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## Dendritic Cells Regulate Extrafollicular Autoreactive B cells via T cells Expressing Fas and Fas Ligand

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### Abstract

The extrafollicular (EF) plasmablast response to self-antigens that contain Toll-like receptor (TLR) ligands is prominent in murine lupus models and some bacterial infections, but the inhibitors and activators involved have not been fully delineated. Here, we used two conventional dendritic cell (cDC) depletion systems to investigate the role of cDCs on a classical TLR-dependent autoreactive EF response elicited in rheumatoid-factor B cells by DNA-containing immune complexes. Contrary to our hypothesis, cDC depletion amplified rather than dampened the EF response in Fas-intact but not Fas-deficient mice. Further, we demonstrated that cDC-dependent regulation requires Fas and Fas ligand (FasL) expression by T cells, but not Fas expression by B cells. Thus, cDCs activate FasL-expressing T cells that regulate Fas-expressing extrafollicular helper T cells ( $T_{\text{effh}}$ ). These studies reveal a regulatory role for cDCs in B cell plasmablast responses and provide a mechanistic explanation for the excess autoantibody production observed in Fas-deficiency.

### Graphical abstract

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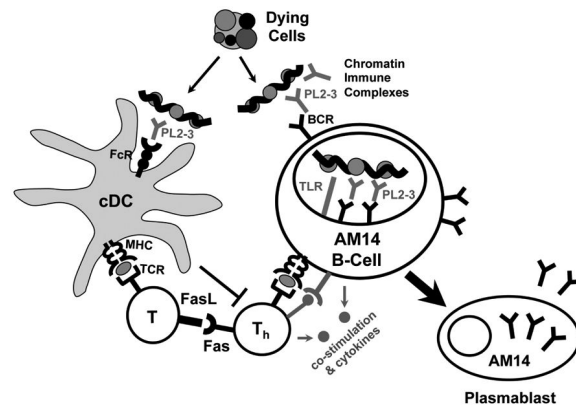
#### SUPPLEMENTAL INFORMATION

Supplemental information includes seven figures and supplemental methods for PD-1 and Bcl-6 staining by flow cytometry.

#### AUTHOR CONTRIBUTIONS

M.L.O. designed and performed experiments, analyzed data, and wrote the manuscript. J.L.C., A.T-N., and J.G. performed experiments and analyzed data. M.J.S. designed experiments, supervised the study, and wrote the manuscript.

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## INTRODUCTION

B cells are pivotal in the development of systemic autoimmune diseases, which are characterized by the selective loss of tolerance to self-Ags such as DNA, RNA, and IgG (Shlomchik, 2009). In addition to autoantibody-mediated damage, B cells promote disease by stimulating T cell activation and expansion, presumably via antigen (Ag) presentation, and possibly through cytokine secretion (Shlomchik, 2009). Autoreactive B cells are often activated in extrafollicular (EF) foci, via co-ligation of B cell receptors (BCRs) and toll-like receptors (TLRs) by antigens (Ags) containing DNA and RNA. These autoreactive, EF-localized, TLR-driven B cell responses consist of short-lived plasmablasts, as found in MRL/MpJ-*Fas<sup>lpr</sup>/J* (MRL.*Fas<sup>lpr</sup>*), and other autoimmune mouse models, such as NZB/W, as well as in human disease (Daridon et al., 2012; Hoyer et al., 2004; Tipton et al., 2015). The EF response is not restricted to autoimmunity, and perhaps evolved to provide pathogen-specific protection. Indeed, EF B cell foci develop in response to various types of T-dependent (TD) and T-independent (TI) Ags, including viruses and bacteria (Cunningham et al., 2007; Di Niro et al., 2015; MacLennan et al., 2003). Such pathogen-induced EF responses are normally transient, indicating that they are tightly regulated. However, in the case of autoimmunity, EF responses persist for the life of the animal, becoming sites of extensive and prolonged clonal expansion, hypermutation, and affinity maturation (William et al., 2002; William et al., 2005b). How normal EF responses become dysregulated in autoimmunity remains unclear.

To study how autoreactive EF B cell responses are regulated, we have used mice transgenic (Tg)—either ectopically inserted or site-directed (sd)—for an immunoglobulin (Ig) heavy chain encoding the anti-IgG2a<sup>a</sup> rheumatoid factor (RF), AM14 (Sweet et al., 2010). In autoimmune prone MRL.*Fas<sup>lpr</sup>* and MRL/MpJ mice, AM14 B cells spontaneously expand, class switch, hypermutate and develop into antibody forming cells (AFCs) within EF foci in the spleen (Sweet et al., 2010; William et al., 2002; William et al., 2005b). This spontaneous, TLR-dependent autoactivation occurs *in vivo* by AM14 BCR binding to DNA- and RNA-containing immune complexes (ICs) (Herlands et al., 2008). We can also induce EF AM14 activation on the BALB/c background by administration of a model physiologic ligand, the IgG2a<sup>a</sup> anti-chromatin antibody, PL2-3 (Herlands et al., 2007), which results in a plasmablast response that is phenotypically and histologically indistinguishable from the

spontaneous model. Like the spontaneous RF and anti-nuclear response, the PL2-3-induced response is TLR7, TLR9, and MyD88 dependent, as well as autoantigen-specific (Herlands et al., 2008). In contrast to the unpredictable onset and variable magnitude of the spontaneous system, the PL2-3 induced response enables precise timing and a directed approach for determination of proximal factors required for autoreactive EF B cell activation. Thus, the PL2-3 induced response has been used by a number of labs to study how a classical autoreactive B cell response is induced (Giltiay et al., 2013; Nundel et al., 2015; Sang et al., 2014).

We initially hypothesized that cDCs are critical supporting cells for the EF response, in part because cDCs are prominent components of such EF foci (MacLennan et al., 2003; William et al., 2002). An activating role for cDCs in the EF response has been inferred from experiments in which anti-CD40 was used to induce cDC proliferation, and appeared to thereby increase EF plasmablast survival in response to a TI-2 Ag (MacLennan et al., 2003). Although cDCs are best known for their interactions with T cells, a small body of literature indicates that cDCs can promote B cell activation. cDCs secrete factors, such as B cell activating factor (BAFF), a proliferation inducing ligand (APRIL), and interleukin-6 (IL-6), which support B cell development, activation and differentiation (MacLennan and Vinuesa, 2002; Mohr et al., 2009). cDCs have also been shown to deliver Ag to, and directly interact with, B cells in lymph nodes (Gonzalez et al., 2010; Qi et al., 2006), and in the spleen (Balázs et al., 2002), thereby promoting B cell activation and the development of humoral immunity. In particular, via a non-degradative Ag uptake and processing pathway, cDCs can present whole Ag on their cell surface to B cells (Bergtold et al., 2005; Zelenay et al., 2012). Although these data suggest that cDCs might aid B cell responses, a more direct cDC ablation study has shown that cDCs are not required for the generation of an EF TI-2 response to 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll (Hebel et al., 2006), calling into question earlier conclusions that DCs played nonredundant roles.

