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Human Naive T Cells Express Functional CXCL8 and Promote Tumorigenesis

Joel Crespo,*† Ke Wu,*‡ Wei Li,*‡ Ilona Kryczek,* Tomasz Maj,* Linda Vatan,* Shuang Wei,* Anthony W. Opipari,§ and Weiping Zou*‡†#

Naive T cells are thought to be functionally quiescent. In this study, we studied and compared the phenotype, cytokine profile, and potential function of human naive CD4+ T cells in umbilical cord and peripheral blood. We found that naive CD4+ T cells, but not memory T cells, expressed high levels of chemokine CXCL8. CXCL8+ naive T cells were preferentially enriched CD31+ T cells and did not express T cell activation markers or typical Th effector cytokines, including IFN-γ, IL-4, IL-17, and IL-22. In addition, upon activation, naive T cells retained high levels of CXCL8 expression. Furthermore, we showed that naive T cell–derived CXCL8 mediated neutrophil migration in the in vitro migration assay, supported tumor sphere formation, and promoted tumor growth in an in vivo human xenograft model. Thus, human naive T cells are phenotypically and functionally heterogeneous and can carry out active functions in immune responses. The Journal of Immunology, 2018, 201: 000–000.

Materials and Methods

Human samples, cell isolation, and flow cytometric analysis (FACS)

Human tonsil was obtained from the tissue procurement laboratory in the University of Michigan. The study was approved by the local institutional review board. Human peripheral blood samples were obtained from healthy volunteers by venipuncture or cytopheresis. Human umbilical cord blood was collected by C.S. Mott Children’s Hospital (University of Michigan) in blood collection tubes (BD Biosciences). Mononuclear cells were collected by Ficoll-Hypaque or Lymphoprep density gradient centrifugation. Peripheral blood naive T cells were isolated by RosetteSep (Stemcell Technologies), followed by CD45RA+ or CD45RO+ MicroBeads (Miltenyi Biotec) column selection. Naive T cells from umbilical cord blood and human tonsil were isolated by first depleting CD14+ monocytes, followed by positive selection of CD4+ T cells using CD14+ monocyte selection EasySep and CD4+ T cell selection EasySep (Stemcell Technologies). CD4+CD45RA+CD31+ naive T cells were sorted from single-cell suspensions by a high-speed cell sorter (FACSArria; BD Immunocytometry Systems). Neutrophils were isolated as described from adult blood (18). Cellular purity was measured by flow cytometry (LSR II; BD Biosciences). Surface and cytokine profiles were measured through surface and intracellular staining, respectively, and analyzed by LSR II, FACSDiva, and FidO software (19–21).

Reagents

Recombinant human cytokines and chemokines were from R&D Systems. Abs for flow cytometry were CCR7, CD3, CD4, CD8, CD25, CD31, CD45RA, CD45RO, CD62L, CD69, CD154, CXCR2, CXCR3, CXCL8, INF-γ, IL-4, IL-10, IL-17, and IL-22 (BD Biosciences) CD57, CD122, and Foxp3 (eBioscience/Thermo Fisher Scientific), and KLRG-1 (BioLegend). Abs for T cell stimulation were CD3 and CD28 (BD Biosciences or AffyMetrix). Small-molecule CXCR1/2 chemical inhibitor reparixin (RPX) was purchased from MedChem Express.

In vitro T cell and OC8 tumor cell line cultures

T cell subsets (1 million cells/ml) were stimulated with oCD3/oCD28 beads (BD Biosciences) for up to 2 wk. Cells were supplemented with fresh medium and recombinant human IL-2 (5 ng/ml) every 3 d and

Abbreviations used in this article: NSG, NOD.Scid.IL-2Rα−/−; RPX, reparixin.

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Abbreviations used in this article: NSG, NOD.Scid.IL-2Rγ−; RPX, reparixin.

