Malignant B Cells Skew the Balance of Regulatory T Cells and $T_H^{17}$ Cells in B-Cell Non-Hodgkin’s Lymphoma

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Abstract

Using biopsy specimens from patients with B-cell non-Hodgkin’s lymphoma, we observed a significantly low frequency of $T_H^{17}$ cells, including several samples with no detectable amount of interleukin (IL-)17–producing cells present in the tumor microenvironment. We found that, in the absence of lymphoma B cells, treatment with IL-13/IL-6 or lipopolysaccharide (LPS) enhanced IL-17 expression in CD4$^+$ T cells and this enhancement was attenuated when CD4$^+$ T cells were cocultured with lymphoma B cells. Blockade of CD27-CD70 or CD28/CD80/86 interactions by anti-CD70 or anti-CD80/86 antibodies restored LPS-mediated induction of IL-17 expression in CD4$^+$ T cells cocultured with lymphoma B cells. Because a subset of lymphoma B cells express IL-2 and given that IL-2 signaling is critically important in the development of regulatory T (Treg) cells, we tested the role of IL-2 signaling in $T_H^{17}$ cell development. We found that treatment with anti-IL-2 antibody to interrupt IL-2 signaling significantly inhibited Foxp3 expression in CD4$^+$ T cells. In contrast, interruption of IL-2 signaling up-regulated IL-17 expression in CD4$^+$ T cells and restored lymphoma-mediated down-regulation of IL-17–producing cells. Furthermore, the reversal of Treg cell activity by LPS or CpG-A resulted in an enhancement of IL-17–producing cells. Taken together, our study indicated that lymphoma B cells play an important role in skewing the balance between Treg and $T_H^{17}$ cells resulting in the establishment of a profoundly inhibitory tumor microenvironment.

Introduction

Newly identified interleukin (IL)-17–secreting CD4$^+$ helper T cells expand the family of $T_H^{17}$ cells into three major lineages, $T_H^{17}$, $T_H^{11}$, and $T_H^{13}$ cells (1–3). CD4$^+$CD25$^+$ regulatory T (Treg) cells form another major lineage of CD4$^+$ T cells (4). $T_H^{17}$ and Treg cells constitute two opposing immune responses that are critically involved in the modulation of inflammation induced by either autoimmunity or bacterial infection. $T_H^{17}$ and Treg cells develop from precursor naive CD4$^+$ T cells and the mechanisms leading to differentiation of $T_H^{17}$ cells have been predominantly described in mice. The data thus far suggest that the presence of transforming growth factor-$eta$ (TGF-$eta$) and IL-6 during activation of precursor CD4$^+$ T cells drives differentiation into $T_H^{17}$ cells (1–3), whereas the presence of TGF-$eta$ alone promotes differentiation of Treg cells (5, 6). The differentiation of precursor CD4$^+$ T cells into Treg or $T_H^{17}$ cells is thought to be mutually exclusive. Compared with the murine system, less is known about human $T_H^{17}$ cell differentiation. However, studies suggest that induction of human $T_H^{17}$ cells is unique in that IL-6 and IL-1$ß$, not TGF-$eta$, drive their differentiation (7–9).

In previous work, we have found that the antitumor response in B-cell non-Hodgkin’s lymphoma is profoundly suppressed by the presence of large numbers of intratumoral Treg cells (10, 11). We have established that non-Hodgkin’s lymphoma B cells induce Foxp3 expression in CD4$^+$CD25$^+$ T cells and contribute to the development of Treg cells in the malignant lymph nodes (12). Given that the generation of effector $T_H^{17}$ and inhibitory Treg cells from naive CD4$^+$ precursors employs reciprocal developmental pathways and that Treg cells are present in substantial numbers in the tumor microenvironment, we explored whether the development of Treg cells induced by tumor cells resulted in inhibition of the differentiation of $T_H^{17}$ cells. We also explored the mechanisms involved in potential tumor cell-mediated supression of $T_H^{17}$ cell differentiation. In the present study, we observed that a low number of $T_H^{17}$ cells are present in the tumor microenvironment of non-Hodgkin’s lymphoma and that an imbalance exists between intratumoral Treg and $T_H^{17}$ cells. Furthermore, we found that non-Hodgkin’s lymphoma B cells play an important role in establishing the imbalance between Treg and $T_H^{17}$ cells by up-regulating Treg cells and inhibiting $T_H^{17}$ cells.

