

Dissociation of Its Opposing Immunologic Effects Is Critical for the Optimization of Antitumor CD8⁺ T-Cell Responses Induced by Interleukin 21

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Abstract **Purpose:** Interleukin 21 (IL-21) is a promising new cytokine, which is undergoing clinical testing as an anticancer agent. Although IL-21 provides potent stimulation of CD8⁺ T cells, it has also been suggested that IL-21 is immunosuppressive by counteracting the maturation of dendritic cells. The dissociation of these two opposing effects may enhance the utility of IL-21 as an immunotherapeutic. In this study, we used a cell-based artificial antigen-presenting cell (aAPC) lacking a functional IL-21 receptor (IL-21R) to investigate the immunostimulatory properties of IL-21. **Experimental Design:** The immunosuppressive activity of IL-21 was studied using human IL-21R⁺ dendritic cells. Antigen-specific CD8⁺ T cells stimulated with human cell-based IL-21R⁻ aAPC were used to isolate the T-cell immunostimulatory effects of IL-21. The functional outcomes, including phenotype, cytokine production, proliferation, and cytotoxicity were evaluated. **Results:** IL-21 limits the immune response by maintaining immunologically immature dendritic cells. However, stimulation of CD8⁺ T cells with IL-21R⁻ aAPC, which secrete IL-21, results in significant expansion. Although priming in the presence of IL-21 temporarily modulated the T-cell phenotype, chronic stimulation abrogated these differences. Importantly, exposure to IL-21 during restimulation promoted the enrichment and expansion of antigen-specific CD8⁺ T cells that maintained IL-2 secretion and gained enhanced IFN- γ secretion. Tumor antigen-specific CTL generated in the presence of IL-21 recognized tumor cells efficiently, demonstrating potent effector functions. **Conclusions:** IL-21 induces opposing effects on antigen-presenting cells and CD8⁺ T cells. Strategic application of IL-21 is required to induce optimal clinical effects and may enable the generation of large numbers of highly avid tumor-specific CTL for adoptive immunotherapy.

Recent clinical success associated with the adoptive transfer of antitumor reactive T cells supports the potential impact of this significant clinical modality (1–3). To generate clinically effective tumor-specific CD8⁺ T cells *ex vivo*, two components are crucially important: antigen-presenting cells (APC) and T-cell growth factors. Dynamic APC-CD8⁺ T-cell interactions drive CD8⁺ T-cell proliferation and differentiation, resulting in the generation of CTL that have diverse phenotypic and

functional characteristics. Several autologous APC such as dendritic cells, CD40 ligand-activated B cells, EBV-lymphoblastoid cell lines, and a number of artificial APC have been used to deliver T-cell receptor engagement and multiple costimulatory and inhibitory signals (4–7).

Once T cells are properly stimulated by APC, CD8⁺ T cells require growth factors such as IL-2, IL-7, and IL-15, which belong to the common γ chain receptor cytokine family, for their expansion and lineage commitment (8). IL-2 has been used extensively by many investigators for the expansion of T cells, and, importantly, has become part of the clinical management of melanoma patients (9). However, accumulating evidence both *in vivo* and *in vitro* suggests that IL-2 also plays a major role in the expansion of regulatory T cells, which inhibit the effector function of antitumor T cells (10, 11). It has been suggested that IL-7 is important for the maintenance of both CD4⁺ and CD8⁺ T cells (12, 13). A recent clinical trial has shown that IL-7 administration in humans results in a rapid and selective increase in circulating CD4⁺ and CD8⁺ T cells (14). IL-15 has also commonly been used for the expansion of T cells *ex vivo*. In stark contrast to IL-2, which induces activation-induced cell death, IL-15 is an antiapoptotic factor for many cell types (15). Studies of IL-15 null mice strongly support the requirement of IL-15 for CD8⁺ T-cell expansion

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Translational Relevance

The immunostimulatory effects of interleukin 21 (IL-21) on effector immune cells such as T cells and natural killer cells have been well documented in both *in vivo* and *in vitro* studies. As a result, IL-21 has been tested in clinical trials for cancer in the hope that antitumor immunity would be enhanced. Unfortunately, although the administration of biologically active doses of IL-21 seems to be safe, only limited clinical benefit has been reported. In this article, we provide a comprehensive analysis of the opposing actions of IL-21 on human dendritic cells and CD8⁺ T cells. We also show that the use of IL-21 can provide large benefit for the *ex vivo* culture and expansion of tumor antigen-specific cytotoxic T cells when an IL-21 receptor negative antigen-presenting cell is used as a stimulator. Our results may be of help in designing future clinical trials involving IL-21 to harness its stimulatory effects on T cells while mitigating its suppressive immunologic effects on antigen-presenting cells.

(15). In mice, IL-15 directs CD8⁺ T cells to preferentially differentiate into central memory T cells whereas IL-2 promotes differentiation into effector memory T cells (16).

