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IL-27 induction of IL-21 from human CD8⁺ T cells induces granzyme B in an autocrine manner

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Abstract

Interleukin (IL)-27 exerts an anti-inflammatory effect on human and mice CD4⁺ T cells by inducing IL-10-producing T regulatory 1 cells through induction of IL-21. However, the role of IL-27 and how it regulates IL-21 from human CD8⁺ T cells is unclear. Here, we show that the IL-27 receptor is expressed on human CD8⁺ T cells and stimulation of human naïve CD8⁺ T cells in the presence of IL-27 leads to an increase in IL-21 and interferon (IFN)- γ production. IL-21 induction in IL-27-stimulated human CD8⁺ T cells correlates specifically with expression of the transcription factor T-bet. IL-27 stimulation of naïve CD8⁺ T cells induces a double-positive T-bet⁺ IL-21⁺ expressing CD8⁺ T-cell population. Furthermore, IL-27 stimulation of human naïve CD8⁺ T cells greatly increases expression of granzyme B. Antibody-mediated neutralization of IL-21 abrogates IL-27-induced granzyme B expression. Moreover, direct addition of IL-21 greatly amplifies granzyme B expression in human naïve CD8⁺ T cells. Our findings identify IL-27-induced IL-21 as a key autocrine regulator of granzyme B expression in human CD8⁺ T cells.

Keywords

Granzyme B; human CD8⁺ T; IL-27; IL-21

Interleukin (IL)-27 is a member of the IL-12 family cytokine and is composed of two chains IL-27p28 and Epstein —Barr virus-induced gene 3 (*EBI3*). IL-27 is mainly produced by antigen-presenting cells such as dendritic cells and macrophages.¹ Dendritic cells secrete IL-27 on exposure to Toll-like Receptor agonists and physiological stimuli such as type I and type II IFNs.^{2,3} Initial animal studies on IL-27 suggested a role for IL-27 in the initiation of Th1 responses by activating the signal transducer and activator of transcription-1-mediated T-bet pathway.⁴ However, studies now indicate that IL-27 is a potent anti-inflammatory factor with broad inhibitory effects on Th1, Th2 and Th17 subsets of T cells as well as antigen-presenting cell function.^{5–8} In addition, IL-27 induces IL-10-producing type 1 regulatory (Tr1) cells both from human and mice CD4⁺ T cells.^{9,10} In contrast to its effects on CD4⁺ T cells IL-27 has been shown to have a proinflammatory role

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on CD8⁺ T cells. In mice, IL-27 stimulation of naïve CD8⁺ T cells augments generation of cytotoxic T lymphocytes with enhanced expression of IFN- γ and granzyme B.¹¹ IL-27 injection or IL-27-transduced autologous tumor cells has been shown to augment CD8⁺ T-cell-mediated tumor clearance *in vivo*.^{12,13}

IL-21 is known to have potent proinflammatory functions. IL-21 is a cytokine produced primarily by activated CD4⁺ T-cell subsets,¹⁴ activated natural killer T cells,¹⁵ and follicular T-helper cells (TFH).¹⁶ IL-21 belongs to the family of IL-2 growth factors and signals via a heterodimeric receptor, formed by the common γ -chain and the specific receptor protein, IL-21R.¹⁴ IL-21 has actions on a wide range of lineages. It has been shown to induce differentiation of Th1, Th2, Th17 and TFH cells in mice and humans under particular activation states and conditions.¹⁷ IL-21 induced through IL-27 stimulation has anti-inflammatory properties as it directs differentiation of Tr1 cells from naïve CD4⁺ T cells, which is critical for IL-10 amplification.^{18,19} IL-21 also promotes expansion of CD8⁺ T cells²⁰ and is critical for normal immunoglobulin G production by B cells.^{21–23} Recently, it has been shown that CD8⁺ T cells specific for HIV-specific peptides produce IL-21.²⁴

Despite a great deal of information on the role of IL-27 and IL-21 on CD4⁺ T cells both in human and mouse, the role of these two cytokines in the regulation of CD8⁺ T cells in humans is not well understood. Furthermore, how IL-27 regulates IL-21 expression in CD8⁺ T cells is unknown. In this study, we show that IL-27-induced IL-21 regulates granzyme B expression in CD8⁺ T cells in an autocrine manner.

