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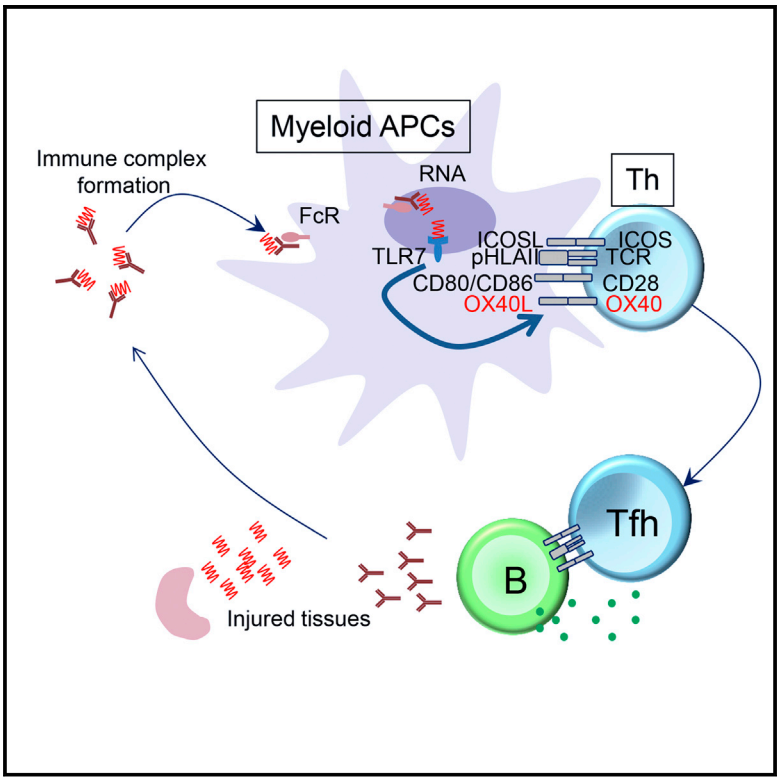
Immunity

OX40 Ligand Contributes to Human Lupus Pathogenesis by Promoting T Follicular Helper Response

Graphical Abstract



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In Brief

Although increased activity of T follicular helper (Tfh) cells plays a pathogenic role in systemic lupus erythematosus (SLE), the mechanism has been unclear. Ueno and colleagues show that exaggerated OX40 signals promote the generation of Tfh cells in SLE.

Highlights

- OX40L is expressed by myeloid antigen-presenting cells in patients with active SLE
- OX40 signals promote the differentiation of human Th cells toward the Tfh lineage
- Strong TCR signals promote the expression of Tfh molecules by human Th cells
- RNP-Anti-RNP immune complexes induce monocytes to express OX40L via TLR7

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OX40 Ligand Contributes to Human Lupus Pathogenesis by Promoting T Follicular Helper Response

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SUMMARY

Increased activity of T follicular helper (Tfh) cells plays a major pathogenic role in systemic lupus erythematosus (SLE). However, the mechanisms that cause aberrant Tfh cell responses in SLE remain elusive. Here we showed the OX40 ligand (OX40L)-OX40 axis contributes to the aberrant Tfh response in SLE. OX40L was expressed by myeloid antigen-presenting cells (APCs), but not B cells, in blood and in inflamed tissues in adult and pediatric SLE patients. The frequency of circulating OX40L-expressing myeloid APCs positively correlated with disease activity and the frequency of ICOS$^+$ blood Tfh cells in SLE. OX40 signals promoted naive and memory CD4$^+$ T cells to express multiple Tfh cell molecules and were sufficient to induce them to become functional B cell helpers. Immune complexes containing RNA induced OX40L expression on myeloid APCs via TLR7 activation. Our study provides a rationale to target the OX40L-OX40 axis as a therapeutic modality for SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic systemic inflammatory autoimmune disease characterized by a breakdown of tolerance to nuclear antigens (Tsokos, 2011). A more comprehensive understanding of SLE pathogenesis is long overdue; in the past 50 years, only one new drug has been approved for SLE treatment (Murphy et al., 2013; Stohl et al., 2012). Genome-wide association studies (GWAS) have identified many susceptibility loci for SLE, confirming that SLE patients display predisposing genetic factors (Cunninghame Graham et al., 2008; Delgado-Vega et al., 2009). Such predisposing genetic factors affect the immune system in particular when challenged with environmental factors and alter the functions of antigen-presenting cells (APCs) and lymphocytes in SLE patients. APCs including dendritic cells (DCs) are aberrantly activated in SLE patients and promote the activation of autoreactive T and B cells (Blanco et al., 2001; Blanco et al., 2008). The developed autoreactive plasma cells produce pathogenic autoantibodies directed against nuclear components and cause tissue injury.

Studies with murine models have demonstrated that T follicular helper cells (Tfh), a CD4$^+$ helper T (Th) cell subset specialized for provision of help to B cells, play a major pathogenic role in lupus (Crotty, 2014; Ueno et al., 2015). Tfh cells are essential for the formation of germinal centers (GCs), the site for the selection of high-affinity B cells, and for the development of B cell memory (Vinuesa and Cyster, 2011). Tfh cells are equipped with multiple features required for B cell help. IL-21 secreted by Tfh cells and their precursors (Bentebibel et al., 2011; Bryant et al., 2007) potently promotes the growth, differentiation, and class-switching of B cells (Tangye et al., 2013). Inducible costimulator (ICOS) is highly expressed by GC Tfh cells and mediates the interaction with B cells (Crotty, 2014). CD40 ligand (CD40L) expressed by Tfh cells provides signals to B cells through CD40 for their differentiation and class-switching (Ueno et al., 2015). The importance of these Tfh molecules in lupus pathogenesis is underscored by the observations in lupus mouse models where inhibition of the function of CD40L (Boumpas et al., 2003; Kalled et al., 1998), ICOS (Odegard et al., 2008), IL-21, and/or IL-21 receptor (Bubier et al., 2009; Herber et al., 2007) delays the disease course and/or improves the clinical symptoms. Furthermore, inhibition of the generation of Tfh cells in lupus prone *sanroque* mice by deleting SAP molecule abrogates the development of renal pathology (Linterman et al.,

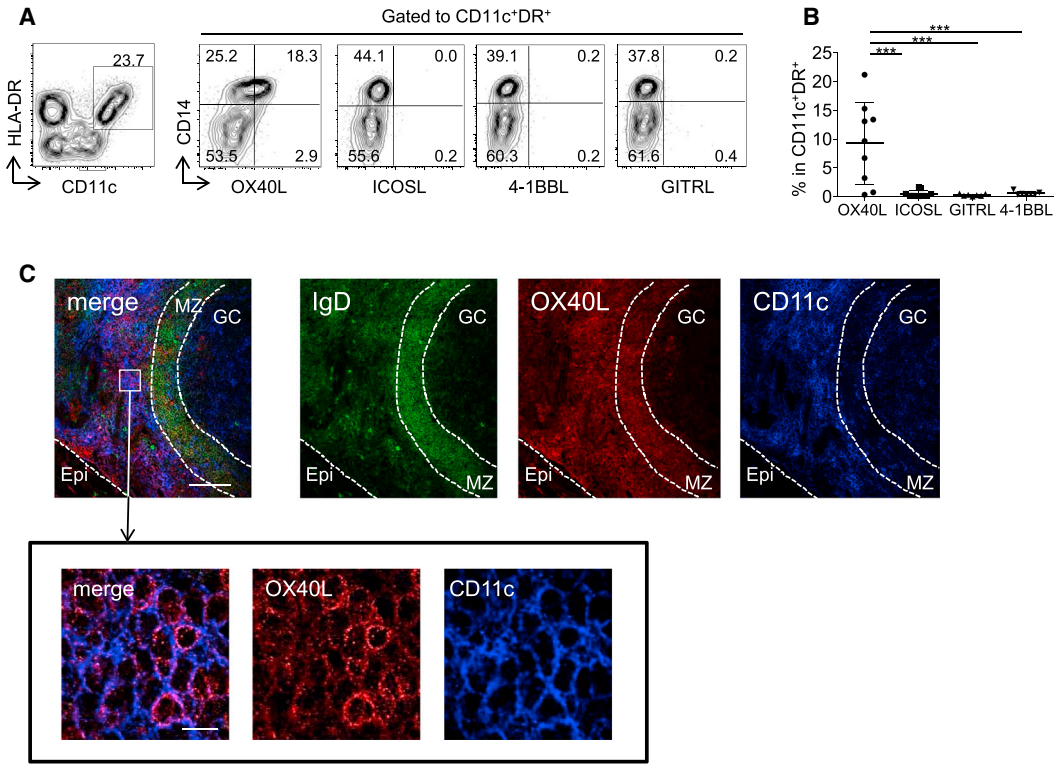

Figure 1. Increased OX40L Expression by Myeloid APCs in Inflammatory Tonsils

(A) Expression of OX40L, ICOSL, 4-1BBL, and GITRL on myeloid CD11c$^+$HLA-DR$^+$ APCs from pediatric tonsils. A representative result out of nine independent experiments.