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restimulated on days 6 and 12. Cytokine expression was measured at 3-d time points through FACS. Umbilical cord blood naive T cells (10 million cells/ml) were cultured for 24 h in medium with PMA/ionomycin, and supernatants were saved and measured for CXCL8 (R&D Systems) and used in the sphere assay. Human tonsil naive T cells were cultured in vitro in the presence of IL-7 (10 ng/ml) and IL-15 (10 ng/ml) for up to 12 d. Human primary OC8 ovarian cancer cells (22, 23) were cultured in vitro in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific).

**Flow cytometry and cytokine detection**

Freshly isolated mononuclear cells were stimulated in vitro with PMA/ionomycin, GolgiStop, and GolgiPlug (BD Biosciences) for 4 h, then stained against extracellular and intracellular Ags. Single-cell suspensions from tissues were stained for extracellular surface Ags with specific Abs, followed by fixation and permeabilization using Fix/Perm solution (Thermo Fisher Scientific), then stained against intracellular Ags. Viable single cells were analyzed.

**Tumor model**

NOD. Scid. IL-2Rγ−/− (NSG) male mice aged 14–18 wk (The Jackson Laboratory) were used (22, 23). Primary human ovarian cancer cells (OCS) were cultured in RPMI 1640 supplemented with 10% FBS (24, 25). NSG mice were s.c. injected with 1 × 10⁶ and 1 × 10⁷ human T cells for 2 wk (25). Mice received peritoneal injection with (200 μl) PBS-solubilized RPX (5 mg/kg) or PBS daily (26). In a separate experiment, OCS cells were cultured in the presence of recombinant human CXCL8 (10 ng/ml) for 24 h, and 1 × 10⁶ single cells were s.c. injected into mice.

**Neutrophil migration assay**

T cells were cultured in vitro for 3 d at 1 million cells/ml in the presence of αCD3/αCD28 beads (BD Biosciences). Supernatants were collected and placed on the bottom of 3-μm pore, 12-mm diameter Transwell supports, whereas neutrophils were placed on the upper chamber. RPX was added as indicated. Neutrophil migration was performed for 2 h (18).

**Sphere formation assay**

Primary ovarian cancer cells (23, 25) were cultured in vitro in X-VIVO medium (Lonza) and ultra-low attachment plates (Corning) for 5 d supplemented with 0, 1, or 10 μg/ml recombinant human CXCL8. Separately, supernatants from umbilical cord blood naive T cells cultured in vitro from above were mixed 1:1 with X-VIVO medium and cultured as described above at a density of 10,000 viable cells per well. Spheres (>100 μm) were counted after 5 d.

**Enzyme-linked immunoassay**

ELISA to detect CXCL8 was carried out as per the manufacturer’s protocol (R&D Systems).

**Statistical analysis**

Student t test was carried out to determine significant difference between expression levels, tumor weight, and neutrophil migration numbers.

**Results**

**Naïve T cells express CXCL8**

To determine whether naïve T cells are functionally quiescent, we studied and compared the cytokine profiles of naïve and memory CD4⁺ T cells in fresh umbilical cord blood and adult peripheral blood. CD45RA⁻/CD45RO⁻ CD62L⁻ CCR7⁻ naïve T cells and CD45RA⁻/CD45RO⁻ memory T cells were defined by flow cytometric analysis (FACS) (Fig. 1A). We found that umbilical cord blood naive CD4⁺ T cells spontaneously expressed 22% CXCL8 and <1% IFN-γ, IL-4, IL-17, IL-10, and IL-22 (Fig. 1B). The expression level of CXCL8 was the highest among detectable cytokines in umbilical cord blood naive CD4⁺ T cells (Fig. 1B). Next, we compared the cytokine expression levels in cord blood and adult peripheral blood (Fig. 1C, 1D). Furthermore, naïve T cells expressed higher levels of CXCL8 than memory T cells (Fig. 1C, 1D). In contrast, the expression levels of IFN-γ and IL-17 were higher in memory T cells than naïve T cells (Fig. 1C, 1E). Thus, human naïve T cells spontaneously and selectively express CXCL8.