RESULTS AND DISCUSSION

IL-27 induces IL-21 expression from naïve CD8⁺ human T cells

We investigated the role of IL-27 in regulating human CD8⁺ T cells. *Ex vivo* total human CD8⁺ T cells from healthy donors express high amounts of surface IL-27R with expression higher on the naïve (CD45RA⁺) fraction compared with the effector memory fraction (Supplementary Figures 1a and b). We observed consistently across several donors that CD8⁺ T cells express higher amounts of the IL-27R compared with CD4⁺ T cells as indicated by mean fluorescence intensity (Supplementary Figure 1c). IL-27 induces IL-21 from both mouse and human CD4 T cells. This secreted IL-21 amplifies IL-10 production from CD4⁺ T cells in an autocrine manner^{18,19} and impacts processes such as B-cell class switching.^{18,21–23} Whether IL-27 stimulation of human CD8⁺ T cells affects IL-21 expression is unknown. As shown in Figure 1a we sorted CD45RA⁺ CCR7⁺ naïve CD8⁺ T cells using flow cytometry and stimulated them with plate-bound anti-CD3 and anti-CD28 in the presence or absence of recombinant human IL-27. As shown in Figure 1b by intracellular staining and Figure 1c by enzyme-linked immunosorbent assay expression, IL-21 is highly expressed when naïve CD8⁺ T cells are stimulated in the presence of IL-27. We observed similar results wherein IL-27 robustly upregulates the expression of IL-21 from total CD8⁺ T cells (Figure 1d) when compared with cells stimulated with anti-CD3+ anti-CD28 alone.

IL-27 has been shown to regulate IFN- γ production in mouse CD8⁺ T cells.¹¹ We thus investigated whether a similar effect occurs in human CD8⁺ T cells. We found that sorted

naïve (Figure 1c) or total (Figure 1d) CD8⁺ T cells stimulated with plate-bound anti-CD3 and anti-CD28 in the presence recombinant human IL-27 greatly increased IFN- γ expression when compared with the anti-CD3⁺ anti-CD28 condition. Thus, we demonstrate that IL-27 induces IL-21 and IFN- γ production from both sorted naïve and total human CD8⁺ T cells.

IL-21 is produced by Th1 and TFH,²⁵ Th2,²⁶ Tr1¹⁸ and Th17^{27–29} populations of CD4⁺ T-helper cells. Although multiple CD4⁺ cell types have been shown to produce IL-21 TFH and Th17 have highest IL-21 expression. Given that, we observe IL-21 production from CD8⁺ T cells we wanted to investigate the transcription factor profile of IL-27-conditioned cells. For this, naïve CD8⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence or absence of IL-27. RNA was extracted and expression of transcription factors was analyzed by real-time PCR. Expression of the following Th lineage commitment transcription factors was analyzed: T-bet (Th1), Eomesodermin (Th1), GATA3 (Th2), c-Maf (Tr1), Bcl6 (TFH), RORC (Th17) and Foxp3 (Treg). Of all transcription factors analyzed, we found that the Th1 lineage commitment transcription factor T-bet (Figure 1e and Supplementary Figure 2) was induced at highest levels in the presence of IL-27. It has been shown that c-Maf and Bcl6 are associated with increased expression of IL-21 in TFH cells. However, we did not observe a correlation of these transcription factors with IL-21 in IL-27-stimulated CD8⁺ T cells. Thus, stimulation of naïve CD8⁺ T cells in the presence of IL-27 leads to an effector cell that increases T-bet expression with increased production of IL-21 and IFN- γ .