Patients, Materials, and Methods

Patient samples. Patients providing written informed consent were eligible for this study if they had a tissue biopsy that on pathologic review showed B-cell non-Hodgkin’s lymphoma and adequate tissue to perform the experiments. Peripheral blood mononuclear cells from healthy donors, normal lymph nodes, and normal tonsils were used as controls. The use of human tissue samples for this study was approved by the Institutional Review Board of the Mayo Clinic/Mayo Foundation.

Cell isolation and purification. Fresh tumor biopsy specimens from patients with B-cell non-Hodgkin’s lymphoma, normal lymph nodes, and normal tonsil tissues were gently minced over a wire-mesh screen to obtain mononuclear cells. CD4$^+$ T cells and CD19$^+$ B cells were isolated using adherence technique described previously (12). Cell suspension or peripheral blood from healthy donors was centrifuged over Ficoll-Hypaque at 500 × g for 15 min to isolate mononuclear cells. CD4$^+$ T cells and CD19$^+$ B cells were isolated using positive selection with CD4 and CD19 microbeads. CD4$^+$CD45RA$^-$ and CD4$^+$CD45RO$^+$ T-cell subsets were purified by using a CD4$^+$CD45RA$^-$ naive T-cell isolation kit (Miltenyi Biotec). CD4$^+$CCR6$^+$ T cells were isolated by CD4$^+$ selection and the resulting CD4$^+$ T cells were incubated with biotin-conjugated CCR6 antibody followed by incubation with streptavidin-conjugated microbeads. Dendritic cells were isolated using a monocyte adherence technique described previously (12).

Intracellular staining and flow cytometry. Intracellular IL-17 staining was done following the manufacturer’s instructions and cells were analyzed on a FACSCalibur flow cytometer. CD4$^+$ T cells were cultured in anti-CD3-coated plates (BD Biosciences) with anti-CD28 (BD Biosciences) for 3 days and then stimulated with phorbol 12-myristate 13-acetate and ionomycin in
the presence of protein transport inhibitor brefeldin A for 5 h. Cells were stained with anti-IL-17-PE and anti-IFN-γ-APC to detect the frequency of T_{H17} cells in each specimen. The same specimens were then used in the subsequent experiments. To ensure that the results were valid for all lymphoma subtypes, each experiment included lymphoma cells from various histologic types.

Where indicated, cultures were supplemented with the following reagents alone or combination: anti-human IFN-γ (2 μg/mL), IL-6 (10 ng/mL), TGF-β (10 ng/mL), IL-1β (10 ng/mL), IL-23 (10 ng/mL), lipopolysaccharide (LPS; 100 ng/mL), or CpG-A/scrambled CpG-A (3 μg/mL). CpG-A (G^*GGGGAC-GATGCAG^*GGAC^*GGGC^*GGG) and scrambled CpG-A (G^*GGGGAG-CATGCTGC^*GGG^*GGG^*GGG) were synthesized by the DNA Sequencing and Synthesis Core Facility at Mayo Clinic as described previously (13).

Foxp3 expression was determined using flow-based intracellular staining following the manufacturer’s instructions. Cells were then stained with Alexa 488-conjugated anti-Foxp3 antibody for 30 min and analyzed by flow cytometry.

**ELISA.** The IL-17 concentration in culture supernatants was measured by ELISA (R&D Systems) according to the manufacturer’s instructions. Briefly, supernatants were collected from the culture of CD4^+ T cells. Assay Diluent R&D1-36 (100 μL) was dispensed into each well in the IL-17 microplate and 100 μL standard or sample was added to the wells. After washing, 200 μL anti-IL-17 conjugate was dispensed into each well. After stopping the reaction, the absorbance of each well was determined using a SpectraMax190 microplate reader (Molecular Devices) set to 450 nm and analyzed using SoftMax Pro 5 software.