The autoreactive response to nucleic acids and to the ICs containing them has characteristics of TI-1, TI-2 (Herlands et al., 2008), and TD responses (Sweet et al., 2011). Such nucleic acid-driven responses are therefore likely to be regulated differently from TI-2 responses. In addition, chromatin-containing immune complexes (ICs) have a distinctive capacity to stimulate myeloid cells through both the Fc gamma receptor (Fc $\gamma$ R) and TLRs (Boulé et al., 2004). To ask whether cDCs are required for the activation of this type of autoreactive B cell response, we employed both acute (Jung et al., 2002) and constitutive (Birnberg et al., 2008; Teichmann et al., 2010) cDC ablation systems. Both cDC-depletion systems yielded the finding that cDCs *negatively* regulate, rather than activate, this autoreactive B cell response. Furthermore, we found that this cDC regulatory effect was dependent on Fas and was not observed in Fas-deficiency. We further determined that cDC-dependent regulation of the EF response required Fas and FasL expression by T cells; whereas, Fas expression on B cells was not relevant for this effect. We thus propose that cDCs activate FasL-expressing T cells to regulate Fas-expressing T cells that, in turn, help B cells in EF sites. In addition to revealing a clear regulatory role for cDCs, these studies elucidate an additional mechanism whereby Fas controls autoreactive EF plasmablast responses, helping explain how Fas-deficiency promotes hypergammaglobulinemia, high autoantibody titers, and autoimmunity.

## RESULTS

### The EF AM14 Rheumatoid Factor Response Does Not Acutely Require, but is Regulated by, cDCs

To determine whether cDCs are required for the generation of the EF AM14 rheumatoid factor B cell response, we made radiation chimeras with bone marrow (BM) from mice expressing the diphtheria toxin (DT) receptor (DTR) and green fluorescent protein (GFP) under a *Itgax* promoter (*Itgax*-DTR) (Jung et al., 2002), administered DT to deplete cDCs, transferred in sd-Tg AM14 B cells, and injected the activating Ag, PL2-3 (schema in Figure S1). Repeated DT treatment is well tolerated in BM chimeras (Zammit et al., 2005). Because the transferred AM14 B cells do not carry the *Itgax*-DTR locus, CD11c<sup>lo</sup> AM14 plasmablasts are not deleted by DT in this system (Hebel et al., 2006). The AM14 cells with rheumatoid factor specificity were tracked by ELISpot analysis and flow cytometry using the anti-idiotypic marker, 4-44.

Notably, we observed 3, 5, 4 and 7-fold increases in the numbers of 4-44<sup>+</sup> AM14 cells, CD22<sup>lo</sup> CD44<sup>hi</sup> plasmablasts, IgM and IgG2a antibody forming cells (AFCs), respectively, in cDC-depleted hosts as compared to controls (Figure 1). The location of the response in the cDC-depleted animals was EF (Figure S2), similar to that observed in untreated controls, and as reported (Herlands et al., 2008; Herlands et al., 2007; Sweet et al., 2011; William et al., 2002). Few, if any 4-44<sup>+</sup> peanut agglutinin (PNA)<sup>+</sup> germinal centers (GCs) could be detected by histology at day 6 (not depicted). These data indicate that cDCs are not required for, and—given the several-fold enhancement in their absence—may regulate, the EF AM14 response.

### cDCs are not Required Constitutively for the AM14 Response

Although acute depletion of cDCs suggested that they are not required for the activation of AM14 B cells, it remained possible that essential cytokines produced by cDCs are retained in the extracellular matrix (Mohr et al., 2009), in which case acute cDC depletion would not reveal an effect of cDCs on B cell activation. We were also concerned that acute local DT-induced cDC death could provide an additional source of chromatin Ag, that could account for the increased AM14 response observed in the acute cDC ablation system. To address these issues, we generated recipient mice constitutively lacking cDCs by crossing mice expressing the *Rosa26*-flox-stop-DTA locus (Ivanova et al., 2005) to mice expressing *Itgax*-Cre (Caton et al., 2007), which we call  $\Delta$ DC mice. We had both strains of these mice fully backcrossed onto the MRL.*Fas*<sup>lpr</sup> background (Teichmann et al., 2010), which were suitable for our studies, because we used young, pre-diseased animals, as previously reported (Herlands et al., 2007). As had been shown for  $\Delta$ DC mice, there was extensive depletion of cDCs and a substantial loss of pDCs as well as increased CD11b<sup>+</sup> cells (Birnberg et al., 2008; Ohnmacht et al., 2009; Teichmann et al., 2010) (Figure S3). We transferred sd-Tg AM14.MRL.*Fas*<sup>lpr</sup> B cells into these recipients on day 0, administered the activating Ag, PL2-3 on days 0, 2 and 4, and assessed the response on day 6. In contrast to our acute ablation experiments in BALB/c mice, EF AM14 B cell responses in  $\Delta$ DC MRL.*Fas*<sup>lpr</sup> mice were similar to those of control recipients (Figure 2). These results confirm that cDCs are not required either acutely or constitutively for the activation of the autoreactive AM14 B

cell response. That cDCs were not required for the EF response was somewhat unexpected, because MacLennan and colleagues have concluded that cDCs are a limiting factor in plasmablast generation and survival in response to TI-2 Ags (MacLennan et al., 2003). Additionally, since we did not observe enhanced EF responses in DC mice, it remained possible that acute DT-induced cDC death within the spleen of *Itgax*-DTR mice enhanced the AM14 B cell response.

### Regulation of the AM14 Response by cDCs Requires Fas

However, another important difference between the acute and the constitutive cDC ablation systems was the genetic background of the mice: BALB/c in the former, and MRL.*Fas*<sup>lpr</sup>, in the latter. Because Fas-deficient mice have prominent spontaneous autoreactive EF responses (Shlomchik, 2009; William et al., 2002), it was possible that Fas could limit this EF response in normal mice. If this role of Fas were cDC-dependent, then depletion of cDCs in Fas-intact BALB/c mice would result in an increased response, as we observed (Figure 1). However, in Fas-deficient mice, cDC elimination would have no effect on the response, as we also observed (Figure 2). This logic is shown schematically in Figure S4. To test this idea, we compared constitutive cDC depletion on the same MRL genetic background, but in the presence or absence of functional Fas. Notably, in Fas-sufficient DC MRL.*Fas*<sup>lpr/+</sup> mice, there was a large enhancement (3, 9, 2, 5, and 4 fold, respectively) of splenic 4-44<sup>+</sup> AM14 B cell and plasmablast numbers, as well as, IgM, IgG2a, and IgG2b AFCs, compared to control recipients (Figure 3). Again, this AM14 response was EF (Figure 3G and S5). Hence, enhancement of the EF AM14 response upon cDC depletion was seen in both acute and constitutive cDC depletion, but only when Fas was intact. Therefore, we conclude that cDCs regulate the EF AM14 plasmablast response via a Fas-dependent mechanism.

### Fas-Dependent cDC Regulation of the AM14 Response is AM14 B Cell Extrinsic

Next, we investigated which cell type was required to express Fas for proper regulation of the response. Because EF helper T cells (Odegard et al., 2008) enhance AM14 B cell expansion (Sweet et al., 2011), it was possible that FasL-killing of Fas-expressing helper T cells could limit EF B cell responses. Alternatively, because B cells are sensitive to FasL-mediated killing (Rothstein et al., 1995), Fas-expressing AM14 B cells could be directly targeted. To distinguish these possibilities, we transferred Fas-deficient sd-Tg AM14.MRL.*Fas*<sup>lpr</sup> B cells into Fas-sufficient DC.MRL.*Fas*<sup>lpr/+</sup> mice or littermate controls, and induced the AM14 response with PL2-3 (Figure 4). Although caution is required in comparing across experiments, as expected, the response size of these Fas-deficient B cells appeared larger than what we had observed in prior experiments. However, if Fas expression on B cells were responsible for cDC-dependent regulation of AM14 B cells, we would have expected to see, upon transfer of Fas-deficient B cells, similar responses within the DC and cDC-intact mice. Rather, we still saw a large enhancement of the AM14 response within DC recipient mice as compared to controls, even when the B cells could not have been influenced by FasL. In fact, the splenic 4-44<sup>+</sup> AM14 B cell, plasmablast, and IgM, IgG2a, and IgG2b AFC numbers were 10, 20, 4, 9, and 9 fold higher, respectively, in recipients lacking cDCs than in controls. Thus, we conclude that cDCs regulate EF autoimmune responses in a Fas-dependent, B cell-extrinsic manner.