We further analyzed expression of IL-21 and IFN- γ in T-bet-expressing cells. As seen in Figure 1f, IL-27 stimulation of sorted naïve CD8⁺ T cells leads to increase in not only single-positive T-bet⁺ and IL-21⁺ cells but also double-positive T-bet⁺ IL-21⁺ cells (shown by intracellular staining). A similar intracellular analysis for expression of IL-21 and IFN- γ shows that IL-27 stimulation leads to an increase in not only single-positive T-bet⁺ and IFN- γ ⁺ cells but also double-positive T-bet⁺ IFN- γ ⁺ cells (Figure 1f). Thus, IL-27 stimulation of naïve CD8 T cells leads to an increase of unique effector T cells expressing both T-bet and IL-21.

IL-27-induced IL-21 directs proliferation of CD8⁺ T cells—IL-21 belongs to the IL-2 family of growth cytokines and has been demonstrated to expand CD8⁺ T cells.²⁰ Our results also demonstrate that IL-27 promotes proliferation of sorted naïve CD8⁺ T cells (Figure 1g). However, addition of an anti-IL-21 antibody to neutralize IL-21 results in a significant decrease in proliferation not seen with the addition of isotype control for the antibody used (Figure 1g). Given this, IL-21 secreted by IL-27-stimulated CD8⁺ T cells may also be an important mechanism to promote self-expansion of CD8⁺ T cells.

IL-27-induced IL-21 directs granzyme B production in activated CD8⁺ T cells in an autocrine manner—Cytotoxic T lymphocyte effector cells express key molecules such as perforin, granzyme A, granzyme B that serve as surrogate biomarkers of cytotoxic activity.³⁰ We thus investigated whether IL-27 impacts production of these key CD8⁺ effector T-cell molecules. We analyzed expression of granzyme B, granzyme A and perforin following IL-27 stimulation (Figures 2a and b). Anti-CD3⁺ anti-CD28 stimulation of sorted

naïve CD8⁺ T cells in the presence of IL-27 lead to an increase in expression of granzyme B compared with anti-CD3⁺ anti-CD28 alone (Figures 2a and b). However, we find no difference in expression of either perforin (Figure 2a) or granzyme A (Figure 2b) on IL-27 stimulation. However, the lack of expression is not due to antibody detection issues as the same antibodies used to detect perforin and granzyme A detect the antigens in a CD8 T-cell clone (Supplementary Figure 3). Thus, IL-27 stimulation of CD8⁺ T cells lead to an increase in effector cytotoxic T lymphocytes with increased IL-21, IFN- γ and granzyme B expression.

IL-21 has been demonstrated to have antitumor activity in the study of certain cancers.^{31,32} In addition, IL-21 in human B cells in the context of viral antigen stimulation is shown to upregulate granzyme B.³³ As shown above, we found that IL-27 amplifies expression of both IL-21 and granzyme B production from naïve CD8⁺ T cells. Given this, we investigated whether IL-27 secreted IL-21 could regulate expression of granzyme B from naïve CD8⁺ T cells. For these experiments we stimulated sorted naïve CD8⁺ T cells with anti-CD3⁺ anti-CD28 antibodies in the presence of IL-27. In some conditions neutralizing antibodies to IL-21 were included. As shown in Figures 2a and b anti-CD3 and anti-CD28 stimulation in the presence of IL-27 increases intracellular granzyme B expression. Neutralizing IL-21 with anti-IL-21 antibodies during IL-27 activation decreases intracellular granzyme B expression from naïve CD8⁺ T cells (Figures 2a and b). We also investigated expression of secreted granzyme B expression from sorted naïve CD8⁺ T cells in response to IL-27 and found similar results (Figure 2c). To investigate whether IL-21 has a direct impact on granzyme B expression we activated sorted naïve CD8⁺ T cells in the presence of IL-21. We found that IL-21 (100 pg ml⁻¹) increased granzyme B production from naïve CD8⁺ T cells and this increase was dose dependent with an increase in granzyme B expression seen upto a 500 pg ml⁻¹ concentration of IL-21 (Figure 2d). Similar increase in granzyme B expression was observed when naïve CD8⁺ T cells were stimulated with higher amount of IL-21 (Figure 2e). Thus, IL-27-induced IL-21 regulates granzyme B expression in CD8⁺ T cells in an autocrine manner.

