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Human T_H17 Cells Are Long-Lived Effector Memory Cells

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Abstract

T helper 17 (T_H17) cells have been shown to contribute to multiple disease systems. However, the functional phenotype and survival pattern of T_H17 cells as well as the underlying mechanisms that control T_H17 cells have been poorly investigated in humans, significantly hampering the clinical targeting of these cells. Here, we studied human T_H17 cells in the pathological microenvironments of graft-versus-host disease, ulcerative colitis, and cancer; T_H17 cell numbers were increased in the chronic phase of these diseases. Human T_H17 cells phenotypically resembled terminally differentiated memory T cells but were distinct from central memory, exhausted, and senescent T cells. Despite their phenotypic markers of terminal differentiation, T_H17 cells mediated and promoted long-term antitumor immunity in in vivo adoptive transfer experiments. Furthermore, T_H17 cells had a high capacity for proliferative self-renewal, potent persistence, and apoptotic resistance in vivo, as well as plasticity—converting into other types of T_H cells. These cells expressed a relatively specific gene signature including abundant antiapoptotic genes. We found that hypoxia-inducible factor–1α and Notch collaboratively controlled key antiapoptosis Bcl-2 family gene expression and function in T_H17 cells. Together, these data indicate that human T_H17 cells may be a long-lived proliferating effector memory T cell population with unique genetic and

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SUPPLEMENTARY MATERIAL

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functional characteristics. Targeting T_H17-associated signaling pathway would be therapeutically meaningful for treating patients with autoimmune disease and advanced tumor.

INTRODUCTION

T helper 17 (T_H17) cells may contribute to many human diseases. A large body of research has mapped out the transcription factors and cytokine milieu necessary for T_H17 development and function (1–4). However, it is poorly understood how human T_H17 cells expand and survive in vivo. Human T_H17 cells are often found in peripheral tissues and organs (5–8), and it has been assumed that human T_H17 cells are effector T cells with a short life span. In support of this notion, mouse T_H17 cells may be short-lived and express low levels of CD27 (9), which is associated with memory T cell survival (10).

However, these observations contrast with the activity of T_H17 cells in multiple cancer settings. In several types of advanced human cancers, although T_H17 cells are a relatively small population compared with other T cell subsets, T_H17 cells are associated with potent antitumor immunity and positively predict improved patient survival (8, 11, 12). These data raise the possibility that T_H17 cells may have a survival and persistence advantage in humans and may contribute to long-lasting antitumor effects in advanced human cancer. In support of this hypothesis, in the adoptive T cell therapy setting where T cell persistence is critical in achieving tumor eradication, mouse T_H17 cells mediate potent tumor regression (13–15).

Here, we studied human T_H17 cells in our well-established human system (16–18) and investigated the underlying mechanisms of controlling T_H17 cell expansion, apoptosis, and survival. Our studies demonstrated that HIF-1α (hypoxia-inducible factor 1α)/Notch/Bcl-2 signaling cascade is crucial for controlling human T_H17 cell survival and apoptosis. Thus, manipulation of this signaling pathway may provide clinical benefit for patients affected by T_H17 cells, including patients with autoimmune disease, tumors, and transplantation rejection.

RESULTS

T_H17 cell numbers are elevated in sites of chronic disease

Multiple chronic human diseases, including chronic organ rejections, autoimmune diseases, and cancers, are thought to be affected by T_H17 cells. To study T_H17 cells in the microenvironments of chronic diseases, we first examined T_H17 cells in the diseased sites of acute and chronic graft-versus-host disease (GVHD), ulcerative colitis, and colon cancer. Immunohistochemistry staining revealed high numbers of interleukin-17–positive (IL-17^+) (Fig. 1A, upper panel) and $CD3^+$ T cells (Fig. 1A, lower panel) in consecutive oral mucosa tissue sections in patients with chronic, but not acute, GVHD (Fig. 1A and fig. S1A). Flow cytometric analysis demonstrated that these IL-17^+ cells were T_H17 cells, but not γδ T cells (fig. S1B). In patients with chronic ulcerative colitis, we detected high percentages of IL-17^+ T cells in diseased intestinal mucosa and adjacent tissues. These IL-17^+ T cells were T_H17, not IL-17^+CD8^+, cells (Fig. 1B). The percentages of T_H17 cells were higher in colitic tissues than in normal colon and blood (Fig. 1B and fig. S1, C and D). Because there were more T cells infiltrating colitic lesions than adjacent tissues (Fig. 1, C and D), the absolute numbers of T_H17 cells were much higher in colitic lesions than in adjacent tissues (Fig. 1D). High percentages of T_H17 cells were also found in colon cancer as demonstrated by flow cytometry analysis (Fig. 1B and fig. S1, C and D) and multiple-color fluorescence staining (Fig. 1E). Increased numbers of T_H17 cells were detected in inflammatory tonsil and spleen compared to blood (Fig. 1, F to H, and fig. S1, C and D).

Primary T_H17 cells exhibit a terminally differentiated phenotype but mediate potent antitumor immunity

We next examined the phenotype and cytokine profile of primary T_H17 cells in the microenvironments of chronic inflammation and cancer and in blood. Blood T_H17 cells were in the $CD45RA^-CD45RO^+$ population (Fig. 2A). Because intracellular staining affected the detection of certain surface antigens, we sorted $CD4^+$ T cells into $CD62L^-CCR7^-$, $CD62L^-CCR7^+$, and $CD62L^+CCR7^+$ populations and subsequently examined IL-17 expression. Fewer than 0.2% of T_H17 cells were found in $CD62L^-CCR7^+$ and $CD62L^+CCR7^+$ populations. IL-17 expression was primarily confined to $CD62L^-CCR7^-$ T cells (Fig. 2B). Further phenotypic analysis demonstrated that T_H17 cells did not express PD-1, KLRG-1, and CD57 (Fig. 2C).

Next, we compared the expression levels of some survival markers on primary T_H0, T_H1, and T_H17 cells. Primary T_H subsets were defined on the basis of specific T_H signature cytokine expression in fresh cells with multiple flow gating (fig. S2). Flow analysis revealed that the percentage and the mean fluorescence intensity (MFI) of CD28 were higher in T_H17 cells than in T_H0 and T_H1 cells (Fig. 2D). T_H17 cells also expressed higher levels of CD127 than T_H0 and T_H1 cells. However, we found that T_H17 cells expressed moderately lower amounts of CD27 and higher amounts of CD95 than their counterpart T_H1 and T_H0 cells (Fig. 2D). We further examined the effector function of primary T_H17 cells. Primary T_H17 cells expressed high levels of IL-2 and TNF-α (tumor necrosis factor–α) and moderate amounts of IFN-γ (interferon-γ) in blood and in the microenvironments of inflammatory tonsil, ulcerative colitic tissue, and colon cancer (Fig. 2, E to H, and fig. S3). The phenotype suggests a terminally differentiated effector phenotype for T_H17 cells.

Because T_H17 cells exhibit a phenotype of terminal differentiation, we hypothesized that T_H17 cells may have poor effector function. To test this hypothesis, we enriched and sorted primary T_H17 cells on the basis of a $CD4^+CD3^+CCR6^+CD161^+CD45RA^-CD45RO^+$ phenotype (figs. S2A and S4A). These primary T_H17 cells were 99% $CD161^+ROR\gamma t^+$ (fig. S4, B and C), produced high amounts of intracellular IL-17 (fig. S4, D and E), and released large amounts of IL-17 (fig. S4F). IL-1 and IL-23 further increased their production of IL-17 (fig. S4F). IL-1– and IL-23–activated primary T_H17 cells expressed moderate levels of effector cytokines (fig. S4G). We next generated tumor antigen–associated specific autologous $CD8^+$ T cells. Activated primary T_H17 cells (fig. S4) or tumor antigen–associated specific autologous $CD8^+$ T cells were transferred to female nonobese diabetic (NOD)/Shi-scid/IL-2Rγnull (NSG) mice bearing ovarian cancer. As expected, $CD8^+$ T cell transfusion resulted in reduced tumor growth. Unexpectedly, T_H17 cells slowed tumor growth as well. Furthermore, the effects of T_H17 cells were superior to that of $CD8^+$ effector T cells from the same donors (Fig. 2I).