**CXCL8⁺ naïve T cells are enriched in CD31⁺ T cells**

We defined naïve T cells based on CD45RA⁻/CD45RO⁻ CD62L⁻ CCR7⁻ surface phenotype. We could not rule out whether CXCL8⁺ naïve T cells were activated and/or differentiated in vivo. To address this possibility, we analyzed the activation and differentiation markers in CXCL8⁺ naïve T cells. CXCL8⁺ naïve T cells expressed none of several activation markers, including CD69, CD122, CD154, and CCRX3 (Fig. 2A), nor did they express differentiation markers, including CD57 and KLRG1 (Fig. 2B). We previously reported that a subset of regulatory T cells expressed CXCL8 in the tumor microenvironment (16). We found that CXCL8⁺ naïve T cells did not express Foxp3 or CD25 (Fig. 2C). Thus, CXCL8⁺ naïve T cells are not regulatory T cells. Interestingly, CXCL8⁺ naïve T cells expressed minimal levels of CXCR2, the receptor for CXCL8 (Fig. 2D), suggesting that CXCL8⁺ naïve T cells may not consume CXCL8 autocrinously or from their microenvironment. CD31 is a marker for recent thymic emigrant cells (27). Adult peripheral blood naive T cells can be divided into CD31⁺ and CD31⁻ mature cells (27). We sorted CD31⁺ and CD31⁻ naïve cells. We found that the levels of CXCL8 were higher in CD31⁺ T cells than CD31⁻ T cells (Fig. 2E). Thus, peripheral blood CXCL8⁺ T cells are enriched in CD31⁺ naïve T cells.

**Activated naïve T cells remain to express CXCL8**

CXCL8⁺ naive T cells are enriched in CD31⁺ T cells. This suggests a potential dynamic expression of CXCL8 in naïve T cells. To test this, we activated naïve T cells from umbilical cord and adult peripheral blood with αCD3/αCD28 stimulation and kinetically analyzed CXCL8 expression. We found that TCR engagement initially maintained and subsequently stimulated CXCL8 expression in naïve T cells from umbilical cord blood (Fig. 3A) and adult peripheral blood (Fig. 3B) naïve CD4⁺ T cells. As expected, the levels of IFN-γ expression were low and gradually increased following T cell activation in CD4⁺ naïve T cells from umbilical cord blood (Fig. 3C) and adult peripheral blood (Fig. 3D). Next, we cultured CD4⁺ naïve T cells enriched from human tonsil with homeostatic cytokines IL-7 and IL-15 and kinetically analyzed CXCL8 expression. We showed that homeostatic cytokines initially maintained and subsequently stimulated CXCL8 expression in these cells (Fig. 3E, 3F). However, homeostatic cytokines did not stimulate IFN-γ expression in human tonsil CD4⁺ naïve T cells within 12 d stimulation (Fig. 3G). The data suggest that CXCL8 expression may be a functional feature for naïve T cells.