(B) Frequency of OX40L$^+$, ICOSL$^+$, GITRL$^+$, and 4-1BBL$^+$ cells within tonsillar myeloid APCs. Mean ± SD, n = 9. One way ANOVA. ***$p < 0.001$.

(C) OX40L$^+$CD11c$^+$ APCs in inflammatory tonsils. GC, germinal center; MZ, mantle zone; Epi, Epithelial layers. The scale bars on the top and the bottom panels shows 100 μm and 10 μm, respectively.

2009). These studies provide a strong rationale that inhibition of the generation and/or activity of Tfh cells is beneficial for the prevention of lupus disease from subjects with susceptible loci and/or for the treatment of lupus patients.

In human SLE, a majority of IgG class autoantibody-producing B cells are somatically mutated (Tiller et al., 2007), suggesting that they are derived from GCs through interactions with Tfh cells. The frequency of blood Tfh cells with active phenotype is increased in active SLE patients (He et al., 2013; Simpson et al., 2010). Furthermore, Tfh cells are also found in T cell and B cell aggregates and ectopic germinal centers in kidneys of patients with lupus nephritis (Chang et al., 2011; Liarski et al., 2014). These observations support the pathogenic role of Tfh cells in human SLE. However, the mechanisms involved in increased Tfh response in SLE patients remains unknown.

Here we show that the OX40 ligand (OX40L)-OX40 axis contributes to the aberrant Tfh cell response in SLE. OX40L was expressed by myeloid APCs, but not by B cells, in blood of adult and pediatric active SLE patients. In inflamed tissues of SLE patients, OX40L was expressed by various types of cells including myeloid APCs, but not B cells. OX40L stimulation induced human Th cells to express Tfh cell-associated molecules and was sufficient to induce them to become functional B cell helpers. Finally, we show that immune complexes (ICs) containing ribonucleoprotein (RNP) present in lupus sera induce OX40L expression by myeloid APCs through activation of TLR7. Thus, our study shows that the RNP IC-OX40L axis likely provides an amplification loop of the generation of autoantibodies in SLE.

RESULTS

OX40L Is Abundantly Expressed in Inflamed Tonsils

We previously demonstrated that dermal CD14$^+$ DCs preferentially induce the generation of Tfh-like cells in vitro (Klechevsky et al., 2008). CD206$^+$ DCs in the lymph nodes, a proposed counterpart of migrating dermal CD14$^+$ DCs, also appear to share this property (Segura et al., 2012). While these observations suggest the involvement of dermal CD14$^+$ DCs in the generation of Tfh cells in draining lymph nodes of skin, the phenotype of APCs associated with Tfh responses in inflammatory lymphoid organs such as tonsils has not been determined.

Previous studies in mouse models demonstrated the importance of ICOS ligand (ICOSL) expressed by DCs for the differentiation of Tfh cells (Choi et al., 2011). We analyzed whether myeloid APCs (CD11c$^+$HLA-DR$^+$) express ICOSL in pediatric tonsils that are enriched with mature Tfh cells along with GCs (Bentebibel et al., 2011). Consistent with a previous report (Aicher et al., 2000), ICOSL was not expressed at detectable levels on either CD11c$^+$ APCs or B cells (Figures 1A and 1B;

data not shown). Staining of tonsil tissue sections with three different anti-ICOSL clones or with an ICOS-Ig chimera protein also failed to detect ICOSL$^+$ cells (data not shown). Because low ICOSL expression might be due to chronic interactions with ICOS$^+$ cells at sites (Witsch et al., 2002), we examined the expression of ICOSL transcripts by RNA-FISH in frozen human tonsil tissues. We found that ICOSL transcript was expressed within GCs at higher density than outside GCs (Figure S1). The expression of ICOSL transcript was largely consistent with the expression of CD19 transcript, suggesting that the major source of ICOSL transcripts was B cells (Figure S1).

We found instead that CD11c$^+$HLA-DR$^+$ myeloid APCs, in particular CD14$^+$ cells, expressed the co-stimulatory molecule OX40L (9.3% ± 7.1% of CD11c$^+$HLA-DR$^+$ cells, mean ± SD, n = 9; Figure 1A). Other tumor necrosis factor (TNF) ligand family molecules such as GITRL and 4-1BBL were undetectable or expressed only minimally (Figures 1A and 1B). OX40L expression by myeloid APCs was nearly absent in spleen (0.3% ± 0.5% of CD11c$^+$HLA-DR$^+$ cells, mean ± SD, n = 4), where Tfh and GC responses are much less evident than in pediatric tonsils (Bentebibel et al., 2013). This suggests that among secondary lymphoid organs, the presence of OX40L$^+$ myeloid APCs is limited to those with strong inflammatory response.

To determine the localization, we stained tonsil tissues with anti-OX40L and anti-CD11c and analyzed them by immunofluorescence microscopy. We found that OX40L was abundantly expressed in tonsils, particularly the subepithelial area, T cell zones, and mantle zones, but less in GCs (Figure 1C). OX40L$^+$ CD11c$^+$ myeloid APCs were mainly found in the T cell zone (Figure 1C). The frequency of OX40L$^+$ cells among myeloid APCs (CD11c$^+$) was 20.5% ± 10.4% (mean ± SD, n = 6. Range 10.7%–35.5%). RNA-FISH analysis also demonstrated the presence of cells expressing both CD14 and OX40L transcript mainly in T cell zone (Figure S1). Consistent with the fact that OX40L can be expressed by a broad range of immune cells including B cells, vascular endothelial cells, mast cells, activated NK cells, and activated Th cells (Croft, 2010), OX40L was also expressed by CD11c$^-$ cells, including B cells. The frequency of OX40L$^+$ B cells largely varied among tonsil tissues and among GCs in a given tonsil tissue (20.2% ± 15.4%; mean ± SD, n = 11. Range 2.3%–51.6%). Our observation suggests that inflammatory environment induces upregulation of OX40L expression on multiple types of cells.

Myeloid APCs from Active SLE Patients Express OX40L

Given prominent expression of OX40L in inflamed tonsils, we wondered whether OX40L was also expressed in inflammatory tissues from SLE patients. We found that OX40L was abundantly expressed by CD11c$^+$ myeloid APCs in inflammatory kidney tissues from active adult SLE patients with nephritis, but absent in tissues from subjects without autoimmune diseases (Figure 2A). OX40L$^+$ myeloid APCs were also found in skin biopsy samples from SLE patients, but not from controls (Figure 2A). ICOSL expression was not detected by any cells (data not shown). Similar to tonsils, OX40L$^+$ CD11c$^-$ cells were also present in both tissues from SLE patients, but no OX40L$^+$ B cells were found in any tissues (Figure S2A).

