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# A T Cell-Dependent Mechanism for the Induction of Human Mucosal Homing Immunoglobulin A-Secreting Plasmablasts

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## SUMMARY

**Mucosal immunoglobulin A (IgA) secreted by local plasma cells (PCs) is a critical component of mucosal immunity. Although IgA class switching can occur at mucosal sites, high-affinity PCs are optimally generated in germinal centers (GCs) in a T cell-dependent fashion. However, how $CD4^+$ helper T cells induce mucosal-homing IgA-PCs remains unclear. Here, we show that transforming growth factor β1 (TGFβ1) and interleukin 21 (IL-21), produced by follicular helper T cells (Tfh), synergized to generate abundant IgA-plasmablasts (PBs). In the presence of IL-21, TGFβ1 promoted naive B cell proliferation and differentiation and overrode IL-21-induced IgG class switching in favor of IgA. Furthermore, TGFβ1 and IL-21 downregulated CXCR5 while upregulating CCR10 on plasmablasts, enabling their exit from GCs and migration toward local mucosa. This was supported by the presence of $CCR10^+IgA^+$PBs in tonsil GCs. These findings show that Tfh contribute to mucosal IgA. Thus, mucosal vaccines should aim to induce robust Tfh responses.**

## INTRODUCTION

Mucosal surfaces are the most frequent port of entry for microorganisms (Brandtzaeg and Johansen, 2005; Fagarasan and Honjo, 2003). Immunoglobulin A (IgA), the predominant antibody isotype in mucosal secretions, is of paramount importance in the immune defense of these surfaces. The main function of IgA is the neutralization of pathogens and toxins without causing inflammation because it does not activate complement (Cerutti, 2008; Fagarasan and Honjo, 2003; Macpherson and Slack, 2007). Unlike IgA1, IgA2 is resistant to bacterial proteases. This makes it of particular importance on mucosal surfaces that are highly colonized by bacteria, such as the lower gastrointestinal tract (He et al., 2007; Kett et al., 1986).

Mucosal IgA-mediated immunity is dependent on the induction of mucosal homing $IgA^+$ plasma cells (IgA-PCs) that secrete antibodies locally. It is not yet completely understood how the abundant IgA-PCs found in the subepithelial regions, especially in gut mucosa, are generated. Recent reports indicate a central role for microbial signals at the epithelial barrier in T cell-independent (TI) induction of IgA-PCs (Fagarasan et al., 2001; He et al., 2007; Macpherson et al., 2000; Uematsu et al., 2008). These innate TI pathways provide an important first line of protection in the time it takes T-dependent (TD) adaptive responses to develop high-affinity antibodies and long-term humoral immunity. TD responses to mucosal antigens take place in germinal centers (GCs) of the mucosa-associated lymphoid tissues (Hornquist et al., 1995; Lycke et al., 1987), which promote clonal expansion and affinity maturation (Liu and Arpin, 1997; Manser, 2004). The GC microenvironment allows intimate interactions between B cells, $CD4^+$ helper T cells, and antigen-presenting cells. $CD4^+$ T cells, in particular follicular helper T cells (Tfh), are central for GC formation, providing CD40 Ligand (CD40L) and multiple cytokines, such as IL-2, IL-4, IL-10, and IL-21 (Breitfeld et al., 2000; King et al., 2008; Moser et al., 2002; Vogelzang et al., 2008). These signals promote B cell proliferation, class switch recombination (CSR), and somatic hypermutation, resulting in highly specific, class-switched plasma cells and long-lived memory B cells (Liu and Arpin, 1997; MacLennan, 1994). IL-21 particularly induces terminal differentiation of naive B cells and also mediates class switching to IgG1 and IgG3 (Kuchen et al., 2007; Ozaki et al., 2002; Pene et al., 2004). $CD4^+$ T cells are also a source of transforming growth factor β1 (TGFβ1) (Li et al., 2006), a known IgA class-switching factor (Cazac and Roes, 2000; Coffman et al., 1989; Islam et al., 1991). IL-10 and other cytokines augment TGFβ1-mediated IgA class switching (Defrance et al., 1992; Fayette et al., 1997; Islam et al., 1991). However, because TGFβ1, as an immunoregulatory cytokine, does not support B cell expansion (Kehrl et al., 1991), this does not explain how GCs can generate abundant IgA-PCs.

For migration into local mucosal areas, PCs need to express mucosal homing receptors. Locally produced vitamin derivatives play a role in the induction of mucosal homing receptors on B cells (Mora et al., 2006; Shirakawa et al., 2008). Mora et al. (2006) demonstrated that vitamin A can induce CCR9 and integrin α4β7 on B cells, which allows their migration toward the small intestine. A recent study (Shirakawa et al., 2008) showed that CCR10, a common mucosal homing marker, can be imprinted on B cells by vitamin D3. These studies indicate that the microenvironment where B cells undergo differentiation can determine their expression of mucosal homing receptors. It is still unclear, however, how $CD4^+$ T cells contribute to the induction of homing receptors on IgA-PCs.