**Statistical analysis.** Statistical analysis was done using Student’s t test. Significance was determined at P < 0.05.

**Results**

**Frequency of T_{H17} cells in biopsy specimens of B-cell non-Hodgkin's lymphoma.** The percentage of T_{H17} cells in B-cell non-Hodgkin’s lymphoma biopsy specimens was determined. Benign biopsy specimens of patients with hyperplasia (benign lymph node), peripheral blood, and tonsils of healthy individuals were used as controls. The frequency of CD4^+ IL-17–producing T cells was significantly lower in biopsy specimens from non-Hodgkin's lymphoma (median, 0.44% of the total CD4^+ T cells; range, 0.02–5.67%; n = 43 non-Hodgkin's lymphoma samples) compared with those from patients with hyperplasia (median, 1.65%; range, 0.51–10%; n = 16 benign lymph nodes; P = 0.009) or peripheral blood (median, 1.25%; range, 0.43–18.1%; n = 17 healthy individuals; P = 0.015) and tonsil (median, 5.3%; range, 2.2–11%; n = 6; P = 0.003) from healthy individuals (Fig. 1A).

Interestingly, we noted three different patterns of IL-17 expression in CD4^+ T cells (Fig. 1B). In some specimens, such as that shown as NHL1, we were not able to detect IL-17 expression in CD4^+ T cells (referred to as undetectable). However, IFN-γ expression could be detected in CD4^+ T cells, suggesting that a lack of IL-17 production in CD4^+ T cells from these particular samples was not due to unresponsiveness to activation. In contrast to NHL1, CD4^+ T cells from both NHL2 and NHL3 were able to produce IL-17 and IFN-γ. In NHL2, we found a second pattern in which IL-17 expression appeared to be independent of IFN-γ expression. In NHL3, unlike NHL2, we found that a subset of CD4^+ IL-17–producing T cells also expresses IFN-γ.

In addition to a low frequency of CD4^+ IL-17–producing T cells, we also observed that the number of specimens with an undetectable amount of IL-17–producing cells was higher in patients with non-Hodgkin’s lymphoma compared with that in normal lymph nodes, tonsil, and peripheral blood. As shown in Fig. 1C, of the 43 non-Hodgkin’s lymphoma specimens, 15 (23%)

Figure 1. Frequency of T_{H17} cells in B-cell non-Hodgkin’s lymphoma biopsy specimens. A, frequency of CD4^+ IL-17–producing cells from B-cell non-Hodgkin’s lymphoma (NHL), benign lymph node (LN), peripheral blood from healthy donors (PB), and benign tonsil tissues (Ton). Cells were cultured in anti-CD3 antibody-coated plates with anti-CD28 antibody for 3 days and restimulated with phorbol 12-myristate 13-acetate/ionomycin in the presence of protein transport inhibitor brefeldin A for 5 h. Cells were subjected to intracellular staining with fluorochrome-conjugated antibodies specific to CD3, CD4, IL-17, and IFN-γ. The frequency of T_{H17} cells was measured as % CD4^+ IL-17–producing T cells. B, IL-17 and IFN-γ expression in CD4^+ T cells from three representative specimens. C, numbers of specimens without detectable IL-17–producing cells. D, frequency of CD4^+ IL-17–producing cells from indicated histologic types of B-cell non-Hodgkin’s lymphoma. FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; CLL/SLL, small lymphocytic lymphoma; MALT, mucosa-associated lymphoid tissue.
were identified as undetectable for IL-17 expression. In contrast, we saw that, of the 16 normal lymph node specimens, only 1 (5.9%) had undetectable IL-17 expression. Furthermore, we were able to detect CD4+ IL-17–producing cells in all of the specimens from normal peripheral blood and tonsils.

We found that there was variability in the frequency of IL-17–producing cells among specimens from different histologic types of lymphoma. Although most lymphoma specimens had low numbers of IL-17–producing cells, it appeared that highest frequency of IL-17–producing cells were seen in patients with small lymphocytic lymphoma (n = 7). Mantle cell lymphoma (n = 7) and lymphomas of mucosa-associated lymphoid tissue (n = 2) contained a considerable number of IL-17–producing cells. In contrast, samples from follicular (n = 11), diffuse large-B-cell (n = 3), and marginal zone (n = 9) lymphomas exhibited a very low frequency of IL-17–producing cells (Fig. 1D).

To determine the overall relevance of T H17 cells in non-Hodgkin's lymphoma, we looked for an association between the number of intratumoral TH17 cells and clinical parameters particularly the risk of progression or overall survival. Although the effect of intratumoral TH17 cells on progression and survival could not be directly assessed in this study, due to small numbers of patients and short follow-up, the number of TH17 cells did correlate with the international prognostic factor index (P = 0.014; ref. 14) and the absolute lymphocyte count in the peripheral blood (P = 0.039; ref. 15), both of which have been shown to be prognostic factors in non-Hodgkin's lymphoma.