We next analyzed whether peripheral myeloid APCs in patients with SLE also express OX40L. OX40L expression was significantly increased on the surface of blood myeloid APCs from adult and pediatric patients with active SLE compared to healthy subjects, inactive SLE patients, and other autoimmune disease patients (Figure 2B; Figure S2B). Similar to tonsillar myeloid APCs (Figures 1A and 1B), we did not observe the expression of ICOSL, GITRL, or 4-1BBL on blood myeloid APCs (Figures S2C and S2D). To determine whether OX40L expression was also increased on B cells in SLE patients, we analyzed the expression of OX40L on blood CD11c$^+$ APCs and B cells side-by-side by including markers in a same staining panel. Both in adult and pediatric SLE blood samples, OX40L expression by B cells was minimal and significantly lower than CD11c$^+$ APCs (percentage of OX40L$^+$ cells in CD11c$^+$ APCs and B cells: 11.0% ± 2.5% versus 0.6% ± 0.3% in adult SLE (mean ± SEM, n = 19, $p < 0.0001$ by paired t test), and 10.5% ± 1.3% versus 2.7% ± 0.6% in pediatric SLE (n = 28, $p < 0.0001$); Figure S2E.

The percentage of OX40L$^+$ myeloid APCs in blood was significantly higher in active patients (assessed by the SLE Disease Activity Index [SLEDAI]) than in inactive patients, both in adult and pediatric SLE (Figure 2C). Furthermore, the frequency of OX40L$^+$ cells within myeloid APCs correlated with disease activity as assessed by the SLEDAI in both adult and pediatric SLE (Figure 2D). OX40L was mainly expressed by CD14$^+$ CD16$^+$ and CD14$^+$CD16$^-$ monocytes in blood (Figure 2E; Figure S2F). In a longitudinal follow-up of ten flaring and previously untreated adult SLE patients, the percentage of OX40L$^+$ myeloid APC substantially decreased after treatment along with the decrease in disease activity (Figure S2G, $p < 0.01$). Taken together, these results show that OX40L is expressed on blood and tissue-infiltrating myeloid APCs, but not on B cells, in active SLE patients.

OX40 Signals Promote the Expression of Tfh Genes in Naive and Memory T Cells

The presence of OX40L$^+$ myeloid APCs in blood and inflamed tissues suggests that OX40L expression is globally increased on myeloid APCs in active SLE patients. In particular, inflamed tissues in SLE patients appear to create an OX40L-rich environment where Th cells receive OX40 signals from multiple cell sources (Figure 2A). While being important for proliferation and survival, OX40 signals also regulate the differentiation of Th cells in collaboration with other factors derived from APCs, microenvironment, and Th cells themselves (Croft, 2010). We hypothesized that OX40 signals might display an intrinsic property to promote the differentiation of human Th cells toward the Tfh lineage. To address this hypothesis, we applied an APC-free system to avoid the contribution of factors from APCs and microenvironment and cultured naive and memory Th cells with anti-CD3 and anti-CD28 in the presence of agonistic soluble OX40L (sOX40L). To minimize the influence of T cell-intrinsic factors, we analyzed the gene-expression profiles at 48 hr of culture by NanoString. We found that OX40 signaling upregulated multiple Tfh genes, including *CXCR5*, *BCL6*, *IL21*, *CXCL13*, and *PDCD1* (encoding PD-1) in both naive and memory Th cells (Figure 3A). Furthermore, OX40L stimulation downregulated the expression of *PRDM1* (encoding Blimp-1), the transcription repressor that inhibits Tfh generation (Crotty, 2014).

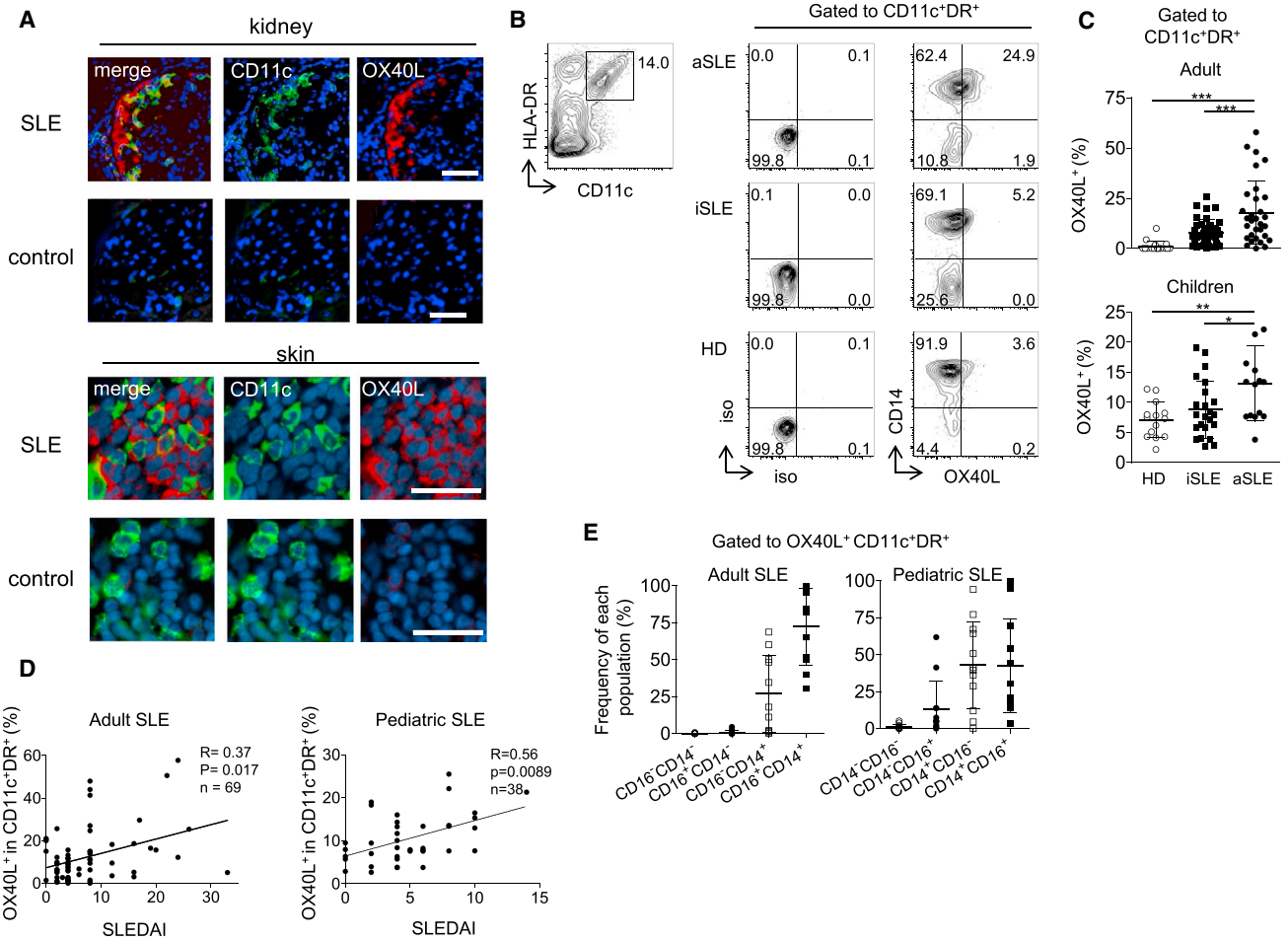

Figure 2. OX40L Expression by Myeloid APCs from SLE Patients

(A) OX40L$^+$ myeloid APCs in skin and kidney biopsies from adult SLE patients and subjects without autoimmune diseases. A representative result of five skin and three kidney biopsy samples from SLE patients and five skin and two kidney biopsy samples from controls. Scale bar represents 100 μm.

(B) Representative flow data on OX40L expression by blood myeloid CD11c$^+$HLA-DR$^+$ APCs from the three groups: healthy donors (HD), inactive (iSLE), and active (aSLE) SLE patients.

(C) Frequency of OX40L$^+$ cells within blood myeloid APCs in the three groups in adult and pediatric cohorts. Top shows the adult cohort; 16 HD, 38 iSLE, and 31 aSLE samples. Bottom shows the children cohort; 14 HD, 20 iSLE, and 14 aSLE samples. One-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(D) Correlation between the percentage of OX40L$^+$ cells within CD11c$^+$HLA-DR$^+$ myeloid APCs (adults: n = 69 and children: n = 38) and disease activity assessed by the SLEDAI. Statistical analysis was performed with the Spearman test.