It is not known whether human T_H17 cells affected $CD8^+$ T cell–mediated antitumor immunity. To test this, we cotransfused T_H17 and $CD8^+$ T cells to NSG mice. We observed that tumor volume was much smaller in mice that received the cotransfusion than in those that received a single transfusion. More markedly, the immune protection mediated by cotransfusion lasted significantly longer than T_H17 or $CD8^+$ cells alone (Fig. 2I). These data indicate that T_H17 and $CD8^+$ T cells collaboratively mediate long-term antitumor immunity.

T_H17 cells give rise to other T_H cell subsets

The plasticity of mouse T_H17 cells may be one of the functional determinants in T_H17 cell biology (19–21). To test human T_H17 cell plasticity, we cultured these cells under polarizing conditions. Under the stimulation of IL-2 and TGFγ (transforming growth factor–β), T_H17 cells expressed Foxp3 (Fig. 3, A and B). Under T_H1 polarization condition, an important

fraction of T_H17 cells expressed IFN-γ (Fig. 3, C and D). In support of this, $Foxp3^+IL\text{-}17^+CD4^+$ T cells (Fig. 3E) and IFN-$\gamma^+IL\text{-}17^+CD4^+$ T cells (Fig. 3F) were observed in pathological environments. These $Foxp3^+IL\text{-}17^+CD4^+$ T cells are functional T regulatory (T_{reg}) cells (22). Furthermore, 40 to 60% of the cells retained IL-17 expression in T_H1 and T_{reg} polarization conditions (Fig. 3, A to D).

T_H17 cells have better persistence in vivo

In vivo persistence is a key feature for effector T cells to mediate antitumor immunity. We compared the in vivo persistence of T_H1, T_H2, and T_H17 cells in the NSG model. We first polarized T_H subsets from the same donor (fig. S5). Then, we equally mixed autologous human leukocyte antigen-A2–positive ($HLA\text{-}A2^+$) T_H17, T_H1, or T_H2 cells with $HLA\text{-}A2^-CD4^+$ T cells, transferred these cells into NSG mice, and followed their persistence in vivo. Five days after transfusion, we showed that there were more T_H17 than T_H1 and T_H2 cells in different organs (Fig. 3G) including spleen (Fig. 3H).

We hypothesized that the in vivo persistence of T_H17 cells may be associated with a particular gene signature. Gene arrays showed that T_H17 cells expressed higher levels of stem cell–associated genes than autologous $IL\text{-}17^-$ control T cells (Fig. 3I). Real-time polymerase chain reaction (PCR) demonstrated that the levels of *NANOG*, *SOX2*, and *OCT3/4* (Fig. 3J); Notch signaling genes (Fig. 3K); Wnt/β-catenin signaling genes (fig. S6A); and *FOXO3*, *MYC*, and *PIM2* (fig. S6B) were higher in T_H17 cells than in control.

T_H17 cells have increased proliferative capacity

Because T_H17 cells exhibit a terminally differentiated phenotype, we hypothesized that T_H17 cells had low proliferating potential. Unexpectedly, upon stimulation, there was more thymidine incorporation in the primary T_H17 cells than in autologous $IL\text{-}17^-$ T cells (Fig. 4A). In a similar setting, carboxyfluorescein succinimidyl ester (CFSE)–labeled primary T_H17 cells underwent more cell divisions than $IL\text{-}17^-$ T cells (Fig. 4B). We also examined the expansion potential of polarized T cells. T cell receptor (TCR) engagement induced the proliferation of polarized T_H1, T_H2, and T_H17 cells. However, the absolute numbers of polarized T_H17 cells were higher than those of T_H1 and T_H2 cells (Fig. 4C). We cultured primary T_H17 cells and autologous $IL\text{-}17^-$ T cells with IL-7 and IL-15. T_H17 cells were more efficiently expanded than control T cells (Fig. 4D). T_H17 and control T cells were separately cultured in this experiment (Fig. 4D) to ensure that all T cells were stimulated in an identical manner. We mixed $HLA\text{-}A2^+$ T_H17 cells with $HLA\text{-}A2^-IL\text{-}17^-$ T cells and cultured these cells with IL-7 plus IL-15. Again, there were more $HLA\text{-}A2^+$ T_H17 cells than $HLA\text{-}A2^-IL\text{-}17^-$ T cells in the culture (Fig. 4E).

We next investigated the capacity of human T_H17 cells to expand in vivo. T_H17 cells were transferred into NSG mice with 5-bromo-2′-deoxyuridine (BrdU) administration. Consistent with the in vitro data, there were more T_H17 cells than control T cells in S phase (Fig. 4F). There were 15% T_H17 cells and 7% control cells in G_1–G_2 phases, indicating that T_H17 cells efficiently entered G_1–G_2 phase. Furthermore, there were more $Ki67^+$ cells in primary T_H17 than T_H1 and T_H0 cell populations in vivo in blood in healthy humans and in colon cancer tissues (Fig. 4G). In accord with this observation, the expression levels of multiple cyclin genes were higher in primary (Fig. 4H) and polarized (Fig. 4I) T_H17 than in the control cells. On the contrary, the expression of multiple cyclin-dependent repressors was lower in primary (Fig. 4J) and polarized (Fig. 4K) T_H17 than in the control cells.

T_H17 cells are resistant to apoptosis

Because T_H17 cells exhibited a terminally differentiated phenotype and expressed CD95 (Fig. 2), T_H17 cells might be more prone to apoptosis. However, in healthy humans, caspase 3^+ T cells were IL-17^- T cells (Fig. 5A).

We next cultured polarized T_H1, T_H2, and T_H17 cells and kinetically measured T cell apoptosis after in vitro TCR activation. We consistently observed lower numbers of apoptotic T_H17 cells than T_H1 and T_H2 cells (Fig. 5B). We also examined T cell apoptosis induced by chemotherapeutic agents. When exposed to an optimal concentration of cisplatin, there were less annexin V^+ T_H17 cells compared to other T cells (Fig. 5C). In patients with ovarian cancer, one cycle of cisplatin treatment in combination with paclitaxel resulted in decreased IFN-γ and IL-6 (Fig. 5, D and E) and increased IL-17 (Fig. 5F) produced by activated blood mononuclear cells. Because IL-17 is primarily derived from T_H17 cells in patients with ovarian cancer (8), the data indicate that primary T_H17 cells are resistant to chemotherapy-mediated cell death in vivo. In further support, after multiple cycles of chemotherapy in a patient with ovarian cancer, the amounts of IL-6 were reduced, whereas IL-17 production was initially increased and then slightly reduced in malignant ascites (Fig. 5G). These data suggest that apoptosis resistance of primary T_H17 cells may not be linked to IL-6 signaling. We further explored whether T_H17 apoptosis resistance was associated with the effects of IL-6 and IL-23 during T_H17 cell differentiation. We polarized T_H17 cells from naïve T cells with antigen-presenting cells (7, 8) (fig. S5A) and blocked IL-6 signaling with anti–IL-6, and IL-23 signaling with small interfering RNA (siRNA)–IL-23 (7). Anti–IL-6 (fig. S7, A and B) and siRNA–IL-23 (fig. S7, C and D) had no effects on T cell viability. Moreover, T_H17 cells expressed CD95 (Fig. 2D); however, CD95 engagement did not alter T_H17 cell apoptosis. In line with these observations, T_H17 cells expressed high amounts of *BCL2* and *BCLXL* (Fig. 5, H and I).