IL-21 is the newest member of the common γ chain receptor cytokine family and is closely related to IL-2 and IL-15 (17). *In vitro* and *in vivo* data suggest that IL-21 plays an important immunostimulatory role by modulating T, B, and natural killer cell-mediated immunity (18–20). Using APC such as dendritic cells, it has been shown *in vitro* that IL-21 can enhance the generation of antigen-specific CD8⁺ T cells with potent effector function (21). Furthermore, the expansion and cytotoxicity of CD8⁺ T cells were impaired in IL-21R^{-/-} mice (22, 23). It is not known whether IL-21 acts by directly stimulating CD8⁺ T cells or by first modulating the APC, which in turn induces a superior T-cell response. Either scenario is possible because, although IL-21 is secreted predominantly by activated CD4⁺ T cells, the IL-21 receptor (IL-21R) is widely expressed by various lineages of immune cells including both T cells and APC.

Other data suggest, however, that the immunostimulatory role of IL-21 may be limited via the immunosuppressive effects on APC. Although not fully established in humans, murine studies support an immunosuppressive role for IL-21 because *in vitro* it inhibits the maturation of dendritic cells and their capacity to stimulate T cells (24). Therefore, the effect of IL-21 may be dependent on whether exposure to IL-21 occurs before or following the initial establishment of an immune response. Understanding the mechanisms of IL-21-mediated immunomodulation in humans is of critical importance clinically as a favorable immune response may be significantly affected by the schedule and dose of IL-21 administered both *in vivo* and *ex vivo*.

In this study, we have shown in the human that IL-21 induces dichotomous immunologic effects on dendritic cells and CD8⁺ T cells. On the one hand, we show in human cells that IL-21 inhibits the maturation and T-cell stimulatory activity of dendritic cells. On the other, we conclusively show that IL-21 can act directly on lymphocytes and significantly

enhance the generation of antigen-specific CD8⁺ T cells by using a novel APC that does not express IL-21R. These results show the importance of dissecting the opposing immunologic effects of IL-21: the immunosuppression of dendritic cells and the immunostimulation of CTL.

Materials and Methods

cDNAs. Human IL-21 and IL-21R cDNA were cloned from normal peripheral blood mononuclear cells by reverse transcription-PCR based on the published sequences. IL-21 cDNA was tandemly fused to IRES-EGFP sequence and ligated to the pMX vector. IL-21R cDNA was cloned into pMXpuro. All the DNA constructs were verified by DNA sequencing.

Cells. K562-based artificial APC (aAPC) expressing HLA-A2, CD80, and CD83 has been reported previously (25). To generate aAPC/IL-21, aAPC was retrovirally transduced with IL-21 and EGFP, and flow cytometry was used to sort EGFP positive cells. Polyclonal cell lines consisting of at least 10⁴ independent clones were used to prevent cloning-induced variations.

The mouse IL-3-dependent pro-B lymphoid cell line BaF3 (a gift from James Griffin, Dana-Farber Cancer Institute) was infected with retrovirus encoding human IL-21R as well as a puromycin resistance gene. After drug selection, BaF3/IL-21R cells were enriched by specific antibody staining followed by magnetic bead guided sorting (Miltenyi Biotec). BaF3/puro, a control cell line transduced with vector alone, was generated by drug selection.

Purified CD8⁺ T cells were stimulated by 10–20 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich) and 1 μ g/mL calcium ionophore A23187 (Sigma-Aldrich) or plate-bound anti-CD3 and anti-CD28 monoclonal antibodies (mAbs; ref. 26). Immature and mature dendritic cells were generated as reported previously (26, 27). Where indicated, 50 ng/mL of recombinant human IL-21 (rIL-21) was added to immature dendritic cells in addition to IL-4 (10 ng/mL) and granulocyte macrophage colony-stimulating factor (50 ng/mL) every 2 d. Maturation was induced with double-stranded RNA (25 μ g/mL) and tumor necrosis factor- α (50 ng/mL) on day 6.

Flow cytometry analysis. mAbs recognizing the following antigens were used: CD54, CD58, CD80, CD83, and CD86 from BD Biosciences; CD45RA, CD45RO, CD28, and CD62L from Beckman Coulter; HLA-class I, HLA-DR, and CD27 from Caltag; CCR7 from R&D Systems; and mouse isotype controls from BD Biosciences and Beckman Coulter. To detect human IL-21R expression, goat anti-human IL-21R antibody or mouse anti-human IL-21R mAb (R&D) and appropriate PE-conjugated secondary antibodies (Jackson ImmunoResearch) were used. Surface molecule staining was done as described elsewhere (27). To avoid detecting the phenotypic changes simply related to the T-cell activation following T-cell receptor engagement and costimulation, all phenotypic analyses of antigen-specific T cells were done at least 7 d after the last stimulation unless indicated otherwise.