(E) Composition of blood OX40L$^+$ myeloid APCs by different subsets (CD14$^+$CD16$^-$, CD14$^+$CD16$^+$, CD14$^-$CD16$^-$, CD14$^-$CD16$^+$) in adult (n = 28) and pediatric (n = 34) SLE patients. Mean ± SD.

Previously, we and others show that IL-12 induces activated human naive Th cells to express multiple Tfh molecules including IL-21, ICOS, CXCR5, and Bcl-6 at higher levels than other cytokines (Schmitt et al., 2013; Schmitt et al., 2014; Schmitt et al., 2009). Subjects deficient of IL-12 receptor β1 (IL-12Rβ1) chain display reduced Tfh and GC responses (in particular children), providing in vivo evidence that signals via IL-12 receptor is essential for the generation of Tfh cell differentiation in humans (Schmitt et al., 2013). Thus, we compared the expression of Tfh genes between OX40- and IL-12-stimulated Th cells. To our surprise, OX40 signals induced naive Th cells to express multiple Tfh genes at equivalent levels with IL-12 signals (Figure S3). Furthermore, overall expression patterns of Tfh genes were similar between OX40- and IL-12-stimulated naive Th cells (Figure 3B, left). While mouse studies suggest the positive role of IFN-γ for the generation of Tfh cells (Lee et al., 2012), the upregulation of Tfh molecules in these cells was not due to IFN-γ secreted in the cultures, as IFN-γ-stimulated naive Th cells did not show the similar gene patterns (Figure 3B, left). The combination of the two signals further increased the expression of *IL21*, but not other Tfh molecules (Figure S3).

Importantly, in contrast to the observation with naive Th cells, OX40 signals were more potent than IL-12 signals at inducing memory Th cells to upregulate Tfh genes (*BCL6*, *CXCR5*, *IL-21*, *CXCL13*, and *PDCD1*) and to downregulate *PRDM1* (Figures 3B and 3C). It was notable that OX40 signals differentially modulated the expression of *MAF* and *BATF*, genes associated

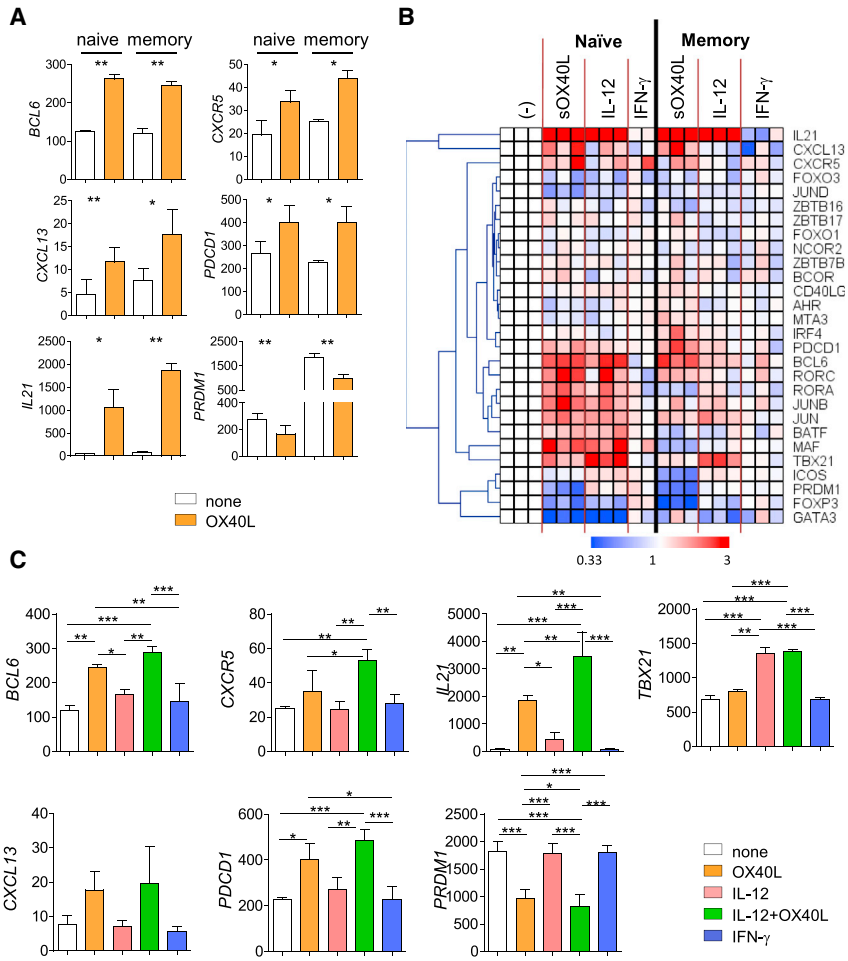

Figure 3. OX40 Signals Induce Upregulation of Tfh Genes

(A) Tfh gene expression by naive and memory Th cells (from three donors) activated with anti-CD3 and anti-CD28 in the presence or absence of sOX40L for 48 hr. Transcript counts in the cultured Th cells are shown after normalization to housekeeping genes. Mean ± SD, n = 3. Paired t test. *$p < 0.05$, **$p < 0.01$.

(B) Tfh gene expression profiles by naive and memory Th cells activated with anti-CD3 and anti-CD28 in the presence of indicated reagents for 48 hr. Transcript counts in Th cells cultured in the presence of the indicated reagents were normalized to those in control Th cells in each donor.

(C) Transcript counts in memory Th cells activated with anti-CD3 and anti-CD28 in the presence of indicated reagents. Mean ± SD, n = 3. One-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

with Tfh development and functions (Crotty, 2014), between naive and memory Th cells. OX40 signals induced upregulation of the two genes in naive Th cells, but downregulation in memory Th cells (Figure 3B). Nonetheless, IL-12 signals cooperated with OX40 signals to increase the expression of *CXCR5* and *IL21* by memory Th cells (Figure 3C).

OX40 Signals Promote the Generation of Functional Helpers

To analyze the expression of Tfh molecules at protein levels, we activated naive and memory Th cells by anti-CD3 and anti-CD28 in the presence or absence of sOX40L for 3 days, and the phenotype was analyzed by flow cytometry. Consistent with transcriptional data (Figures 3A and 3B), OX40 signals promoted both naive and memory Th cells to express Tfh molecules including CXCR5, CD40L, and IL-21, and increased the generation of $CXCR5^+$ cells co-expressing IL-21, CD40L, ICOS, and Bcl-6 (Figures 4A and 4B; Figure S4A). Of note, in addition to IL-21, OX40 signals induced the expression of IL-2 and TNF-α, but not IFN-γ or IL-4 (Figure S4B) despite increased T-bet expression (Figures 4A and 4B). OX40 signals also weakly increased the expression of RORγt, but did not induce IL-17A expression (Figure S4C). OX40L stimulation also induced naive Th cells to downregulate the expression of CCR7 on $CXCR5^+$ cells (Figure S4A), and increased the generation of $CXCR5^+CCR7^-$ cells, a chemokine receptor expression profile required for homing to B cell follicles (Haynes et al., 2007). This was not due to an enhanced expression of achaete-scute homolog 2 (Ascl2), the transcription factor important for initiation of the murine Tfh cell development (Liu et al., 2014), because Ascl2 transcript expression was completely absent in any culture conditions (data not shown).

Strikingly, OX40 signals induced memory Th cells to express Tfh molecules including CXCR5, CD40L, and IL-21 more efficiently than IL-12 signals (Figure 4B). We noticed that OX40 signals decreased the expression of ICOS on memory Th cells compared to the control culture (Figure S4A), which was consistent with the transcriptional data (Figure 3B). However, ICOS expression levels remained high, and more than 80% of $CXCR5^+$ cells stimulated with OX40 signals expressed ICOS.