## Recipient Mice Lacking Fas, cDCs, or Both Have Higher Numbers of Activated CD4<sup>+</sup> T Cells than Control Mice

Since Fas-dependent killing of B cells was not the mechanism for Fas-dependent regulation of the AM14 B cell response by cDCs, regulation must depend on a different cDC-dependent, Fas-expressing cell. T cells that help the EF response (Lee et al., 2011; Odegard et al., 2008) would be a leading candidate for such a Fas-expressing cell. Reciprocally, FasL expression could be required either on other T cells or on cDCs (Lu et al., 1997; Süß and Shortman, 1996; Van Parijs and Abbas, 1996) in order to kill the Fas-expressing EF helper T cell (T<sub>effh</sub>). The notion that cDCs could activate both Fas-sensitive T<sub>effh</sub> and FasL-expressing killer T cells is reasonable, because T cells are regulated by Fas-FasL interactions (Van Parijs and Abbas, 1996), and cDCs can both activate and regulate T cell responses (Reis e Sousa, 2006). Furthermore, T cells can promote AM14 activation and differentiation via CD40L and IL-21 (Sweet et al., 2011). With these possible cDC-T and T-B interactions in mind, we first assessed our three systems of AM14 B cell transfer into DC mice for cDC and Fas-dependent alterations in T cell activation (Figure 5). Fas-intact DC MRL mice had a higher proportion of CD4<sup>+</sup> T cells that were CD44<sup>hi</sup>, CD62L<sup>lo</sup> than the cDC-sufficient littermate controls. Absolute numbers of this T cell subset were also elevated. These cDC-dependent differences in the T cell compartment were Fas-dependent, because the increases were only seen in Fas-sufficient and not Fas-deficient MRL recipients. Of note, similar differences were present even in mice that had not received Ag or B cell transfer, although Ag injection with B cell transfer also enhanced T cell activation above controls (Figure S6). Further analysis of DT-treated *Igax*-DTR.BALB/c bone marrow chimeras revealed that the increase in CD4<sup>+</sup> T cells observed in DC-depleted mice included an increase in the PD-1<sup>+</sup>, Bcl-6<sup>+</sup> T<sub>effh</sub> cell subset (Figure S6). These data imply that cDC-dependent regulation of AM14 B cells involves the elimination of Fas-expressing T<sub>effh</sub> cells.

## Regulation of AM14 Plasmablasts by cDCs Requires T Cells Expressing Fas and FasL

To more definitively determine cell-specific roles for Fas and FasL in limiting autoreactive EF plasmablast responses, we performed a series of mixed bone marrow (BM) chimera experiments. First, we made mice in which irradiated BALB/c recipients received a mix of donor BM that was 80% from BALB/c. *Tcra*<sup>-/-</sup> mice and 20% from BALB/c. *Fas*<sup>dpr</sup> mice. Thus, all T cells within the chimeric animals lacked Fas, while other cell types had normal Fas expression on the great majority of their cells (Figure 6). Control animals received BM that was either 100% BALB/c or a mix of 80% BALB/c. *Tcra*<sup>-/-</sup> and 20% BALB/c. In these experiments, when T cells lacked Fas, the AM14 B cell response was enhanced by 3, 7, and 10 fold for IgM, IgG2a, and IgG2b AFCs, respectively. Finally, we made mice in which irradiated BALB/c recipients received a mix of donor BM that was 80% from BALB/c. *Tcra*<sup>-/-</sup> mice and 20% from BALB/c. *FasL*<sup>mut</sup> mice (Gregory et al., 2011). In these chimeras, no T cells had active FasL, while other cell types were normal (Figure 7A-F). Control mice received BM that was either 100% BALB/c or a mix of 80% BALB/c. *Tcra*<sup>-/-</sup> and 20% BALB/c. Here, when T cells could not signal via FasL, AM14 IgM, IgG2a, and IgG2b AFC numbers were 2, 3, and 8 fold higher, respectively. As expected, when T cells lacked either Fas or FasL, an activated T cell subset was elevated in frequency (Figures 6F, G & 7F, G). These experiments confirm that cDC-dependent regulation of autoreactive plasmablasts requires FasL-expressing T cells to regulate Fas-expressing T<sub>effh</sub> cells.

## Loss of cDCs Reduces Cell Death of Splenic CD4<sup>+</sup> T Cells During the AM14 B Cell Response

To determine if cDCs increase the ability of FasL-expressing T cells to kill Fas-expressing helper T cells during the AM14 B cell response, we used the TUNEL assay to detect apoptotic cells in tissue sections of DT-treated *Itgax*-DTR.BALB/c bone marrow chimeras. Indeed, less TUNEL<sup>+</sup> CD4<sup>+</sup> cells were detected in cDC-depleted than control mice (Figure 7G,H). These data further support the idea that cDC-dependent regulation of AM14 B cells involves the killing of Fas-expressing helper T cells by FasL-expressing T cells.

## DISCUSSION

In normal responses to non-replicating TI and TD Ags, the EF response is transient (MacLennan et al., 2003), though the mechanisms that constrain it are not yet defined. A limiting component is likely the inherent propensity of plasmablasts to die, which does not depend on Fas (Do et al., 2000; Ursini-Siegel et al., 2002; William et al., 2005a). Although regulatory T (Treg) cells can restrain humoral immunity, they have been implicated in GC, rather than EF responses (Sage and Sharpe, 2015), and Treg cell neutralization with the anti-CD25 Ab PC61 had no effect in our system (not depicted). Determining the identity and function of the negative regulators of EF plasmablasts is important for understanding both normal (Di Niro et al., 2015) and pathogenic, autoimmune responses. That the EF plasmablast response was greatly enhanced in the absence of cDCs was an important and unexpected finding, as it revealed a regulatory rather than stimulatory role for cDCs in the humoral immune response.

That cDCs were not even *required* for the EF response was also somewhat unexpected based on prior reports, albeit in different contexts. MacLennan and colleagues have concluded that cDCs are a limiting factor in plasmablast generation and survival in response to TI-2 Ags (MacLennan et al., 2003). CD11c<sup>+</sup> cells are also prominently seen in juxtaposition with dividing and differentiating EF AM14 B cells (William et al., 2002), supporting the notion that cDCs are critical in promoting the response. However, an essential role of cDCs in the EF response has been called into question by Jung, *et al.*, who found that cDC depletion does not alter the extent of a TI-2 response elicited by NP-Ficoll (Jung et al., 2002). Of note, the response we tested was not a TI-2 response, but one with qualities of TI-1 (given the TLR requirement), TI-2 (given the polymeric nature of the Ag) and TD (given the ability of T cells, when present, to enhance it). Because EF responses are typical of autoreactive and probably bacterial (Di Niro et al., 2015) and viral pathogen responses, the EF response we studied may be more physiologically relevant than idealized TI or TD protein antigens given in artificial adjuvant.

