transfusion, and requires DC-intrinsic CCR7. Therefore, we tested whether in vivo functional DC specialization could be explained by the spatial segregation of migratory DCs within the spleen during an immune response. By combining immunofluorescence microscopy using recently identified DC subset markers with in vivo flow-cytometry-based cell tracking, we discovered that the two major DC subsets segregated into non-overlapping regions of the T cell zone. 33D1+ DCs migrated to the periphery of the T cell zone (bordering the B cell area), whereas MZ XCR1+ DCs migrated into the center of the T cell zone. Thus, the post-immunization location of each DC subset correlates with the T cell it most efficiently primes. By defining the T cell response in vivo rather than in vitro, we elucidated how movement of individual DC subsets selectively generates a CD4+ versus CD8+ T cell response to blood-borne antigens.

RESULTS

33D1+ and XCR1+ DC Subsets Survey Different Regions of the Spleen for Antigen at Steady State

To identify the two primary cDC subsets by flow cytometry and immunofluorescence, we stained for XCR1 (the chemokine receptor for XCL1) and for the C-type lectin receptor DCIR2 (DC-inhibitory receptor 2) with the antibody 33D1. Unlike other subset markers such as CD8α or CD11b, these markers are unique to DCs, do not stain T cells or other myeloid populations, and detect almost all cDCs in the spleen (Figure S1A) (Dorner et al., 2009; Dudziak et al., 2007; Yi and Cyster, 2013). Using these markers and the staining of MZ metallophilic macrophages (Chang et al., 1995) with MOMA-1 (anti-CD169) to delineate the WP border (Figure 1A), we found that the two primary DC subsets segregated at steady state (Figures 1B–1E) (Idoyaga et al., 2009; Pack et al., 2008; Yi and Cyster, 2013). Consistent with previous reports, 33D1+ DCs are concentrated in the bridging channel (Figures 1B and 1D) (Yi and Cyster, 2013), a unique region of the spleen that spans the interface between the RP and WP and can be identified by a break in MOMA-1 staining (Figures 1A and 1D). It was originally believed that CD8+ DCs were solely located within the T cell zone of the WP, based on DEC205 staining (Pack et al., 2008). However, DEC205 is not expressed on all CD8+ DCs; the recent identification of XCR1 as a subset-defining marker for CD8+ and CD103+ DCs in multiple tissues facilitated the tracking of all splenic DCs within this subset, including those from the MZ (Crozat et al., 2011; Dorner et al., 2009). We found that the majority of XCR1+ DCs are located in the RP/MZ at steady state (Figures 1C and 1E) and that only about 30% of CD8+XCR1+ DCs reside in the WP at steady state (Figure 2A). This finding is consistent with more recent work demonstrating

that some XCR1+ DCs—in particular, those marked by CD103 or langerin—reside in the MZ (Alexandre et al., 2016; Dorner et al., 2009; Idoyaga et al., 2009; Qiu et al., 2009). This configuration may enable the two cDC subsets to survey distinct regions of the spleen for potential antigens, analogous to distinct regions scanned by particular cDC subsets in LNs (Gerner et al., 2015).

33D1+ and XCR1+ DCs Move into Distinct Regions of the T Cell Zone following Immunization

We sought to address whether only particular DC subsets migrate within the spleen and the effect of DC migration on T cell priming to bloodborne antigens. We chose two models to study systemic immunization: (1) the CD4+ and CD8+ T cell responses to an i.v. soluble protein, ovalbumin (OVA), in the presence of an innate stimulus, lipopolysaccharide (LPS); and (2) the CD4+ T-dependent alloantibody response to transfused RBCs. Despite matching the ABO blood group antigens, approximately 3% of all transfusion recipients and up to 45% of patients requiring chronic transfusion generate alloantibodies against minor antigens on RBCs, with potentially detrimental outcomes, such as hemolysis and renal failure (Tormey et al., 2008; Vichinsky et al., 1990). Unlike previous studies using xenogeneic sheep RBCs, we used a model to study the alloimmune response to foreign antigens on transfused murine RBCs: transgenic “HOD” RBCs expressing a membrane-bound, allogeneic antigen incorporating hen egg lysozyme (HEL), OVA, and $Duffy^b$ (Hendrickson et al., 2011). This facilitates antigen tracking and uptake by different cell populations in the spleen, along with the measurement of RBC-specific T and B cell responses in vivo. When RBCs from HOD transgenic mice are collected and stored in human anticoagulant solutions, processed to remove leukocytes, and transfused into congenic recipients, they induce a rapid innate and delayed adaptive immune response (Figure S2A) (Hendrickson et al., 2011). In this model, RBC alloimmunization is CD4+ T cell dependent, and requires 33D1+ DCs for CD4+ T cell activation and subsequent antibody induction (Calabro et al., 2016). We used i.v. immunization with these two forms of antigen to address the relative roles of the primary cDC subsets in the spleen in regulating adaptive immunity.

We adapted a flow-cytometry-based methodology to first identify the gross location of particular DC subsets pre/post-RBC transfusion using in vivo intravascular antibody labeling immediately before harvesting spleen samples (Ander son et al., 2014). This allowed us to track the movement within the spleen of both 33D1+ and XCR1+ DCs from the antibody-accessible RP to antibody-inaccessible WP (Figure 2A). We confirmed by immunofluorescence that intravascular anti-CD11c antibody stained only DCs located in the RP/MZ and in the outer rim of the bridging channel, but not those located in the WP (Figure 2B). In naive mice, we found that almost 60% of 33D1+ and 70% of XCR1+ DCs were outside of the WP at steady state (Figure 2A). Our previous work demonstrated that transfused GFP+ RBCs were excluded from the WP immediately after transfusion (Calabro et al., 2016). By transfusing GFP-labeled RBCs, we could, thereby, track antigen-carrying DCs into the WP and found that the majority of 33D1+ and XCR1+ DCs migrated into the WP, including those carrying RBCs. Similar results were found following LPS infusion (data not shown). Mass DC migration in transfused mice was also evident by the loss of immunofluorescence staining of intravascular-bound anti-CD11c (Figure S2B, lower panel),

as compared with the naive state, which demonstrates extensive staining in the MZ, RP, and bridging channel (Figure S2B, upper panel). Therefore, i.v. immunization induces mass DC migration of both antigen-positive and -negative DCs into the T cell zone of the WP.

We then evaluated the location of 33D1+ and XCR1+ DCs by immunofluorescence in wild-type mice transfused either with RBCs or LPS over time. Four hours after immunization, 33D1+ DC migration commences from the bridging channel, and 6–8 hr after immunization, most 33D1+ and a large fraction of XCR1+ DCs migrate to the T cell area. Strikingly, these two DC subsets migrated into non-overlapping regions of the T cell area of the WP (Figures 2C, 2D, and S2C). The 33D1+ DCs migrated to the periphery of the T cell zone and remained near the border of the B cell area. In contrast, XCR1+ DCs migrated from the MZ/RP into the center of the T cell zone. Measurement of the average distance of all DCs within each subset to the MOMA-1 ring across multiple spleens confirmed that 33D1+ DCs are located proximally to the MZ, whereas XCR1+ DCs are located distally to the MZ (Figure 2E). 12 hr after immunization, both 33D1+ and XCR1+ DCs completed migration, and at around 16 hr, both the MOMA-1+ ring (Gaya et al., 2015) and 33D1+ DC staining began to dissolve. Simultaneously, some 33D1+ staining began to appear in the bridging channel and XCR1+ staining began to appear in the RP, consistent with observed turnover rates by bromodeoxyuridine (BrdU) studies, suggesting the influx of new DC populations to their steady-state locations (data not shown) (Kamath et al., 2000). These results demonstrate that, after immunization, both 33D1+ and XCR1+ DCs migrate into the T cell area but segregate into distinct zones.

The Location of Each DC Subset Corresponds to the T Cell Lineage Preferentially Primed by that Subset

Given the known preferential activation of CD4+ versus CD8+ T cells by 33D1+ versus XCR1+ DCs, respectively, we assessed the location of the two T cell lineages in comparison with each DC subset in adjacent spleen sections following immunization. Use of CD8β allowed us to label all CD8+ T cells, but not DCs, which express a CD8αα homodimer. TCRβ +CD8β − (Figure 3A) or TCRβ+CD4+ co-staining (Figure 3B) was used to identify CD4+ T cells. Analysis revealed that the two T cell lineages occupy preferential domains within the T cell zone at steady state and after immunization (Figures 3A and B; Figure S3A). CD4+ T cells formed a ring concentrated at the periphery of the T cell zone along the B cell border. In contrast, CD8+ T cells were concentrated in the center of the T cell zone, often surrounding the central arteriole. Intensity analysis of CD4 versus CD8β staining within a single WP highlighted the gradient of T cell lineages (Figures 3C and S3B). Simultaneous analysis of DC subsets in this same WP demonstrated that the 33D1+ DC location coincided with the most intense CD4+ T cell staining; in contrast, XCR1+ DCs from the same region coincided with the highest CD8β+ T cell staining (Figures 3B and S3A). This cellular organization after immunization places each DC subset in proximity to the T cell that it is known to prime most efficiently. To gauge the importance of this co-localization to adaptive immunity, we disrupted DC subset and T cell pairing using *Ccr7*-, *Batf3*-, and *Dock8*-deficient mice.