We wondered whether OX40 signals are sufficient to induce Th cells to become functional helpers. To this end, stimulated Th cells were co-cultured with autologous B cells and the produced IgG were measured at day 14. OX40 signals were sufficient to induce both naive and memory Th cells to become B cell helpers (Figure 4C). Notably, OX40 signals were more efficient than IL-12 signals to induce memory Th cells to become helpers (Figure 4C). These results show that OX40L stimulation promotes naive and memory Th cells to differentiate into Tfh-like cells.

Collectively, these results show that OX40 signals display an intrinsic property to induce human naive and memory Th cells to express multiple Tfh molecules and to become functional B cell helpers.

OX40 Signals Promote the Expression of Tfh Molecules by Enhancing TCR Signals

OX40 signals activate canonical and non-canonical NF-κB pathways (Croft, 2010). To determine the mechanism by which

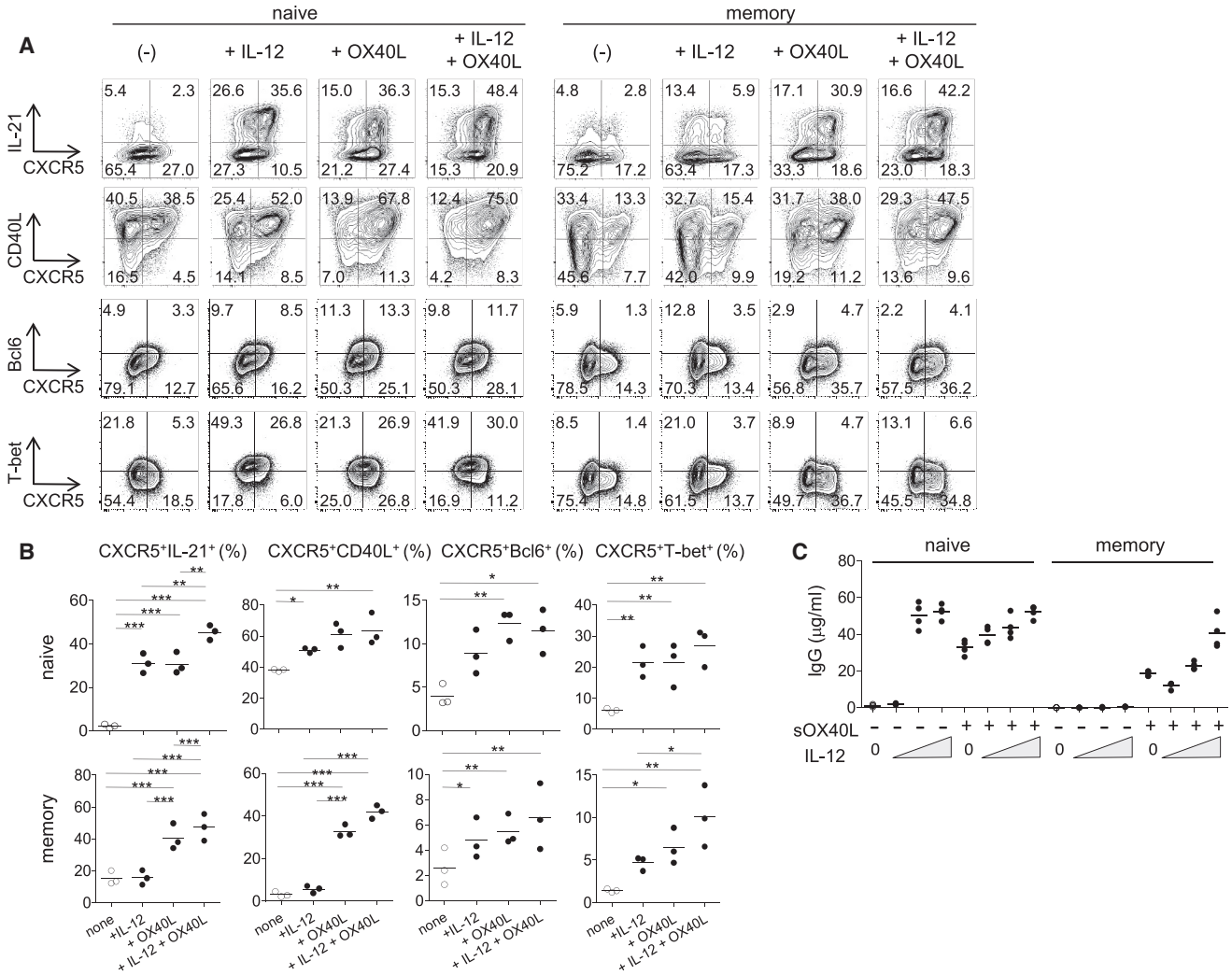

Figure 4. OX40L Stimulation Promotes the Differentiation of Naive and Memory T Cells into Tfh-like Cells

(A) Expression of CXCR5, IL-21, CD40L, Bcl-6, and T-bet by naive and memory Th cells activated with anti-CD3 and anti-CD28 in the presence or absence of sOX40L and/or IL-12. Gated to $FSC^{hi}SSC^{hi}$ activated cells. A representative result out of three independent experiments is shown.

(B) Frequency of $CXCR5^+IL\text{-}21^+$,$CXCR5^+CD40L^+$, $CXCR5^+Bcl\text{-}6^+$, and $CXCR5^+T\text{-}bet^+$ cells developed in naive or memory Th cells after activation with anti-CD3 and anti-CD28 in the presence or absence of sOX40L and/or IL-12. One-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$.

(C) Naive or memory Th cells were activated for 4 days with anti-CD3 and anti-CD28 in the presence of sOX40L and/or IL-12, and then cultured with autologous memory B cells. IgG concentrations in the supernatant of each well are shown. A representative result out of two independent experiments is shown.

OX40 signals promote the expression of Tfh molecules, we first analyzed whether inhibition of either canonical or non-canonical NF-κB pathway affects the expression of Tfh molecules by OX40-stimulated human naive Th cells. We inhibited the expression of NF-κB signaling molecules by transfecting specific siRNA, including NF-κB1, RelA (included in canonical pathway), NF-κB2, and RelB (involved in non-canonical pathway). Because a previous mouse study demonstrated that TRAF6 was essential for OX40 signals to promote the generation of Th9 cells (Xiao et al., 2012), we also tested the role of TRAF6. We confirmed that siRNA transfection substantially inhibited the expression of the target protein (Figure S5A). Naive Th cells that transfected siRNA were stimulated with CD3-CD28 mAbs in the presence or absence of sOX40L, and the expression of Tfh molecules was analyzed by flow cytometry.

Blocking molecules of canonical (NF-κB1, RelA) and non-canonical (NF-κB2) pathways weakly but significantly inhibited the Bcl-6 expression by OX40-stimulated $CD4^+$ T cells (Figure 5A). However, this did not appear to depend on OX40 signals, because a similar trend was observed with the Th cells cultured in the control condition (no sOX40L). Similarly, while inhibition of NF-κB2 upregulated the expression of CXCR5 and IL-21 and downregulated the expression of CD40L and ICOS, this was independent of OX40 signals (Figure S5B). These results suggest that while NF-κB pathway can regulate the expression of Tfh molecules, this might not be the dominant

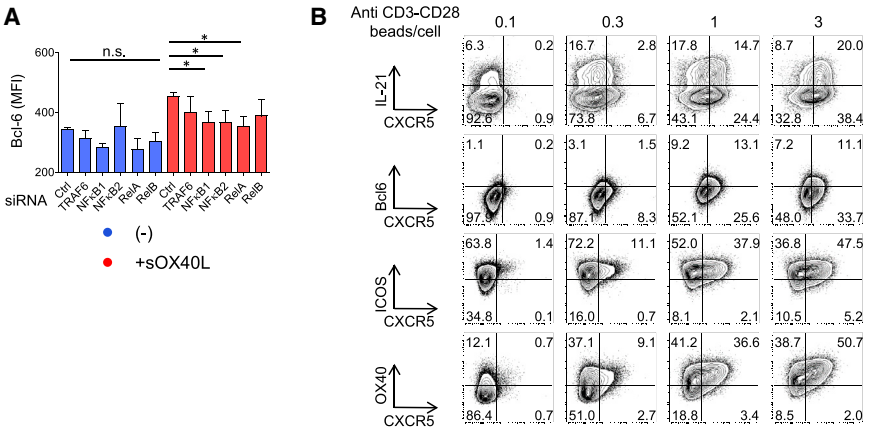

Figure 5. Strong TCR stimulation induce naive Th cells to express Tfh molecules.