HIF-1α regulates T_H17 cell apoptosis and persistence

We next examined why T_H17 cells were less apoptotic and efficiently persisted in vivo. Low oxygen pressures exist in many solid tissues. One key element in cellular adaptation for survival to hypoxia is induced expression of HIF-1. We investigated whether apoptosis resistance and persistence of T_H17 cells are associated with HIF-1α. As expected, primary (Fig. 6A) and polarized T_H17 cells (Fig. 6B) expressed higher amounts of HIF-1α compared to IL-17^- T, T_H1, and T_H2 cells.

We tested the role of HIF-1α in T_H17 cell persistence in vivo in NSG mice. We equally mixed IL-17^+HLA-$A2^+$ T_H17 cells and IL-17^-HLA-$A2^-CD4^+$ T cells and treated these cells with phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), and the HIF-1 inhibitor echinomycin (fig. S8A) (23). The mixed cells were transfused into NSG mice. In the HIF-1 inhibitor–treated group, the number of IL-17^+HLA-$A2^+$ T_H17 cells recovered from mouse spleen was reduced by 20% within 36 hours (Fig. 6C). These data suggest that HIF-1 blockade reduces T_H17 cell persistence in vivo. We confirmed that the recovered human T cells from mouse spleen expressed limited *HIF1A* (Fig. 6D) and reduced *IL17A* (Fig. 6E), whereas the expression of human *IFNG* and *CD3* was not changed (fig. S9, A and B).

We further dissected whether HIF-1 blockade affected T_H17 cell apoptosis in vivo. T_H17 cells were initially treated with DMSO or HIF-1 inhibitor and subsequently transfused into NSG mice. After 48 hours, we showed that there were more annexin V^+ T_H17 cells in the HIF-1 inhibitor–treated group than in the control group (Fig. 6F). Furthermore, HIF-1 blockade reduced *BCL2* expression in T_H17 cells (Fig. 6G). Thus, HIF-1 blockade increases T_H17 cell apoptosis in vivo.

We further examined whether HIF-1 blockade had an impact on T_H17 cell apoptosis during T cell activation and chemotherapy (Fig. 5). T_H17 and T_H1 cells were activated in the presence of HIF-1 inhibitor for 3 days. The number of T_H17 cells was reduced by 28%, whereas there was only 5% reduction for T_H1 cells (Fig. 6H).

We genetically blocked HIF-1α with small hairpin HIF-1 (shHIF-1) (fig. S8B) and examined T_H17 cell apoptosis in response to cisplatin treatment. Genetic HIF-1 blockade increased the number of annexin V^+ T_H17 cells compared to scramble controls (Fig. 6I). In line with this observation, the expression of *BCL2* (Fig. 6J) and *BCLXL* (Fig. 6K) was inhibited by genetic HIF-1 blockade. These data indicate that HIF-1 is crucial for controlling the survival/apoptosis and persistence of T_H17 cells in multiple experimental settings.

HIF-1α is linked to Notch and Bcl-2 family and regulates T_H17 cell biology

We further hypothesized that HIF-1α targeted the Bcl-2 family and in turn controlled T_H17 cell survival and apoptosis. Unexpectedly, in the promoter binding and activity assays, HIF-1α and Myc (control) were not capable of activating the *BCL2* proximal promoter (Fig. 7A, lower panel). Because T_H17 cells expressed high amounts of Notch signaling genes (Fig. 3K), we next examined whether the Notch pathway was involved in the regulation of Bcl-2 family gene expression and function. The intracellular active domain of Notch (Notch-IC) stimulated *BCL2* promoter activities (Fig. 7A, lower panel). As confirmation, Notch-IC activated key Notch signaling genes (fig. S10). In line with this, there were multiple Notch binding sites in the *BCL2* promoter area (Fig. 7A, upper panel). Furthermore, Notch inhibition resulted in reduced expression of *BCL2* and *BCLXL* (Fig. 7, B and C) and more annexin V^+ T_H17 cells (Fig. 7D). We confirmed that Notch activation induced the expression of Bcl-2 on T_H17 cells (Fig. 7E).

Because both HIF-1 (Fig. 6) and Notch (Fig. 7, A to E) signaling pathways regulate T_H17 cell survival and apoptosis, we further examined the mechanistic relationship between these two pathways. It is possible that HIF-1 might directly activate and maintain Notch signaling gene expression and in turn regulate Bcl-2 family gene expression and function through Notch signaling pathway. In support of this, genetic blockade of HIF-1α reduced the expression of key Notch signaling genes in T_H17 cells (Fig. 7F). Furthermore, althoughHIF-1 blockade induced T_H17 cell apoptosis (Figs. 6 and 7G), when T_H17 cells were cotransfected with shHIF-1 and Notch-IC, Notch activation partially but importantly reduced T_H17 cell apoptosis mediated by HIF-1 blockade (Fig. 7G). The results indicate that Notch activation may independently regulate T_H17 cell survival and apoptosis, whereas HIF-1 may regulate T_H17 cells through activating and maintaining the Notch signaling pathway.

DISCUSSION

Here, we have reported several findings: (i) human T_H17 cells are long-lived cells with a high capacity for expansion and are resistant to apoptosis, (ii) HIF-1α/Notch/Bcl-2 is a key signaling pathway controlling the T_H17 cell survival and apoptosis pattern, and (iii) human T_H17 cells persist in vivo and mediate or promote long-term antitumor immunity.

Regardless of their tissue origins, T_H17 cells are confined to the memory T cell compartment with a phenotype of $CD28^+CD127^+PD\text{-}1^-Foxp3^-KLRG\text{-}1^-CD57^-IL\text{-}10^-$. This phenotype makes them unlikely candidates for being exhausted $PD\text{-}1^+$ T cells, suppressive $Foxp3^+$ or $IL\text{-}10^+$ T cells, or senescent $CD28^-CD57^+KLRG\text{-}1^+$ T cells. However, T_H17 cells express high amounts of CD95 and lower amounts of CD27 and produce effector cytokines (8). Thus, T_H17 cells exhibit a terminally differentiated effector cell phenotype.

Although T_H17 cells have a terminal differentiation phenotype, they express high levels of Ki67 and efficiently expand. T_H17 cells are resistant to apoptosis induced by different stimuli, have better persistence in vivo, and mediate potent antitumor immunity. Under specific conditions, in line with mouse studies (19–21), T_H17 cells can be differentiated into T_H1 and T_{reg} cells, indicating their high plasticity. These characteristics endow unique features for human T_H17 cells. The long-lived capacity can help maintain a constant repertoire of memory T_H17 cells for a human lifetime despite the finite life span of individual effector cells and reduced thymus function.

Genetic and molecular experiments have demonstrated that *BCL* family genes control T_H17 cell survival and apoptosis resistance. Human T_H17 cells express high levels of Bcl-2 family members. In support of our observations on human T_H17 cells, high amounts of *BCL2* expression are associated with long-term survival in viral-specific $CD8^+$ T cells in mice (24) and with memory $CD8^+$ T cell renewal in a mouse bone marrow transplantation model (25).

We have further explored the molecular mechanisms controlling the expression and function of the Bcl-2 family in T_H17 cells. It has been proposed that hypoxic environment is required for cancer stem cell function and HIF-1 is important for maintaining an active niche for long-term hematopoietic stem cells (HSCs) (26). Hypoxia is a common phenomenon in inflammatory and tumor tissues (27, 28). Human tissue T_H17 cells express high amounts of *HIF1A.* Our gain- and loss-of-function experiments demonstrate that the HIF-1α signaling pathway is crucial for the expression of Bcl-2 family members. However, HIF-1α does not directly target the *BCL2* promoter and control its expression; instead, the promoter activity assay revealed that Notch, but not HIF-1α, activates *BCL2* promoter. There were multiple binding sites for Notch binding on the *BCL2* promoter. Furthermore, similar to HIF-1α, Notch controlled the expression and functions of the Bcl-2 family in T_H17 cells and affected T_H17 cell survival and apoptosis. The data point toward an interaction between HIF-1α and Notch signaling pathways in regulating T_H17 cell biology. In support of this, we have uncovered that HIF-1α controlled Notch signaling gene expression. However, genetic HIF-1α blockade did not disable the effects of Notch activation on the expression and function of the Bcl-2 family on T_H17 cells. These data suggest that Notch signaling may independently regulate T_H17 cell function. Therefore, HIF-1α activation is important for maintaining T_H17 cell survival and apoptosis through activating the Notch signaling pathway, whereas the collaboration between HIF-1α and Notch signaling pathways promotes and maintains T_H17 functional integrity.