There have been few reports of negative regulation of B cells by cDCs. Three groups have observed inhibition of B cell responses by cDCs *in vitro* via either an IL-6 and soluble CD40L-dependent mechanism (Gilbert et al., 2007), or a mechanism requiring CD22 expression by the B cells (Santos et al., 2008; Sindhava et al., 2012). None of these systems was translated *in vivo*. Moreover, it is unlikely that they relate directly to our *in vivo* observations. In the former case only anergic B cells are affected, but AM14 B cells are not

anergic (Hannum et al., 1996). In the latter cases CD22 is required, but CD22 is rapidly downregulated early in plasmablast differentiation (William et al., 2005b).

Use of multiple systems for cDC depletion allowed us to rule out some alternate explanations for the enhanced EF response observed in the absence of cDC. Because we saw an elevated AM14 response in both the transient and constitutive cDC depletion systems the acute release of Ag (*ie.* chromatin) from dying CD11c<sup>+</sup> cells that might occur upon DT treatment in the *Itgax*-DTR system was not the primary cause of the enhanced AM14 responses seen in the absence of cDCs. Macrophage hyperplasia, which we observed to some degree in both cDC depletion systems and as noted by others (Birnberg et al., 2008), was also likely not the cause of enhanced EF responses, because this macrophage expansion was also present within cDC-deficient MRL.*Fas*<sup>Jpr</sup> mice, which did not have an altered frequency of AM14 AFCs. Finally, a role for pDCs is unlikely, because pDCs were not depleted in the CD11c-DTR system, in which acute cDC depletion substantially increased the AM14 AFC response.

We found that the mechanism by which cDCs regulate the EF autoimmune response *in vivo* was dependent on Fas: cDCs did not regulate the AM14 response in Fas-deficient animals. In this case, the regulatory pathway was short-circuited because the Fas-signaling was already “off.” Depleting cDCs in addition to Fas, therefore, had no additional effect. Fas has previously been implicated in controlling autoimmunity via a number of mechanisms, but the pathway revealed by our studies appears different. For example, FasL and Fas are required for elimination of anergic B cells by CD4<sup>+</sup> T cells *in vivo* (Rathmell et al., 1995). However, AM14 B cells are not anergic (but remain ignorant), and do not need to escape anergy to break tolerance (Hannum et al., 1996; Wang and Shlomchik, 1999). GC B cells upregulate Fas, which regulates the duration and extent of the GC reaction and the generation of long-lived plasma cells (Butt et al., 2015; Hao et al., 2008; Takahashi et al., 2001). In contrast, the response we are studying is EF and generates short-lived plasmablasts.

cDC regulation did not depend on Fas expression by the B cell itself, even though activated B cells can be sensitive to FasL-mediated killing (Rothstein et al., 1995). Rather, we demonstrated that the FasL targets were Fas-expressing T cells that help amplify the EF B cell response, namely Tfh cells (Lee et al., 2011; Odegard et al., 2008; Sweet et al., 2011). Because Tfh cells express Fas (Rasheed et al., 2006), we would expect that the Tfh cells in our system would also be sensitive to elimination by a FasL-expressing cell: either another T cell, the activation of which is cDC-dependent, or the cDC itself (Lu et al., 1997; Süss and Shortman, 1996). We also showed that the FasL-expressing cells required in this system were indeed T cells. There is precedent for such a mechanism, because regulation of T cells by Fas-FasL interactions is well documented (Van Parijs and Abbas, 1996), and T cell restricted Fas- or FasL-deficiency leads to autoantibody production (Mabrouk et al., 2008; Stranges et al., 2007). Seo *et al* also noted that B cell-specific Fas expression is not required to regulate autoreactive B cell responses to strong T cell help (Seo et al., 2002). Thus, our observations are consistent with prior studies, but extend our knowledge by demonstrating that cDCs can regulate an EF plasmablast response via a mechanism that requires Fas ligation on T cells—most likely Tfh cells—rather than on B cells directly.



We previously showed, using constitutive cDC-depletion, that cDCs can amplify disease in aged MRL.*Fas<sup>lpr</sup>* mice, although they are not required for disease initiation (Teichmann et al., 2010). These studies did not reveal a regulatory role for cDCs, and in fact, certain types of autoantibodies were reduced in aged cDC-deficient MRL.*Fas<sup>lpr</sup>* mice. Similarly, Fas expression on cDCs suppresses autoimmunity in older mice (Stranges et al., 2007). This presents the paradox that, in the short term, cDCs negatively regulate the initiation of the EF response, whereas in the long-term cDCs promote specific, chronic aspects of disease. We suggest two ways to understand this paradox. First, as in many complex cellular networks *in vivo*, cDCs likely have both positive and negative effects. When integrated over the life of an autoimmune-prone animal, these effects are seen as mainly promoting disease, in particular, by aiding the growth of tissue cellular infiltrates (Teichmann et al., 2015; Teichmann et al., 2010). Over time cDC-driven positive feedback cycles of T cell activation could override any short-term negative effects. Second, and most relevant to the present context, our previous study of constitutively cDC-deficient mice was on the Fas-deficient MRL.*Fas<sup>lpr</sup>* background. In that model, the regulatory effects of cDCs would not have been observed, because the Fas-dependent mechanism was already short-circuited.

These insights into the Fas-dependent regulatory role of cDCs in the generation of autoantibodies could apply to other EF plasmablast responses. The EF response can be driven by simultaneous TLR and BCR signals. In the AM14 system, chromatin-containing ICs promote the EF response in a TLR7- and TLR9-dependent manner (Herlands et al., 2008; Leadbetter et al., 2002). A more general effect of combined TLR and BCR stimulation in directing the EF response has been demonstrated in elegant studies in which a BCR signal was engineered to have a TLR9 signal associated with it on the same synthetic bead (Eckl-Dorna and Batista, 2009). Similarly, many bacteria and viruses contain Ags that can directly stimulate B cell-expressed TLR4, 7, and 9 as well as ligate relevant BCRs. This dual recognition promotes vigorous EF plasmablast responses that can undergo isotype switch, and is probably critical for the early control of pathogens like *Salmonella* (Cunningham et al., 2007; Di Niro et al., 2015; Neves et al., 2010). Thus, the cDC-regulated pathway we have observed could also be important during the primary response to pathogens.

Initial EF responses are typically supplanted by the GC response, which provides definitive B cell memory and long-lived AFCs. GC and T-dependent responses engage multiple mechanisms to enforce self-tolerance. In contrast, the primary EF response is less selective and partially T-independent, thus making it more liable to be subverted into autoimmunity. Here we have demonstrated a Fas- and cDC-dependent pathway that reduces the magnitude of the EF response by 3-6 fold over the course of a week. cDCs are, therefore, potent regulators of the autoreactive EF response, and possibly of EF responses in general.