**Phenotypic characterization of TH17 cells in non-Hodgkin's lymphoma.** As shown in Fig. 2A, both CD4+ and CD8+ T cells were able to express IL-17, which is consistent with the results found in various types of cancers in mice (16). CD9+ B cells or other cell types, including macrophages and dendritic cells, did not express IL-17 (Fig. 2A, right). Because the frequency of CD8+ T cells in lymphoma specimens is low (17), it is likely that TH17 cells are the primary source of IL-17 production in the tumor microenvironment. We next wanted to determine if naive or memory CD4+ T cells produced IL-17 (Fig. 2B). We found that CD4+CD45RO+ memory T cells expressed more IL-17 than CD4+CD45RA+ naive T cells as detected by both intracellular staining (1.7% versus 0.3%, respectively; n = 12 non-Hodgkin's lymphoma samples; P = 0.001) and ELISA (125 versus 12 pg/mL; n = 6 non-Hodgkin's lymphoma samples; P = 0.01). This is in keeping with the observation that IL-17–producing cells are memory T cells (18, 19).

Recent studies have shown that the vast majority of IL-17–producing cells are CD4+CCR6+ T cells (8, 9, 20). To determine if this holds true in non-Hodgkin's lymphoma specimens, we enriched for CD4+CCR6+ T cells and measured IL-17 expression by both intracellular staining and ELISA (Fig. 2C). We found that CD4+CCR6+ T cells produced higher levels of IL-17 than CD4+CCR6- T cells measured by both intracellular staining and ELISA.

**Cytokine regulation of TH17 cell generation in non-Hodgkin's lymphoma.** To determine whether CD4+ T cells from non-Hodgkin's lymphoma specimens could respond to cytokines permissive for the generation of TH17 cells, we measured IL-17 expression in CD4+ T cells treated with a panel of cytokines known to influence TH17 generation. As shown in Fig. 3A, we observed that IL-1β or IL-6 alone or in combination increased the number of CD4+ IL-17–producing cells. We found that treatment with TGF-β alone was not sufficient to induce IL-17 expression and, in most cases, slightly inhibited the expression of IL-17 in CD4+ T cells (data not shown). We then measured the number of IL-17–producing cells and the level of IL-17 concentration in CD4+ T cells treated without or with IL-1β plus IL-6 in multiple samples (n = 10 non-Hodgkin's lymphoma samples) and confirmed that IL-1β/IL-6 promoted IL-17 production as determined by both intracellular staining and ELISA (Fig. 3B).

To exclude the possibility that low numbers of TH17 cells are due to low levels of IL-1β, IL-2, or IL-6, we measured the concentration of IL-1β, IL-2, and IL-6 in serum of patients with follicular lymphoma as well as normal controls. We found that IL-1β levels in the serum from patients with follicular lymphoma (median, 0.047 ng/mL; range, undetectable to 0.52 ng/mL; n = 32) were similar to that of normal controls (median, 0.061 ng/mL; range, undetectable to 1.37 ng/mL; n = 49; P = 0.72). The IL-6 concentration was in fact higher in serum from patients with follicular lymphoma (median, 17.5 ng/mL; range, undetectable to 80 ng/mL; n = 32) than in normal controls (median, 6.94 ng/mL; range, undetectable to 120 ng/mL; n = 49; P = 0.045). IL-2 levels were similar in patients (median, 0.012 ng/mL; range, undetectable to 0.18 ng/mL; n = 32) and controls (median, 0.026 ng/mL; range, undetectable to 0.28 ng/mL; n = 24; P = 0.29). These results suggested that the low number of TH17 cells is not due to decreased levels of IL-1β and IL-6 in patients.

As shown in Fig. 3C, we observed that treatment with IL-1β/IL-6 not only increased the numbers of CD4+ IL-17–producing cells but also induced IL-17 expression in IFN-γ-producing cells. Interestingly, treatment with TGF-β decreased IFN-γ expression in CD4+ IL-17–producing T cells induced by IL-1β/IL-6. It appeared that TGF-β completely blocked the expression of IFN-γ in IL-17–producing cells induced by IL-1β/IL-6 and
“purified” IL-17–producing cells into a T_{H}17 subset similar to that originally described in mice.