The next question is to identify the key downstream gene targets of the HIF/Notch/Bcl-2 signaling pathway, which may be important to determine T_H17 cell biology. T_H17 cells express high levels of cyclins and reduced CDK repressors. CDK repressors contribute to multiple types of cellular senescence and exhaustion, and suppression of $p16^{Ink4a}$ and $p19^{Arf}$ is essential for HSC self-renewal (29). T_H17 cells also express high amounts of Wnt/β-catenin–associated genes. Certain mouse $CD8^+$ memory T cells have increased activity ofWnt/β-catenin pathway and mediate potent antitumor immunity (30). Further experiments are warranted to examine the importance of the Wnt/β-catenin pathway in human T_H17 cell biology.

T_H17 cells express high amounts of CD95, which may be a stem cell–associated gene (31). Indeed, CD95 engagement does not induce T_H17 cell apoptosis. T_H17 cells express relatively lower amounts of CD27. This observation is consistent with levels reported on mouse T_H17 cells (9). Given the importance of CD27 in T cell survival (10), it is thought that mouse T_H17 cells are a short-lived population (9). In humans, after activation, human primary T_H17 cells quickly acquire high levels of CD27 expression. CD27 expression would not be a limiting factor for T_H17 cell survival. Furthermore, T_H17 cells express high

amounts of the IL-7 receptorα, CD127. IL-7 and IL-15 induce T_H17 cell expansion. Because these two cytokines maintain and promote the memory $CD8^+$ T cell pool, the data suggest that T_H17 cells share some properties with memory $CD8^+$ T cells.

The genetic and functional features of human T_H17 cells we described here may be therapeutically important in T cell–based immunotherapy for cancer and in molecule-based immunotherapy for autoimmune disease. Mouse T_H17 cells induce tumor eradication (13, 14, 32), and ICOS (inducible costimulator)–expanded human T_H17 cells mediate antitumor activity (33). In line with these reports, we have demonstrated that human T_H17 cells execute/promote potent long-term antitumor effects in vivo and are positively associated with patient survival in human ovarian cancer (8). Experimental and clinical evidence indicates that expansion potential, persistence, survival, and capacity for apoptosis are key factors that determine the therapeutic efficacy of adoptive $CD8^+$ cell therapy (15). We analogically reason that potent antitumor immunity mediated and promoted by T_H17 cells may be partially due to their important functional features including high expansion potential, resistance to apoptosis, long-lived capacity, and high plasticity. However, the in vivo T_H17 cell dynamics and evolution and the relative contribution of each functional feature to human diseases remain to be dissected. The importance of HIF/Notch/Bcl-2 signaling pathway in T_H17 cell biology suggests that enforced HIF/Notch/Bcl-2 expression and activation may further promote this interesting feature and improve T_H17-mediated antitumor immunity. Thus, human T_H17 cells may be used to treat patients with advanced cancer in combination with $CD8^+$ T cell therapy or other immune therapeutic regimens. In a similar vein, disruption of HIF/Notch/Bcl-2 expression and activation may promote T_H17 cell apoptosis and in turn disable the pathologic effects of T_H17 cells in patients with GVHD and autoimmune diseases.

In conclusion, we have provided evidence at the genetic, molecular, and functional level that human T_H17 cells are long-lived cells. This property may be critically important for controlling T_H17 cell biology. We suggest that manipulation of T_H17 cell apoptosis and survival may be therapeutically interesting for treating patients with chronic diseases affected by T_H17 cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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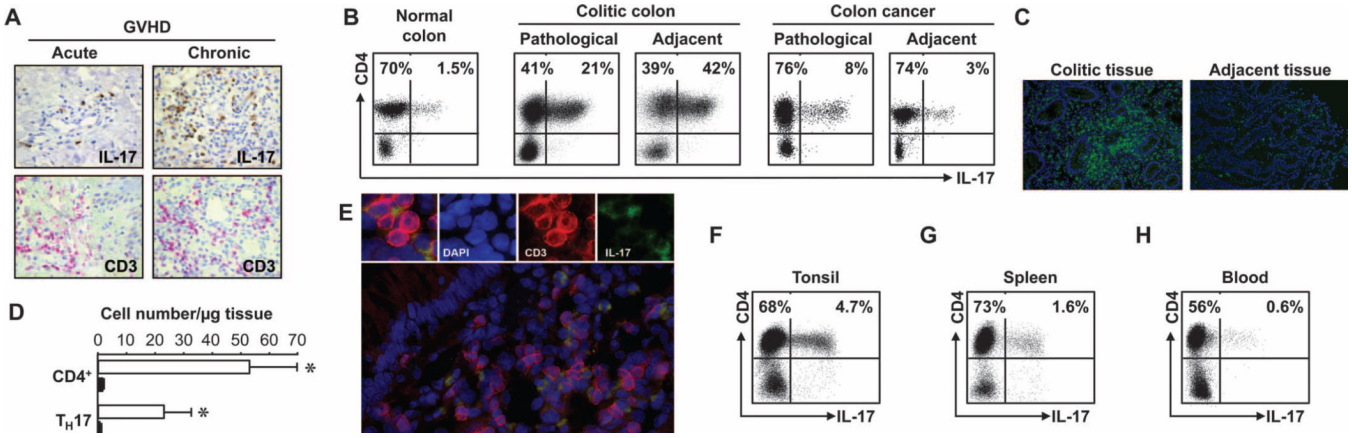

Fig. 1.

High amounts of T_H17 cells are found in sites of chronic diseases. Case numbers: blood (31), oral mucosa (6), spleen (5), tonsil (6), colon cancer (21), and colitis (12). (**A**) High numbers of IL-17$^+$ cells were detected in oral mucosa tissues in patients with GVHD. Consecutive sections of oral mucosa biopsies were stained for IL-17 (brown) or CD3 (red). $P < 0.001$, acute versus chronic. (**B**) High percentages of T_H17 cells were found in colitic colon and colon cancer tissues. Tissue single cells were stained for T_H17 cell markers, analyzed by flow cytometry, and gated on CD3$^+$ cells. (**C** and **D**) CD4$^+$ and T_H17 cells were found in colitic colon tissues. Colon tissues were stained for CD3 (green) (C). Absolute numbers of CD4$^+$ and T_H17 cells were quantified based on flow cytometry analysis. Results are expressed as the absolute numbers of CD4$^+$ or T_H17 per microgram tissue ± SEM (D). $P < 0.001$, colitic lesion (empty bars) versus adjacent tissue (filled bars). (**E**) Colon tissues were stained for CD3 and IL-17, and analyzed with a fluorescence microscope. Red, CD3; green, IL-17. DAPI, 4′,6-diamidino-2-phenylindole. (**F** to **H**) T_H17 cells were found in different organs. Single cells were stained for T_H17 cell markers and analyzed by flow cytometry. $P < 0.001$, tonsil and spleen as compared with blood.