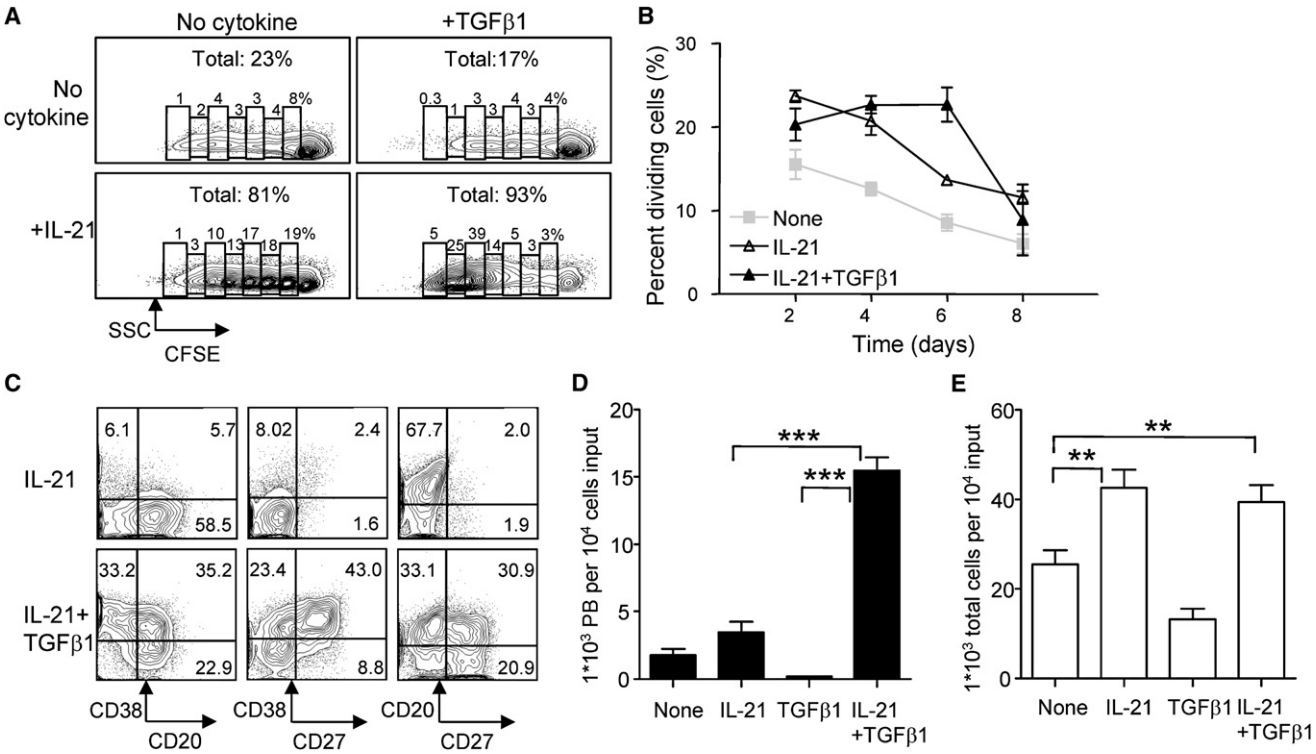


**Figure 1. TGFβ1 Synergizes with IL-21 to Promote Naive B Cell Proliferation and Differentiation**

CFSE-labeled naive B cells were activated with anti-IgM and then cultured with the indicated cytokines in the presence of anti-CD40, CpG, and IL-2.

(A) Proliferation was measured by CFSE dilution on day 6. Data presented are one out of six independent experiments showing similar results.

(B) Cell-cycle analysis was performed with a DNA-binding dye on different time points of culture. The proportion of cells in G2+S phase, representing dividing cells, is shown for B cells cultured in the indicated conditions.

(C) Expression of differentiation markers on day 6 as determined by flow cytometry.

(D) The number of $CD38^+CD20^-$ cells (PB) per 1 × $10^4$ naive B cells input as calculated by (number of cells/1 × $10^4$ input) × (%$CD38^+CD20^-$) from eight independent experiments.

(E) Total number of cells recovered per 1 × $10^4$ cells input on day 6.

Error bars in (B), (D), and (E) represent mean ± SEM.

With the aim of establishing human vaccines that induce potent mucosal immunity, we studied the role of Tfh in the generation of mucosal homing IgA-PCs. We identified IL-21 and TGFβ1 as important Tfh-derived cytokines that promoted the differentiation of naive B cells into IgA-secreting plasmablasts (PBs). When combined with IL-21, TGFβ1 furthermore upregulated CCR10 while downregulating CXCR5, which would enable migration of the PBs from the GC toward the mucosal surfaces.

## RESULTS

### TGFβ1 Synergizes Uniquely with IL-21 to Promote Naive B Cell Proliferation and Differentiation

In order to identify T cell-dependent factors involved in IgA-PC differentiation, we mimicked the different steps of a TD B cell response in an in vitro culture system. Naive $IgD^+CD27^-$ B cells were activated with a combination of anti-IgM (to mimic BCR engagement by antigens), CpG (TLR9 engagement by microbial DNA), anti-CD40, and IL-2 (cognate interactions with $CD4^+$ T cells). These activated B cells underwent significant though limited proliferation, as measured by dilution of CFSE (Figure 1A). As expected, IL-21, a known B cell proliferation and differentiation factor (Bryant et al., 2007; King et al., 2008; Kuchen et al., 2007), induced enhanced proliferation of CD40+BCR+TLR9-activated naive B cells. On the other hand, TGFβ1, an IgA-switching factor (Cazac and Roes, 2000; Coffman et al., 1989), slightly inhibited B cell proliferation (Figure 1A), which is in line with previous reports (Li et al., 2006; Kehrl et al., 1991). Strikingly, when combined with IL-21, TGFβ1 resulted in considerably enhanced B cell proliferation. The majority (≈70%) of cells proliferating with IL-21 and TGFβ1 underwent more than five cycles of cell division, whereas less than 20% of the cells did in response to IL-21 alone (Figure 1A). Both IL-21R and TGFβRII were upregulated by anti-CD40, anti-BCR, and CpG stimulation (Figure S1 available online).

Cell-cycle analysis with a DNA-binding dye revealed that naive B cells cultured with IL-21 and TGFβ1 maintained a higher ratio of division over time (Figure 1B). This prolonged division lasted until day 6, whereas IL-21-induced B cell division peaked on day 2 and then decreased steadily. This shows that TGFβ1 prolongs IL-21-induced proliferation of activated naive B cells, allowing the cells to undergo more divisions, as observed in Figure 1A.

Phenotypic analysis showed that a large proportion (30%–40%) of the cells cultured with IL-21 and TGFβ1 were $CD20^-CD38^+$,