Cell-Intrinsic CCR7 Is Crucial for DC Migration within the Spleen, and Its Loss Results in Disorganized DC and T Cell Co-localization

CCR7 is needed for T cells to home properly to T cell zones within both LNs and the spleen (Braun et al., 2011). It is also required for peripheral DCs to home to LNs (Förster et al., 1999; Ohl et al., 2004). The role of CCR7-directed DC migration within the spleen is less clear, with different studies drawing opposite conclusions (Czeloth et al., 2005; Gunn et al., 1999; Junt et al., 2004; Ritter et al., 2004; Yi and Cyster, 2013). We found that T cells in *Ccr7*$^{-/-}$ mice were scattered throughout the RP, analogous to the mislocalized T cells identified in LNs from these mice (Figure 4A; Figure S4A) (Förster et al., 1999). Although *Ccr7*$^{-/-}$ mice had a normal percentage of both total DCs and DC subsets (data not shown), all DCs were scattered around the RP and lacked structured organization in bridging channels and MZ rings (Figure 4A). Upon immunization, *Ccr7*$^{-/-}$ DCs failed to move inside the MOMA-1 ring (Figures 4A and S4A). Accordingly, there was a lack of 33D1+ and XCR1+ DC migration into the respective areas of the T cell zone after immunization (Figure S4B). To determine whether this was due to lack of CCR7 expression on DCs or simply a disorganized splenic structure, we created mixed bone marrow chimeras from CD11c-YFP (yellow fluorescent protein) and *Ccr7*$^{-/-}$ mice. In these chimeras, YFP^{+} DCs express CCR7, while YFP^{-} DCs lack CCR7. Anti-CD11c in vivo labeling revealed that *Ccr7*-deficient DCs failed to migrate into WP, as compared with wild-type YFP+ DCs (Figure 4B). These data indicate that cell-intrinsic CCR7 expression is essential for intrasplenic DC migration.

To test the immunological consequence of impaired DC migration in *Ccr7*$^{-/-}$ mice and a disorganized niche for DC subset-T cell interactions, we adoptively transferred *Ccr7*-sufficient transgenic OT-II T cells that carry an OVA-specific, MHCII-restricted T cell receptor into wild-type and *Ccr7*$^{-/-}$ mice. OVA-expressing HOD RBCs were transfused into recipient mice 24 hr later and T cell proliferation was assessed by CFSE dilution after 3 days. In contrast to wild-type recipients, OT-II proliferation in *Ccr7*$^{-/-}$ mice was almost completely absent (Figure 4C). In this model, CD8+ T cells are not significantly activated; therefore, we tested transgenic CD4+ (OT-II) and CD8+ (OT-I) T cell activation, using i.v. soluble OVA with the DC maturation stimulus LPS. OVA induced significant activation of both OT-I and OT-II cells in wild-type mice, but this was significantly impaired in *Ccr7*$^{-/-}$ mice (Figure 4D). These findings indicate that, even if DCs and T cells occupy the same general area within the spleen, it is insufficient to induce T cell activation unless line-age-specific spatial organization is achieved.

XCR1+ DCs Are Required for CD8+, but Not CD4+, T Cell Activation, but Only In Vivo

We showed that different DC subsets co-localize with their respective T cell subsets within subdomains of the T cell zone (Figure 3) and that disruption of DC-T cell niches impairs subsequent adaptive immunity (Figure 4). However, *Ccr7* deficiency impairs overall splenic architecture, making delineation of the isolated role of each DC subset difficult. To evaluate DC subset function in vivo, one approach is to target antigen to a DC subset-specific surface receptor, such as DEC205 (Dudziak et al., 2007), but this can also deliver antigen to unintended DCs (Kamphorst et al., 2010). Another approach is to inject antigen in vivo and sort DCs to stimulate T cells in vitro (Pooley et al., 2001; Schnorrer et al., 2006). This ex vivo system evaluates the ability of a DC to phagocytose and present antigen but does not

necessarily correlate with the ability to activate a T cell in vivo. Indeed, both DC subsets can activate CD4+ and CD8+ T cells in vitro for most types of antigens, albeit with different efficiency, implying that restricted processing of antigen on either MHCI or MHCII does not sufficiently explain the in vivo preferences observed for T cell lineage activation (Itano and Jenkins, 2003; Lewis et al., 2011; Schulz and Reis e Sousa, 2002).

The recent development of mice lacking DC-lineage-specific transcription factors has enabled the evaluation of the in vivo role of specific DC subsets (Satpathy et al., 2012). We took advantage of *Batf3*$^{-/-}$ mice, which are deficient in the basic leucine zipper transcription factor 3 (*Batf3*), to evaluate the role of XCR1+ DCs on instructing its corresponding CD8+ T cell line-age. *Batf3*$^{-/-}$ mice have a selective loss of CD8αα +XCR1+ DCs in the spleen and have defects in mounting antigen-specific CD8+ T cell responses to viral infections or cell-associated antigens (Hildner et al., 2008; Seillet et al., 2013) (Figure 5A). Whether cross-presentation of soluble antigens is impaired in *Batf3*$^{-/-}$ mice is unclear, as a recent study showed that CD8+ T cell activation was impaired in *Batf3*-deficient mice with particulate, but not soluble, antigen (Seillet et al., 2013). To test the impact of XCR1+ DC loss on both CD4+ and CD8+ T cells in vivo, we adoptively co-transferred CFSE-labeled OT-I and OT-II T cells into wild-type and *Batf3*$^{-/-}$ recipient mice. Three days after OVA/LPS immunization (i.v.), we observed a dramatic loss of CD8+, but not CD4+, T cell activation in *Batf3*$^{-/-}$ mice (Figure 5B). During chronic inflammation, compensatory transcription factors can partially rescue CD8αα+ XCR1+ DC development and might rescue the ability to prime CD8+ T cells (Tussiwand et al., 2012); therefore differences in CD8+ T cell activation observed between studies might relate to a complete versus partial loss of XCR1+ DCs in the mice used. Given that we found an almost complete absence of XCR1+ DCs in naive *Batf3*$^{-/-}$ mice by immunofluorescence and by flow cytometry (Figure 5A) and that CD8+ T cell responses were abrogated in vivo (Figure 5B), we tested whether the remaining splenic DCs could cross-present soluble protein by performing an in vitro T cell stimulation assay. Splenic DCs from wild-type and *Batf3*$^{-/-}$ mice co-cultured with OT-I or OT-II cells in vitro induced activation of both T cell lineages (Figure 5C), further demonstrating that CD8+ T cell activation in vivo requires XCR1+ DCs, despite the ability of 33D1+ DCs to cross-present soluble antigens (Hildner et al., 2008). This suggests that the location of a DC subset in vivo might dictate whether it can present antigen to a CD4+ or CD8+ T cells. This work also suggests that experiments conducted ex vivo that bypass differential cellular locations within secondary lymphoid regions provide a limited picture of DC-T cell interactions.

33D1+ DC Migration in the Spleen Is Required for CD4+, but Not CD8+, T Cell Activation

Mice lacking *Dock8* (Dedicator of cytokinesis 8), an atypical guanine nucleotide exchange factor that regulates Rho GTPases, have impaired DC migration to LNs that results in loss of CD4+ T cell activation to subcutaneous immunization (Harada et al., 2012; Krishnaswamy et al., 2015). In vivo labeling of splenic DC location demonstrated that *Dock8*$^{-/-}$ mice have only partially impaired DC migration into the WP after RBC transfusion (Figure 6A) or LPS (data not shown). Immunofluorescence visualization revealed that only one of the two DC subsets failed to migrate. Although the location of the two DC subsets is not altered at steady state (data not shown), following immunization, 33D1+ DCs remained in bridging

channels, while the majority of XCR1+ DCs migrated normally into T cell zones (Figures 6B and S5A). Using in vivo labeling to detect migration of each of the two DC subsets into the WP, we indeed found that XCR1+, but not 33D1+, DCs migrated into the WP after immunization (Figure 6C).