In this study, we demonstrate that IL-27 induces IL-21 and IFN- γ expression from naïve CD8⁺ T cells. This IL-21 functions in an autocrinemanner to induce the effector molecule granzyme B from CD8⁺ T cells. Our observations indicate that IL-27 stimulation specifically induces granzyme B without altering other CD8 effector molecules granzyme A and perforin in human CD8⁺ T cells. It is possible that IL-27 stimulation impacts the transcriptional locus of granzyme B without affecting those of granzyme A and perforin.

IL-27 has been shown to induce T-bet and granzyme B in mouse CD8 T cells.¹¹ Recently, a publication from *Schneider et al.*³⁴ demonstrated that IL-27 induces T-bet, IFN- γ and granzyme B expression in CD8 T cells. Williams *et al.*²⁴ have also shown that CD8⁺ T cells were able to produce IL-21 in response to HIV-1 infection. In addition, we have previously reported that IL-27 could induce the production of IFN- γ , from human CD4⁺ T cells.³⁵ However, the role of IL-27 on IL-21 induction and its impact on granzyme B expression in human CD8⁺ T cells unknown. We demonstrate that IL-27-induced IL-21 has a crucial role granzyme B expression by CD8⁺ T cells. Our results show consistency and indicate IL-21 production by CD8⁺ T cells with previously published results.

IL-27 has been demonstrated to have an impact on the tumor development in several pre-clinical models.^{12,13,36–38} IL-21 enhances antitumor activity in animal models by enhancing tumor-specific cytotoxic T lymphocyte responses by increasing proliferation, survival and the specific killing ability of T cells.³⁹ This has led to its use in the first phase of clinical trials in advanced cancer patients.⁴⁰ It has been demonstrated that IL-21 has effects on several cell types: B cells, T cells and dendritic cells.¹⁴ Our results that (IL-27 directed) IL-21 is secreted by effector CD8⁺ T cells may therefore impact the tumor microenvironment, T and B cells, as well as have direct effects on CD8⁺ T cells as demonstrated by the upregulation of granzyme B. These findings thus identify an important mechanism by which human CD8⁺ T cells may influence disease processes.

METHODS

Subjects

Peripheral blood drawn from healthy subjects was obtained after informed consent according to the Institutional Ethics Review Board Protocols.

Antibodies and reagents

Cells were maintained with serum free X-VIVO 20 medium (BioWhittaker, Walkersville, MD, USA). Anti-IL-21 was obtained from Peprotech (Rocky Hill, NJ, USA). Antibodies to granzyme B (MHGB) were from Invitrogen (Grand Island, NY, USA). Perforin-APC (dG9) was obtained from Biolegend (San Diego, CA, USA). Antibodies to CD3, CD28, granzyme A (CB9) and IFN- γ (B27) were obtained from BD Biosciences (San Diego, CA, USA). Antibodies to IL-21 (eBio3A3-N2) were obtained from eBiosciences (San Diego, CA, USA). Recombinant human IL-27 and IL-27 receptor antibodies were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human IL-21 was obtained from Miltenyi Biotec (Cambridge, MA, USA). All reverse transcription PCR primers and reagents were obtained from Applied Biosystems (Carlsbad, CA, USA).