Production of HLA class I peptide-specific CD8⁺ T cells. Peripheral blood samples from normal donors were collected in compliance with protocols approved by the Institutional Review Board of the Dana-Farber Cancer Institute. Peptide-specific cytotoxic CD8⁺ T cell lines were generated as described previously (25–28). Briefly, aAPC and aAPC/IL-21 cells were pulsed with synthetic peptide [⁵⁸GILGFVFL⁶⁶ of the influenza virus matrix antigen at 0.1 μ g/mL, ²⁷AAGIGILT³⁵ of MART1 or ⁸⁵ELTLEFLK⁹⁴ of survivin both at 10 μ g/mL; (New England Peptides)] for 6 h at room temperature. APC were then irradiated, washed, and added to CD8⁺ T cells (1:20 APC T cells). Between stimulations, IL-2 (10 IU/mL; Chiron) and IL-15 (10 ng/mL; Peprotech) were added to the cultures unless indicated otherwise.

HLA/peptide multimer analysis. HLA/peptide multimer analysis was done as described previously (25–28). HLA/peptide multimer was generated in-house or purchased from ProImmune.

Endocytic activity assay. Endocytosis was measured as the cellular uptake of FITC-dextran and was quantified by flow cytometry. Briefly, dendritic cells were incubated with FITC-dextran (1 mg/mL; molecular weight 3,000; Invitrogen) for 10, 30, or 60 min. After incubation, the cells were washed twice with cold PBS to stop endocytosis and to remove excess dextran.

Cytotoxicity assay. Cytotoxicity assay was done as described previously (25–28).

Intracellular cytokine release assay. T cells were cocultured with peptide-loaded T2 cells at the ratio of 4:1 for 6 h in the presence of 10 µg/mL brefeldin-A. Cells were then collected, washed, fixed, permeabilized, and stained with PC5-conjugated anti-CD8 mAb