To test whether this selective 33D1+ DC migration defect impacted CD4+ or CD8+ T cell activation, we adoptively co-transferred CFSE-labeled OT-I and OT-II T cells into wild-type and *Dock8*$^{-/-}$ mice and tested the immune response to OVA i.v. Systemic OVA administration failed to induce antigen-specific CD4+ T cell proliferation, while CD8+ T cell responses remained intact (Figure 7A). Therefore, CD4+ T activation was impaired in *Dock8*$^{-/-}$, mice despite intact XCR1+ DC migration, suggesting non-redundancy between these two cDC subsets in T cell lineage activation in vivo. In the absence of 33D1+ DC migration into WP, antigen-specific CD4+ T cell proliferation was also completely abrogated to allogeneic RBCs in *Dock8*$^{-/-}$ mice (Figure 7B). Accordingly, T-dependent induction of alloantibodies was also impaired (Figure S5B). However, in addition to failed DC migration, *Dock8*$^{-/-}$ mice lack MZ B cells and have intrinsic defects in the function of T and B cells (Krishnaswamy et al., 2015; Randall et al., 2009), thus confounding interpretation of failed antibody responses. To exclude the role of other cell types, we generated *Dock8*$^{-/-}$/*Zbtb46*-DTR mixed bone marrow chimeras. *Zbtb46*-DTR mice express the diphtheria toxin (DT) receptor in cells that express the zinc finger transcription factor ZBTB46 (Meredith et al., 2012). In these chimeric mice, after DT treatment, T and B cells develop normally, including MZ B cells, but cDCs are exclusively *Dock8* deficient (Figures S5C and S5D). This allowed us to specifically study 33D1+ DC function during alloimmune responses to transfused RBCs. Three weeks after allogeneic RBC transfusion, DT-treated *Dock8*$^{-/-}$/*Zbtb46*-DTR mice failed to generate alloantibodies, whereas control mice mounted a robust response (Figure 7C). Therefore, mislocalized 33D1+ DCs are unable to initiate a T-dependent B cell antibody response.

Using the RBC alloimmunization model, we conclude that migration of RBC-carrying XCR1+ DCs (Figure 2A) into the WP (Figure 6B) is insufficient for CD4+ T cell activation (Figure 7A), potentially because these DCs are primarily co-localized with CD8+ instead of CD4+ T cells (Figure 3). These results also imply that simple differences in antigen presentation on MHCI versus MHCII does not solely account for the DC division of labor. These findings provide compelling evidence that efficient adaptive immune responses rely on specific spatial distributions within lymphoid tissues.

DISCUSSION

We demonstrate the existence of a dynamic architecture of immune cells in the spleen that regulates T and B cell responses to blood-borne antigens. Although DC movement within the spleen after activation has long been appreciated, it was not recognized that the two major DC subsets segregate into non-overlapping regions or, surprisingly, that CD4+ and CD8+ T cells form a cellular gradient across the T cell zone at steady state. 33D1+ DCs migrate into the CD4+ T cell area, and XCR1+ DCs migrate into the CD8+ T cell area of the WP (Figures 2 and 3), uncovering the existence of both T cell lineage niches and the intricate orchestration of DC migration within the spleen. Accordingly, if 33D1+ DC

migration into the T cell zone is abrogated (in *Dock8*-deficient mice), CD4+ T-cell-dependent immune responses to blood-borne antigens are lost, but CD8+ T cell activation is unimpaired (Figures 6 and 7). Conversely, loss of XCR1+ DCs in *Batf3*-deficient mice impairs CD8+ T cell activation in vivo while strikingly leaving CD4+ T cell activation intact (Figure 5). However, simply having DCs and T cells occupying overlapping but disorganized regions of the spleen is insufficient to generate T cell activation, as demonstrated by experiments using *Ccr7*-deficient mice (Figure 4). We used two different types of stimulation to induce segregated DC migration into the WP, including LPS and transfused allogeneic RBCs; our ongoing work using a range of immune stimuli, including systemic live viral infection, replicates these findings (data not shown), suggesting that this is a universal phenomenon. Similarly, we chose OVA/LPS and allogeneic RBCs to study differential activation of CD4+ and CD8+ T cells, but we also observe the same pattern of selective loss of CD4+ or CD8+ T cell activation with other forms of antigen, including heat-killed *Listeria monocytogenes* (data not shown), again arguing that this model applies to a wide range of immune responses. Therefore, establishment of niches of T cell lineages with their corresponding DC subsets appears to be a fundamental step in the generation of an efficient T cell response.

How is this differential migration achieved? Fibroblastic reticular cells (FRCs) are present in the bridging channel and T cell zone and are thought to act as “roads” in the spleen, directing lymphocyte migration (Bajénoff et al., 2008; Steiniger et al., 2001). FRCs are lined by the CCR7 ligand CCL21, which acts to guide T cells into the WP. Although it is not known whether they similarly direct activated DCs within the spleen, they are crucial for directing peripheral tissue DCs into and within LNs (Acton et al., 2012). Our demonstration of a DC-intrinsic CCR7-dependent pathway regulating DC orchestration (Figure 4B) suggests that a similar situation might exist in the spleen but cannot explain the differential DC subset segregation we observed. The XCR1 ligand, XCL1, is made by activated CD8+ T cells and presumably helps localize XCR1+ DCs during an immune response (Crozat et al., 2011; Dorner et al., 2009), it but might only act at later time points during an ongoing immune response. Sphingosine-1-phosphate (S1P) and EBI2 (GPR183) both have been shown to direct 33D1+ DCs to the bridging channel; disruption of either by pharmacological inhibitors or genetic deletion impairs DC organization and capture of particulate antigen from the bloodstream (Czeloth et al., 2005; Gatto et al., 2013; Yi and Cyster, 2013). Future work will need to determine whether a balanced combination of these and other localization signals determine both the steady-state and post-immunization DC positions, as has been shown for naive versus activated B cells in the spleen (Reif et al., 2002).

Recent work on DC subsets and their interactions with T cells in LNs following viral infection found that initial activation of CD4+ versus CD8+ T cells was spatially segregated, and this segregation correlated with the location of migratory versus LN-resident DCs (Eickhoff et al., 2015; Hor et al., 2015). Whether the two migratory DCs segregated once reaching the LN was not analyzed. Furthermore, XCR1 staining was used to identify LN-resident DCs, which marks both migratory CD103+ and LN-resident CD8+ DCs (Crozat et al., 2011). Therefore, it is difficult to know whether XCR1+ DCs in LNs after infection are derived from tissue migratory DCs, LN-resident DCs, or both. Nevertheless, XCR1+ DCs in the LN were shown to activate CD8+ T cells during a viral infection and, interestingly, at

later time points (>30 hr), to also co-localize with CD4+ T cells. Future work analyzing the relative location of CD4+ versus CD8+ T cells in the spleen as the immune response ensues will be important to determine whether T cell segregation dissipates as the immune response evolves (Gerner et al., 2015); in order to access CD4+ T cell help for DC licensing and fulminant CD8+ T cell activation, it is possible that CD4+ T cells leave the peripheral T cell zone, as identified in our work, and migrate to an XCR1+ DC subset, where we show CD8+ T cells are concentrated. Whether T cell segregation exists at the steady state in LNs has not been addressed, but given our direct evidence for subdomain organization in the spleen between DC subsets and T cell lineages, we propose that this pattern might be universal across all secondary lymphoid organs.

Although human and murine splenic architecture is different in a number of respects, the basic principle of DC subset segregation and then re-organization to promote appropriate adaptive immune responses is likely to be conserved (Pack et al., 2008; Steiniger et al., 2001). DC subsets with overlapping markers and function exist in both species (Mittag et al., 2011). Future work will examine whether similar DC subset segregation exists within human spleens. If this proves to be the case, interventions aimed at blocking DC migration might ameliorate unwanted immune responses, such as those to allogeneic RBC transfusion. Despite matching the ABO blood group antigens, alloimmunization rates approach 45% in chronically transfused patients, such as those with sickle cell disease or thalassemia (Vichinsky et al., 1990). We previously demonstrated that 33D1+ DCs were required for inducing CD4+ T-cell-dependent RBC alloimmunization using *Irf4*-deficient mice lacking this DC subset (Calabro et al., 2016). However, we also found that both XCR1+ and 33D1+ DCs phagocytose transfused RBCs, so it was not clear why XCR1+ DCs could not compensate to activate CD4+ T cells. Our finding of segregated 33D1+ and XCR1+ DC subsets within the T cell zone after RBC transfusion suggests that XCR1+ DCs are, in fact, in the wrong location to induce CD4+ T cell priming to RBC antigens. Accordingly, we now demonstrate that just the loss of 33D1+ DC migration into the T cell zone in *Dock8*-deficient mice is sufficient to prevent the induction of RBC allo-antibodies. This suggests that blocking DC migration therapeutically during prophylactic RBC transfusion could ameliorate the potentially life-threatening impact of alloantibodies in patients who require lifelong RBC support.