## EXPERIMENTAL PROCEDURES

### Mice

BALB/c, BALB/c.*Rag1<sup>-/-</sup>*, MRL/MpJ-*Fas<sup>lpr</sup>*/J (MRL.*Fas<sup>lpr</sup>*), MRL/MpJ and BALB/c FVB-Tg(*Itgax*-DTR/EGFP)57Lan/J (*Itgax*-DTR.BALB/c) (Jung et al., 2002) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). BALB/c sd-Tg AM14 mice were previously described (Sweet et al., 2011), and were also backcrossed greater than 10 times

onto MRL.*Fas<sup>lpr</sup>*. AM14.BALB/c mice were used at 7-9 weeks of age. AM14.MRL.*Fas<sup>lpr</sup>* mice were used at 5-7 weeks of age. AM14.MRL.*Fas<sup>lpr/+</sup>* mice were used at 5-10 weeks of age. C57BL/6 CD11c-Cre BAC transgenic (Caton et al., 2007) and Rosa26-eGFP-flox-stop-DTA transgenic (Ivanova et al., 2005) mice, gifts from Boris Reizis (Columbia University) and Juan Martinez-Barbera (University College London), respectively, were backcrossed greater than 10 times onto MRL.*Fas<sup>lpr</sup>* (Teichmann et al., 2010). These two strains were then intercrossed to obtain cDC-deficient (DC MRL.*Fas<sup>lpr</sup>*), CD11c-Cre<sup>+</sup> Rosa26-eGFP-DTA<sup>+</sup> mice. Single positive and WT littermates were used as controls. Mice were used at 6-7, 5-9.5 and 7-9 weeks of age in three experiments with equivalent results. To obtain Fas-sufficient cDC-deficient mice (DC.MRL.*Fas<sup>lpr/+</sup>*) mice and controls, double positive,

DC.MRL.*Fas<sup>lpr</sup>* females were outcrossed to MRL/MpJ males for one generation. Mice were used at 6-7 weeks of age in three experiments and 6-10 weeks of age in two experiments, with equivalent results. BALB/c.*Fas<sup>lpr</sup>* mice were obtained from Thomas Ferguson (Washington University) and used at 4-9 weeks of age. BALB/c.*Tcra<sup>-/-</sup>* mice (Sweet et al., 2011) were obtained from Kim Bottomly (Yale University) and used at 5-7 weeks of age. BALB/c.*FasL<sup>mut</sup>* mice, gifted from Ann Marshak-Rothstein (Gregory et al., 2011) were used at 7-9 weeks of age. All animals were housed under SPF conditions and handled according to IACUC approved protocols.

## B cell Purification and Adoptive Cell Transfer

Splenocyte suspensions were made in 2% fetal calf serum (FCS), 1mM EDTA, phosphate buffered saline (PBS). B cells were purified using an EasySep Mouse B cell Enrichment Kit (Stemcell Technologies).  $3-12 \times 10^6$  cells suspended in PBS per mouse (as indicated) were injected intravenously (IV) on day 0.

## Anti-Chromatin Preparation and Immunization

PL2-3, an IgG2a<sup>a</sup> anti-chromatin hybridoma (Losman et al., 1992) was prepared as ascites in *Rag<sup>-/-</sup>* mice as described (Herlands et al., 2008; Sweet et al., 2011). PL2-3 protein concentration was determined by IgG2a ELISA. Mice were immunized intraperitoneally (IP) with 0.5 mg equivalent of PL2-3 on days 0, 2, and 4. AM14 B cell activation was assayed on day 6.

## Bone Marrow Chimeras and Diphtheria Toxin Treatment

BM from CD11c-DTR donors or Tg-negative littermates was processed in 0.5% bovine serum albumin (BSA), 5mM EDTA, penicillin, streptomycin in PBS. After red blood cell lysis with ACK solution, BM cells were washed with BSA, PBS and resuspended in injection buffer (2.5% v/v ACD-A, 10mM HEPES, penicillin, streptomycin, PBS). Eight week old BALB/c recipients received 450 Rad of irradiation twice from a <sup>137</sup>Cs source with a 3-4 h rest period between doses. 1-2 h after the second dose,  $8-10 \times 10^6$  BM cells were injected IV. Animals were allowed to recover for 6 weeks prior to cDC depletion or B cell transfer. Diphtheria toxin (Sigma) was administered IP at 4-8 ng/g weight on days -2 and -1 prior to and on days 1, 3 and 5 after B cell transfer. Effectiveness of chimerism and depletion was assessed by flow cytometry of splenocytes (Figure S1).

## ELISpot Assay

ELISpot assays were performed as described (Hannum et al., 1996). Spots were counted using a dissecting microscope.

## Flow Cytometry

Splenocytes were suspended in staining media (SM: 3% FCS, 5 mM EDTA, 0.05% sodium azide, PBS) with the FcR blocking antibody, 2.4G2 and ethidium monoazide (EMA) for live/dead discrimination. Reagents used for staining were either prepared in house: 4-44-biotin, PDCA1 (927)-Alexa 647, CD62L (MEL-14)-Alexa 647; or purchased from Biolegend: CD44 (1M7)-APC/Cy7, CD11c (N418)-PE/Cy7, CD4 (GK1.5)-PE/Cy7, Gr1 (RB6.8C5)-biotin; from BD: CD22.2 (Cy34.1)-PE, TCR $\beta$  (H57-597)-PE, I-A/I-E (M5/114.15.2)-PE; from eBioscience: CD11b (MAC-1)-PE, streptavidin-PE/Cy7; or from Invitrogen: streptavidin-PacBlue. Surface stained cells were fixed with 1% PFA, washed and resuspended in SM. For intracellular staining, cells were fixed in 4% PFA in BD Perm/Wash Buffer, washed, blocked (with 5% rat serum), and stained with 4-44-Alexa 647 or GFP-FITC (Rockland) in the BD Perm/Wash Buffer, with a final wash in SM. Cytometry was performed on a LSRII (BD) and data analyzed with FlowJo software.

## Immunofluorescence Histology

CD11c-DTR and control spleens were fixed in paraformaldehyde-lysine-periodate (PLP) solution (1% paraformaldehyde, 95 mM L-lysine, 10 mM sodium M-periodate, 0.1 M phosphate buffer (PB), pH 7.2) for 1-7 days at 4°C. They were then washed three times with PB, dehydrated stepwise in 10%, 20%, and 30% sucrose/PB and frozen in OCT (TissueTek). Alternatively, for Bcl-6 and TUNEL staining, spleens were fixed with 4% paraformaldehyde for 2-4h at 4°C and washed for 2-4h in cold PBS and frozen in OCT medium. Spleens from CD11c-cre  $\times$  Rosa26-eGFP-flox-stop-DTA mice were first frozen in OCT and fixed after cryostat sectioning with 4°C acetone for 10 min. 7-10  $\mu$ m cryostat sections were cut, rehydrated with PBS, blocked for 20 min with 10% rat serum, 1% BSA, 0.1% Tween-20 in PBS, stained for 1 h with respective antibodies in BSA, Tween-20, PBS, washed three times with BSA, Tween-20, PBS and once with PBS. Staining reagents were either prepared in house: 4-44-biotin, B220 (RA3-6B2)-488, B220 (RA3-6B2)-Dylight 405, B220 (RA3-6B2)-PE, CD4 (GK1.5)-Alexa 647, F4/80 (BM8)-Alexa 647, PNA-Alexa 647, CD19 (1D3.2)-Alexa 647; or purchased from Invitrogen: streptavidin-Alexa 555; from Vector Laboratories: PNA-FITC; from BD: anti-Bcl-6 (K112-91)-Alexa 647; from BioLegend: anti-CD4 (GK1.5)-PE. For the visualization of apoptotic cells by the TUNEL method, ApoAlert DNA Fragmentation Assay Kit (Clontech) was used as per manufacturer's instructions. After air-drying, slides were mounted with Prolong Antifade (Molecular Probes). Images were captured either with a 10  $\times$  lens on a Nikon Exlipse Ti automated wide field microscope with a QImaging Retiga 200R CCD camera (Figures 3, S2, & S5) using NIS Elements software or with a 20  $\times$  lens on an Olympus IX83 microscope (Figures 7 & 6S) with an ORCA-flash 4.0 camera (Hamamatsu) using Olympus CellSens Dimension 1.15 software. Images were further processed with Adobe Photoshop software, and CellSens software assisted in TUNEL<sup>+</sup> cell quantitation.