(A) Bcl-6 expression by naive Th cells transfected with the indicated siRNA and cultured for 3 days with anti-CD3 and CD28 ± sOX40L. Gated to $FSC^{hi}SSC^{hi}$ activated cells. Mean ± SEM, n = 3.

(B) Expression of the indicated markers by naive Th cells activated for 4 days with the indicated number of anti-CD3 and anti-CD28-coated beads. Gated to $FSC^{hi}SSC^{hi}$ activated cells. A representative result out of three independent experiments is shown.

one by which OX40 signals induce naive Th cells to upregulate Tfh molecules.

Recent studies demonstrate that the strength and the duration of signals through T cell receptor (TCR) play a major role in determining the fate of primed Th cells (Tubo et al., 2013; van Panhuys et al., 2014). In this regard, strong and durable TCR signals promote Th differentiation toward the Tfh lineage and their proliferation (Deenick et al., 2010; Fazilleau et al., 2009; Tubo et al., 2013). It is known that OX40 signals augment TCR signals via the PI3K-Akt pathway (So et al., 2011). Furthermore, a recent study shows that blood memory Th cells in pediatric SLE patients constitutively express higher levels of phosphorylated Akt, and OX40 signals further enhance Akt activation of these cells (Kshirsagar et al., 2013). Therefore, it is possible that OX40 signals promote the expression of Tfh molecules by enhancing TCR signals. However, whether strong TCR signals promote human Th cells to express Tfh molecules or not remains unknown. Therefore, we stimulated naive Th cells with titrated numbers of anti-CD3 and anti-CD28-coated beads and analyzed the expression of Tfh molecules. We found that the stimulation with anti-CD3-CD28 beads promoted the expression of multiple Tfh molecules including CXCR5, IL-21, CD40L, and Bcl-6 in a dose-dependent manner (Figure 5B). Stronger TCR signals also increased OX40 expression. In contrast, the expression of IFN-γ, IL-4, and IL-17A was not modified by the number of anti-CD3-CD28 beads (Figure S5C). Thus, strong TCR signals promote human naive Th cells to express multiple Tfh molecules, but not other Th molecules.

Collectively, these results suggest that OX40 signals promote the expression of Tfh molecules by enhancing TCR signals.

The Frequency of $OX40L^+$ APCs Correlates with That of $ICOS^+CXCR5^+$ Tfh Cells in Blood

Previous studies showed that active SLE patients display an increased frequency of blood Tfh cells with active phenotype ($ICOS^+CXCR5^+$) (He et al., 2013; Simpson et al., 2010). We were able to confirm this observation in our cohort (Figure 6A). Importantly, we found that the frequency of $ICOS^+$ cells within blood Tfh cells positively correlated with the frequency of $OX40L^+$ cells within blood myeloid APCs (Figure 6B). The frequency of $OX40L^+$ APCs also positively correlated with the frequency of blood Tfh cells ($CXCR5^+$ in total Th cells) (Figure S6A), but showed no correlation with the frequency of blood $CXCR5^-$ Th1 ($CXCR3^+CCR6^-$), Th2 ($CXCR3^-CCR6^-$) Th17 ($CXCR3^-CCR6^+$) cells (Morita et al., 2011) (Figure S6B). These results suggest that OX40L-expressing myeloid APCs from SLE patients promote the development and/or the activation of Tfh cells.

RNP-Anti-RNP ICs Promote OX40L Expression through TLR7 Activation

We wondered which mechanism is involved in OX40L expression by myeloid APCs in active SLE patients. We previously demonstrated that SLE sera induce monocytes to acquire the properties of DCs (Blanco et al., 2001). Therefore, we hypothesized that SLE sera might contain components that induce OX40L expression by myeloid APCs. Accordingly, we found that SLE sera, but not control sera, induced OX40L expression on healthy donor monocytes at variable levels (Figure 7A). Upon co-culture with allogeneic naive Th cells, monocytes exposed to SLE sera promoted the expression of IL-21 in a manner partly dependent on OX40L (Figures S7A and S7B). We suspected the involvement of immune complexes (ICs) containing self-nucleic acid, because activation of APCs through endosomal nucleic acid sensors play a key role in SLE pathogenesis (Barrat and Coffman, 2008). Indeed, stimulation with agonist of TLR7, but not TLR9 and TLR3, induced healthy donor monocytes to express OX40L (Figure 7B). Of note, B cells did not express OX40L in response to stimulation with any of these TLR ligands (Figure S7C; data not shown). While previous mouse and human studies show that CD40 signal induces DCs to upregulate OX40L expression (Fillatreau and Gray, 2003; Murata et al., 2000; Ohshima et al., 1997), CD40 signal by itself was insufficient to induce monocytes to express OX40L (Figure S7D). To test whether TLR7 was directly implicated in OX40L upregulation by SLE sera, we exposed monocytes to SLE sera in the presence of a specific TLR7 inhibitor IRS-661 (Barrat et al., 2005) or RNase. Both TLR7 inhibitor and RNase significantly reduced the ability of SLE sera to induce OX40L expression (Figure 7C; Figure S7E), suggesting the major role by ICs containing RNA. In agreement with this hypothesis, we observed that the presence of anti-ribonucleoprotein (RNP), but not anti-DNA, antibodies in SLE sera was associated with the increased ability to promote OX40L expression on monocytes (Figure 7D).

To validate whether RNP-anti-RNP ICs were directly involved in OX40L expression, we cultured healthy donor monocytes with

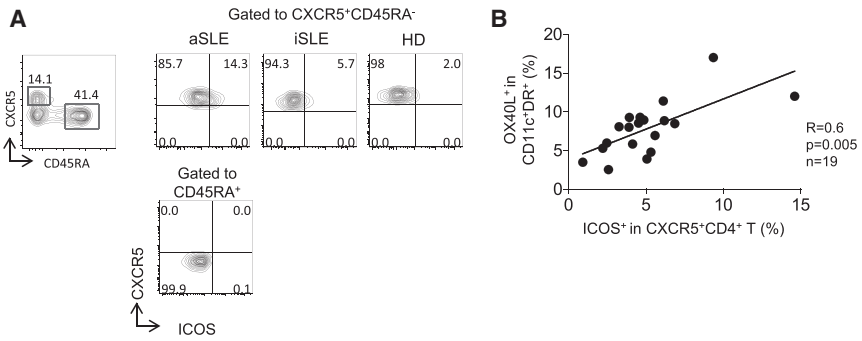

Figure 6. The Frequency of OX40L$^+$ Myeloid APCs Correlates with the Frequency of ICOS$^+$ Blood Tfh Cells in Human SLE

(A) Expression of ICOS on blood Tfh cells in the three groups; aSLE, iSLE, and HD. A representative flow result is shown.

(B) Correlation between the frequency of OX40L$^+$ cells within blood myeloid APCs and the frequency of ICOS$^+$ cells within blood Tfh cells in SLE patients. Spearman correlation test, n = 19.

anti-RNP negative SLE sera and spiked purified IgG-containing RNP-anti-RNP ICs into the cultures. We found that the supplementation with RNP-anti-RNP ICs rendered anti-RNP negative SLE sera able to promote OX40L expression (Figure 7E). This effect was dependent on TLR7 because addition of TLR7-specific inhibitor abrogated the upregulation of OX40L (Figure 7E).

These data show that RNP-anti-RNP ICs promote OX40L expression through TLR7 activation in myeloid APCs in active SLE.

DISCUSSION

Autoreactive antibody production is a hallmark of a variety of autoimmune diseases including SLE. Our study provides evidence that the OX40L-OX40 axis contributes to lupus pathogenesis by promoting the generation of Tfh cells.