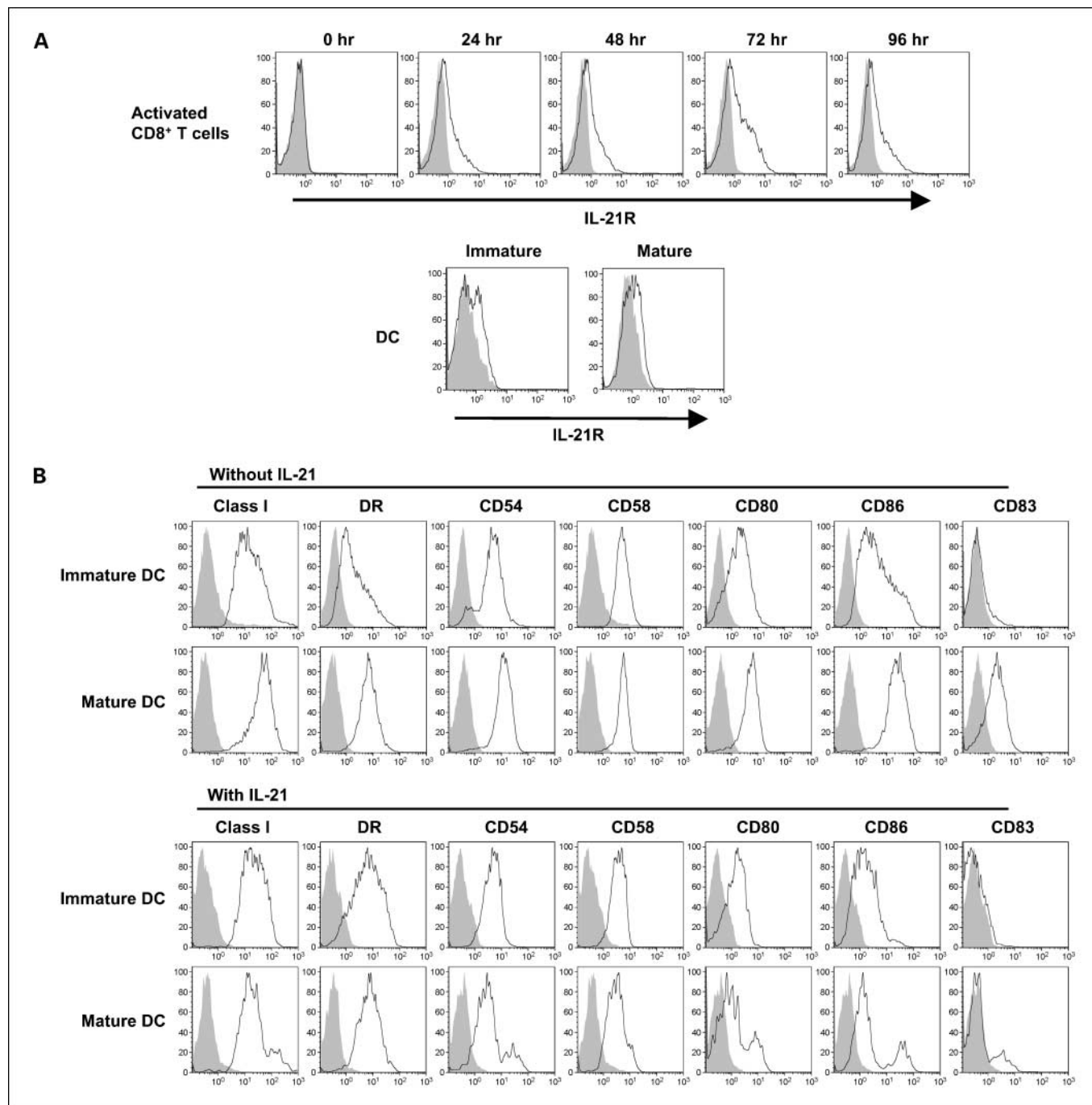


Fig. 1. IL-21 counteracts the full maturation of dendritic cells. **A**, IL-21R is not expressed on resting CD8⁺ T cells but induced upon activation by phorbol myristate acetate and calcium ionophore A23187. In contrast, dendritic cells constitutively express IL-21R. Surface expression of IL-21R on purified CD8⁺ T cells, and immature and mature dendritic cells from healthy donors, was determined by flow cytometry with specific antibody (open curves) and isotype control (shaded curves). One result of at least six (CD8⁺ T cells) and four (dendritic cells) independent experiments is shown. **B**, IL-21 inhibits the up-regulation of adhesion and costimulatory molecules by dendritic cells upon exposure to maturation signals. Immature dendritic cells were generated from purified monocytes using IL-4 and granulocyte macrophage colony-stimulating factor. Where indicated, dendritic cell cultures were also supplemented with rIL-21 at 50 ng/mL every 2 d. Surface phenotype of immature and mature dendritic cells generated in the presence or absence of IL-21 is shown. Open curves, staining for indicated molecules; shaded curves, isotype controls. A representative result of four independent experiments is given.

(Beckman Coulter), FITC-conjugated anti-IFN- γ and PE-conjugated anti-IL-2 (BD Biosciences).

Cell proliferation assay. Duplicates of BaF3/IL-21R cells were plated in 96-well plates at 50,000 per well. Serially increasing concentrations of rIL-21 (0-400 ng/mL) or conditioned medium of aAPC or aAPC/IL-21 were added to the cells. Cells without rIL-21 or conditioned medium were used to assess the baseline growth for the duration of the assay. After incubation for 6 h at 37°C, each well was pulsed with 1 μ Ci [³H]thymidine (Amersham Biosciences), and plates were collected and counted 18 h later.

Purified CD8⁺ T cells from allogeneic healthy donors were cocultured with irradiated immature or mature dendritic cells generated in the presence or absence of IL-21. After incubation for 72 h at 37°C, each well was pulsed with 1 μ Ci [³H]thymidine, and plates were collected and counted 18 h later.

Statistical analysis. Allogeneic T-cell proliferation was analyzed as a ratio of responses to the following APC: mature versus immature dendritic cells, mature dendritic cells without versus with IL-21, and immature dendritic cells without versus with IL-21. T-cell data were analyzed as the ratio of CD8⁺ T cells, and the percentage and total number of MART1-specific CTL generated with aAPC/IL-21 compared with aAPC. The one-sided Student's *t* test was used to assess whether proliferation was significantly reduced with IL-21 treatment or was increased with dendritic cell maturation. Similarly, an increase in T-cell generation was assessed following IL-21 treatment. All statistical tests were conducted at the 0.05 significance level.

Results

IL-21R is expressed by activated T cells and dendritic cells. Others have reported that IL-21R is expressed by T cells as well as by APC such as dendritic cells and activated B cells

(17, 24). Purified CD8⁺ T cells from healthy donors were optimally stimulated and cell surface IL-21R expression was studied every 24 hours. IL-21R expression was absent on resting CD8⁺ T cells and was up-regulated upon stimulation (Fig. 1A). Maximal expression was observed after either 72 or 96 hours depending on the donors. We next evaluated the expression of IL-21R on dendritic cells. Immature dendritic cells were generated from peripheral blood monocytes in the presence of granulocyte macrophage colony-stimulating factor and IL-4. To obtain mature dendritic cells, we used tumor necrosis factor- α and double-stranded RNA, a combination that has been shown to be a potent method of dendritic cell activation (29). As shown in Fig. 1A, IL-21R was expressed by both immature and mature dendritic cells at the protein level, suggesting that IL-21 may modulate the phenotype and function of dendritic cells.

IL-21 mediates immunosuppression of human dendritic cells by counteracting maturation. Next we investigated the effect of IL-21 treatment on the phenotype of immature and mature dendritic cells (Fig. 1B). On immature dendritic cells, IL-21 treatment consistently suppressed CD86 expression as previously observed in the mouse. In contrast to the mouse, however, IL-21 treatment consistently increased the expression of HLA-DR suggesting that IL-21 selectively modulates the dendritic cell phenotype. When dendritic cells were exposed to maturation signals, significant differences were observed. Maturation induced up-regulation of adhesion molecules (CD54 and CD58), costimulatory molecules (CD80 and CD86), and the maturation marker, CD83, was inhibited when dendritic cells were generated in the presence of IL-21. In