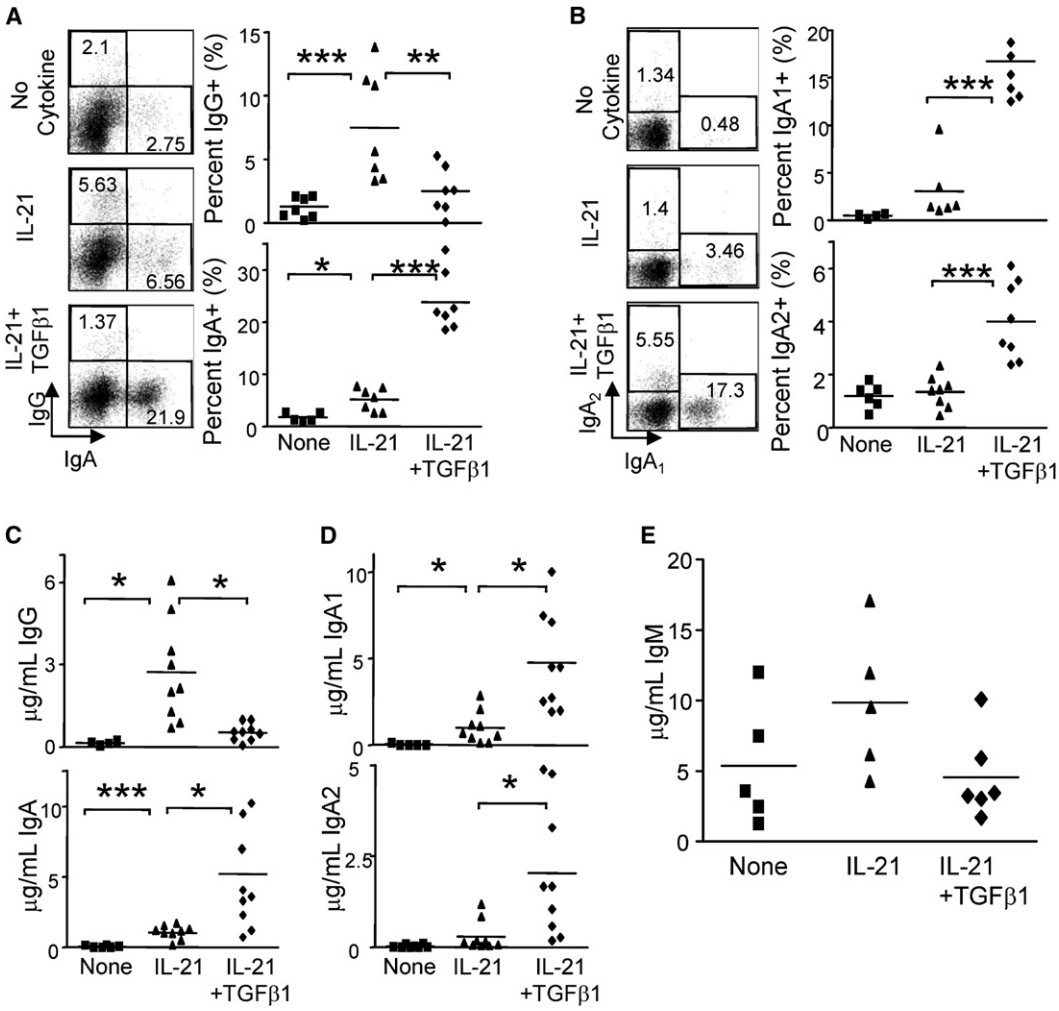


**Figure 2. TGFβ1 Skews IL-21-Mediated Class Switching toward IgA1 and IgA2**

Naive B cells activated with anti-IgM and anti-CD40 were cultured in the presence of IL-2 and CpG with either IL-21 alone or a combination of IL-21 and TGFβ1.

(A and B) On day 6, surface immunoglobulin expression was determined by staining the cells with anti-IgG, IgA, IgA1, and IgA2. The left panels show the dot plots. The right panels show the results of multiple experiments with mean.

(C and D) On day 12, IgG, IgA, IgA1, and IgA2 secreted in the culture supernatants were measured by ELISA.

(E) IgM secreted in the culture supernatants on day 12 as measured by ELISA.

a phenotype of PBs, whereas only about 10% of the cells cultured with IL-21 alone were $CD20^{-}CD38^{+}$ (Figure 1C). The absolute number of PBs recovered on day 6 was significantly higher after culture with IL-21 and TGFβ1 when compared to IL-21 or TGFβ1 alone (both $p < 0.0001$) (Figure 1D). The total number of cells recovered on day 6 was comparable between IL-21 alone or IL-21 with TGFβ1 (Figure 1E), suggesting that the combination of IL-21 and TGFβ1 induces enhanced differentiation of naive B cells into PBs. Approximately 25%–30% of the cells cultured with IL-21 and TGFβ1 expressed CD27 and CD20 (Figure 1C), reminiscent of a memory B cell phenotype. Of note, we did not observe a similar synergy between TGFβ1 and IL-10 (Defrance et al., 1992; Fayette et al., 1997). When CD40+BCR+ TLR9-activated naive B cells were cultured with a combination of TGFβ1 and IL-10, the proliferation was similar to that induced by IL-10 alone (30%–40%) (Figure S2A) but lower than that induced by IL-21 or a combination of IL-21 and TGFβ1. The number of PBs induced by IL-10 alone or IL-10 plus TGFβ1 was also not significantly different ($p = 0.86$) (Figure S2B). Thus, TGFβ1 synergizes uniquely with IL-21 to promote naive B cell proliferation and PB differentiation.

## TGFβ1 Skews IL-21-Mediated Class Switching toward IgA1 and IgA2

Naive B cells stimulated through CD40, BCR, and TLR9 expressed low amounts of surface IgG (sIgG) and sIgA (Figure 2A). Addition of IL-21 resulted in increased amounts of sIgG and sIgA (Figure 2A), as previously described (Kuchen et al., 2007; Ozaki et al., 2002; Pene et al., 2004; Avery et al., 2008). The combination of IL-21 and TGFβ1 considerably increased the frequency of $sIgA^{+}$ B cells (mean 22%), and it decreased $sIgG^{+}$ B cells (mean 2.5%). Surface IgA induced by IL-21 and TGFβ1 consisted of both IgA1 (mean 17%) and IgA2 (mean 4%), whereas IL-21 alone induced only $sIgA1^{+}$ B cells (Figure 2B).

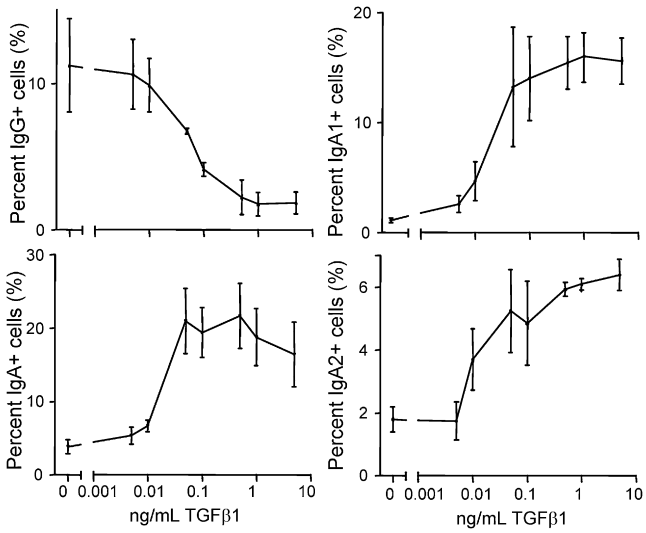


**Figure 3. Surface IgA Expression Is Directly Related to TGFβ1 Concentration**

Naive B cells were activated with anti-IgM, anti-CD40, and CpG and cultured with 20 ng/ml IL-21 and increasing concentrations of TGFβ1. Surface immunoglobulin expression was determined on day 6. Mean ± SEM of three independent experiments is shown.

Activated naive B cells secreted large amounts of IgG (mean 3 μg/ml) and some IgA (mean 1.2 μg/ml) in response to IL-21 alone (Figure 2C). The combination of IL-21 and TGFβ1, however, induced activated naive B cells to secrete high amounts of IgA (mean 6 μg/ml), consisting of both IgA1 (mean 4.5 μg/ml) and IgA2 (mean 2.0 μg/ml) but low amounts of IgG (mean 0.5 μg/ml) (Figures 2C and 2D). The amounts of IgM secreted were not different between the different conditions (Figure 2E).

A dose titration of TGFβ1 in naive B cell cultures containing IL-21 showed that the frequency of $sIgA1^+$ and $sIgA2^+$ B cells correlated with TGFβ1 concentration and that the frequency of $sIgG^+$ cells was inversely related to the TGFβ1 dose (Figure 3). Increasing the dose of IL-21 did not affect the proportion of $IgA^+$ cells (data not shown).

The combination of IL-10 with TGFβ1, as previously described (Defrance et al., 1992; Fayette et al., 1997), could also induce $sIgA^+$ B cells (Figure S2C), including IgA1 and IgA2, but to a lower extent compared to IL-21 with TGFβ1. In line with the lower frequency of PBs induced by IL-10 plus TGFβ1 (Figure S2B), the amount of secreted IgA was relatively low in those cultures (Figure S2D).