CD8⁺ T-cell isolation, flow cytometry sorting and stimulation

Peripheral blood mononuclear cells were obtained by Ficoll density gradient. Total CD8⁺ T cells were isolated using the magnetic bead kits (Miltenyi Biotec). For naïve CD8⁺ T cells, magnetically purified total CD8⁺ T cells were stained with antibodies to CD8, CD45RA and CCR7 (BD Biosciences) and Naïve CD8⁺ CCR7⁺ CD45RA⁺ were isolated by flow cytometry sorting. The purity of sorted naïve CD8⁺ T cells used in all experiments was >99%. CD8⁺ T cells were cultured at a concentration of 1.5×10^6 cells ml⁻¹ in 24-well plates or at a density of 2×10^5 cells per well in a 96-well plate coated with anti-CD3 and anti-CD28 (1 μ g ml⁻¹) in the presence or absence of 100ng ml⁻¹ of recombinant human IL-27. In some culture conditions, anti-IL-21 antibody was added at a concentration of 20 μ g ml⁻¹.

Proliferative responses of T-cell and cytokine analysis

Cells were cultured at 0.2 million per well in 96-well U-bottom plates. Plates were pulsed after 60 h of culture with thymidine (H3) at 1 μ Ci per well for the final 18 h, harvested and assayed for proliferation. Mean incorporation of thymidine was measured in triplicate wells

and is indicated as counts per minute (c.p.m.). For enzyme-linked immunosorbent assay, supernatants from T cells were harvested after 72 h of stimulation and stored at -80°C . Granzyme B (Abcam, Cambridge, MA, USA) and IL-21 (eBioscience) expression was quantified by using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions.

Flow cytometric analysis

Flow cytometric analysis was conducted as described previously.⁴¹ Samples were acquired on FACSCalibur or LSR II flow cytometers (Sanjose, CA, USA). Data analysis was done using the FloJo software (Orlando, FL, USA).

Real-time quantitative reverse transcription PCR

Real-time quantitative reverse transcription PCR was performed as described previously.⁴¹ For each sample, mRNA abundance was normalized to the amount of the housekeeping gene *GAPDH*.

Statistical analysis

Statistical analysis was performed using the unpaired *t*-test. A value of $P < 0.05$ was considered significant. Data are presented as mean s.e.m.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

IL-27 induces IL-21 and IFN- γ expression from naïve and total CD8⁺ human T cells. Flow cytometry sorted CD45RA⁺ CCR7⁺ naïve CD8⁺ T cells (**a**) were stimulated with anti-CD3 and anti-CD28 antibodies with or without IL-27 (100ng ml⁻¹). (**b**) Intracellular analysis of IL-21 expression in naïve CD8⁺ T cells stimulated with or without IL-27. (**c, d**) Naïve or total CD8⁺ T cell were stimulated with or without IL-27, and supernatants were analyzed 72 h subsequent to culture for expression of IL-21 and IFN- γ . (**e**) Real-time reverse transcription PCR analysis of T-bet expression CD8⁺ T cells stimulated in the presence or absence of IL-27. (**f**) Intracellular analysis of IL-21, T-bet and IFN- γ expression in naïve CD8⁺ T cells stimulated with or without IL-27. Upper and lower panel of (**f**) are from two different donors. (**g**) Naïve CD8⁺ T cells were stimulated with plate-bound anti-CD3 and CD28 with or without recombinant human IL-27 (100ng ml⁻¹) and proliferation was measured at day 3. Data are representative of at least four to seven experiments and in case of (**c**), (**d**) and (**e**) eight donors.

Figure 2. IL-27-induced IL-21 directs granzyme B production in activated CD8⁺ T cells through an autocrine manner. Naïve CD8⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies with or without IL-27 (100ng ml⁻¹). (a, b) In some cultures anti-IL-21 antibody was used at 20 µg ml⁻¹. Cells were analyzed for granzyme B, granzyme A and perforin expression by flow cytometry. (c) Supernatants were analyzed for expression of granzyme B. (d, e) The direct effect of IL-21 on granzyme B expression was analyzed by intracellular expression and real-time reverse transcription PCR analysis.