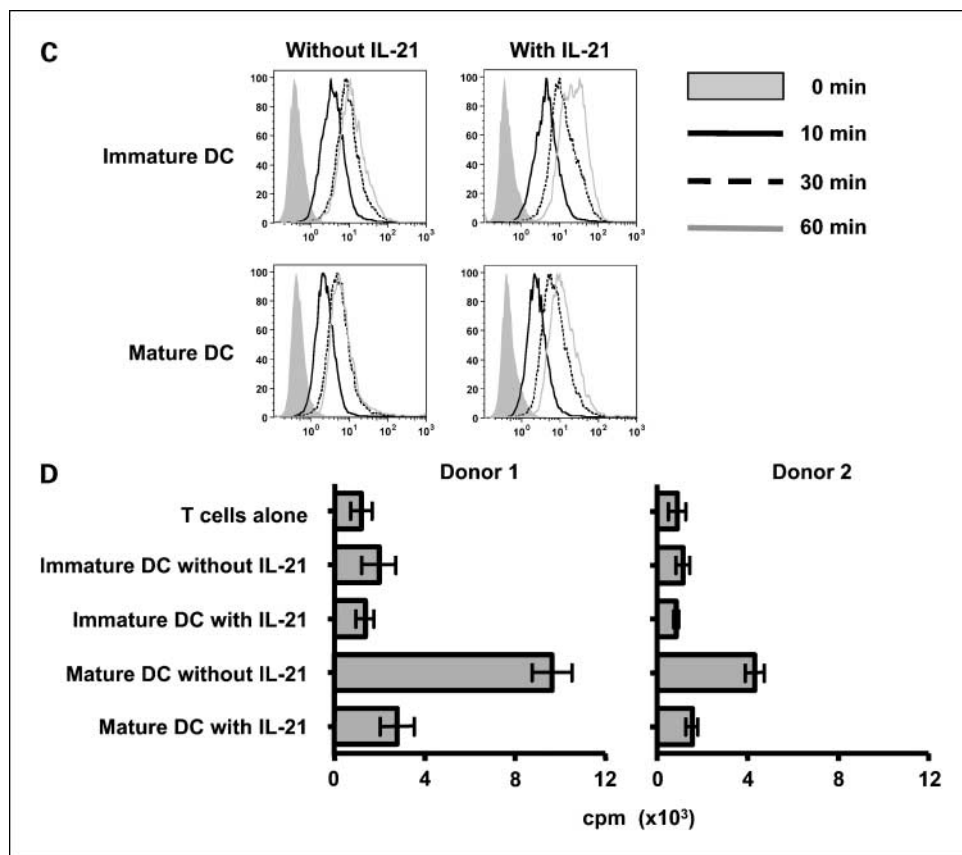


Fig. 1 Continued. C, IL-21 enhances the endocytic activity of dendritic cells. Endocytic activity of immature and mature dendritic cells generated in the presence or absence of IL-21 was measured by FITC-dextran uptake and quantified by flow cytometry. Note that dendritic cells lose endocytic activity in response to maturation signals despite the presence of IL-21. Shaded curve, 0 min; solid black line, 10 min; dashed line, 30 min; solid gray line, 60 min. Three independent experiments were done with similar results. **D,** T-cell stimulatory capacity of IL-21 – treated mature dendritic cells is markedly suppressed. Purified CD8⁺ T cells from four healthy donors were cocultured with irradiated allogeneic immature or mature dendritic cells that had been generated in the absence or presence of IL-21. Allogeneic CD8⁺ T-cell response was measured using a standard [³H]thymidine proliferation assay. The results from two representative donors are shown, and each value displayed is the average of six replicates (\pm SD). Note that because purified CD8⁺ T cells are used as responders, the measured values are lower in comparison with values typically obtained when CD4⁺ or CD3⁺ T cells are used as responder cells. However, the results obtained for CD8⁺ T-cell proliferation in this assay are reproducible.

contrast, the increased HLA-DR expression was retained, further demonstrating selective modulation by IL-21. It should be noted that the viability of dendritic cells was not affected by IL-21 treatment. These results suggest that IL-21 may block the maturation of dendritic cells by counteracting maturation signals.

We studied the effect of IL-21 treatment on dendritic cell function. Consistent with an increased capacity to take up antigen, exposure to IL-21 enhances the endocytic activity of immature dendritic cells. However, IL-21 did not inhibit the ability of maturation signals to down-regulate dendritic cell endocytic activity to levels observed in cells not treated with IL-21 (Fig. 1C). Next we evaluated the ability of dendritic cells treated with or without IL-21 to stimulate CD8⁺ T cells. Allogeneic CD8⁺ T cells from normal donors were stimulated with irradiated dendritic cells, and the allogeneic response was measured. As expected, significantly higher proliferation was induced with mature than with immature dendritic cells by an average of 3.4-fold (1.3-4.9; $P = 0.02$). As predicted by the failed induction of immunostimulatory molecules on IL-21-treated mature dendritic cells, significant suppression of proliferation by allogeneic CD8⁺ was observed so that proliferation induced by untreated mature dendritic cells was 2.7-fold higher (1.6-3.5; $P = 0.01$) than by IL-21-treated mature dendritic cells (Fig. 1D). IL-21 treatment of immature dendritic cells did not significantly reduce proliferation. Taken together, these results suggest that dendritic cells express a functional IL-21R, and that IL-21 signaling blocks the immunogenicity of dendritic cells by suppressing the maturation signal-induced up-regulation of costimulatory molecules.