To confirm the induction of isotype switching, we measured expression of activation-induced cytidine deaminase (*AICDA*, the gene encoding AID), circle transcripts, germline transcripts, and mature transcripts, as illustrated in Figure S3. Quantitative real-time PCR data show that activated naive B cells cultured with IL-21 alone or a combination of IL-21 and TGFβ1 upregulated *AICDA* (Figure 4A), a hallmark of cells undergoing active class switching (Muramatsu et al., 2000). Figure 4B shows that IL-21 upregulated $I\gamma_3$-$C\gamma_3$ germline transcription, confirming its role as an IgG3 switch factor (Ozaki et al., 2002; Pene et al., 2004). In addition, IL-21 also moderately increased Iα1-Cα1 and Iα2-Cα2 germline transcription. However, the combination

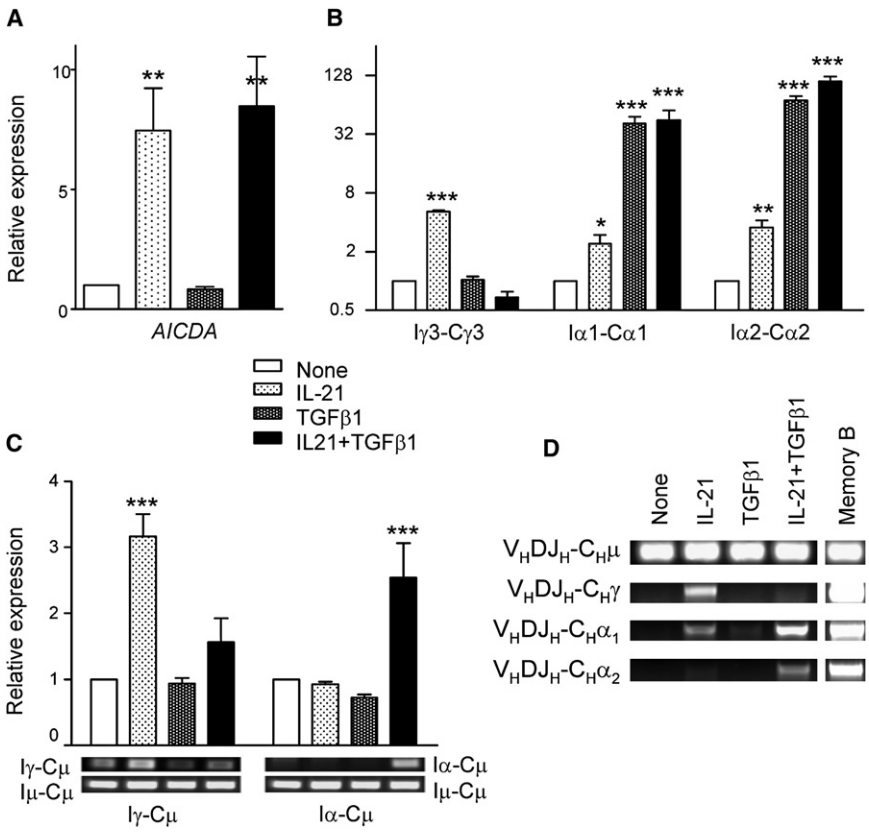


**Figure 4. Molecular Events in IL-21+TGFβ1-Induced IgA Class Switching**

(A–C) CD40+BCR+TLR9-activated naive B cells cultured with the indicated cytokines were harvested on day 4. mRNA was isolated, and cDNA was synthesized. Real-time PCR was performed for activation-induced cytidine deaminase (*AICDA*) (A); germline transcripts $I\gamma_3$-$C\gamma_3$, $I\alpha_1$-$C\alpha_1$, and $I\alpha_2$-$C\alpha_2$ (B); and switch circle transcripts Iγ-Cμ and Iα-Cμ (C). The expression of *AICDA*, $I\gamma_3$-$C\gamma_3$, $I\alpha_1$-$C\alpha_1$, and $I\alpha_2$-$C\alpha_2$ was normalized to *ACTB* expression. Iγ-Cμ and Iα-μ expression was normalized to Iμ-Cμ expression (He et al., 2007). Below (C), agarose gel electrophoresis of RT-PCR amplified Iγ-Cμ and Iα-μ transcripts compared to Iμ-Cμ (loading control). Data shown are mean ± SEM of three independent experiments with cells from three different healthy donors.

(D) Conventional RT-PCR was used to detect mature Ig transcripts. One out of three similar results is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

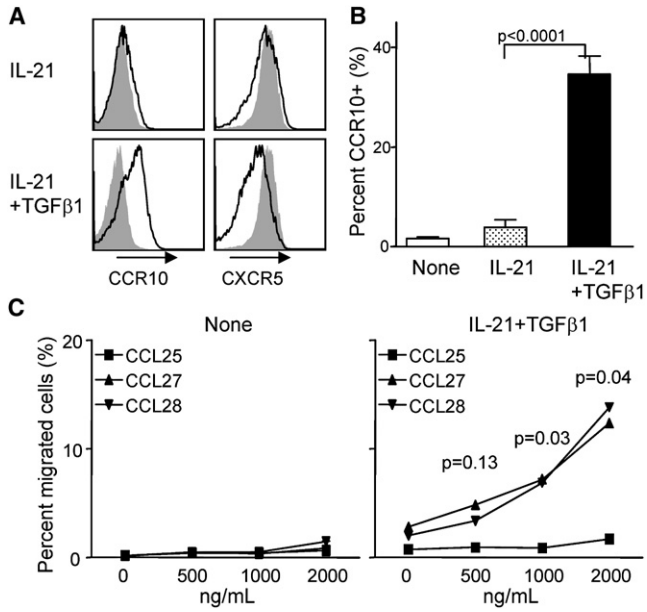


**Figure 5. In Combination with IL-21, TGFβ1 Upregulates CCR10 but Downregulates CXCR5**

CD40+BCR+TLR9-activated naive B cells were cultured for 6 days with the indicated cytokines.

(A) B cells were stained for CCR10 and CXCR5. Histogram overlays are shown with staining of activated B cells cultured without additional cytokines. Data from one out of eight independent experiments are shown.

(B) The average percentage of CCR10$^+$ ± SEM of eight independent experiments is shown.

(C) Migration assay. On day 6 of culture, B cells were added to the upper wells of transwells containing the indicated concentrations of CCL25, CCL27, and CCL28 in the lower wells. After 2 hr, the number of cells that migrated into the lower wells was determined with flow cytometry with counting beads. Results shown are the mean of three independent experiments.

of IL-21 and TGFβ1 more strongly upregulated the transcription of both Iα1-Cα1 and Iα2-Cα2 germlines. On the other hand, B cells cultured with IL-21 alone expressed increased Iγ-Cμ, but not Iα-Cμ switch circle transcripts, whereas B cells cultured with the combination of IL-21 and TGFβ1 expressed more Iα-Cμ circle transcripts (Figure 4C). Consistently, IL-21 upregulated $V_HDJ_H$-$C_H\gamma$ and $V_HDJ_H$-$C_H\alpha1$ mature transcripts, whereas IL-21 and TGFβ1 together induced increased expression of both $V_HDJ_H$-$C_H\alpha1$ and $V_HDJ_H$-$C_H\alpha2$ transcripts (Figure 4D). B cells cultured with TGFβ1 alone showed upregulation of both α germlines, but not of *AICDA*, which explains the absence of Iα-Cμ circle transcripts and mature $V_HDJ_H$-$C_H\alpha$ transcripts (Figures 4A–4C).