The expression of OX40L by myeloid APCs, but not B cells, was increased in blood in active SLE patients. OX40L$^+$ myeloid APCs in blood of active SLE patients were largely confined to CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$ monocyte populations. OX40L$^+$ myeloid APCs in pediatric tonsils were also largely limited to the CD14$^+$ population. Increased OX40L expression on blood monocyte populations was also reported in patients with sepsis (Karulf et al., 2010) and patients with chronic hepatitis C (Zhang et al., 2013). Interestingly, both disease conditions are known to be often associated with hyper gammaglobulinemia. These observations suggest that monocytes and macrophages upregulate OX40L in inflammatory environment and contribute to antibody responses in humans. Furthermore, various types of cells upregulate OX40L expression in tonsils and inflammatory tissues of SLE patients, and therefore might also provide OX40 signals to T cells.

The pathogenic roles of ICs containing self-nucleic acid are well established in SLE. The ICs activate plasmacytoid DCs via TLR9 and TLR7 and induce them to produce large amounts of type I interferons (Lövgren et al., 2006). Type I IFN induces neutrophils to upregulate TLR7 and renders them able to respond to RNP-anti-RNP ICs. Then neutrophils produce DNA-containing components that activate pDCs (Garcia-Romo et al., 2011; Lande et al., 2011). RNP-anti-RNP ICs also target the CD16$^+$ CD14^{dim} monocyte population and induce these cells to produce cytokines that damage the endothelium, including TNF-α, IL-1, and CCL3 (Cros et al., 2010). While these mechanisms involve the activation of the innate immune system and consequent inflammation, our study shows that RNP-anti-RNP ICs also activate the adaptive immune system. We found that RNP-anti-RNP ICs contribute to OX40L expression by monocytes and macrophages via TLR7. Tfh responses increased by the RNP-anti-RNP IC-OX40L axis further accelerate the generation of autoantibodies including those against self-nucleic acid. Therefore, the RNP-anti-RNP IC-OX40L axis appears to provide an amplification loop of the generation of autoantibodies in SLE.

We showed that OX40 signals together with TCR and CD28 signals promote naive and memory Th cells to express multiple Tfh molecules, including CXCR5, IL-21, and Bcl-6. Remarkably, OX40 signals were more potent than IL-12 signals to induce memory Th cells to express Tfh molecules and were sufficient to render them to become efficient B cell helpers. These results show that OX40 signals display intrinsic property to promote Th differentiation toward the Tfh lineage in humans. Our study further suggests that this property is mainly mediated by an enhancement of TCR signals rather than that of the NF-κB pathway. In this line, strong TCR signals induced human naive Th cells to express multiple Tfh molecules including CXCR5, IL-21, CD40L, and Bcl-6, but not other Th molecules. Thus, the OX40L-OX40 axis contributes to Tfh development in a manner independent of cytokine signals that activate STAT3 and STAT4 (Schmitt et al., 2014). These two mechanisms likely cooperate, because human Th cells stimulated with both OX40 signals and IL-12 signals further upregulated IL-21 expression.

While previous mouse studies demonstrated a fundamental role of ICOSL-expressing DCs for the development of Tfh cells (Choi et al., 2011), to our surprise, we were not able to detect myeloid APCs or B cells highly expressing ICOSL in inflamed tonsils or SLE samples. Nonetheless, considering that ICOS deficiency in humans results in absence of mature Tfh cells and GCs (Bossaller et al., 2006), we do not argue the contribution of ICOSL$^+$ APCs for Tfh cell development in humans or for lupus pathogenesis. Rather, our study highlights the additional contribution of OX40L$^+$ DCs to the development of Tfh response in humans. Whether, when, and how OX40 signals and ICOS signals contribute to the development and/or maintenance of aberrant Tfh response in human autoimmune diseases remain to be addressed. Nonetheless, given that both OX40 signals and ICOS signals enhance the PI3K-Akt pathway, which plays an important role for the expression of IL-21 (Gigoux et al., 2009), these two pathways might cooperate and/or be complementary to each other for the development of Tfh cells. Importantly, in contrast to ICOS deficiency, a recent report shows that human OX40 deficiency seems to have intact Tfh and antibody responses (Byun et al., 2013). This suggests that OX40 signals are not essential for Tfh cell development or

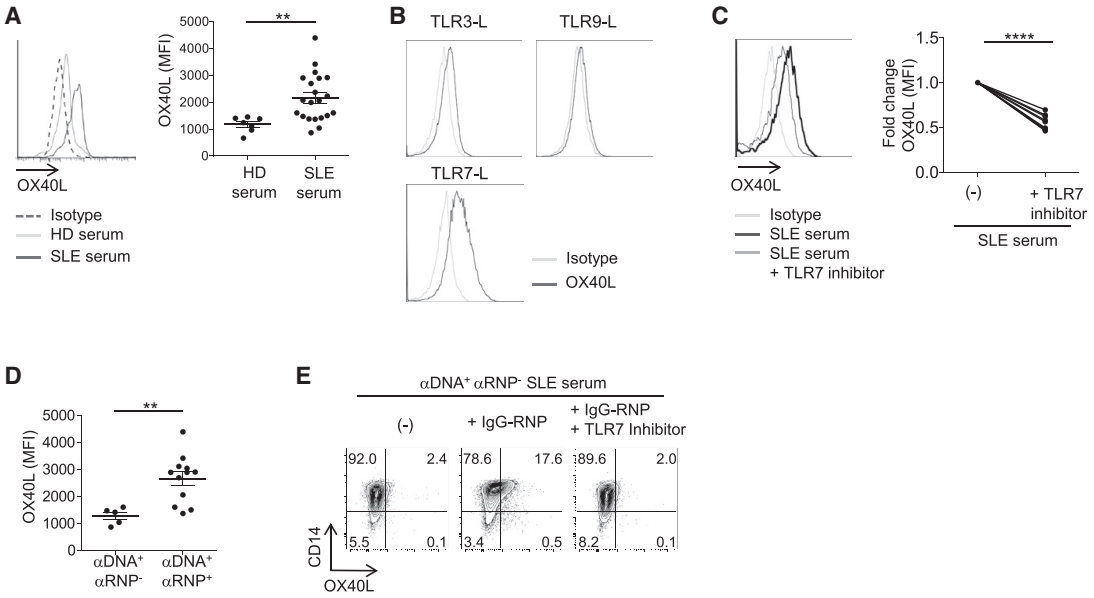

Figure 7. RNP-anti-RNP ICs Promote OX40L Expression by Myeloid APCs in a TLR7-Dependent Manner

(A) Expression of OX40L (MFI) by purified healthy donor monocytes exposed to control sera (n = 7) or SLE sera (n = 21). Mann-Whitney U-test. $^{**}p < 0.01$. A representative staining is shown on the left panel.

(B) OX40L expression upon stimulation of purified healthy donor monocytes by TLR3, TLR7, or TLR9 agonists. A representative staining out of four different experiments is shown.

(C) Fold change in OX40L expression (MFI) in healthy donor monocytes exposed to SLE sera (n = 7) in the presence or not of a TLR7 inhibitor. Paired t test. $^{****}p < 0.0001$. A representative staining is shown on the left panel.

(D) OX40L expression (MFI) in healthy donor monocytes exposed to anti-RNP^{neg} SLE sera (n = 5) or anti-RNP^{pos} SLE sera (n = 16). Mann-Whitney U-test. $^{**}p < 0.01$. Mean ± SEM.

(E) OX40L expression of purified healthy donor monocytes exposed to anti-RNP^{neg} SLE serum, the serum supplemented with anti-RNP-containing IgG, the serum spiked with anti-RNP-containing IgG in the presence of a TLR7 inhibitor. A representative staining out of three independent experiments is shown.

sufficient to compensate ICOS deficiency in vivo in humans. Thus, we surmise that OX40 signals cause aberrant Tfh response and autoimmunity in humans only when excessive. The positive correlation between the frequency of ICOS$^+$ blood Tfh cells and the frequency of OX40L$^+$ myeloid APCs in active SLE patients supports this hypothesis.