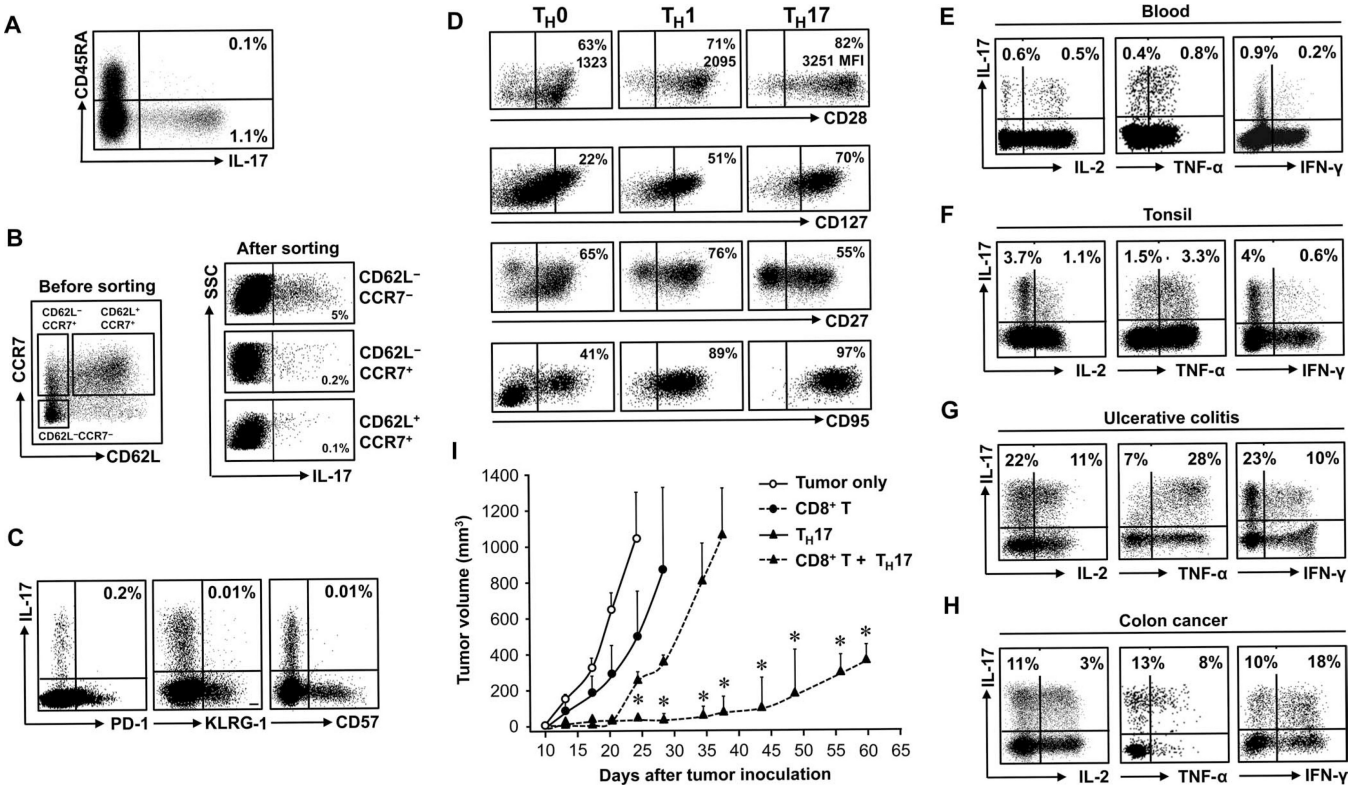

Fig. 2.

Primary T_H17 cells exhibit a terminally differentiated phenotype but mediate potent antitumor immunity. (**A** to **D**) Tissue or blood cells were stained for the indicated markers and cytokines and analyzed by flow cytometry. Results are expressed as the percentage of the specific subset of $CD4^+$ T cells. $n = 10$ to 25. (A) T_H17 cells were in the $CD45RO^+CD45RA^-$ memory T subset. (B) Relationship between T_H17 cells and expression of CCR7 and CD62L. Three $CD4^+$ T cell subsets were sorted and stained for IL-17. (C) Relationship between T_H17 cells and expression of CD57, KLRG-1, and PD-1. (D) Expression of the described markers on primary T_H0, T_H1, and T_H17 cells. $P < 0.001$, for CD28 and CD95 expression on T_H17 as compared with T_H1 and T_H0 cells, and $P < 0.05$, for CD127 and CD27 expression on T_H17 as compared with T_H1 and T_H0 cells. (**E** to **H**) Effector cytokine profile of primary T_H17 cells in different tissues gated on $CD4^+$ T cells. (**I**) T_H17 cells mediate and promote tumor regression in vivo. Ovarian cancer–bearing NSG mice (16, 17) received PBS, T_H17, and/or $CD8^+$ T cells. Mean ± SEM of tumor volumes are shown ($n = 4$ to 6 mice per group). $^*P < 0.05$.

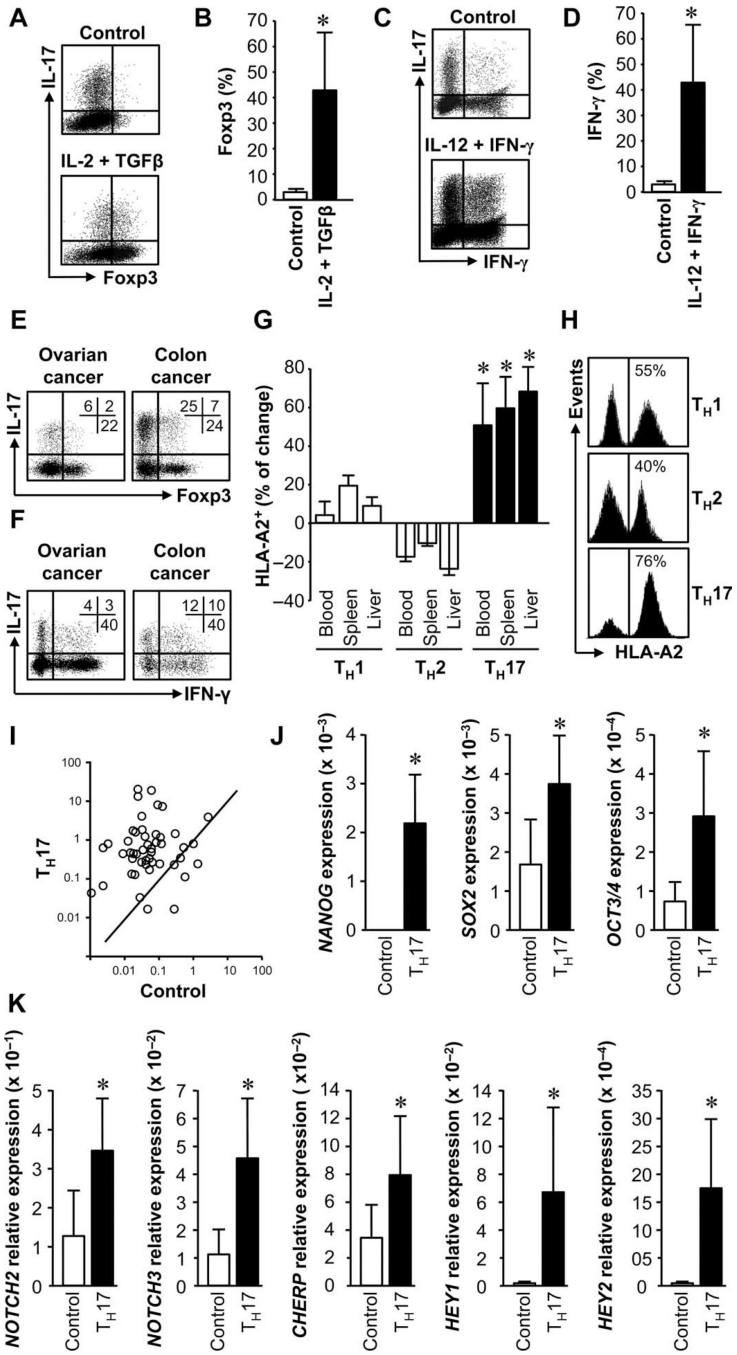

Fig. 3.