Due to the dual expression of IL-17 and IFN-γ in some cells, we next determined if altering IFN-γ expression had an effect on the development of T_{H}17 cells (Fig. 3D). Similar to TGF-β, anti-IFN-γ antibody treatment cleared the population of IL-17/IFN-γ+ T cells but further enhanced the up-regulation of IL-17/IFN-γ− T cells. This suggested that the generation of T_{H}17 cells can be affected by the development of T_{H}1 cells, consistent with the concept that the development of TH cell subsets is mutually and exclusively regulated.

Effect of malignant B cells on the generation of T_{H}17 cells. It is unknown whether lymphoma cells play a role in the generation of T_{H}17 cells. We therefore first used a CD19 depletion approach to determine the effect of non-Hodgkin’s lymphoma cells on T_{H}17 cell generation. We found that, in the presence of lymphoma cells, IL-1β/IL-6-mediated T_{H}17 cell generation was inhibited (<0.6% of total CD4+ T cells; n = 6 non-Hodgkin’s lymphoma samples). In contrast, depletion of lymphoma cells resulted in the enhancement of IL-17–producing T cells by IL-1β/IL-6 (4.5% of total CD4+ T cells; n = 8 non-Hodgkin’s lymphoma samples; Fig. 4A). These results suggested that lymphoma B cells suppress T_{H}17 cell generation.

We also employed a coculture system in which CD4+ T cells isolated from tumor biopsy specimens were cultured with or without CD19+ lymphoma cells (IL-1β and IL-6 were used as before and LPS was used as an additional positive control). As shown in Fig. 4B, in the absence of stimulation, there was no significant difference in the number of IL-17–producing cells between CD4+ T cells cocultured with or without lymphoma cells (2.3% versus 2.7%). As expected, treatment of T cells with IL-1β/IL-6 enhanced the number of IL-17–producing cells from 2.7% to 7.5%. However, in the presence of lymphoma cells, IL-1β/IL-6 treatment was unable to induce IL-17 expression in CD4+ T cells (2.3% versus 0.8%). Similar to cytokines, LPS treatment increased the frequency of IL-17–producing cells from 2.7% to 15.2% in CD4+ T cells. However, coculture with lymphoma cells decreased LPS-mediated enhancement of IL-17 expression in CD4+ T cells from 15.2% to 7.6%. As a control, we examined the effect of normal B cells on the generation of T_{H}17 cells. CD4+ cells, in the presence or absence of normal CD19+ B cells, were stimulated with or without IL-1β plus IL-6 and

Figure 3. Cytokine regulation of T_{H}17 cells. A, IL-17 expression in CD4+ T cells treated with indicated cytokine. B, left, summary of IL-17 induction in CD4+ T cells incubated without (NIL) or with IL-1β/IL-6 (CKs). Horizontal bars, median. Right, summary of IL-17 concentration in culture supernatants in CD4+ T cells incubated without or with IL-1β/IL-6. C, IL-17 expression in CD4+ T cells treated without or with IL-1β/IL-6 in the absence or presence of TGF-β. D, IL-17 expression in CD4+ T cells treated without or with IL-1β/IL-6 in the absence or presence of anti-IFN-γ antibody.
subjected to IL-17 intracellular staining. In the presence or absence of normal B cells, treatment of IL-1β plus IL-6 induced a similar number of IL-17–expressing cells (1.5% versus 5.8% and 1.8% versus 6.2%, respectively). These results further confirm that lymphoma cells specifically suppress Th17 cell generation.

We next investigated the potential mechanisms that may account for the interactions between lymphoma and intratumoral Th17 cells. In previous work, we have shown that costimulatory molecules were involved in non-Hodgkin’s lymphoma-mediated Treg cell development (12). To test whether CD27/CD70 or CD28/CD80/86 interactions were involved in non-Hodgkin’s lymphoma-mediated suppression of Th17 cells, we cocultured CD4+ T cells isolated from tumor biopsy specimens in the presence or absence of CD19+ lymphoma cells with or without blocking antibodies specific to CD70, CD80, or CD86. As shown in Fig. 4C (top), T-cell activation in the presence of mIgG (isotype control) significantly increased IL-17 expression in CD4+ T cells in the absence of lymphoma cells. Under the same culture conditions, treatment with CD27 or CD80 or CD86 antibody had no effect on IL-17 expression in CD4+ T cells when compared with isotype control. In the presence of lymphoma cells, CD4+ T-cell activation modestly up-regulated IL-17 expression, similar to the results shown in Fig. 4B. However, in the presence of anti-CD70, CD80, or CD86 antibody, LPS-mediated up-regulation of IL-17 was restored to that seen in CD4+ T cells cocultured without lymphoma cells. These results confirmed an inhibitory effect of lymphoma cells on Th17 cell generation and suggest that CD70, CD80, and CD86 are involved in the process.