We propose a unified model of DC function in regulating T cell immunity. Upon activation by an innate immune stimulus, DC migration from the “peripheral tissue” of the spleen (MZ and RP) to the “lymph node” of the spleen (WP) follows the classic rules now well established for other tissues: DC maturation includes upregulation of CCR7, which directs mature DCs into the T cell zone of the WP (Figures 2 and 4). This relocalization to the T cell area is clearly dependent on innate immune stimuli such as LPS, although how stored allogeneic RBCs trigger innate immune receptors is still under investigation (Gibb et al., 2016). This paradigm, then, would hold universally true for all migratory DCs and all naive T cell responses, regardless of the site of antigen encounter. Furthermore, it suggests that inappropriate immune responses to systemic antigens are avoided by setting a threshold for T cell activation in the spleen by restricting antigen exposure to migrated DCs, analogous to what is proposed in the periphery. The kinetics of DC migration we observed into the splenic T cell zone is similar to that observed in the LNs of intra-lymphatically injected DCs; once

DCs reach the sub-capsular sinus of a LN, it takes less than 12 hr to migrate into the inner T cell zone (Braun et al., 2011). Once migrated, both DC subsets can process antigen for MHCI or MHCII, but 33D1+ DCs preferentially interact with CD4+ T cells in one region of the T cell zone, and XCR1+ DCs interact with CD8+ T cells in a different region. These findings suggest a developing paradigm in which specialized migratory DC subsets preferentially interact in domains enriched for CD4+ or CD8+ T cells in the spleen and LNs to selectively promote adaptive immune responses.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from Charles River Laboratories. *Dock8*$^{-/-}$ and HOD mice were previously described (Hendrickson et al., 2011; Krishnaswamy et al., 2015); OT-I, OT-II, CD11c-YFP, UBC-GFP, *Batf3*$^{-/-}$, and *Ccr7*$^{-/-}$ mice were purchased from The Jackson Laboratory. All protocols used in this study were approved by Yale Institutional Animal Care and Use Committee.

Immunofluorescence Analysis

Fresh splenic tissue was dehydrated through sequential exposure to solutions of 10%, 20%, and 30% sucrose; mounted in a cryomold with OCT (optimal cutting temperature) compound (Tissue-Tek, Sakura); and stored at −80°C prior to 7-μm sectioning and staining. The following antibodies were used for staining different cell subsets: CD11c (N418), 33D1 (33D1), XCR1 (ZET), CD169 (MOMA-1), TCRβ (H57–597), CD8β (YTS156.7.7), CD4 (RM4–5), and B220 (RA3–6B2). The images were acquired immediately after staining with the Nikon Eclipse Ti microscope using either 4× or 20× objectives.

Intravascular Antibody Labeling

Mice were injected i.v. with 15 μg LPS or 1 U of HOD RBCs. At 6–8 hr later, 1.5 μg of anti-CD11c-APC was injected i.v. Three minutes later, the mice were sacrificed, and spleens were collected in cold PBS. To obtain optimal DC staining for injected antibodies, we prepared single-splenocyte suspension by mechanically chopping the spleens.

RBC Transfusion Model

RBCs were collected from HOD transgenic mice in 12% CPDA-1 anticoagulant and leukoreduced using a Pall neonatal filter, followed by 4°C storage for 12 days (Hendrickson et al., 2011). Before transfusion, the RBCs were washed by centrifugation, and the packed RBCs were diluted 1:2 with sterile PBS; 200 μl diluted blood was transfused i.v. into recipient mice (the equivalent of 1–2 U human RBCs). A dilution of 1:2 was used for alloantibody induction or ex vivo antigen presentation, and a ratio of 1:20 was used for in vivo OT-II proliferation.

Statistical Analysis

All statistical analyses were performed using Prism (GraphPad Software). Data were analyzed with the unpaired t test using Welch’s correction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- CD4+ and CD8+ T cells preferentially occupy separate areas of the splenic T cell zone
- DC-intrinsic CCR7 is required for migration within the spleen after i.v. immunization
- 33D1+ and XCR1+ DCs migrate into CD4+ and CD8+ T cell areas, respectively
- Loss of DC subset and T-cell-lineage pairing abrogates either CD4+ or CD8+ T cell immunity

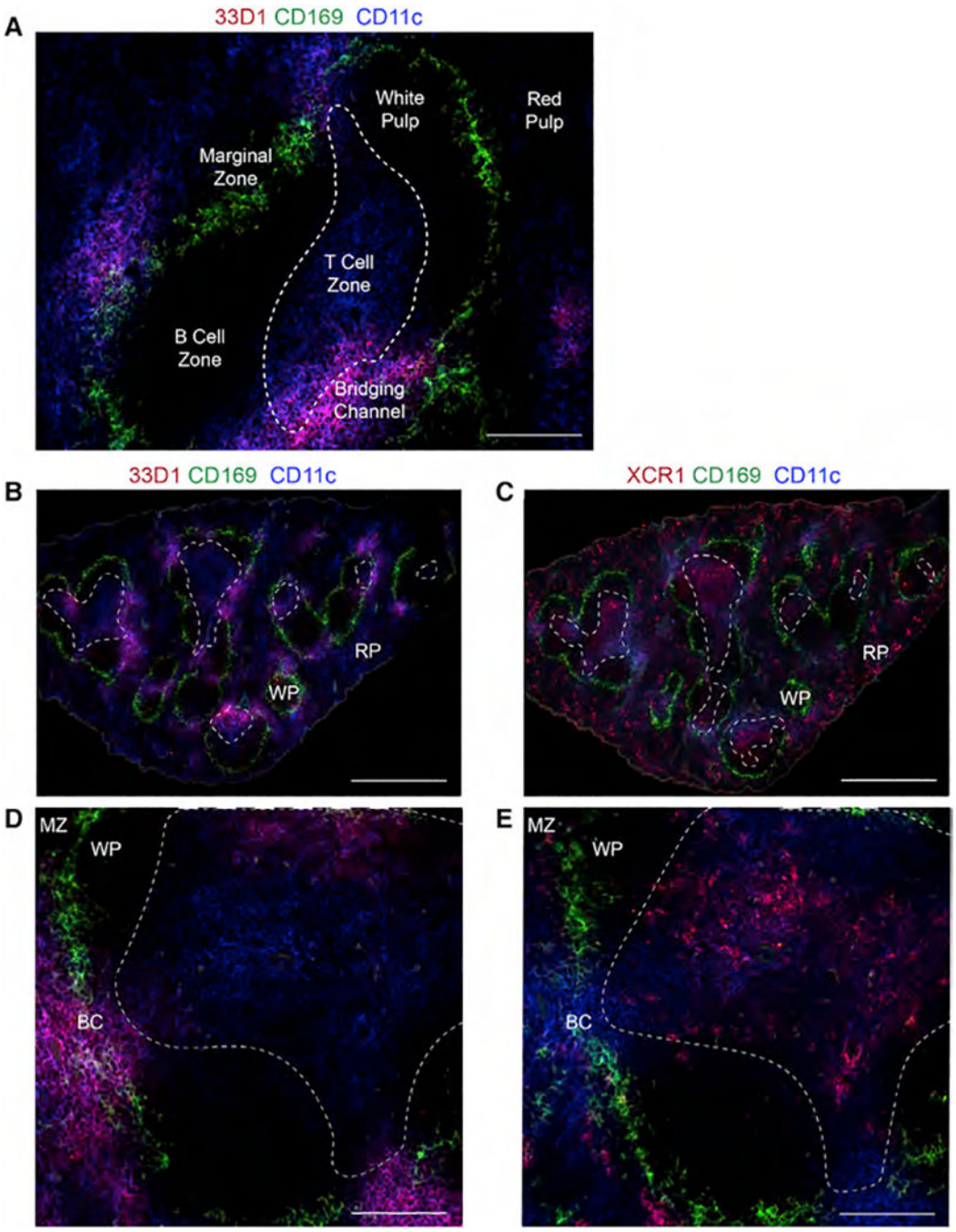

Figure 1. 33D1+ and XCR1+ DC Subsets Survey Different Regions of the Spleen

(A) Fluorescence image of a spleen from a naive wild-type mouse highlighting splenic architecture. Red indicates 33D1, green indicates CD169, and blue indicates CD11c antibodies. Scale bar, 100 μm. Dotted line demarcates the T cell zone (TCRβ).