Taken together, our data indicate that IL-21 alone induces mainly IgG class switching, whereas the combination of IL-21 and TGFβ1 strongly skews switching toward IgA1 and IgA2.

## In Combination with IL-21, TGFβ1 Upregulates CCR10 but Downregulates CXCR5

For migration into local mucosal areas, PBs need to express appropriate mucosal homing receptors. Remarkably, the combination of IL-21 and TGFβ1 could induce 30%–50% of activated naive B cells to express CCR10 (Figures 5A and 5B). This is a common mucosal homing receptor (Brandtzaeg and Johansen, 2005; Fagarasan and Honjo, 2003; Kunkel et al., 2003; Salmi and Jalkanen, 2005) that allows migration toward most mucosal areas, including the intestinal tract and extraintestinal mucosal sites (Hieshima et al., 2004; Kunkel et al., 2003). The induced CCR10 was functional as the B cells migrated, in a chemotaxis assay, toward the two CCR10 ligands: CCL28 and CCL27 (Figure 5C). There was no upregulation of CCR9 or integrin α4β7, homing receptors for the small intestine (data not shown). Consequently, B cells cultured with IL-21 and TGFβ1 did not migrate in response to CCL25, a ligand for CCR9. The combination of IL-21 and TGFβ1, but neither IL-21 nor TGFβ1 alone, downregulated CXCR5 (Figure 5A), which mediates migration to and retention in the GCs. Thus, upregulation of CCR10 along with downregulation of CXCR5 by IL-21 and TGFβ1 could allow PBs to migrate out of the GCs en route toward most mucosal sites.

## Tonsil Tfh Induce IgA$^+$CCR10$^+$ Plasmablasts in an IL-21-Dependent Manner

To determine whether the above findings made with recombinant cytokines reflected TD responses in GCs, BCR+TLR9-activated naive B cells were cocultured with autologous peripheral blood CXCR5$^+$CD4$^+$ Tfh cells (Breitfeld et al., 2000). Tfh cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin secreted high amounts of IL-21 (mean 42 ng/ml) compared to CXCR5$^-$ T cells (3 ng/ml). They also produced more TGFβ1 upon activation (mean 230 pg/ml) compared to CXCR5$^-$ T cells (150 pg/ml) (Figure 6A). Intracellular staining showed that all T cells producing IL-21 also made TGFβ1 (Figure 6B). Activated naive B cells proliferated considerably when cocultured with Tfh cells (Figure 6C, upper panels). Blocking IL-21 by a recombinant fusion protein IL-21R-Fc limited the proliferation beyond five cycles of cell division (Figure 6C, lower panels). Only B cells that underwent more than five cycles expressed CD38 and CCR10. Accordingly, blocking IL-21 decreased the frequency of CD38$^+$ and CCR10$^+$ cells. Adding IL-21R-Fc also decreased expression of γ (Iγ-Cμ) as well as α (Iα-Cμ) circle transcripts (Figures 6D and 6E) and IgA secretion (Figure 6F). IgG secretion could not be determined due to the IgG-Fc domain in the blocking agent. Because TGFβ1 also affects CD4$^+$ T cell development, we could not address the specific effects of TGFβ1 on B cell differentiation by blocking studies. Altogether, CXCR5$^+$CD4$^+$ T cells can stimulate activated naive B cells to become CCR10-expressing IgA-PBs in an IL-21-dependent manner.

## IgA$^+$CCR10$^+$ Cells Are Present in Tonsil GCs

To investigate the presence of CCR10-expressing IgA-PBs in GCs, we stained tonsil sections with anti-IgA and anti-CCR10 antibodies. Figure 7A shows that the majority of IgA$^+$ cells in a GC express CCR10. Figure 7B shows the presence of IgA$^+$ cells in the subepithelial area of the tonsil, which are brighter for CCR10 than the cells found in the GCs. This indicates that, after induction in the GCs, these IgA$^+$CCR10$^+$ could migrate out of the GCs to populate mucosal surfaces.

## DISCUSSION

This study demonstrates that T cell-dependent B cell responses can generate abundant IgA-PBs with mucosal homing properties.