**Effect of interruption of IL-2 signaling on Th17 cells in B-cell non-Hodgkin’s lymphoma.** Because IL-2 signaling plays a critical role in the maintenance and activity of Treg cells (21) and is also involved in the regulation of Th17 cells (22), we explored whether inhibiting IL-2 affected Th17 cells. We therefore first induced Foxp3 expression in CD4+ T cells from lymphoma specimens by treating them with TGF-β (Fig. 5A). We found that the TGF-β-mediated induction of Foxp3 in intratumoral CD4+ T cells was completely abolished when the cells were treated with an anti-IL-2 antibody, indicating that IL-2 is critically important in the generation of Treg cells. We then determined whether the inhibition of Treg generation by anti-IL-2 antibody results in the enhancement of Th17 generation. As shown in Fig. 5B (top), blockade of IL-2 by anti-IL-2 antibody increased IL-17 expression in CD4+ T cells, suggesting that IL-2 is involved in the regulation of Th17 cell generation. In addition to using an anti-IL-2 antibody, we also used antibodies blocking IL-2Rα or IL-2Rβ (Fig. 5B). Treatment with anti-IL-2Rα (middle) or IL-2Rβ (bottom) led to an increase in CD4+ IL-17–producing cells. An increase in both IL-17+ IFN-γ and IL-17+ IFN-γ+ cells was seen after treatment with IL-2Rα and IL-2Rβ blocking antibodies. Taken together, these findings indicate that activation of IL-2 signaling plays a role in the inhibition of Th17 cell generation.

**Figure 4.** Effect of malignant B cells on Th17 cells. A, IL-17 expression in CD4+ T cells from non-Hodgkin’s lymphoma tissue without depletion (U, unsorted) or with depletion (D, CD19-depleted) of B cells. Right, summarized data (n = 8 patient samples). B, IL-17 expression in CD4+ T cells isolated from non-Hodgkin’s lymphoma tissue and cocultured without (top) or with (bottom) CD19+ lymphoma cells (LB) in the absence (NIL) or presence of IL-1β/IL-6 (CKs) or LPS. C, IL-17 and IFN-γ expression in CD4+ T cells cocultured without or with CD19+ lymphoma cells in the absence or presence of LPS plus blocking antibody specific to CD70 or CD80 or CD86.
Reciprocal regulation of TH17 and Treg cells in biopsy specimens of B-cell non-Hodgkin’s lymphoma. It has been shown that CpG-A (a type D CpG oligonucleotide) and LPS are able to reverse the inhibitory activity of Treg cells (13, 23). We, therefore, wanted to test whether the reversal of Treg cell function by CpG-A or LPS resulted in the enhancement of TH17 cell numbers. As shown in Fig. 6A, treatment with CpG-A, but not a scrambled CpG-A control, enhanced IL-17 expression in CD4+ T cells (5.7% versus 3.3%; n = 3 non-Hodgkin’s lymphoma samples). Similar findings were observed in CD4+ T cells treated with LPS. The number of IL-17+ T cells was increased in CD4+ T cells treated with LPS (6.4% versus 3.8%; n = 5 non-Hodgkin’s lymphoma samples). Furthermore, we tested the effect of LPS-activated dendritic cells on IL-17 production by CD4+ T cells because LPS-matured dendritic cells have been shown to be able to abrogate Treg cell suppressive function (24, 25). As shown in Supplementary Fig. S1, coculture with LPS-activated dendritic cells increased IL-17 production in intratumoral CD4+ T cells. These results indicate that inhibition of Treg cells is associated with enhanced generation of TH17 cells.

We also tested whether changes in Foxp3 expression correlated with the number of IL-17-producing cells (Fig. 6B). CD4+ T cells were treated with TGF-β in the presence or absence of IL-23, a cytokine that has been shown to be able to promote TH17 cell expansion and stain for IL-17 or Foxp3 expression. As shown in Fig. 6B, treatment with TGF-β induced Foxp3 in CD4+ T cells and this up-regulation of Foxp3 expression was reversed by IL-23, consistent with other studies (26). Conversely, treatment with TGF-β inhibited IL-17 expression in CD4+ T cells and this down-regulation of IL-17 expression was relieved by IL-23. Similar results were seen when CD4+ T cells were treated with anti-IL-2 antibody (data not shown). These results indicate that the expression of Foxp3 and IL-17 is reciprocally regulated in intratumoral CD4+ T cells.