(B–E) Fluorescence images of serial sections from one spleen from a naive wild-type mouse demonstrating distinct DC subset regions. Dotted lines demarcate the T cell zone (see Figures S1B and S1C). (B) Red indicates 33D1, green indicates CD169, and blue indicates CD11c. Scale bar, 500 μm. (C) Red indicates XCR1, green indicates CD169, and blue

indicates CD11c. Scale bar, 500 μm. (D) Red indicates 33D1, green indicates CD169, and blue indicates CD11c. Scale bar, 100 μm. (E) Red indicates XCR1, green indicates CD169, and blue indicates CD11c. Scale bar, 100 μm. A representative spleen from 18 different WT mice. RP, red pulp; WP, white pulp; BC, bridging channels; MZ, marginal zone.
See also Figures S1B and S1C.

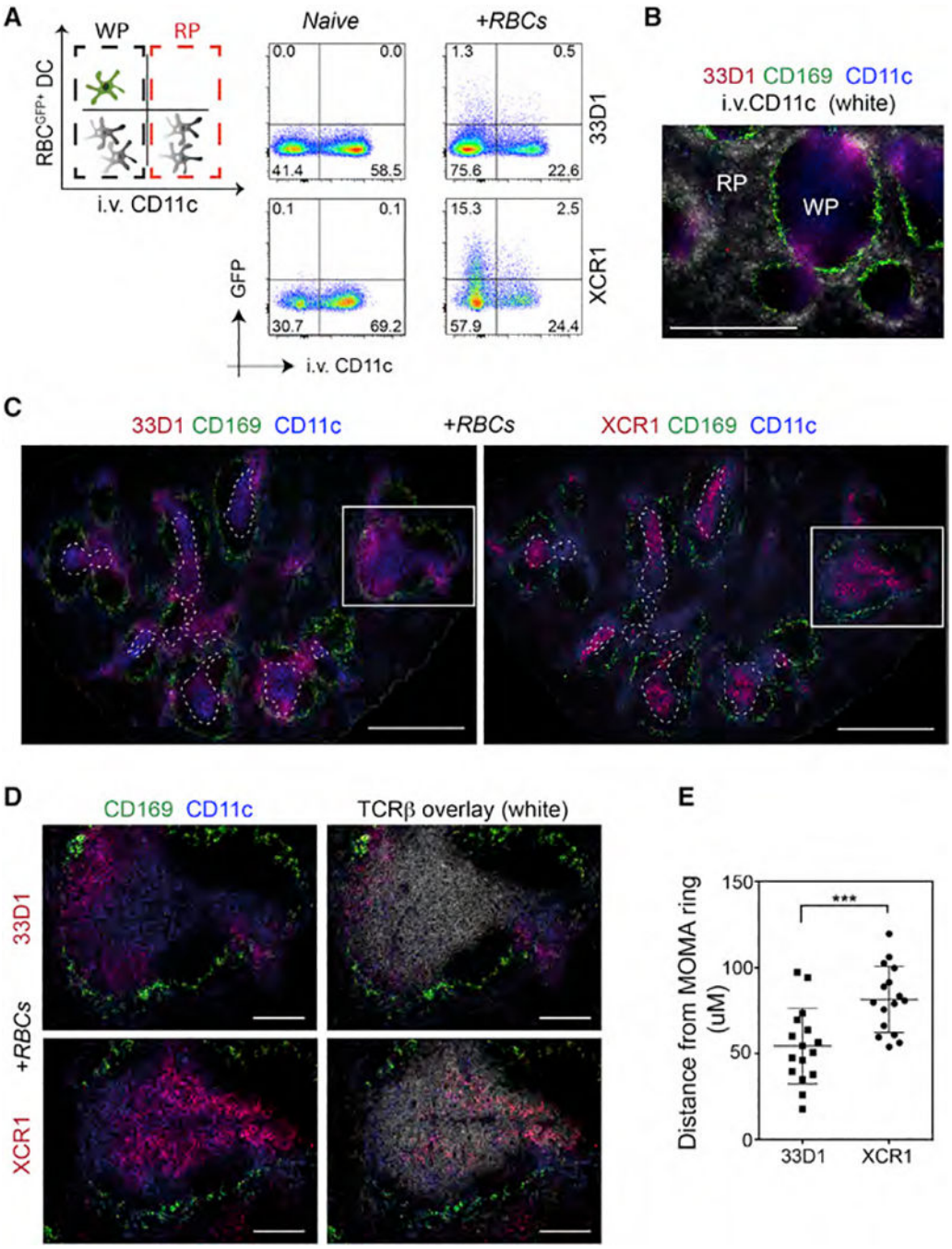

Figure 2. 33D1+ and XCR1+ DCs Migrate into Distinct Regions of the T Cell Zone following Immunization

(A) Tracking of 33D1+ (top) or XCR1+ (bottom) splenic DCs into the white pulp (WP) before (naive) or 6–8 hr after (+RBC) transfusion with GFP+ RBCs, using in vivo labeling. Injection of anti-CD11c i.v. 3 min before sacrifice marks DCs in the red pulp (RP) but not the WP. DCs that have phagocytosed RBCs are GFP+.

(B) Fluorescence image of a spleen from a naive wild-type mouse transfused 3 min prior to sacrifice with anti-CD11c (white overlay). Red indicates 33D1, green indicates CD169, and blue indicates CD11c in vitro stain. Scale bar, 100 μm.

(C and D) Fluorescence image from a section of an entire spleen from a wild-type mouse transfused with RBCs 6–8 hr earlier, demonstrating the differential location of 33D1+ (left) or XCR1+ (right) DCs (red). Green indicates CD169, and blue indicates CD11c. Scale bars, 500 μm.White box highlights the regions in (D) showing a single white pulp. 33D1+ (top) and XCR1+ (bottom) DC staining. (Right) T cell zone is demarcated by an overlay of TCRβ (white). Scale bars, 100 μm. Representative spleen from one of three mice in seven independent experiments.

(E) Images of DC subsets within 16 distinct WPs from five independent experiments were analyzed by Imaris software to determine the average distance of DC centroids to the WP border as defined by location of MOMA-1+ cells. Each data point represents the average distance of all 33D1+ (squares) or XCR1+ (circles) DCs within an individual MOMA-1 ring. ***$p < 0.001$.