## Statistical Analysis

P-values were calculated by one-way ANOVA with Tukey's multiple comparisons test, two-tailed Mann-Whitney U-test, or unpaired T-test, as indicated, using Prism software (Graphpad).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### HIGHLIGHTS

- cDCs are not required for extrafollicular (EF) autoreactive B cell activation
- Loss of cDCs markedly enhances the EF B cell response
- cDC regulation of the EF response requires B cell extrinsic Fas
- Loss of Fas or FasL on T cells enhances autoantibody production



**IN BRIEF**

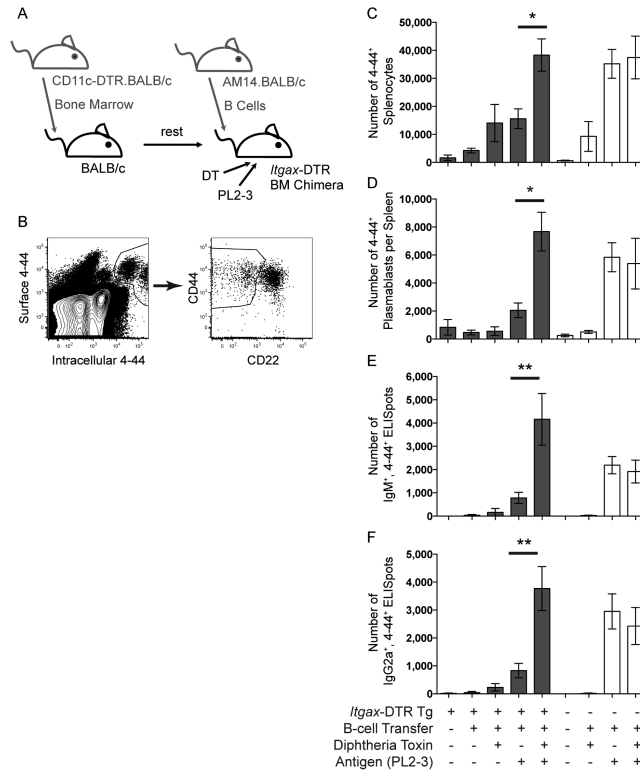
cDCs negatively regulate extrafollicular B activation and autoantibody production. Using two cDC ablation systems, Ols and colleagues observe elevated autoantibody production in the absence of cDCs. They further show that the regulatory effect of cDCs on B cells requires Fas and FasL expression by T cells.

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**Figure 1. The EF AM14 Rheumatoid Factor Response does not Acutely Require, but is Regulated by cDCs**

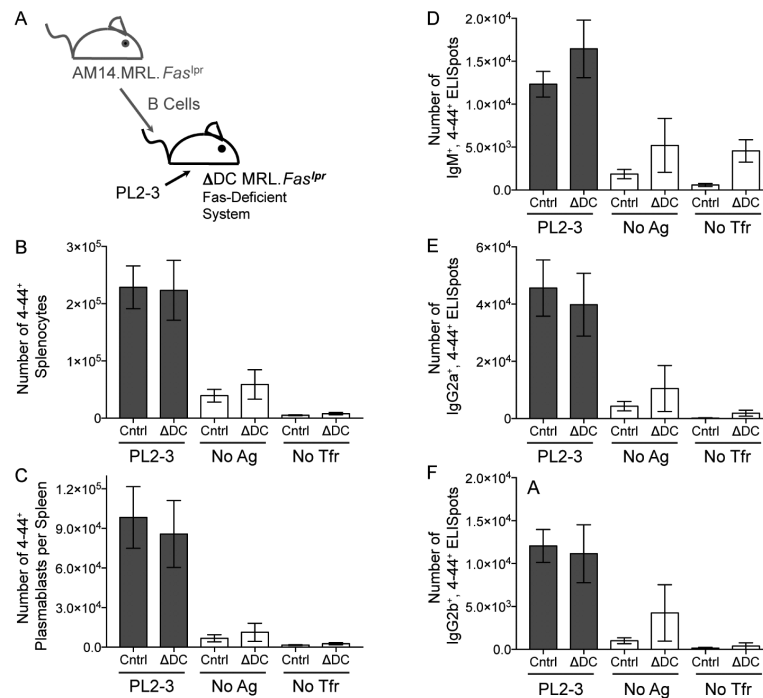
(A) Experiment design. *Itgax-DTR.BALB/c* or Tg-negative littermates were used to make BM chimeras. cDCs were depleted with DT on days -1, -2, 1, 3 and 5.  $9-10 \times 10^6$  AM14.BALB/c B cells were transferred into the mice on day 0, and activated with PL2-3 on days 0, 2 and 4. Spleens were harvested on day 6.

(B) Representative flow cytometry gating of live AM14 B cells by surface and intracellular 4-44 (anti-idiotype) staining, and further subgating for CD22<sup>lo</sup>, CD44<sup>hi</sup> plasmablasts.

(C) Number of AM14 B cells (surface and intracellular 4-44+) per spleen.

(D) Number of AM14 plasmablasts (4-44<sup>+</sup>, CD22<sup>lo</sup>, CD44<sup>hi</sup>) per spleen.

(E & F) ELISpot assays for the number of 4-44<sup>+</sup>, IgM<sup>+</sup> or IgG2a<sup>+</sup> AFCs per spleen. Data were combined from 2 experiments to obtain 4-8 mice per group. Bars represent mean and SEM. \*p < 0.05; \*\*p < 0.01 by ANOVA. See also Figures S1 and S2.



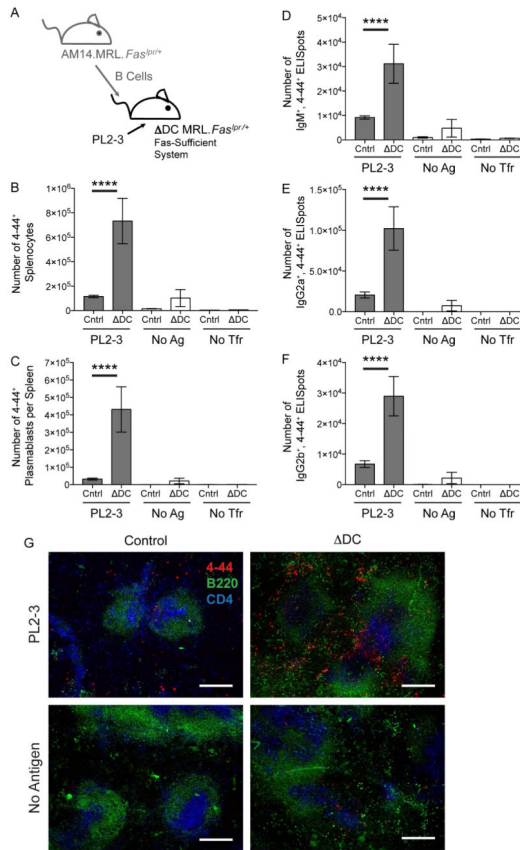
### Figure 2. cDCs are not Required Constitutively for the AM14 Response

(A) Experiment design.  $4 \times 10^6$  AM14.MRL.Fas<sup>lpr</sup> B cells were transferred into MRL.Fas<sup>lpr</sup> recipients that were either double positive for both the CD11c-cre and Rosa26-flox-stop-DTA Tgs, and thus lacked cDCs (ΔDC mice), or into single Tg positive littermates (Cntrl) on day 0. The AM14 B cell response was activated with PL2-3 on days 0, 2 and 4, or left untreated (no Ag). As additional controls, some mice did not receive cell transfer or Ag (no Tfr). Spleens were harvested on day 6.