Next, we determined whether a reverse correlation between Treg and TH17 cells could be observed in vivo. As shown in Fig. 6C, an increased number of Foxp3+ cells and a low number of IL-17+ cells were found in tonsils involved by non-Hodgkin’s lymphoma, whereas a low number of Foxp3+ cells and a relatively high number of IL-17+ cells were found in benign tonsils. Tonsil tissue was used as a representative sample because it has a higher number of TH17 cells. We then compared the expression of Foxp3 and IL-17 in vivo. As shown in Fig. 6D, we again observed that stimulation with IL-1β/IL-6 was not able to enhance IL-17 expression in CD4+ T cells when cocultured with lymphoma cells. However, we found that the number of IL-17-producing cells was increased when CD4+ T cells were cocultured with lymphoma cells treated with an IL-2 blocking antibody (Fig. 5D). These results suggest that IL-2 generated by lymphoma cells is involved in the inhibition of T17 cells.

Reciprocal regulation of TH17 and Treg cells in biopsy specimens of B-cell non-Hodgkin’s lymphoma.

Figure 5. Effect of IL-2 signaling on TH17 cells. A, Foxp3 expression in CD4+ T cells isolated from tumor biopsy specimens treated without or with TGF-β in the absence or presence of anti-IL-2 antibody (n = 3). B, IL-17 and IFN-γ expression in CD4+ T cells treated without or with IL-1β/IL-6 in the presence or absence of anti-IL-2 (top), anti-IL-2Rα (middle), or anti-IL-2Rβ (bottom) antibody (n = 5). Each panel represents a patient specimen. C, IL-2 expression in CD19+ cells activated without (Unstim) or with LPS and phorbol 12-myristate 13-acetate/ionomycin (Stim; n = 4 non-Hodgkin’s lymphoma samples). D, IL-17 and IFN-γ expression in CD4+ T cells cocultured with CD19+ lymphoma B cells in the absence or presence of IL-1β/IL-6 plus blocking antibody specific to IL-2 (aIL-2) or isotype control (IgG; n = 3 non-Hodgkin’s lymphoma samples).

We next wanted to test whether IL-2 contributed to lymphoma cell-mediated inhibition of TH17 cell generation. We first confirmed that lymphoma cells were able to produce IL-2 (Fig. 5C). We then cultured CD4+ T cells in the presence of lymphoma cells with IL-1β/IL-6 or with IL-1β/IL-6 plus an anti-IL-2 antibody. As shown in Fig. 5D, we again observed that stimulation with IL-1β/IL-6 was not able to enhance IL-17 expression in CD4+ T cells when cocultured with lymphoma cells. However, we found that the number of IL-17-producing cells was increased when CD4+ T cells were cocultured with lymphoma cells treated with an IL-2 blocking antibody (Fig. 5D). These results suggest that IL-2 generated by lymphoma cells is involved in the inhibition of TH17 cells.
induced Foxp3 expression and inhibited IL-17 production in intratumoral CD4+ T cells. Similarly, coculture with malignant B cells resulted in up-regulation of Foxp3 expression and down-regulation of IL-17 production.

Discussion

Although Th17 cells have been intensively studied in mouse models of autoimmune and infectious diseases, there are very few studies attempting to explore the role of Th17 cells in tumor models either in mice or humans (16, 27–29). Previous studies (16, 30–36) have found that expression levels of IL-17 mRNA or protein differ in different types of tumors with the range from undetectable to abundant. However, it is not clear in most of these studies whether IL-17 detected in tumor tissues is produced by Th17 cells. In the present study, we found that IL-17 production was predominantly from Th17 cells. We also found that the frequency of Th17 cells varies among specimens with a range from undetectable to a considerable amount. When we quantified the number of Th17 cells in non-Hodgkin's lymphoma biopsy specimens, we found a significantly low percentage of Th17 cells present in lymphoma specimens. This led us to explore the underlying mechanism that accounted for the low percentage of Th17 cells.