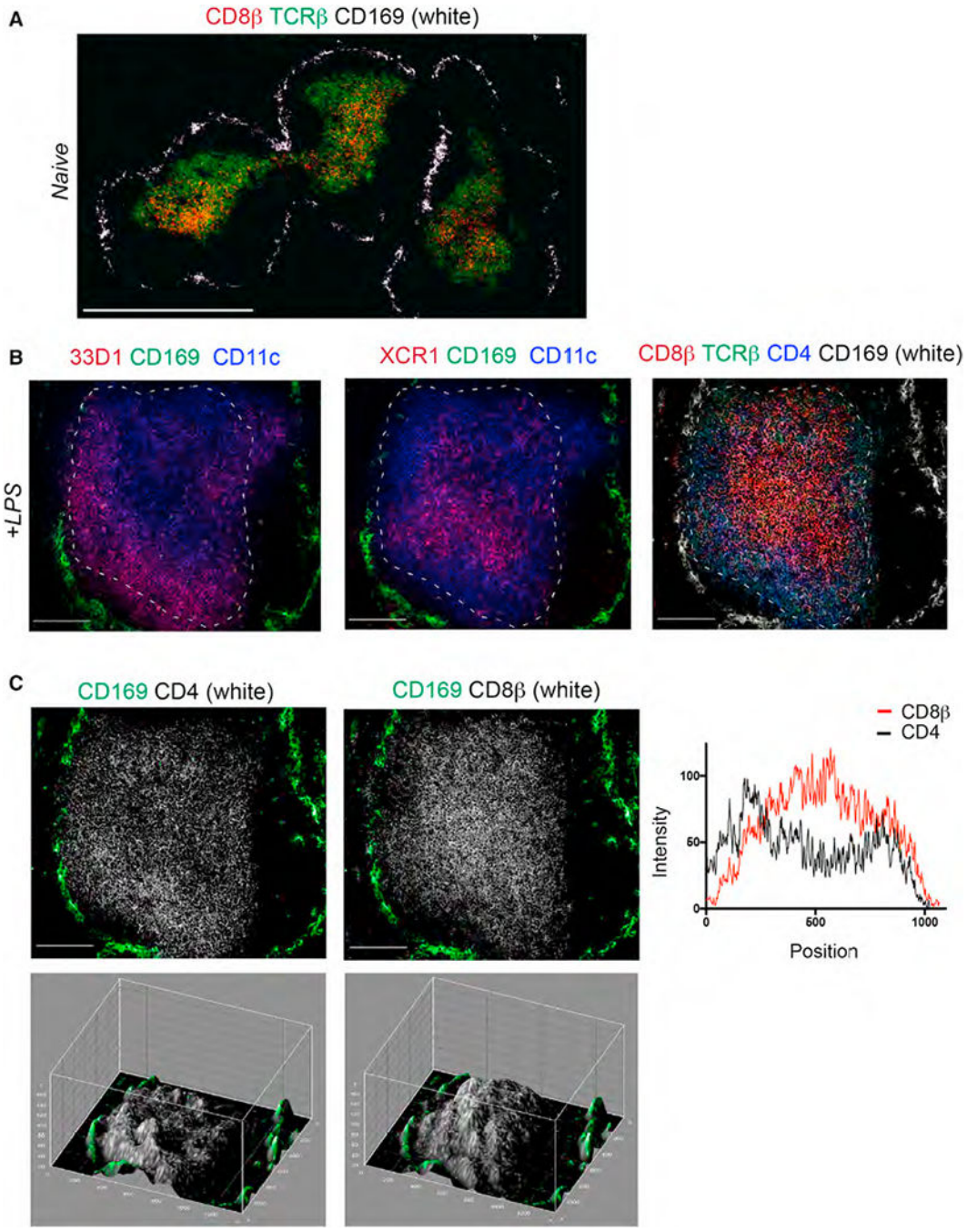

Figure 3. 33D1+ and XCR1+ DCs Co-localize with CD4+ and CD8+ T Cells, Respectively

(A) Fluorescence image of a spleen from a naive wild-type mouse. Red indicates CD8β, green indicates TCRβ, and white indicates CD169. Scale bar, 500 μm. Representative spleen from eight independent experiments.

(B) Fluorescence images of a single follicle of a spleen from a mouse 6–8 hr after challenge i.v. with 25 μg LPS. Left panel: red indicates 33D1, green indicates CD169, and blue indicates CD11c. Middle panel: red indicates XCR1, green indicates CD169, and blue indicates CD11c. Right panel: red indicates CD8β, green indicates TCRβ, blue indicates

CD4; and white indicates CD169. Dotted line indicates T cell region. Scale bars, 100 μm. Representative spleen from six independent experiments.

(C) Follicle shown in (B) demonstrating the single stain for CD4 (left) or CD8β (middle) with the CD169 ring (green) overlaid. Right panel: histogram showing relative intensity of a cross-section of the CD4 versus CD8β staining (see Figure S3). Below each image is a 3D surface plot displaying relative staining intensity. The x and y axes represent image coordinates, and the z axis represents the relative luminance of each pixel. Scale bar, 100 μm.

See also Figure S3.

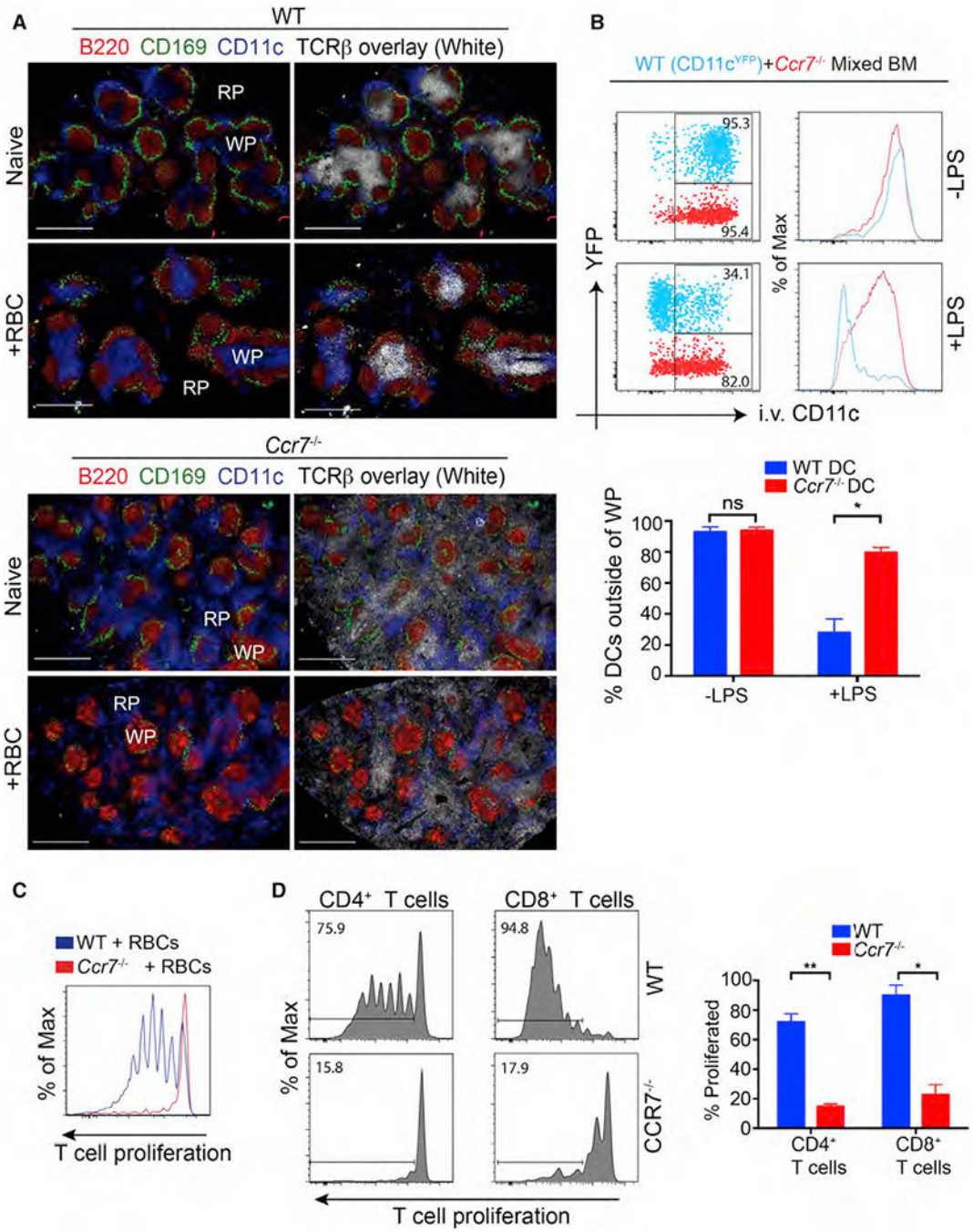

Figure 4. Cell-Intrinsic CCR7 Is Crucial for DC Migration within the Spleen and Subsequent T Cell Activation

(A) Fluorescence image of a spleen from wild-type (WT) and *Ccr7*$^{-/-}$ mouse without transfusion (naive) or 6–8 hr after RBC transfusion (+ RBC). Red indicates B220, green indicates CD169, blue indicates CD11c, and white overlay indicates TCRβ. RP, red pulp; WP, white pulp. Scale bars, = 200 μm. n = 2–3 mice per group. Representative of three independent experiments.

(B) CD45.1 mice were irradiated and reconstituted with a mixture of bone marrow cells from CD11c-YFP (50%) and wild-type or *Ccr7*$^{-/-}$ (50%) mice. Eight weeks later, chimeric

mice were injected i.v. with 15 μg LPS or PBS. 6–8 hr later, in vivo labeling was used to track the migration of splenic DCs into the white pulp. (Top) Flow cytometry plots of splenic DCs, with gates indicating DCs outside of the WP; blue indicates wild-type DCs, and red indicates *Ccr7*$^{-/-}$ DCs. (Bottom) Percentage of DCs outside of white pulp (WP) in the presence or absence of LPS treatment in each group. *$p < 0.05$; ns, not significant; results representative of three independent experiments. Error bars indicate SD.

(C) Wild-type and *Ccr7*$^{-/-}$ mice received 10^6 CFSE-labeled CD45.1$^+$CD4$^+$ OT-II cells. 24 hr after T cell transfer, mice were transfused with HOD RBCs, and proliferation in the spleen was measured by CFSE dilution in wild-type (blue) and *Ccr7*$^{-/-}$ (red) mice 3 days after RBC transfusion.

(D) CFSE-labeled purified CD45.1$^+$ naive 10^6 OT-I (CD8$^+$ T cells) and 10^6 OT-II (CD4$^+$ T cells) were co-transferred into naive wild-type and *Ccr7*$^{-/-}$ mice, and 1 day later, the recipient mice were immunized i.v. with 5 μg OVA with 1 μg of LPS. (Left) Three days later, OT-I and OT-II proliferation was assessed in the spleen by measuring CFSE dilution by flow cytometry. Proliferation gates were set using unimmunized mice. (Right) Percentage of OT-I and OT-II T cells proliferated in each group. *$p < 0.05$; **$p < 0.01$; results are representative of five independent experiments. Error bars indicate SD.