Mouse models so far provided mixed results regarding the role of the OX40-OX40L axis on the regulation of Tfh cell responses. Early studies showed that OX40L stimulation promotes mouse naive Th cells to express CXCR5 (Flynn et al., 1998), and their migration into B cell follicles (Brocker et al., 1999; Fillatreau and Gray, 2003). Furthermore, an OX40L-transgenic mice model (T cell-specific overexpression) showed development an autoimmune-like disease characterized by interstitial pneumonia, colitis, and high levels of anti-nuclear antibodies (Murata et al., 2002). Recent studies show that the mutation of *Roquin* gene in *sanroque* mice causes upregulation of OX40 on Th cells, suggesting the positive role of OX40 signals for the generation of Tfh cells (Pratama et al., 2013; Vogel et al., 2013). On the other hand, at least two studies concluded that the absence of OX40 signals did not affect CXCR5 expression by Th cells, Tfh differentiation, GC development, or antibody generation (Akiba et al., 2005; Kopf et al., 1999). Furthermore, in vivo treatment with agonistic OX40 mAb inhibited Tfh cell generation in mice in an acute viral infection model (Boettler et al., 2013) and in a listeria infection model (Marriott et al., 2014). Boettler et al. showed that agonistic anti-OX40 mAb enhanced the expression of Blimp-1 by specific Th cells while suppressing the expression of Bcl-6 in vivo (Boettler et al., 2013), contrary to our observations with human Th cells in vitro. Given that OX40 signals regulate Th differentiation in collaboration with other factors derived from APCs, microenvironment, and Th cells themselves (Croft, 2010), it is possible that OX40 signals promote or suppress Tfh cell differentiation according to the microenvironment where Th cells interact with APCs. For example, in an acute viral infection model, it is possible that OX40 signals enhanced Blimp-1 expression due to the co-presence of Type I interferons, which strongly promote Blimp-1 expression (Schmitt et al., 2014). It is yet possible that OX40 signals differentially modulate the expression of Tfh molecules between human and mouse Th cells.

Our conclusion is also supported by the findings in GWAS in autoimmune diseases. *TNFSF4* (encoding OX40L) polymorphism has been found to confer susceptibility to SLE (Cunninghame Graham et al., 2008; Delgado-Vega et al., 2009) and other autoimmune diseases, such as Sjögren syndrome, and rheumatoid arthritis (Kim et al., 2015; Nordmark et al., 2011). Furthermore, copy number variations and/or polymorphism at the *TLR7* locus has been shown to associate with SLE susceptibility (Shen et al., 2010). Our study provides a rationale that therapeutic modalities targeting the RNP-containing IC-OX40L-OX40 axis and TLR7 could impact the development of autoantibodies and therefore be beneficial for human SLE.

EXPERIMENTAL PROCEDURES

Patient Samples

For adult SLE samples, blood samples from routine lab analysis were used after informed consent was obtained. For pediatric SLE samples, the study was approved by the Institutional Review Board of Baylor Research Institute and informed consent was obtained from all the participants or their legal guardian. Adult SLE patients (total 61: 53 female and 8 male) and pediatric SLE patients (total 38: 34 female and 4 male) who met the American College of Rheumatology revised criteria for SLE were enrolled. All clinical and biologically relevant information of the patients is shown in Tables S1 and S2. Clinical disease activity was assessed using the SLE Disease Activity Index (SLEDAI). Active patients were defined as SLEDAI score $\geq$ 6.

Phenotyping of Blood Immune Cells by Flow Cytometry

For the analysis of OX40L expression, whole blood samples were stained with anti-CD14-PC5, CD16-FITC, CD11c-APC, HLA-DR-PC7, and OX40L-PE mAbs, and red blood cells were lysed with Versalyse (Beckman Coulter). For the analysis of blood Tfh cells, whole blood samples were stained with anti-CXCR5-AF488, CCR6-PE, CXCR3- PC5, CCR4-PC7, CD3-AF700, CD8-APCH7, CD4-Pacific Blue (all from Becton Dickinson), CD45RA-ECD (Beckman Coulter), ICOS-APC (Biolegend), and CD45-Pacific Orange (Invitrogen). Data were collected using a BD LSR II instrument (BD Biosciences) and analyzed with Flowjo software (Tree Star).

Culture of Th Cells

Naive ($CD45RA^+CCR7^+$) and memory ($CD45RA^-$) Th cells were sorted by flow cytometry as described before (Schmitt et al., 2009). Th cells were stimulated overnight with CD3/CD28 Dynabeads (Invitrogen) in RPMI complete medium supplemented with 10% FCS. In some experiments, overnight stimulated naive Th cells were transfected with either control or TRAF6 (s14389), NFκB1 (s9504), NFκB2 (s9507), RelA (s11916), or RelB (s11919)-specific siRNA (Life Technologies) as previously described (Schmitt et al., 2009). Cells were then transferred to flat-bottomed 96 well plates coated with CD3 mAb (5 μg /ml, OKT3) supplemented with soluble CD28 mAb (1 μg/ml, CD28.2), in the presence or absence of recombinant IL-12 (100 pg/ml), and/or soluble OX40L (100 ng/ml, R&D systems). T cells were harvested at day 4 (for CD3/CD28 stimulated T cells) or at day 7 (for monocyte-T co-culture) for phenotyping with anti-CXCR5 AF647, anti-CD40L APC-eFluor 780, and anti-ICOS biotin/Streptavidin-PerCP; and for co-culture with B cells. For the assessment of IL-21 expression (with anti-IL21-PE), cultured cells were re-stimulated with 25 ng/ml PMA, 1 μg/ml ionomycin for 6 hr in the presence of brefeldine and monensin for the last 4 hr.

Co-Culture of Th and B Cells

Activated Th cells were co-cultured with autologous memory B cells (5×10^3 T cells for 40×10^3 memory B cells per well) in 96-well round-bottom plates in Yssel medium/10% FBS in the presence of endotoxin-reduced SEB (0.25 ng/ml; Toxin Technology). IgG produced in the cultures were analyzed by ELISA at day 14.

Culture of Monocytes

$CD14^+$ monocytes were purified from blood samples from healthy donors by negative selection (Stemcell) and then exposed to SLE serum (10%) or control serum for 3 days in a 6 well-plate. The phenotype was analyzed by FACS with anti-CD14-PC5, anti-HLA-DR-PC7, and anti-OX40L-PE. TLR3 (poly-IC, 10 μg/ml), TLR7 (R837, 5 μg/ml), TLR9 (ODN2216, 10 μg/ml) agonists were purchased from InvivoGen. The TLR7 inhibitor IRS-661 (1 μM) (Barrat et al., 2005) was incubated for 10 min with the monocytes before the addition of SLE serum or anti-RNP IgG (50 μg/ml).

Statistical Analysis

When the normality of the distribution was rejected, non-parametric paired Wilcoxon test or unpaired Mann-Whitney U tests were used. One-way ANOVA with multiple comparison tests was used to compare more than three parameters. Correlation between variables was determined by using the Spearman test.

Additional Supplemental Experimental Procedures are available online.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.05.012.

AUTHOR CONTRIBUTIONS

C.J., C. C.-B., J.S., T.M., D.D., E.S.D., and L.R. analyzed the phenotype and performed the statistical analysis of blood samples. N.S. performed the in vitro experiments with naive and memory $CD4^+$ T cells. Y.L. performed tissue staining and RNA-FISH. P.N., C.J., and N.S. performed tonsillar cell analysis. T.M., E.S.D., and I.D. performed experiments with monocytes. H.D. and S.M. were involved in experimental design. L.R., C.R., E.L., P.D., M.-E.T., L.K., P. Mercié, L.C., P. Merville, T.S., J.-F.V., J.-L.P., and J.-F.M. provided adult SLE samples and clinical information. V.P. provided pediatric SLE blood samples and clinical information. S.Z. generated anti-OX40L mAb. R.L.C. provided a TLR7 inhibitor and contributed to the design of the experiments. C.J., N.S., H.U., and P.B. wrote the manuscript. H.U. and P.B. conceived the project and oversaw the entire work.

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