Although it has been convincingly shown that TGF-β plus IL-6 induces the development of murine Th17 cells, the development of human Th17 cells turns out to be more complex due to inability of TGF-β and IL-6 to induce Th17 cells in human tissues (37). Two groups (7, 38) independently observed that IL-1β but not TGF-β was essential for the differentiation of human IL-17–producing T cells, and this has been confirmed in human tissues by several studies (39–41). In this study in human B-cell lymphomas, we found that Th17 cell generation can be induced in vitro by IL-1β and/or IL-6, but not TGF-β, confirming the important role of inflammatory cytokines in the development of Th17 cells. Although TGF-β alone had little effect on the development of Th17 cells in our samples, TGF-β was able to inhibit IFN-γ expression in CD4+ T cells.
T cells including IL-17+ T cells induced by IL-1β/IL-6 and cleared the population of IL-17+ IFN-γ+ cells. This resulted in a subset of IL-17-producing cells similar to Th17 cells originally identified and described in mice. In addition to TGF-β, attenuation of IFN-γ expression by anti-IFN-γ antibody resulted in the disappearance of the IL-17+ IFN-γ+ population and an enhancement of IL-17+ IFN-γ- T cells, suggesting a reciprocal regulation between development of Treg1 and Th17 cells.

The underlying mechanism for the significantly low percentage of Treg1 cells present in lymphoma specimens is unknown. We found that malignant transformation of B cells plays an important role in CD4+ T-cell differentiation favoring Treg1 cells and leading to down-regulation of Th17 cell generation. In the present study, we found that lymphoma cells inhibited cytokine- and LPS-mediated enhancement of Th17 cell generation accounting for the significantly low percentage of Th17 cells present in lymphoma specimens. Our results differ from those of a recent study that showed that ovarian tumor cells promote Th17 cell generation by secreting IL-1β and IL-6 (39). However, this report also showed that ovarian tumor cells secreted TGF-β, thereby leading to an inhibition of Treg1 cell generation and an induction of Treg1 cells.

Interaction between costimulatory molecules and their ligands plays an important role in T-cell differentiation and development. Lymphoma B cells aberrantly express abundant CD70, CD80, or CD86 when compared with normal B cells and interaction between CD27–CD70 and CD28–CD80/86 interactions between lymphoma cells and CD4+ T cells would be able to promote Th17 cell generation. Our data clearly showed that the administration of antibodies blocking the costimulatory molecules CD70, CD80, or CD86 increased the number of IL-17–producing cells.

The IL-2 signaling pathway has been shown to be critically involved in the maintenance of the Treg1 cell phenotype and activity. The role of the IL-2 signaling pathway in the generation of Th17 cells, however, is controversial. By using peripheral blood mononuclear cells from healthy subjects and patients with uveitis or uveitis, Amadi-Obi and colleagues found that IL-2 induced the production of IL-17 in CD4+ T cells and contributed to the progression of uveitis and scleritis (43). In contrast to this finding, other reports showed that addition of exogenous IL-2 suppressed the up-regulation of IL-17–producing cells mediated by TGF-β/IL-6. Furthermore, IL-2 signaling interruption using an anti-IL-2 antibody resulted in the up-regulation of IL-17 expression in CD4+ T cells in a mouse model (16, 22), suggesting that IL-2 has a direct inhibitory effect on Th17 generation. Supporting this finding, our data clearly showed that interruption of IL-2 signaling enhanced the up-regulation of cytokine-mediated IL-17 expression in intratumoral CD4+ T cells.

Throughout the experiments, we noted that the development of Treg or Th17 cells in the non-Hodgkin’s lymphoma tumor microenvironment was reciprocally regulated. Several factors including cytokines (22, 44), retinoic acid (45), and rapamycin (46) are able to mediate a reciprocal relationship between Treg and Th17 cells. This relationship has been reported in patients with acute coronary syndrome (47) and described in studies on synovial fluid from children with arthritis (48). However, to the best of our knowledge, this relationship has not been reported in any malignancy. We have presented a line of evidence showing that up-regulation of Treg cells was associated with down-regulation of Th17 and vice versa. These findings confirmed that the development of Treg and Th17 cells is reciprocally regulated and, more importantly, that lymphoma B cells generate an inhibitory environment that favors the development of Treg cells rather than Th17 cells. This lack of Th17 cells may therefore contribute to the suboptimal immune response in non-Hodgkin’s lymphoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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