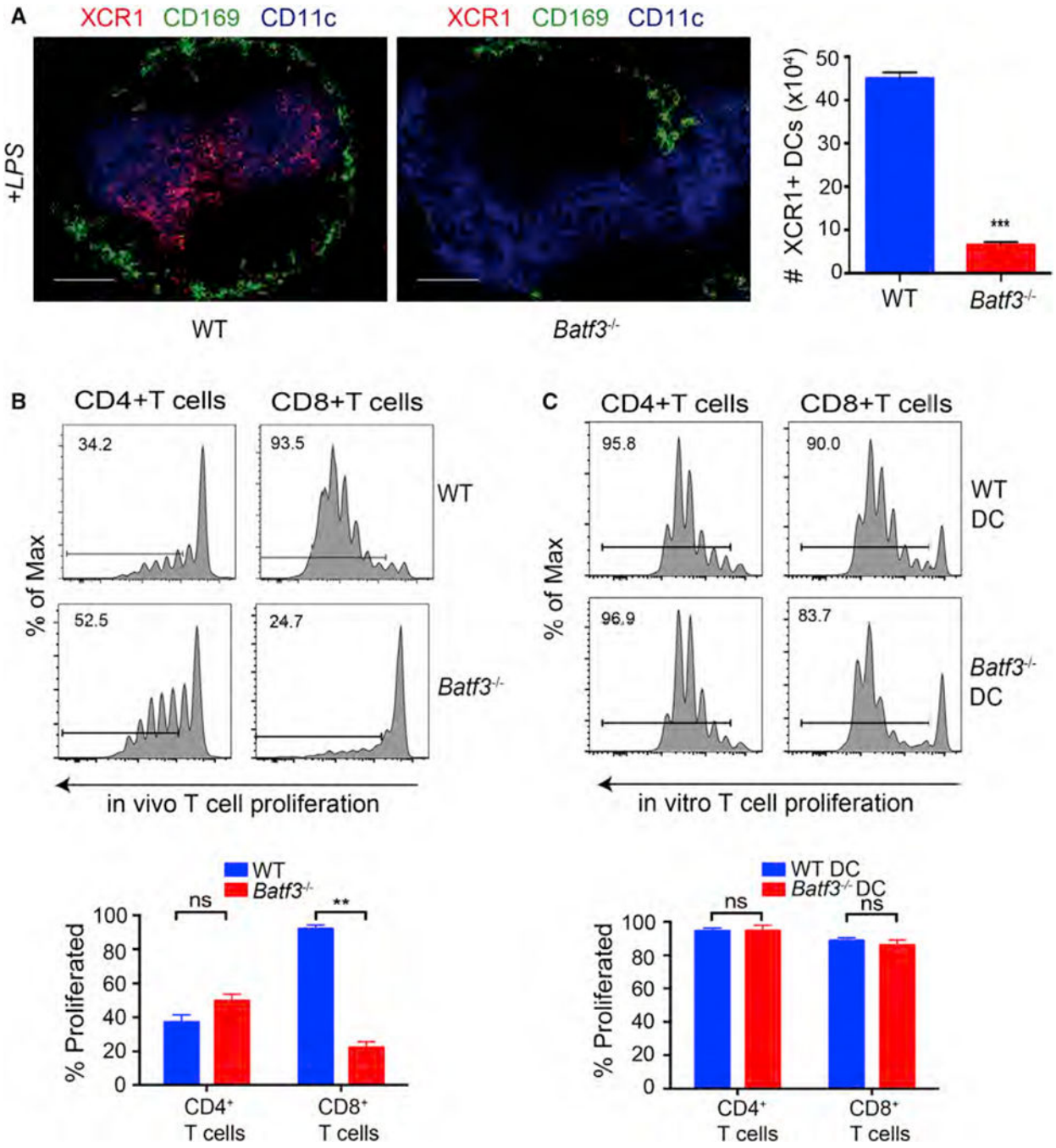

Figure 5. XCR1+ DCs Are Required for CD8+, but Not for CD4+, T Cell Activation Only In Vivo

(A) Fluorescence image of spleens from wild-type (WT) (left) and *Batf3*$^{-/-}$ (middle) mice 6–8 hr after injection i.v. of 15 μg of LPS. Red indicates XCR1, green indicates CD169, and blue indicates CD11c. Scale bar, 100 μm. (Right) Quantitation of number of XCR1+ DCs in the spleen of naive wild-type versus *Batf3*$^{-/-}$ mice by flow cytometry. Less than 2% of all DCs in the spleen of *Batf3*$^{-/-}$ mice expressed XCR1. ***$p < 0.001$; n = 2–3 mice per group. Representative of three independent experiments. Error bars indicate SD.

(B) 10^6 CFSE-labeled CD45.1$^+$ OT-I and OT-II cells were co-transferred into wild-type and *Batf3*$^{-/-}$ mice, and 24 hr later, mice were immunized i.v. with 5 μg OVA with 1 μg of LPS. Three days later, OT-I and OT-II proliferation was assessed by measuring CFSE dilution by flow cytometry. (Bottom) Percentage of OT-I and OT-II T cells proliferating in the spleen in groups of wild-type and *Batf3*$^{-/-}$ mice after immunization **$p < 0.01$; ns, not significant. n = 2–3 mice per group. Representative of six independent experiments. Error bars indicate SD.

(C) Splenic DCs from wild-type or *Batf3*$^{-/-}$ mice were enriched by MACS purification. After pulsing with 100 μg/ml OVA for 1 hr at 37°C, the purified DCs were co-cultured with CFSE-labeled purified OT-I and OT-II T cells for 72 hr, respectively. OT-I and OT-II proliferation was assessed by measuring CFSE dilution by flow cytometry. Numbers indicate the percentage of proliferating cells in the indicated gates. Representative of two independent experiments. (Bottom) Percentage of OT-I and OT-II T cells proliferating in vitro after co-culture with pulsed wild-type or *Batf3*$^{-/-}$ DCs. ns, not significant. Error bars indicate SD.