Generation of IL-21R-negative aAPC which constitutively secretes IL-21. Using IL-21R-positive APC such as dendritic cells, others have reported that IL-21 can enhance the generation of antigen-specific CD8⁺ T cells with potent effector function and higher avidity. Whether IL-21 acts directly on CD8⁺ T cells or first modulates IL-21R⁺ APC, which subsequently stimulate CD8⁺ T cells, has not yet been determined. Therefore, to study the immunologic functions of IL-21 on CD8⁺ T cells, it is mandatory to separate the effects of IL-21 on IL-21R⁺ APC from its effects on T cells because IL-21 can modulate the function of APC.

Previously, we reported the generation and characterization of a novel aAPC that expresses HLA-A2, CD80, and CD83 (25, 27). We showed that aAPC is able to support the priming and prolonged expansion of peptide-specific CD8⁺ CTL with potent effector functions. Using an IL-21R-specific antibody, we confirmed that K562, the parental cell line, lacks IL-21R expression (Fig. 2A). In contrast, the same antibody specifically recognized BaF3/IL-21R, a BaF3 cell line transduced with human IL-21R. Using IL-21R negative aAPC, we then generated an APC that constitutively secretes IL-21 (aAPC/IL-21) by further transducing aAPC with human IL-21 cDNA. IL-21 transduction did not affect the expression of A2, CD80, nor CD83 previously transduced (data not shown). To verify that IL-21 secreted by aAPC/IL-21 is biologically active, we did a proliferation assay using BaF3/IL-21R cells (17, 30). Graded amounts of supernatant harvested from aAPC/IL-21 were added to BaF3/IL-21R, and proliferation was measured by [³H]thymidine incorporation. As shown in Fig. 2B, conditioned medium derived from aAPC/IL-21, but not from parental aAPC, supported the growth of BaF3/IL-21R in a dose-

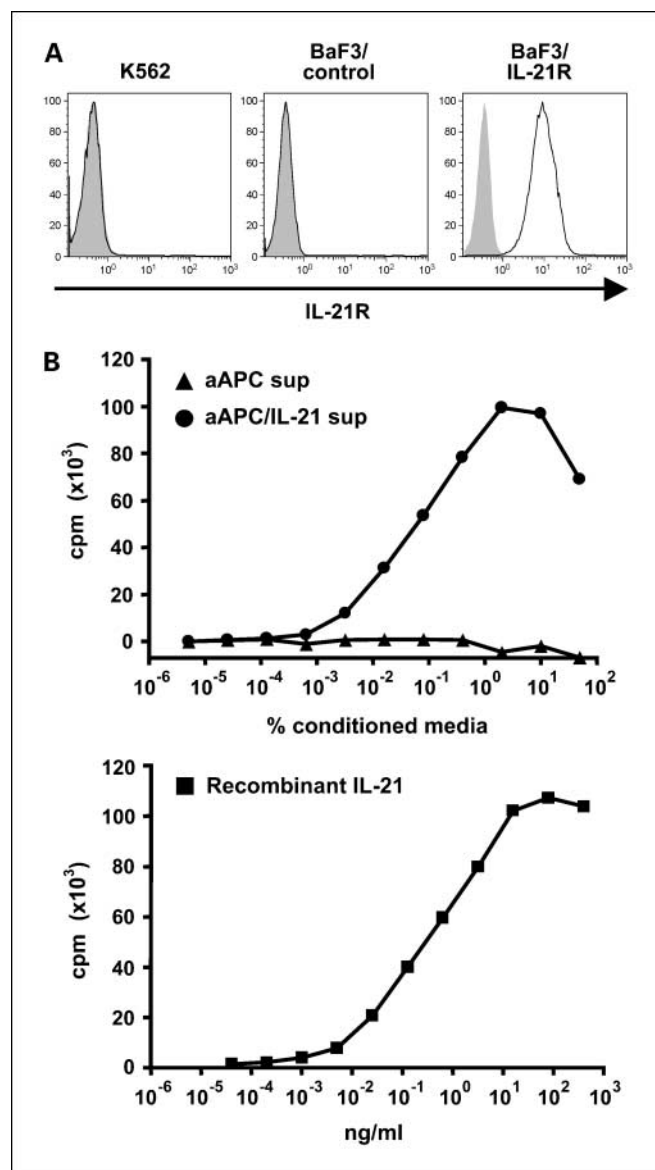


Fig. 2. Generation of IL-21R negative aAPC constitutively secreting IL-21. *A*, K562, the parental cell line of aAPC and aAPC/IL-21, lacks the expression of IL-21R. To create an IL-21 dependent cell line, mouse BaF3 cells were engineered to express human IL-21R. Surface expression of IL-21R on wild-type K562, BaF3 transduced with vector (BaF3 control), and BaF3 cells transduced with human IL-21R (BaF3/IL-21R) was determined by flow cytometry with specific antibody (open curves) and isotype control (shaded curves). *B*, aAPC/IL-21 but not aAPC constitutively secretes large amounts of biologically active IL-21. Induction of BaF3/IL-21R proliferation by supernatants derived from aAPC and aAPC/IL-21, and rIL-21 was determined using a standard thymidine proliferation assay. Baseline values for proliferation observed in the absence of conditioned medium or rIL-21 were subtracted. Neither supernatant from aAPC nor aAPC/IL-21 was able to support the growth of BaF3/control cells (data not shown).