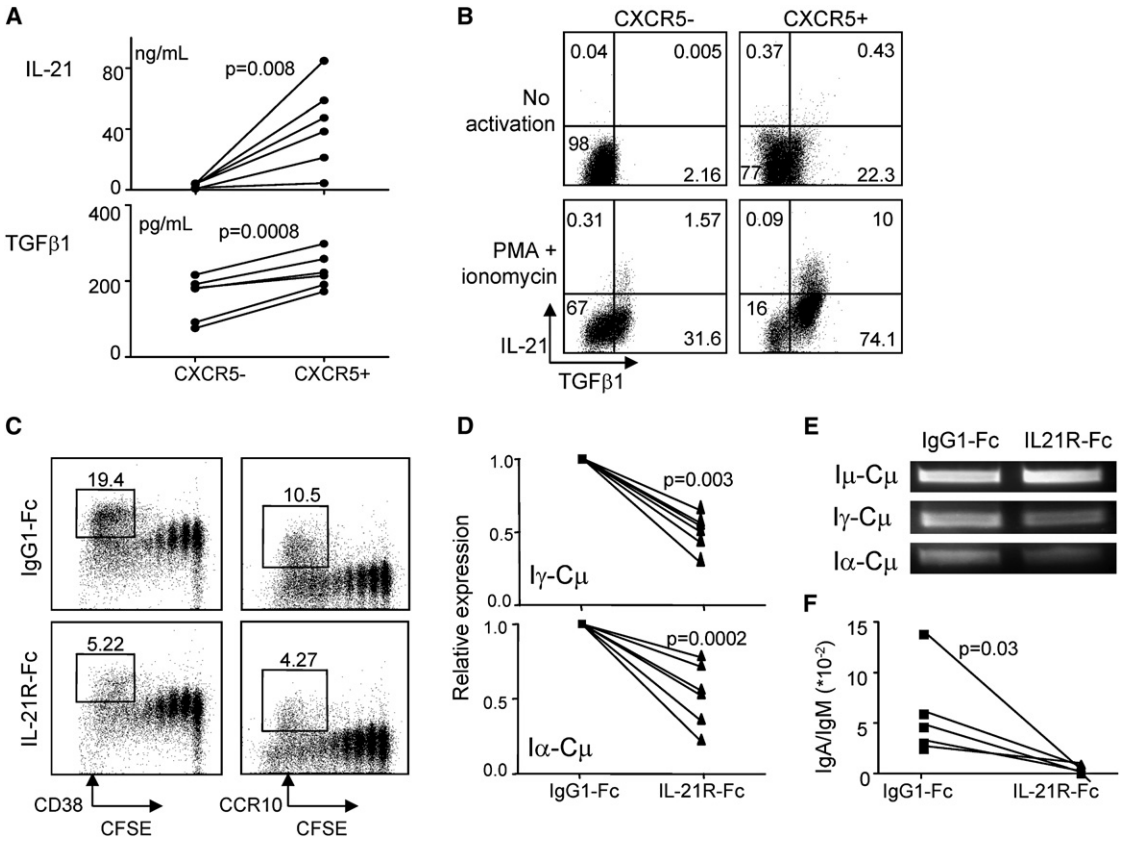


**Figure 6. Tonsil Tfh Induce IgA$^+$CCR10$^+$ Plasmablasts in an IL-21-Dependent Manner**

(A) Sorted peripheral blood CXCR5$^+$ and CXCR5$^-$ CD4$^+$ T cells were cultured with PMA + ionomycin ($10^6$/ml). After 48 hr, concentrations of IL-21 and TGFβ1 in the supernatant were measured by Luminex.

(B) Total peripheral blood CD4$^+$ T cells were cultured with or without PMA and ionomycin for 20 hr, the last 4 hr in the presence of brefeldin A. Cells were stained for CXCR5, intracellular IL-21, and TGFβ1. Plots shown are gated on CXCR5$^+$ and CXCR5$^-$ populations. One out of two similar experiments is shown.

(C–F) Naive B cells ($5 \times 10^4$) were cocultured with autologous CXCR5$^+$CD4$^+$ T cells ($2 \times 10^4$) in the presence of IL-21R-Fc or control IgG1-Fc.

(C) B cell proliferation, CD38 expression, and CCR10 expression were measured on day 8.

(D) On day 4, cells were harvested, RNA was isolated, and cDNA was synthesized. Real-time PCR was performed for switch circle transcripts Iγ-Cμ and Iα-Cμ. Expression was normalized to Iμ-Cμ expression.

(E) Agarose gel electrophoresis showing absolute expression of Iμ-Cμ, Iγ-Cμ, and Iα-Cμ.

(F) On day 8, the supernatants from the cocultures were harvested, and IgM and IgA were measured by ELISA. IgA is normalized for IgM secretion with IgA/IgM ratio.

We identified TGFβ1 and IL-21 as critical Tfh-derived cytokines that synergize at three different points in B cell development: (1) proliferation and differentiation, (2) IgA class switching, and (3) imprinting of homing receptors.

This study shows a positive effect of TGFβ1 on B cell proliferation and PB differentiation. We found that it prolonged IL-21-induced proliferation and enhanced PB differentiation. The molecular basis of this phenomenon remains to be elucidated. This effect is strictly dependent on the TGFβ1 dose, with an optimal effect between 0.01 and 1 ng/ml. Lower doses of TGFβ1 do not support proliferation and PB development, whereas higher doses induce cell death. Our data support the model by Tangye and Hodgkin (2004), proposing that the likelihood of a B cell to switch to a certain isotype is related to the number of divisions it has undergone. We observed that B cells became IgA$^+$ only after they had undergone five or more divisions. Thus, the capacity of TGFβ1 to induce IgA class switching could be partly dependent upon its ability to drive B cell proliferation in the presence of IL-21.

An extensive body of literature from the 1990s exists on TD IgA class switching. CD40 ligation is a first essential step for TD IgA switching. This is highlighted by IgA deficiency in mice and patients with defects in CD40 or CD40L (Allen et al., 1993; Kawabe et al., 1994). TGFβ1 cooperates with CD40L to induce IgA switching in vitro (Cerutti et al., 1998; Defrance et al., 1992; Nakamura et al., 1996). In line with that, TGFβ1-responsive elements were identified both in the mouse (Zhang and Derynck, 2000) and human Cα promoters (Pardali et al., 2000). The combination of TGFβ1 with IL-10 in human (Fayette et al., 1997) or LPS in mouse B cells (Coffman et al., 1989) enhances the generation of IgA-PBs. IL-21 was more recently identified as an important Tfh-derived cytokine that promotes B cell proliferation, differentiation (Bryant et al., 2007; Kuchen et al., 2007; Ozaki et al., 2002), and class switching toward IgG1 and IgG3 (Pene et al.,

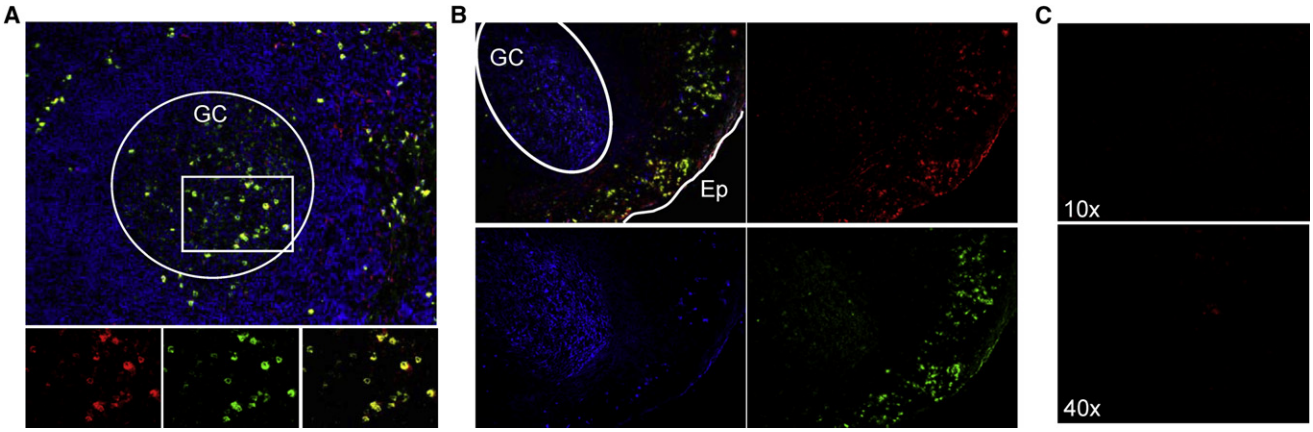


**Figure 7. IgA$^+$CCR10$^+$ Cells Are Present in Tonsil Germinal Centers**

(A) Fixed frozen tonsil sections were stained with DAPI (blue), anti-IgA (green), and anti-CCR10 (red). The three-color overview picture (10×) shows the presence of IgA$^+$CCR10$^+$ cells in the germinal center (GC). The rectangle indicates the magnified area (40×).

(B) Fixed frozen tonsil sections were stained with anti-IgM (blue), anti-IgA (green), and anti-CCR10 (red), showing the presence of IgA$^+$CCR10$^+$ cells in the subepithelial area (Ep) as well as in the GC (10×).

(C) Adjacent tonsil sections stained with control antibodies.