(B) Number of AM14 B cells (4-44<sup>+</sup>) per spleen.

(C) Number of AM14 plasmablasts (4-44<sup>+</sup>, CD22<sup>lo</sup>, CD44<sup>hi</sup>) per spleen.

(D, E, & F) ELISpot assays for the number of 4-44<sup>+</sup>, IgM<sup>+</sup>, IgG2a<sup>+</sup>, or IgG2b<sup>+</sup> AFCs per spleen. Data were combined from 3 experiments to obtain 12-24 mice per group receiving PL2-3 and 2-12 mice in the untreated groups. Bars represent mean and SEM. See also Figure S3.



### Figure 3. Regulation of the AM14 Response by cDCs Requires Fas

(A) Experiment design.  $8-9 \times 10^6$  Fas-sufficient AM14.MRL.Fas<sup>lpr/+</sup> B cells were transferred into Fas-sufficient MRL.Fas<sup>lpr/+</sup> recipients that either lacked cDCs (ΔDC) or into littermate controls (Cntrl) on day 0. The AM14 B cell response was activated with PL2-3 on days 0, 2 and 4, or left untreated (no Ag). Some mice did not receive cell transfer or Ag (no Tfr). Spleens were harvested on day 6.

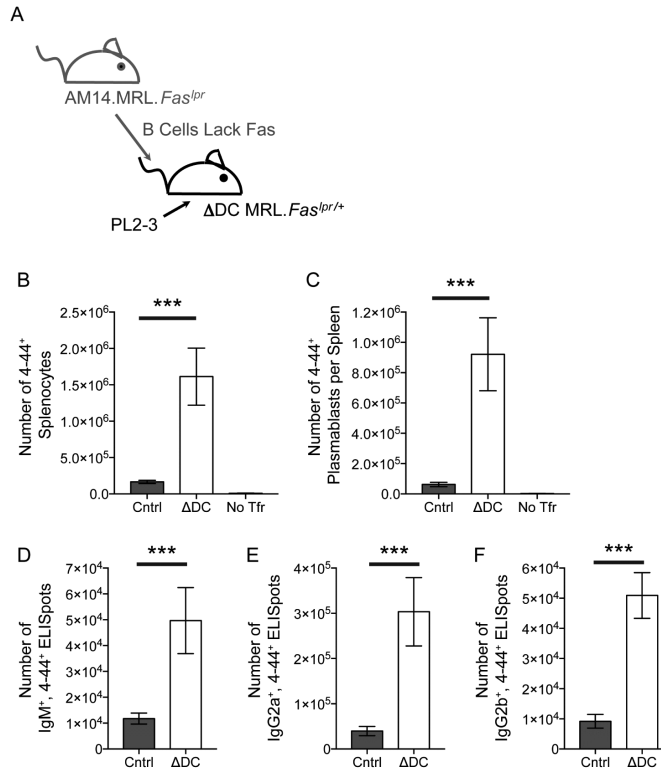
(B) Number of AM14 B cells (4-44<sup>+</sup>) per spleen.

(C) Number of AM14 plasmablasts (4-44<sup>+</sup>, CD22<sup>lo</sup>, CD44<sup>hi</sup>) per spleen.

(D, E, & F) ELISpot assays for the number of 4-44<sup>+</sup>, IgM<sup>+</sup>, IgG2a<sup>+</sup>, or IgG2b<sup>+</sup> AFCs per spleen. Data were combined from 5 experiments to obtain 15-29 mice per group receiving PL2-3 and 2-7 mice in the other groups. Bars represent mean and SEM. \*\*\*\*p < 0.0001 by Mann-Whitney U test.

(G) Immunofluorescence histology was performed on spleens from experimental mice.

Images were taken at a magnification of 100x. Red, AM14 B cells (4-44<sup>+</sup>); green, B cell follicles (B220<sup>+</sup>); blue, T cell zones (CD4<sup>+</sup>). White scale bars are 200 μm. See also Figures S4 and S5.



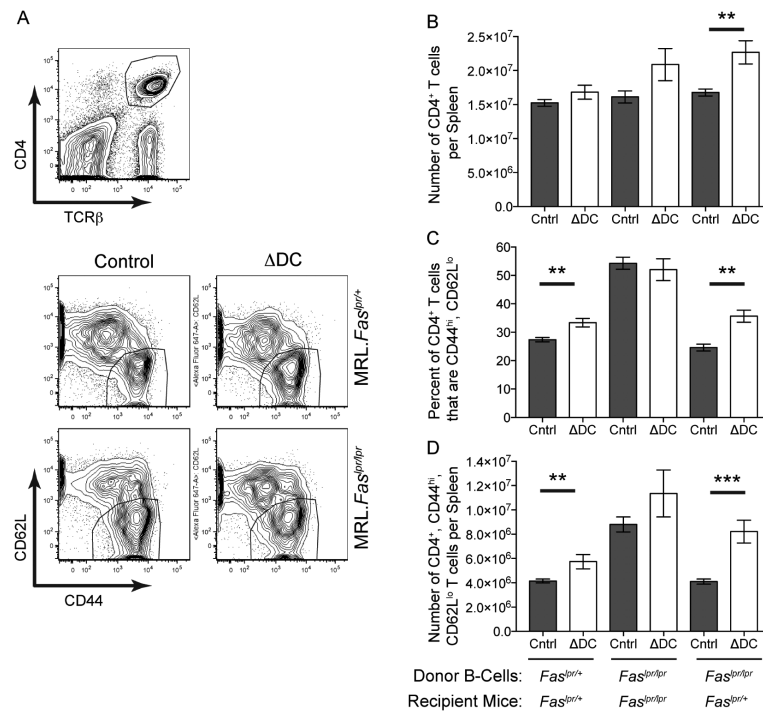
**Figure 4. Fas-Dependent cDC Regulation of the AM14 Response is AM14 B Cell Extrinsic**  
 (A) Experiment design.  $8 \times 10^6$  Fas-deficient AM14.MRL.Fas<sup>Ipr</sup> B cells were transferred into either Fas-sufficient, cDC-deficient (ΔDC) MRL.Fas<sup>Ipr/+</sup> recipients or into littermate controls (Cntrl) on day 0. The AM14 B cell response was activated with PL2-3 on days 0, 2 and 4. Some mice did not receive cell transfer or Ag (no Tfr). Spleens were harvested on day 6.

(B) Number of AM14 B cells (surface and intracellular 4-44<sup>+</sup>) per spleen.

(C) Number of AM14 plasmablasts (4-44<sup>+</sup>, CD22<sup>lo</sup>, CD44<sup>hi</sup>) per spleen.