T_H17 cells have functional and genetic stem cell–like features. (**A** to **D**) T_H17 cells gave rise to other T_H cells. Primary T_H17 cells were stimulated with the cytokine milieu for T_{reg} (A and B) or T_H1 (C and D) induction for 3 days and analyzed by flow cytometry. Results are expressed as the percentage of $Foxp3^+$ (B) or IFN-g^+ cells (D) in T_H17 cells. $n = 5$. $^*P < 0.05$. (**E** and **F**) IL-$17^+$$Foxp3^+$(E) and IL-$17^+$IFN-$\gamma^+$(F) T cells in cancers. Colon ($n = 9$) and ovarian cancer ($n = 25$) T cells were analyzed by flow cytometry. (**G** and **H**) T_H17 cells had superior in vivo persistence. HLA-$A2^+$ T_H subsets were mixed with HLA-$A2^-$IL-$17^-$$CD4^+$ T cells and transfused to NSG mice. On day 5, human T cells were analyzed. (**I**) The

percentage of changes between the initial and the recovered ratios of HLA-$A2^+$ and HLA-$A2^-$ cells was calculated. Three paired donors. *$P < 0.001$. (**J**) Representative histogram shows different T subsets (HLA-$A2^+$) in spleen. (I to **K**) T_H17 cells expressed stem cell genes. SuperArray was performed in primary T_H17 and controls (I). Selective stem cell genes were quantified (J and K). $n = 8$. $P < 0.05$.

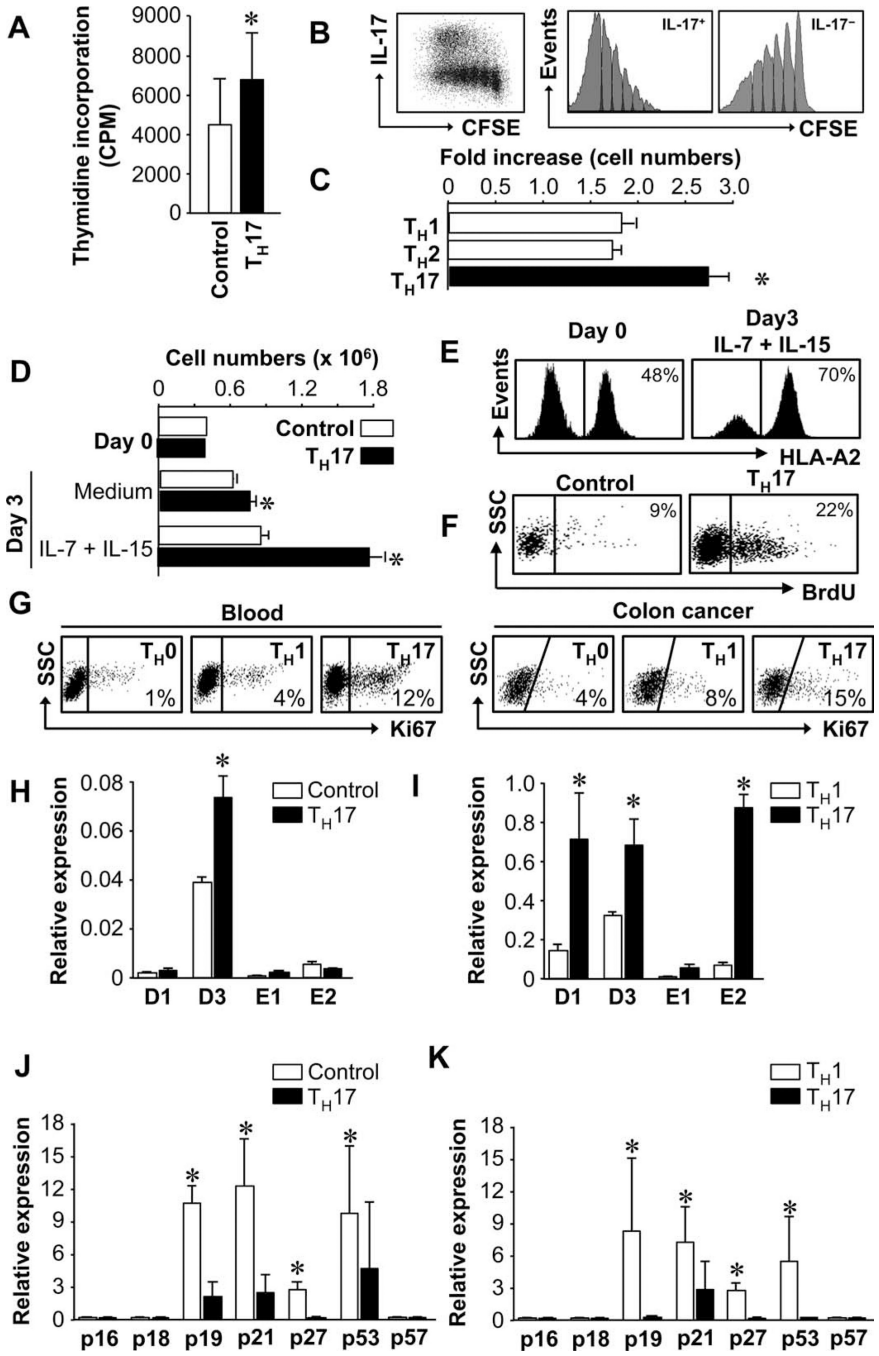

Fig. 4.

T_H17 cells have increased proliferative capacity. (**A** to **C**) T_H17 cells had potent expansion capacity. Primary (A and B) and polarized T_H subsets (C) were stimulated with TCR for 3 (A and C) or 14 (B) days. Cell proliferation/expansion was detected by [^{3}H]thymidine incorporation (A), CFSE dilution (B), or increased cell numbers (C). $n = 3$. *$P < 0.05$. (**D** and **E**) Cytokines stimulated T_H17 expansion. Primary HLA-A2$^+$ T_H17 and HLA-A2$^-$IL-17$^-$ control T cells were separately cultured (D) or initially mixed and cocultured (E) for 3 days with IL-7 plus IL-15. The absolute cell numbers (D) or the percentage (E) of T_H17 cells was determined by flow cytometry. $n = 3$. *$P < 0.05$. (**F**) T_H17 cells were in S

phase. NSG mice received T_H17 or control cells and BrdU. Cell cycling phase was analyzed on day 5 by flow cytometry to determine $BrdU^+$ human T cells in mouse spleen. $n = 2$. (**G**) T_H17 cells expressed increased Ki67 expression in blood and colon cancer. Eight to 10 donors. $P < 0.05$, T_H17 as compared with T_H1/T_H0. (**H** to **K**) T_H17 cells expressed different amounts of cell cycling genes. Expression of multiple cyclin genes (H and I) and CDK inhibitors (J and K) was quantified in primary (H and J) and polarized T_H17 cells (I and K). $n = 8$. $*P < 0.05$.