dependent manner. Using rIL-21, we were able to determine that 10⁶ aAPC/IL-21 are capable of constitutively secreting IL-21 at 0.8 μg/mL per 24 hours. These results show that aAPC/IL-21 lacks the functional receptor for IL-21 and constitutively secretes bioactive IL-21, and can serve as a useful APC for dissecting the immunologic effects of IL-21 on CD8⁺ T cells.

IL-21 can enrich and expand large numbers of CD8⁺ T cells. Using IL-21R null aAPC/IL-21, we first examined the immunologic effects of IL-21 on the expansion of newly primed

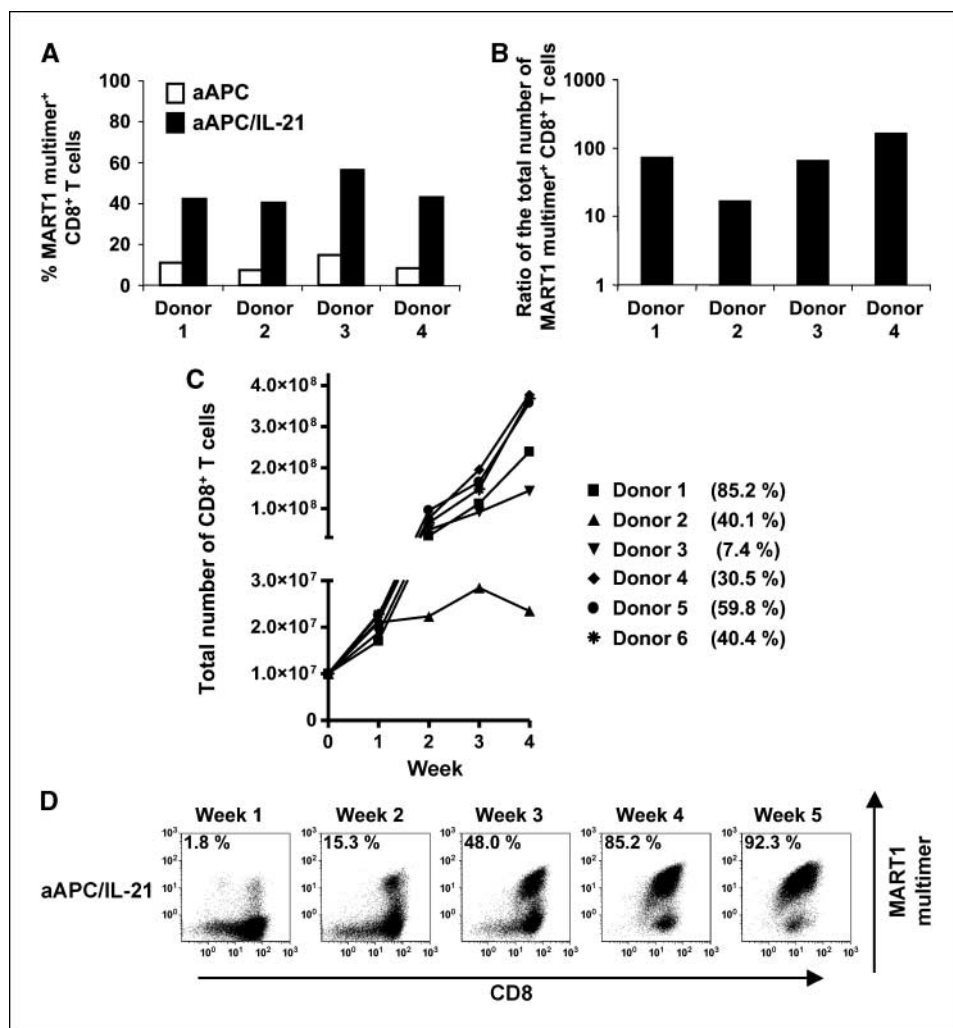


Fig. 3. Stimulation in the presence of IL-2 induces robust enrichment and expansion of antigen-specific CD8⁺ T cells. **A**, IL-21 augments the expansion and enrichment of antigen-specific CD8⁺ T cells. Purified CD8⁺ T cells from HLA-A2⁺ healthy donors were primed with aAPC pulsed with MART1 peptide in the absence of IL-21. After 1 wk, T-cell cultures were split by half and stimulated thrice on a weekly basis with either peptide-pulsed aAPC or aAPC/IL-21. Between stimulations, T cells were supplemented with IL-2 (10 IU/mL) and IL-15 (10 ng/mL). Resulting T-cell cultures were stained with HLA/peptide multimer to determine the percentage of MART1-specific CD8⁺ T cells. **B**, the number of MART1-specific T cells was determined by calculating the product of the total number of T cells and the percentage of multimer positive cells. Shown here is the ratio of the total number of MART1-specific CD8⁺ T cells generated in the presence versus the absence of IL-21 for each donor. **C**, generation of CTL by both priming and restimulation with MART1 peptide-pulsed aAPC/IL-21 was done. Purified CD8⁺ T cells from six A2⁺ healthy donors were stimulated once a week. Between stimulations, T cells were supplemented with IL-2 (10 IU/mL) and IL-15 (10 ng/mL). The calculated total number of CD8⁺ T cells generated is shown. The percentage of MART1 multimer positive CD8⁺ T cells after four stimulations is also presented. **D**, representative MART1 multimer staining done weekly after each stimulation is presented (donor 1 from C).

antigen-specific CD8⁺ T cells. Purified CD8⁺ T cells from HLA-A2⁺ healthy donors were initially primed with MART1 peptide-pulsed aAPC. Each week thereafter, cultures were split and stimulated thrice with either MART1 peptide-pulsed aAPC or aAPC/IL-21. The ratio of the total number of CD8⁺ T cells generated by aAPC/IL-21 compared with aAPC was increased by an average of 17.8-fold (3.2-31.4; *n* = 4; *P* = 0.03; data not shown). Stimulation with aAPC/IL-21 consistently generated a higher percentage of MART1 multimer positive CTL, averaging 45.4% (40.4-55.9%; *n* = 4), which, compared with aAPC, is increased by 31.2 to 40.9 percentage points (*P* = 0.002; Fig. 3A). As a result, stimulation with aAPC/IL-21 yielded an increase of MART1-specific CTL by an average of 79.8-fold (17.0-163.5; *n* = 4; *P* = 0.04) compared with aAPC (Fig. 3B). In summary, starting with 6.5×10^6 to 7.8×10^6 purified CD8⁺ T cells (*n* = 4), we generated a total of 0.14×10^8 to 1.3×10^8 MART1-specific T cells within 4 weeks. These results suggest that exposure to IL-21 during restimulation promotes the robust expansion of antigen-specific CD8⁺ T cells initially primed in the absence of IL-21.