(D, E, & F) ELISpot assays for the number of 4-44<sup>+</sup>, IgM<sup>+</sup>, IgG2a<sup>+</sup>, or IgG2b<sup>+</sup> AFCs per spleen. Data were combined from 2 experiments to obtain 8-15 mice per group receiving PL2-3 and 2 no Tfr controls. Bars represent mean and SEM. \*\*\*p < 0.001 by Mann-Whitney U test.

See also Figure S7.



**Figure 5. Recipient Mice Lacking Fas and/or Dendritic Cells Have Higher Numbers of Activated CD4<sup>+</sup> T Cells than Control Mice**

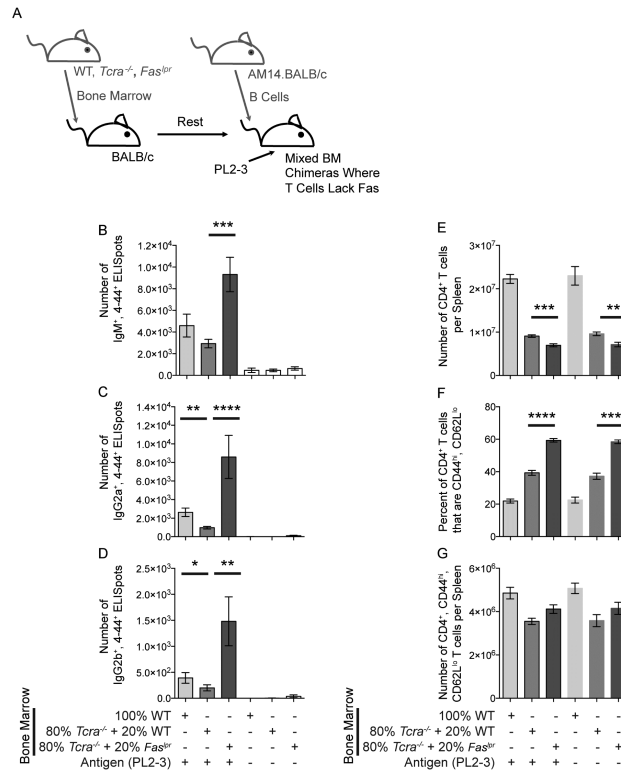
Splenic T cells from experimental mice shown in Figures 2-4 were analyzed by flow cytometry.

(A) Representative sample showing the gating of live splenocytes for CD4<sup>+</sup>, TCRβ<sup>+</sup> cells (top) that were further sub-gated (bottom) by their activation markers (CD44<sup>hi</sup>, CD62L<sup>lo</sup>).

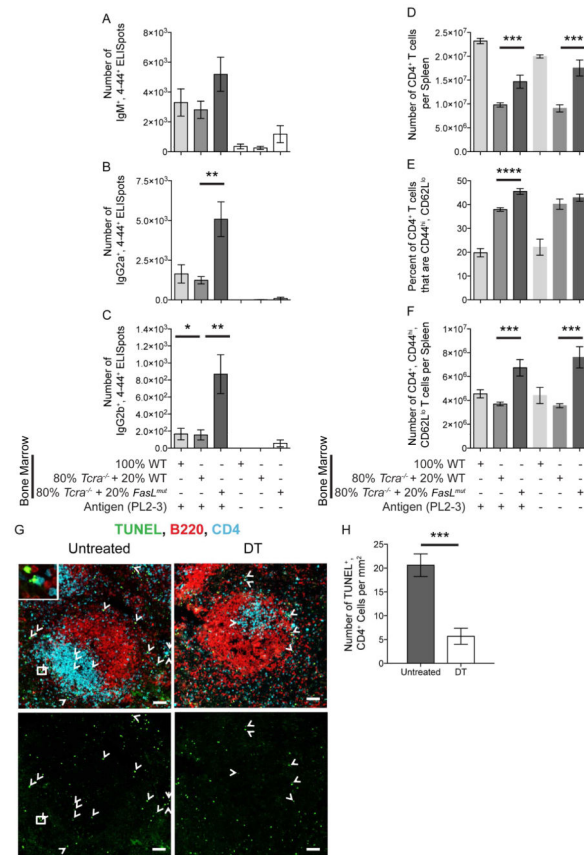
(B) Number of CD4<sup>+</sup>, TCRβ<sup>+</sup> cells per spleen.

(C) Percent of CD4<sup>+</sup>, TCRβ<sup>+</sup> cells that are activated (CD44<sup>hi</sup>, CD62L<sup>lo</sup>).

(D) Number of CD4<sup>+</sup>, TCRβ<sup>+</sup>, CD44<sup>hi</sup>, CD62L<sup>lo</sup> cells per spleen. DC, mice that lack cDCs; Cntrl, littermate controls. Bars represent mean and SEM. \*\*p < 0.01; \*\*\*p < 0.001 by Mann-Whitney U test. See also Figure S6.



**Figure 6. Regulation of AM14 Plasmablasts by cDCs Requires T Cells Expressing Fas**  
 (A) Experiment design. BALB/c mice were used to make chimeras with BM mixed from WT, *Tcrα*<sup>-/-</sup> or *Fas*<sup>Lpr</sup> mice.  $3 \times 10^6$  AM14.BALB/c B cells were transferred into the mice on day 0, and activated with PL2-3 on days 0, 2 and 4. Spleens were harvested on day 6.  
 (B, C, & D) ELISpot assays for the number of 4-44<sup>+</sup>, IgM<sup>+</sup>, IgG2a<sup>+</sup>, or IgG2b<sup>+</sup> AFCs per spleen.  
 (E) Number of CD4<sup>+</sup>, TCRβ<sup>+</sup> cells per spleen.  
 (F) Percent of CD4<sup>+</sup>, TCRβ<sup>+</sup> cells that are activated (CD44<sup>hi</sup>, CD62L<sup>lo</sup>).  
 (G) Number of CD4<sup>+</sup>, TCRβ<sup>+</sup>, CD44<sup>hi</sup>, CD62L<sup>lo</sup> cells per spleen.  
 Data were combined from 2 experiments to obtain 6-12 mice per group receiving PL2-3 and 4-8 controls. Bars represent mean and SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001 by Mann-Whitney U test.  
 See also Figure S7.



### Figure 7. Regulation of AM14 Plasmablasts by cDCs Requires T Cells Expressing FasL and Increases the Death of CD4 T Cells

(A-F) Experiments performed as described and arranged in same order as in Figure 6, except BM was mixed from WT, *Tcra*<sup>-/-</sup> or *FasL*<sup>mut</sup> mice. Data were combined from 2 experiments to obtain 6-12 mice per group receiving PL2-3 and 3-8 controls.

(G & H) As described in Figure 1, CD11c-DTR.BALB/c BM chimeras were generated and depleted of cDCs using 16 ng per g weight doses of DT.  $8 \times 10^6$  AM14.BALB/c B cells were transferred and activated with PL2-3. Spleens were harvested on day 7. Data for untreated versus DT-treated mice are shown. (G) Representative histology of the spleen, taken at a magnification of 200x. Arrowheads indicate TUNEL<sup>+</sup>, CD4<sup>+</sup> cells. Inset upper left shows a digital magnification of a TUNEL<sup>+</sup> CD4<sup>+</sup> cell in the white box of the larger image. Scale bars are 50  $\mu$ m. (H) Quantification of TUNEL<sup>+</sup>, CD4<sup>+</sup> cells from a total of 8 fields combined from 2 representative mice per group. Bars represent mean and SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  by Mann-Whitney U test.

See also Figures S6 and S7.