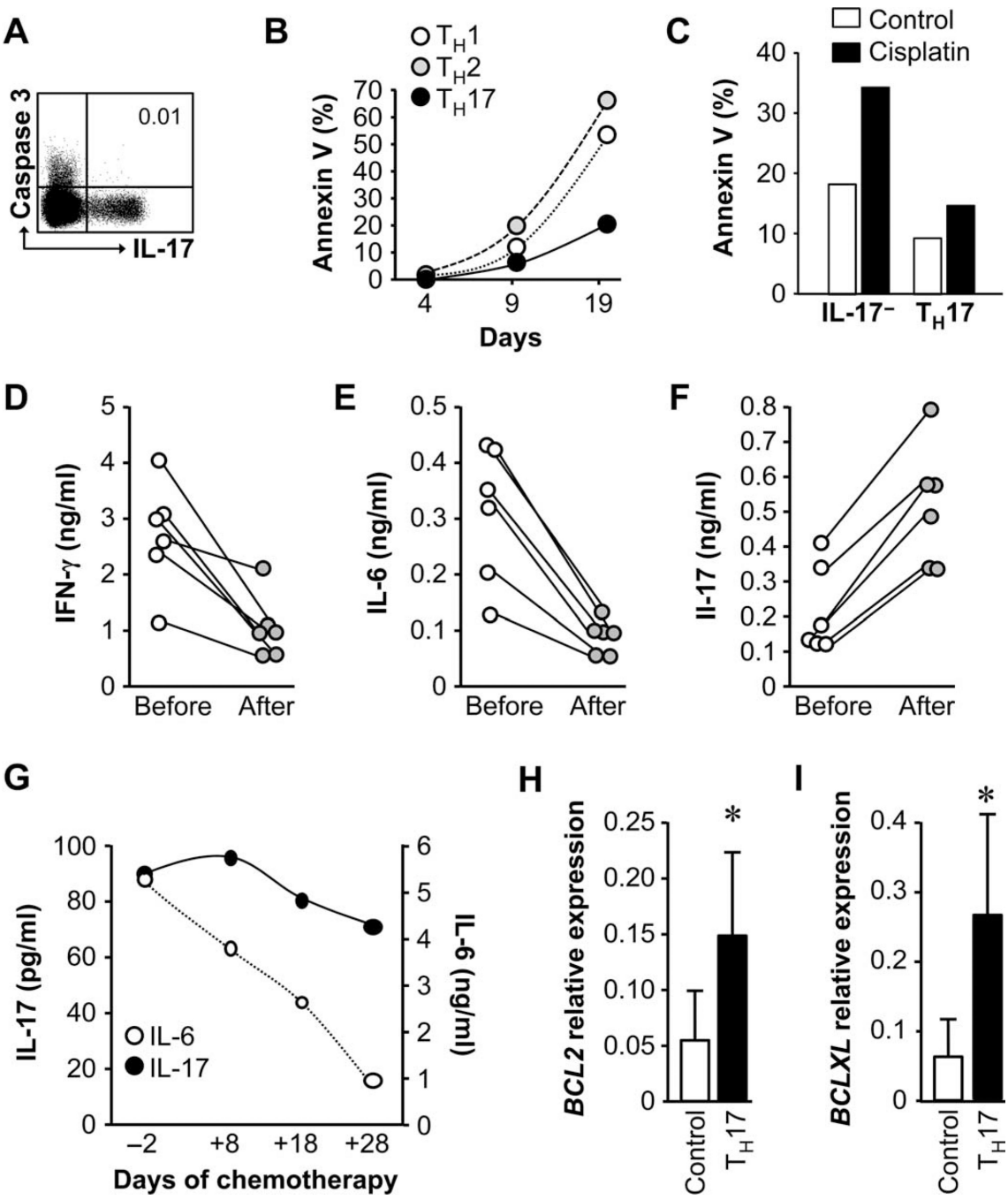

Fig. 5.

T_H17 cells are resistant to apoptosis. (**A**) T_H17 cells did not express caspase 3. The expression of caspase 3 in T cells was analyzed by flow cytometry. $n = 4$. (**B**) T_H17 cells were resistant to apoptosis induced by TCR activation. T subsets were stimulated with anti-CD3, and cell apoptosis was analyzed with annexin V staining. $n = 5$. $P < 0.01$, T_H17 as compared with T_H1/T_H2 on day 19. (**C**) T_H17 cells were resistant to apoptosis induced by in vitro cisplatin. Primary T_H17 and control cells were treated with cisplatin for 48 hours. Annexin V^+ cells were analyzed. $n = 4$. $P < 0.01$. (**D** to **F**) Chemotherapy increased IL-17 production. Ovarian cancer patients received one cycle of cisplatin therapy. Blood

mononuclear cells were activated, and cytokines were measured in the supernatants with enzyme-linked immunosorbent assay (ELISA). $n = 6$. $P < 0.001$. (**G**) Chemotherapy reduced IL-6 but not IL-17 production in ovarian cancer ascites. Ovarian cancer patient was treated with cisplatin. Cytokines were measured in the ascites fluid by ELISA. Left scale shows IL-17 (filled circles), and right scale shows IL-6 (empty circles). (**H** and **I**) T_H17 cells spontaneously expressed high levels of *BCL2* family genes. Real-time PCR was performed in primary T_H17 and control cells for *BCL2* (H) and *BCLXL* (I). $n = 5$. $*P < 0.001$.

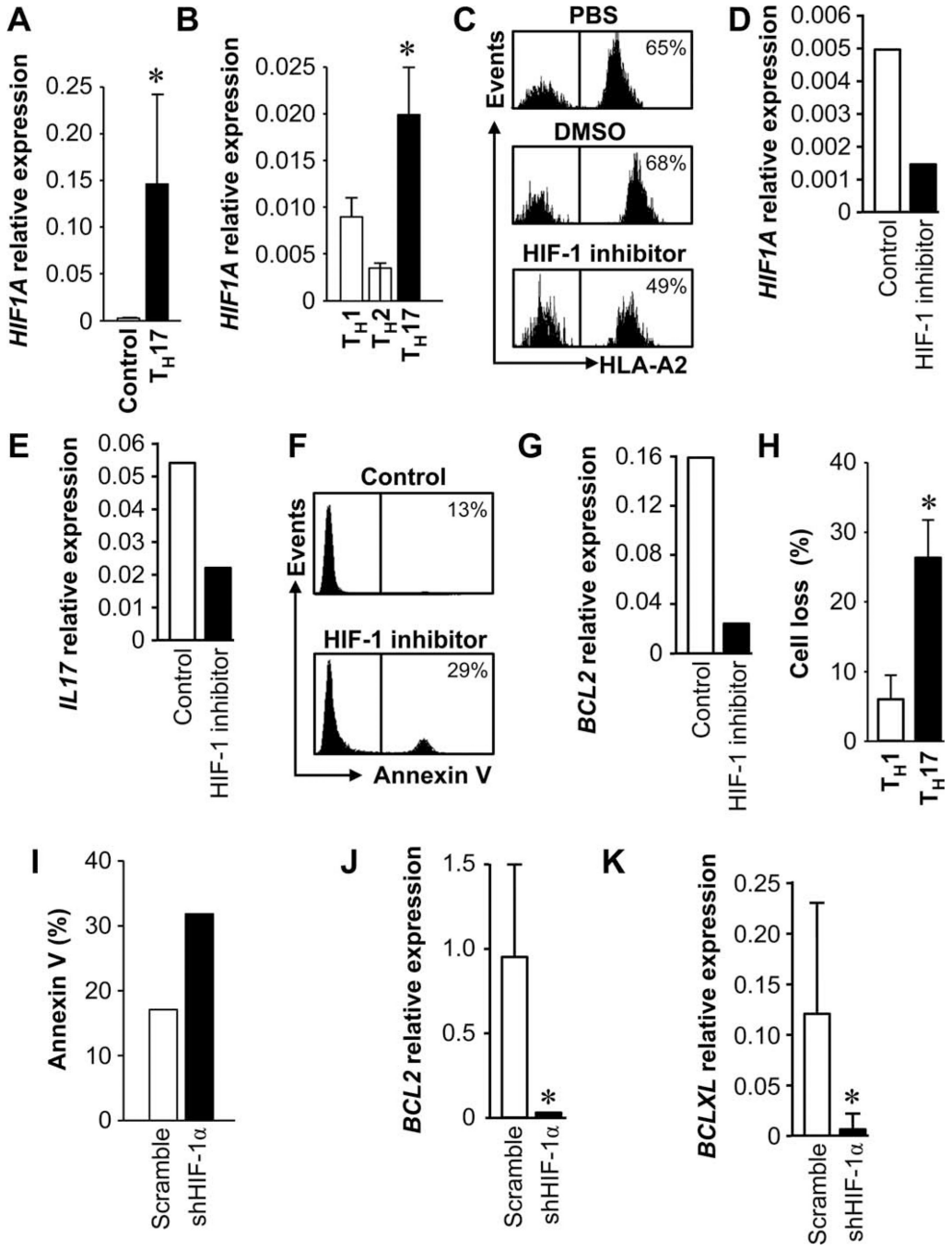

Fig. 6.