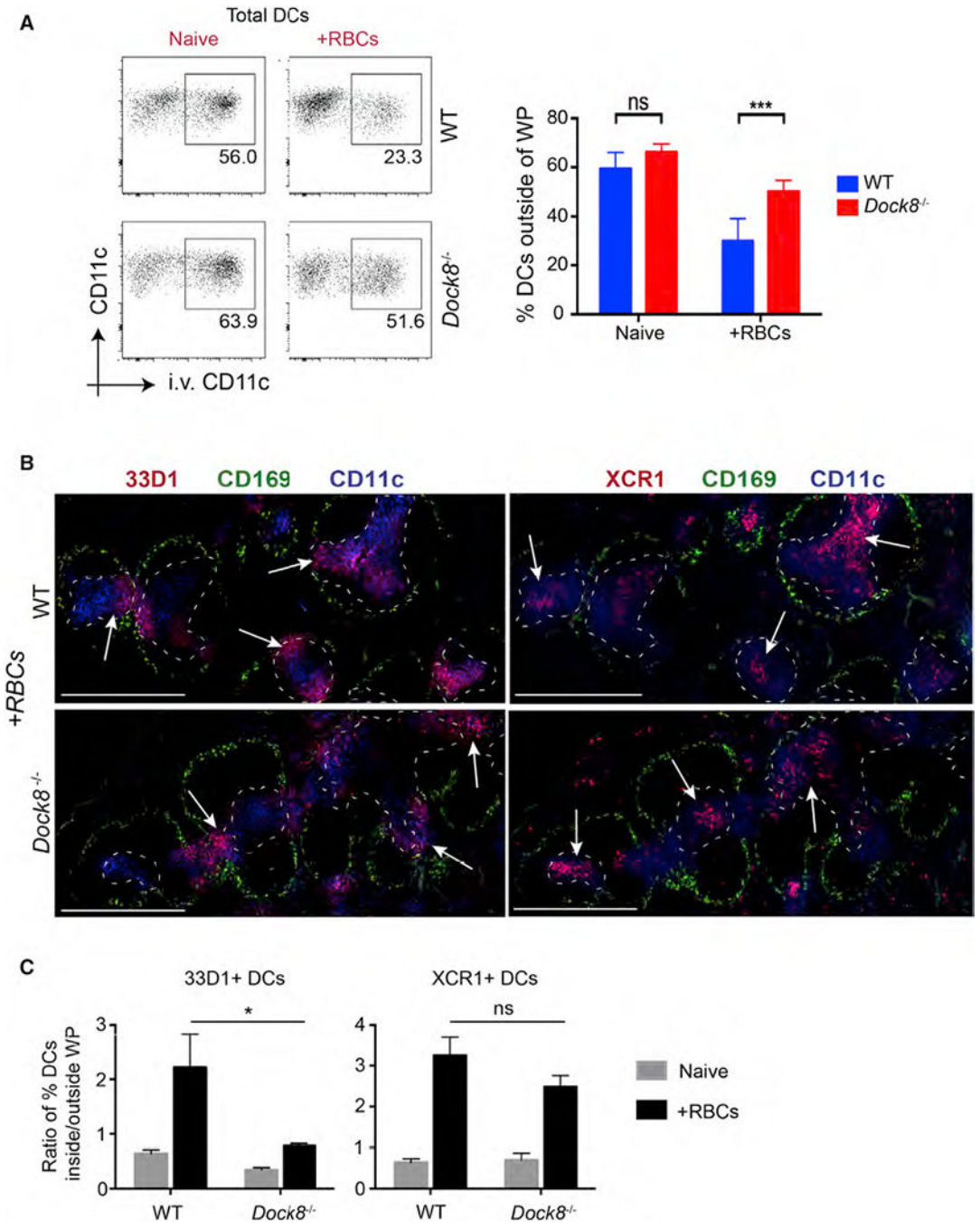

Figure 6. Selective Splenic 33D1+ DC Migration Defect in Dock8-Deficient Mice

(A) Wild-type (WT) and *Dock8*$^{-/-}$ mice were transfused with HOD RBCs. (Left) 6–8 hr later, in vivo labeling was used to track the migration of splenic DCs into the white pulp (WP). DCs were analyzed in wild-type and *Dock8*$^{-/-}$ mice before (naive) or after (+RBCs) RBC transfusion. (Right) Pooled percentages of DCs outside of WP of wild-type and *Dock8*$^{-/-}$ mice before or after RBC transfusion from three separate experiments. ***p < 0.001. Error bars indicate SD.

(B) Fluorescence images of spleens from wild-type (top) and *Dock8*$^{-/-}$ (bottom) mice 6–8 hr after RBC transfusion. Red indicates 33D1 or XCR1, green indicates CD169, and blue indicates CD11c. T cell area (from TCRβ stain) outlined to assess DC migration into the T cell zone (dotted line). Arrows highlight the presence of 33D1+ DCs inside the T cell zone of wild-type, but not *Dock8*-deficient, mice (left). Arrows highlight the presence of XCR1+ DCs inside the T cell zone of both wild-type and *Dock8*-deficient mice (right). Scale bars, 500 μm. n = 2–3 mice per group. Representative of six independent experiments.
(C) Average ratio of percentage of DCs inside:outside of WP of either 33D1+ (left) or XCR1+ (right) DCs in naive (gray) or RBC transfused (black) mice. Shown is one representative experiment of three. n = 2–4 mice per group. *$p < 0.05$; ns, not significant. Error bars indicate SD.

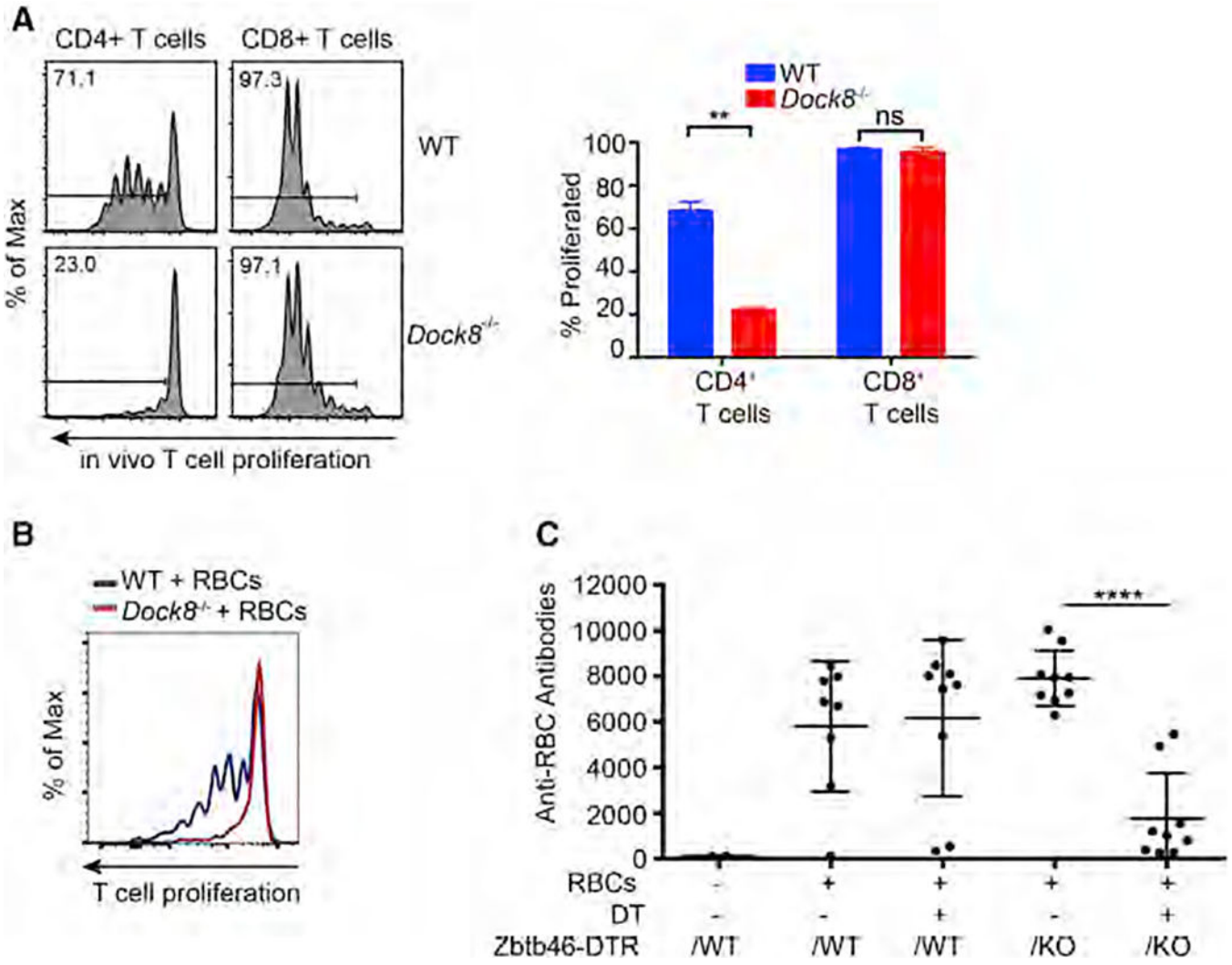

Figure 7. Loss of 33D1+ DC Migration in Dock8-Deficient Mice Impairs CD4+, but Not CD8+, T Cell Responses to Multiple Antigens

(A) Wild-type (WT) and *Dock8*$^{-/-}$ mice received 10^6 CFSE-labeled CD45.1+CD4+ OT-II cells and CD45.1+CD8+ OT-I cells. 24 hr later, mice were immunized i.v. with 5 μg of OVA with 1 μg LPS, and proliferation in the spleen was measured by CFSE dilution by gating on CD45.1+ OT-I or OT-II cells, respectively. (Left) Numbers indicate the percentage of proliferating cells in the indicated gates. (Right) Proliferation of OT-I and OT-II T cells in the spleens of each group of wild-type or *Dock8*$^{-/-}$ mice after immunization. **$p < 0.01$; ns, not significant; representative of six independent experiments. Error bars indicate SD.

(B) Wild-type and *Dock8*$^{-/-}$ mice received 10^6 CFSE-labeled CD45.1$^+$CD4$^+$ OT-II cells. 24 hr later, mice were transfused with HOD RBCs, and OT-II proliferation in the spleen was measured by CFSE dilution 3 days later in wild-type (blue) and *Dock8*$^{-/-}$ (red) mice. Representative of four independent experiments.

(C) Anti-RBC antibodies in the sera of *Zbtb46*-DTR/*Dock8*$^{-/-}$ mixed bone marrow chimeric mice with or without treatment with diphtheria toxin (DT) 21 days following HOD RBC transfusion. Zbtbt46-DTR bone marrow was mixed 1:1 with either C57BL/6 (WT) or

Dock8$^{-/-}$ (KO) bone marrow as indicated. Zbtbt46-DTR/KO chimeric mice administered DT have only *Dock8*-deficient DCs during RBC transfusion. Each dot represents an individual mouse. ****$p < 0.0001$. n = 6–9 mice per group. Representative of three independent experiments. Error bars indicate SD.