2004). Compared to IL-10 plus TGFβ1, the combination of IL-21 with TGFβ1 showed a more pronounced synergy for generation of IgA PBs. This might be explained by the superior capacity of IL-21 compared to IL-10 to induce *AICDA* (M.D., unpublished data), which is needed for class switching, and Blimp-1 (Bryant et al., 2007), which is essential for PC development. Our observations indicate a dominant role for TGFβ1 in IL-21+TGFβ1-mediated generation of IgA$^+$CCR10$^+$ PBs. Adding TGFβ1 to B cells cultured with IL-21 as many as 4 days later still resulted in predominant IgA switching and CCR10 upregulation (data not shown). Furthermore, IgA expression was directly related to TGFβ1, but not to IL-21 concentration. However, increased expression of AID and α germline transcripts by IL-21 alone indicate that IL-21 contributes to IgA class switching. In mice, the importance of IL-21 for IgA CSR is less clear. Ozaki et al. found that specific serum IgA titers after immunization were not much different in IL-21R-deficient mice compared to wild-type mice, unless they were also deficient for IL-4 (Ozaki et al., 2002). This may reflect an interspecies difference and/or the difference between mucosal and systemic sites of IgA expression. A number of recent studies have provided evidence for the importance of intestinal flora-driven TI IgA class switching in the lower intestinal tract (Mora et al., 2006; He et al., 2007; Uematsu et al., 2008). The capacity for TI IgA switching may represent a functional specialization in B cells (Cerutti, 2008). Through TI pathways, these B cells can rapidly yield low-affinity polyreactive antibodies (IgM, IgG, and IgA) (Bendelac et al., 2001; Wardemann et al., 2003) that form an important first line of protection against infections. However, these innate antibodies can not completely substitute for antigen-specific high-affinity IgA produced by TD responses in GCs, which also generate memory B cells (McHeyzer-Williams and Ahmed, 1999).

Because mucosal IgA is secreted locally in subepithelial areas, IgA-PCs need to acquire appropriate homing receptors and migrate toward the effector site. CCL28, a ligand for CCR10, is a common mucosal chemoattractant produced by epithelial cells in the intestinal tract, salivary glands, airways, mammary glands, uterine tract, and palatine tonsils (Brandtzaeg and Johansen, 2005; Fagarasan and Honjo, 2003; Kunkel et al., 2003). CCL27, another CCR10 ligand, is produced by epidermal keratinocytes (Reiss et al., 2001). The CCR9 ligand, CCL25, is produced largely in the small intestine (Kunkel et al., 2000). It has been shown that vitamin D (produced locally in the skin) can induce B cells to express CCR10 (Shirakawa et al., 2008), whereas vitamin A (absorbed from food in the small intestine) imprints CCR9 and integrin α4β7 (Mora et al., 2006). Here, we provide evidence that B cell homing capacity can be imprinted by follicular T helper cells. The Tfh-derived cytokines IL-21 and TGFβ1 induced the concomitant upregulation of CCR10 and downregulation of CXCR5. Together, this allows B cells to leave the GCs and migrate toward CCL27-expressing and CCL28-expressing mucosal tissues. The fact that we could find CCR10$^+$IgA$^+$ cells in tonsil GCs supports the imprinting of mucosal homing in a TD fashion.

TGFβ1 is produced in varying amounts by Tfh and other cells in lymphoid organs, such as stromal cells, DCs, and B cells themselves (Li et al., 2006). Thus, IL-21 is likely the critical factor for the generation of large numbers of CCR10-expressing IgA-PBs. Because Tfh are the main source of IL-21, we conclude that the induction of potent Tfh responses is crucial for a solid IgA-mediated mucosal immunity. Therefore, future vaccines, especially those against mucosal infections, like influenza or HIV, should aim to induce strong antigen-specific Tfh responses.

## EXPERIMENTAL PROCEDURES

### Cells and Cell Cultures

Study subjects were recruited at the Baylor University Medical Center of Dallas. The study was approved by the Institutional Review Board of Baylor Research Institute, and informed consent was obtained from all subjects. Tonsil tissue samples, obtained from patients under 12 years old with tonsillitis, were frozen in OCT for tissue sections.

Peripheral blood mononuclear cells (PBMC) of healthy volunteers were fractionated by elutriation. B cells were isolated from the lymphocyte-rich fractions with Human B cell Enrichment Set (BD Biosciences, CA). B cells were stained

for IgD and CD27. $CD27^{-}IgD^{+}$ (naive) and $CD27^{+}IgD^{-}$ (memory) cells were sorted on a FACSAria (BD). The purity of sorted B cells was generally > 98%.

Naive B cells were cultured with anti-IgM-coated beads for 2 hr at 4°C (500 ng/ml, Immunobead Rabbit anti-Human IgM; IrvineScientific, CA). Then, cells were cultured in complemented RPMI containing 10% FCS at $2 \times 10^4$/200 μl/well in the presence of IL-2 (20 units/ml; R&D Systems, CA), anti-CD40 (500 ng/ml; in-house preparation, clone 12E12), and CpG (50 nM; ODN2006, InvivoGen, CA).

Peripheral blood $CD4^+$ T cells were isolated from lymphocyte-rich PBMC fractions with EasySep Human $CD4^+$ T cell enrichment kit (StemCell Technologies, BC, Canada). For cytokine production, we cultured sorted peripheral blood $CXCR5^+$ and $CXCR5^-$ $CD4^+$ T cells at $10^6$/ml in 200 μl in 96-well U bottom plates with or without phorbol 12-myristate13-acetate (PMA) (50 ng/ml) and ionomycin (1 μM). Naive B cells ($5 \times 10^4$) were cocultured with autologous $CXCR5^+$ $CD4^+$ T cells ($2 \times 10^4$) in the presence of 20 units/ml IL-2, 50 nM CpG, and 1 μg/ml Staphylococcus enterotoxin B (SEB, Sigma-Aldrich, MO).

### Reagents and Antibodies

Human recombinant IL-2 (R&D Systems), IL-10 (Peprotech, NJ), and IL-21 (R&D Systems) were used at 20 units/ml, 20 ng/ml, and 20 ng/ml, respectively. Human recombinant TGFβ1 (R&D Systems) was used at 0.5 ng/ml or as indicated.

The following fluorochrome-labeled antibodies were used for flow cytometry and FACS sorting: anti-CD19, anti-CD27, anti-CD38, and anti-CD20 (BD Biosciences) and anti-CXCR5 (R&D Systems); anti-human IgD, IgM, IgG, IgA, IgA1, and IgA2 (Southern Biotech, AL); and anti-human CCR4, CCR6, CCR7, CCR9, CCR10, CXCR4, CXCR5, anti-TGFβRecII, and anti-IL-21Rec (R&D Systems). Intracellular staining of $CD4^+$ T cells was performed with PermFix and PermWash (BD Biosciences), anti-IL-21-AlexaFluor647 (eBioscience, CA), and anti-TGFβ1-PE (R&D Systems).

IL-21R-Fc and IgG1-Fc control (both R&D Systems) were used to block IL-21 in naive B and CD4 cocultures.