HIF-1α regulates T_H17 cell apoptosis and persistence. (**A** and **B**) T_H17 cells expressed *HIF1A*. *HIF1A* expression was quantified by real-time PCR in primary T_H17 (A) and polarized T cell subsets (B). $n = 5$ to 8. $^*P < 0.05$. (**C** to **E**) HIF-1 blockade reduced T_H17 persistence in vivo. Primary HLA-$A2^+$ T_H17 cells and HLA-$A2^-$IL-17^-CD4^+ T cells were mixed and pretreated with PBS, DMSO, and the HIF-1 inhibitor echinomycin for 48 hours and transfused to NSG mice. Human T cells were stained and analyzed for HLA-A2 and human CD5 (C), and human *HIF1A* (D) and *IL17A* (E) were quantified in the spleen. $n = 5$. (**F** and **G**) HIF-1 blockade increased T_H17 apoptosis in vivo. Primary T_H17 cells were

pretreated with HIF-1 inhibitor or DMSO and transferred to NSG mice. After 2 days, annexin V expression (F) and *BCL2* gene expression (G) were analyzed in human T cells in mouse spleen. $n = 6$. $P < 0.05$. (**H**) HIF-1 blockade increased T_H17 apoptosis in vitro. Enriched primary T_H17 cells were transfected with lentiviral vector encoding shHIF-1α and then activated with TCR for 3 days. T_H17 and T_H1 cell numbers were counted, and the percentage of cell loss was calculated. $n = 3$. $P < 0.05$. (**I** to **K**) HIF-1 blockade increased T_H17 apoptosis induced by chemotherapy. Primary T_H17 cells were transfected with lentiviral vector encoding shHIF-1 or scramble and cultured with cisplatin for 48 hours. Cell apoptosis was analyzed with annexin V expression (I). *BCL2* (J) and *BCLXL* (K) genes were quantified. $n = 7$. $P < 0.01$.

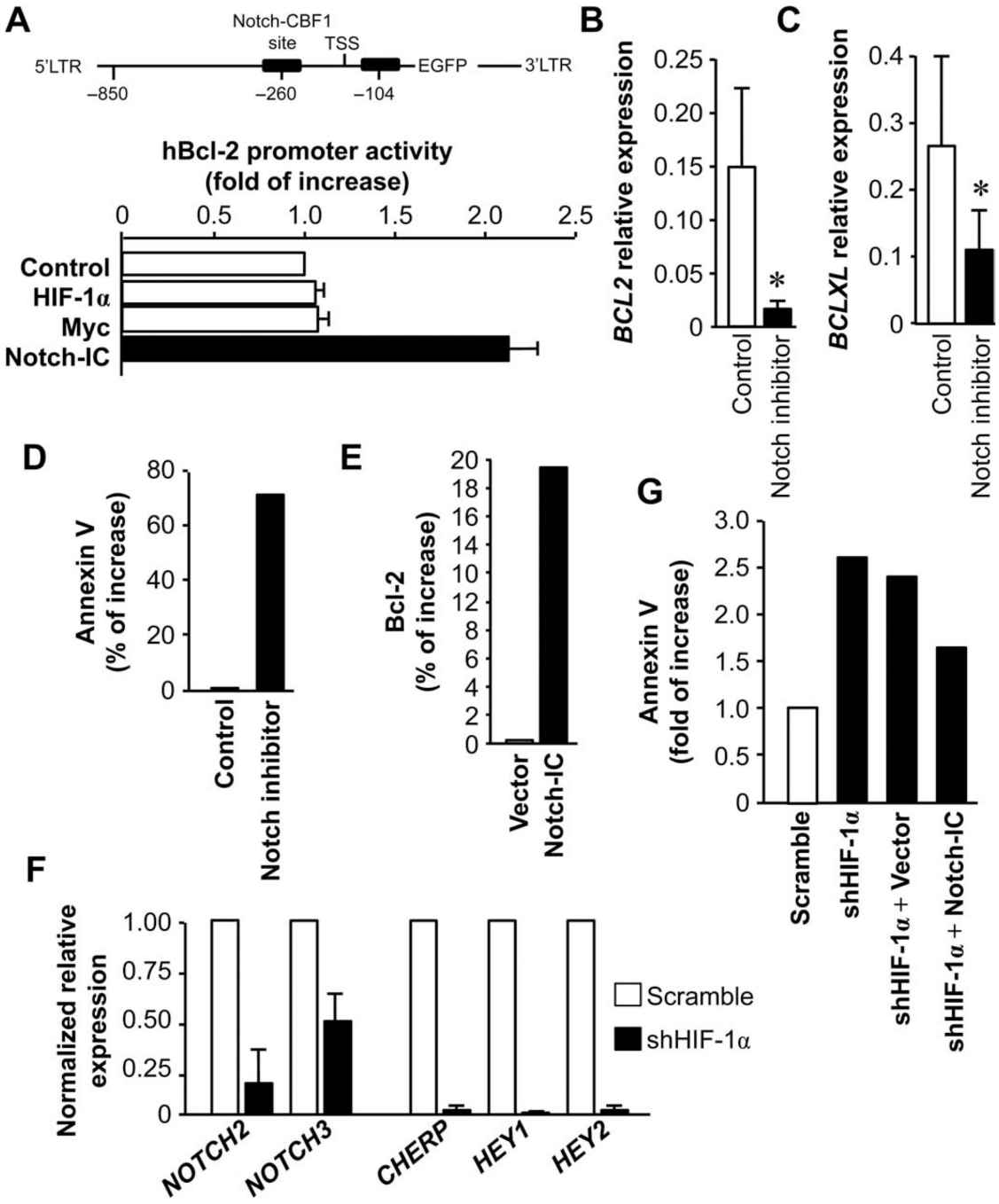

Fig. 7.

Notch and Bcl-2 family regulate the stem cell-like feature of the T_H17 cells. (**A**) Notch bound to and activated the *Bcl-2* promoter. Black boxes show Notch binding sites in the *BCL2* promoter (upper panel). Human embryonic kidney (HEK) 293 cells were cotransfected with the h*BCL2*-EGFP (enhanced green fluorescent protein) promoter with the indicated plasmids for 38 hours. The intensity of EGFP was measured with flow cytometry. LTR, long terminal repeat; TSS, transcription start site. (**B** and **C**) Notch blockade suppressed *BCL2* and *BCLXL* expression. Primary T cells were treated with Notch inhibitor for 24 hours. *BCL2* (B) and *BCLXL* (C) expression was quantified by real-time PCR. $n = 3$.

$P < 0.05$. (**D**) Notch blockade increased T_H17 cell apoptosis. Primary T_H17 cells were cultured with the Notch inhibitor for 3 days. Annexin V expression was analyzed by flow cytometry. $n = 3$. (**E**) Notch activation increased *BCL2* expression on T_H17 cells. Primary T_H17 cells were transfected with Notch-IC. After 24 hours, Bcl-2 expression was analyzed by flow cytometry. $n = 3$. (**F**) HIF blockade reduced Notch signaling gene expression in T_H17 cells. Primary T_H17 cells were transfected with shHIF-1α. After 24 hours, Notch signaling gene expression was quantified. $n = 3$. (**G**) Notch activation rescued T_H17 cell apoptosis induced by HIF blockade. Primary T_H17 cells were transfected with lentiviral vectors encoding shHIF-1α and/or Notch-IC. After 24 hours, annexin V expression was analyzed by flow cytometry. $n = 3$.