**Naïve T cell–derived CXCL8 is biologically active**

To study the potential role of naïve CD4⁺ T cell–derived CXCL8, we carried out an in vivo model of tumor growth in the NSG mice. We inoculated primary human ovarian cancer cells into the NSG mice with or without umbilical cord blood naïve CD4⁺ T cells at a 1:1 ratio. We treated the mice with RPX, a small-molecule chemical inhibitor for CXCL8 receptors CXCR1 and CXCR2 (28, 29). We observed that mice that received umbilical cord blood naïve CD4⁺ T cells showed enhanced tumor volume and weight (Fig. 4A, 4B) as compared with mice that did not receive naïve CD4⁺ T cells. This effect was abrogated by RPX administration (Fig. 4A, 4B). In support of this observation, we incubated primary human ovarian cancer cells with recombinant human CXCL8 for 24 h and subsequently injected them into the NSG mice. CXCL8 treatment resulted in increased tumor weight.
FIGURE 1. Human naive T cells spontaneously express CXCL8. (A) Gating strategy used to identify T cell subsets. Naive T cells: CD3+CD4+CD45RA+CD45RO−CD62L+CCR7+. Memory T cells: CD3+CD4+CD45RA−CD45RO+CD62L−CCR7−. (B-E) Cytokine profile of cord blood and peripheral T cells. Intracellular cytokines were analyzed by FACS. Representative FACS plots and percentages are shown in (B) and (C). (D and E) Graphical summary of CXCL8 (D) and IFN-γ (E) FACS values in human T cell subsets (n = 4–8). *p < 0.05.
FIGURE 2. CXCL8⁺ naive T cells are enriched in CD31⁺ T cells. (A–D) Fresh CXCL8⁺ umbilical cord blood naive T cells were analyzed for expression of surface and intracellular marks of activation and differentiation. Representative FACS plots and percentages are shown. Gated on CXCL8⁺ naive T cells. (E) Graphical summary of CXCL8 expression in CD31⁺ and CD31⁻ T cells (E) (n = 4–8). *p < 0.05.
Thus, naive T cell–derived CXCL8 is biologically active. CXCL8 is known to promote stemness qualities in cancer cells (30, 31). We performed a sphere assay by culturing primary ovarian cancer cells (OC8) (24, 25) in vitro with recombinant human CXCL8. We found that CXCL8 promoted tumor sphere formation (Fig. 4D). We further tested whether naive T cells could...
promote sphere formation in a CXCL8-dependent manner. We observed an increase in tumor sphere numbers in the presence of naive T cell supernatants. This effect was abolished by RPX (Fig. 4E). In addition, we tested the potential effect of naive T cell–derived CXCL8 on neutrophil migration in an in vitro Transwell assay. We observed that neutrophils migrated toward naive T cell supernatants. This effect was blocked by RPX (Fig. 4F). As confirmation, we detected high levels of CXCL8 in umbilical cord blood naive CD4 + T cells (Fig. 4G). Thus, naive T cell–derived CXCL8 may be functionally important in vivo.

**Discussion**

Naive T cells are thought to require activation to acquire effector functions. Recent reports suggest that naive T cells carry out active functions such as maintaining lymph node integrity prior to their activation (1). Previous studies have shown that human umbilical cord blood CD4 + naive T cells express CXCL8, and naive T cell CXCL8 expression is lost in adulthood (7, 8). In this article, we show that human CD4 + naive T cells spontaneously express high levels of CXCL8 and are poised to express CXCL8 upon activation. Furthermore, adult human tonsil naive T cells express CXCL8 spontaneously, and this expression may be a functional feature of these cells. Typical Th effector cytokines, including IFN-γ, IL-4, IL-17, and IL-22, are not coexpressed with CXCL8 in naive CD4 + T cells. Phenotypic experiments reveal that these cells are enriched in the CD4 +CD45RA +RO − CD62L +CCR7 +CD31 + population. These CXCL8 producers show no signs of prior activation or differentiation, indicating that they are naive T cells.

Interestingly, although naive T cells in both fresh umbilical cord blood and adult peripheral blood express high levels of CXCL8, the percentage of CXCL8 + T cells is substantially higher in umbilical cord blood than in adult peripheral blood. Naive T cells from human tonsil also expressed CXCL8 under homeostatic conditions. In addition, ex vivo memory T cells express high levels of other effector cytokines, including IFN-γ, rather than CXCL8. The data suggest that spontaneous CXCL8 expression may be a functional feature for naive T cells. In support of this, we have found that naive T cell–derived CXCL8 mediates neutrophil migration in vitro and promotes primary ovarian cancer growth in the NSG model. CXCL8 expression in naive T cells may allow humans to be prepared against pathogens encountered early in life. Thus, CXCL8 + naive T cells may regulate innate cell trafficking and shape early immune responses. Given the well-defined role of CXCL8 in angiogenesis, CXCL8 + naive T cells may support vascularization in newborn tissues. Of course, these cells may represent a remnant of the fetal immune system, and their potential role in fetus development has yet to be determined (32). Moreover, the protumor role of CXCL8 has been well-defined in many types of tumor (33, 34). As the CXCL8, CXCRL1, and CXCRL2 signaling is involved in the regulation of cancer stem cells (30), and tumor often metastasizes into lymph nodes, it is speculated that CXCL8 + naive T cells may support tumor lymphoid...
metastasis via CXCL8 production. Altogether, naive T cell–derived CXCL8 may play an important role in physiologic homeostasis and specific pathologic conditions.

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Disclosures

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