### ELISAs

Sandwich ELISAs were performed to measure total IgM, IgG, IgA, IgA1, and IgA2 in the culture supernatant. Capturing and detection antibodies were purchased from Southern Biotech (AL). Human reference serum (Bethyl, TX) containing known amounts of the different immunoglobulin isotypes was used as a standard.

### Proliferation and Cell-Cycle Analysis

Cells were labeled with CFSE (Molecular Probes, CA), and proliferation was monitored by measuring CFSE dilution on a FACSCalibur. Cell-cycle analysis was performed with the DNA-binding dye Vybrant DyeCycle Orange according to the manufacturer's instructions (Molecular Probes). The resulting histogram allows for the identification of the different phases of the cell cycle: G0/G1 (one set of paired chromosomes per cell), S (DNA synthesis with variable amount of DNA), G2/M (two sets of paired chromosomes, prior to cell division) and sub-G1 (DNA in digestion, apoptotic cells).

### Migration Assay

Migration assays were performed with B cells on day 6 after stimulation. Transwell inserts (5 μM pores, Corning Costar, MA) containing $2–5 \times 10^5$ B cells in 100 μl were placed in the wells of 24-well plates in contact with 600 μl medium (complemented RPMI + 0.5% BSA) containing the indicated concentration of chemokines. CCL25 (CCR9 ligand) and CCL27 and CCL28 (both CCR10 ligands) were purchased from Peprotech. The cells were left to migrate at 37°C for 2 hr and were, subsequently, enumerated with Countbright Beads (Invitrogen, CA) on a FacsCalibur.

### Conventional and Quantitative Real-Time PCRs

RNA was isolated from stimulated B cells on day 4 with Trizol (Invitrogen), and cDNA was synthesized with Reverse Transcription System (Promega, CA). Conventional RT-PCR was performed for mature immunoglobulin transcripts $V_HDJ_H$-$C_H$ μ $V_HDJ_H$-$C_H\gamma$ $V_HDJ_H$-$C_H\alpha_1$ and $V_HDJ_H$-$C_H\alpha_2$ with the following primers: framework 3 (FR3) forward, 5′-GACACGGCTGTGTATTACTGTGC G-3′; $C_H\mu$ reverse, 5′-CCGAATTCAGACGAGGGGGAAAAGGGTT; $C_H\gamma$ reverse, 5′-TTGTGTCACCAAGTGGGGTTTTGAGC-3′; $C_H\alpha_1$ reverse, 5′-GGGTGGCG GTTAGCGGGGTCTTGG-3′; and $C_H\alpha_2$ reverse, 5′-TGTTGGCGGTTAGTGGGG TCTTGCA-3′.

Germline transcripts $I\gamma_3$-$C\gamma_3$, $I\alpha_1$-$C\alpha_1$, and $I\alpha_2$-$C\alpha_2$ and switch circle transcripts Iμ-Cμ, Iγ-Cμ, Iα-Cμ, and AICDA (AID gene) were quantified through quantitative real-time PCR with the following primers: $I\gamma_3$ forward, 5′-G CCATGGGGTGATGCCAGGATGGGCAT-3′; $C\gamma_3$ reverse, 5′-GAAGACCGAT GGGCCCTTGGTGGA-3′; $I\alpha_2$ forward, 5′-CTCAGCACTGCGGGCCCTCCA-3′; $C\alpha_2$ reverse, 5′-GTTCCCATCTTGGGGGGTGCTGTC-3′; $I\alpha_1$ forward, 5′-C TCAGCACTGCGGGCCCTCCA-3′; $C\alpha_1$ reverse, 5′-GTTCCCATCTGGCTGG GTGCTGCA-3′; Iμ forward, 5′-GTGATTAAGGAGAAACACTTTGAT-3′; Cμ reverse, 5′-AGACGAGGGGGAAAAGGGTT-3′; Iγ forward, 5′-GGGCTTCCAA GCCAACAGGGCAGGACA-3′; Iα forward, 5′-CAGCAGCCCTCTTGGCAGGC AGCCAG-3′; AICDA forward, 5′-AGAGGCGTGACAGTGCTACA-3′; AICDA reverse, 5′-TGTAGCGGAGGAAGAGCAAT-3′; ACTB forward, 5′-GGATGCAG AAGGAGATCACT-3′; and ACTB reverse, 5′-CGATCCACACGGAGTACTTG-3′.

Real-time PCR was performed on a Lightcycler 480 machine (Roche Applied Bioscience, IN) with SYBR Green master mix (Roche). $I\gamma_3$-$C\gamma_3$, $I\alpha_1$-$C\alpha_1$, $I\alpha_2$-$C\alpha_2$, and *AICDA* expression were normalized to *ACTB* (β-actin) mRNA, and Iγ-Cμ and Iα-Cμ were normalized to Iμ-Cμ expression. The relative expression (RE) of a target gene was calculated with the following formula: RE*n* = $2^{-(\Delta Ctn - \Delta Ct1)}$, in which ΔC*tn* (change in cycle threshold) is the cycle threshold of the test gene minus the cycle threshold of the reference gene (*ACTB* or Iμ-Cμ); *n* is a specific sample; and *1* is the nontreatment sample. Switch circle transcript Iμ-Cμ, Iγ-Cμ, and Iα-Cμ PCR products were run on an agarose gel to confirm up and downregulation.

### Cytokine Multiplex Analysis

Cell-culture supernatants were analyzed for TGF-β1 with the BeadLyte cytokine assay kit (Upstate) as per manufacturer's protocol. Anti-IL-21 capture and detector antibodies were generated by the Hybridoma core facility at BIIR. Amounts of IL-21 were measured with an IL-21 capture antibody conjugated to seroMAP beads (Luminex Corp., Austin, TX) and detected with a second biotinylated IL-21 antibody according to the manufacturer's protocol. Fluorescence was analyzed with a Bio-Plex Luminex 100 XYP instrument (Bio-Rad, CA), and cytokine concentrations were calculated with Bio-Plex Manager 4.1 software with a 5 parameter curve fitting algorithm applied for standard curve calculations.

### Tissue Sections and Immunofluorescence

Frozen tissue was cut into 6 μm thick sections that were fixed with paraformaldehyde before staining. The following antibodies were used for staining: rabbit-anti-human CCR10 (Imgenex, CA), goat-anti-rabbit IgG-AF568 (Molecular Probes), goat-anti-human IgM-FITC, and goat-anti-human IgA-AF647 (in-house conjugation with Molecular Probes Alexa Fluor kit) both from Southern Biotech. DAPI (Molecular Probes) was used to counterstain the nuclei. Slides were analyzed with an Olympus BX51 microscope with Metamorph 6 software (Universal Imaging Corporation, UK).

### Statistical Analysis

All bar graphs represent mean ± SEM. Data were analyzed with GraphPad Prism 4 software. The significance of difference between experimental variables was determined with the Student's t test, and significance was set at $p < 0.05$.

## SUPPLEMENTAL DATA

Supplemental Data include three figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00550-5.

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