To study the maximal effect of IL-21 on the generation of antigen-specific CD8⁺ T cells, purified CD8⁺ T cells from A2⁺ healthy donors were both primed and restimulated with MART1 peptide-pulsed aAPC/IL-21. As shown in Fig. 3C,

starting with 10^7 purified CD8⁺ T cells, we generated 0.23×10^8 to 3.8×10^8 (*n* = 6) CD8⁺ T cells within 4 weeks, and the mean HLA/peptide multimer positivity was 43.9% (7.4-85.2%; *n* = 6). MART1 multimer staining after each stimulation for one representative donor is shown in Fig. 3D.

Priming in the presence of IL-21 modulates the phenotype of antigen-specific CD8⁺ T cells. It has been postulated that critical signaling during priming determines the fate of T cells. Therefore, we studied the effects of exposing CD8⁺ T cells with IL-21 during priming. Purified CD8⁺ T cells obtained from A2⁺ normal donors were stimulated once with either MART1 peptide-pulsed aAPC or aAPC/IL-21. Cultures were supplemented with IL-2 and IL-15 every 3 days without any further stimulation, and phenotypic analysis of MART1 multimer positive T cells was done 10, 12, and 14 days later. CTL primed with aAPC/IL-21 showed an average of 1.8% multimer positivity (1.0-2.3%; *n* = 3; day 14), which is higher than the average of 1.1% seen in aAPC-primed CTL (0.6-1.4%; *n* = 3; day 14). As published previously (21), priming in the presence of IL-21 resulted in higher expression of CD28 by MART1-specific CTL, which might explain the more robust expansion of antigen-specific CD8⁺ T cells primed with aAPC/IL-21. In variance with this report, however, a higher percentage of MART1-specific CTL generated with aAPC/IL-21 expressed

CD45RA and fewer displayed CD45RO (Fig. 4A). Both aAPC/IL-21 and aAPC resulted in similarly high CD27 expression by MART1-specific T cells. Compared with aAPC, aAPC/IL-21 generated MART1-specific CTL with a decrease in the percentage of CCR7⁺ cells, although the effect of IL-21 on the level of CD62L expression was variable. The differential expression of CD28, CD45RA, CD45RO, and CCR7 was consistently observed in all three donors on all three days tested (Fig. 4B). Furthermore, using peptide-pulsed aAPC and exogenously

added rIL-21 (10-30 ng/mL), we were able to recapitulate these observations, underscoring that these phenotypic alterations are reproducible and specific to IL-21 (data not shown).

Chronic stimulation of antigen-specific CD8⁺ T cells abrogates the phenotypic differences induced by IL-21, resulting in a convergence of the immunophenotype. Although the above results clearly revealed the effect of IL-21 on the priming phase, it is important to investigate the effect of IL-21 in the context of chronic stimulations. Purified CD8⁺ T cells were

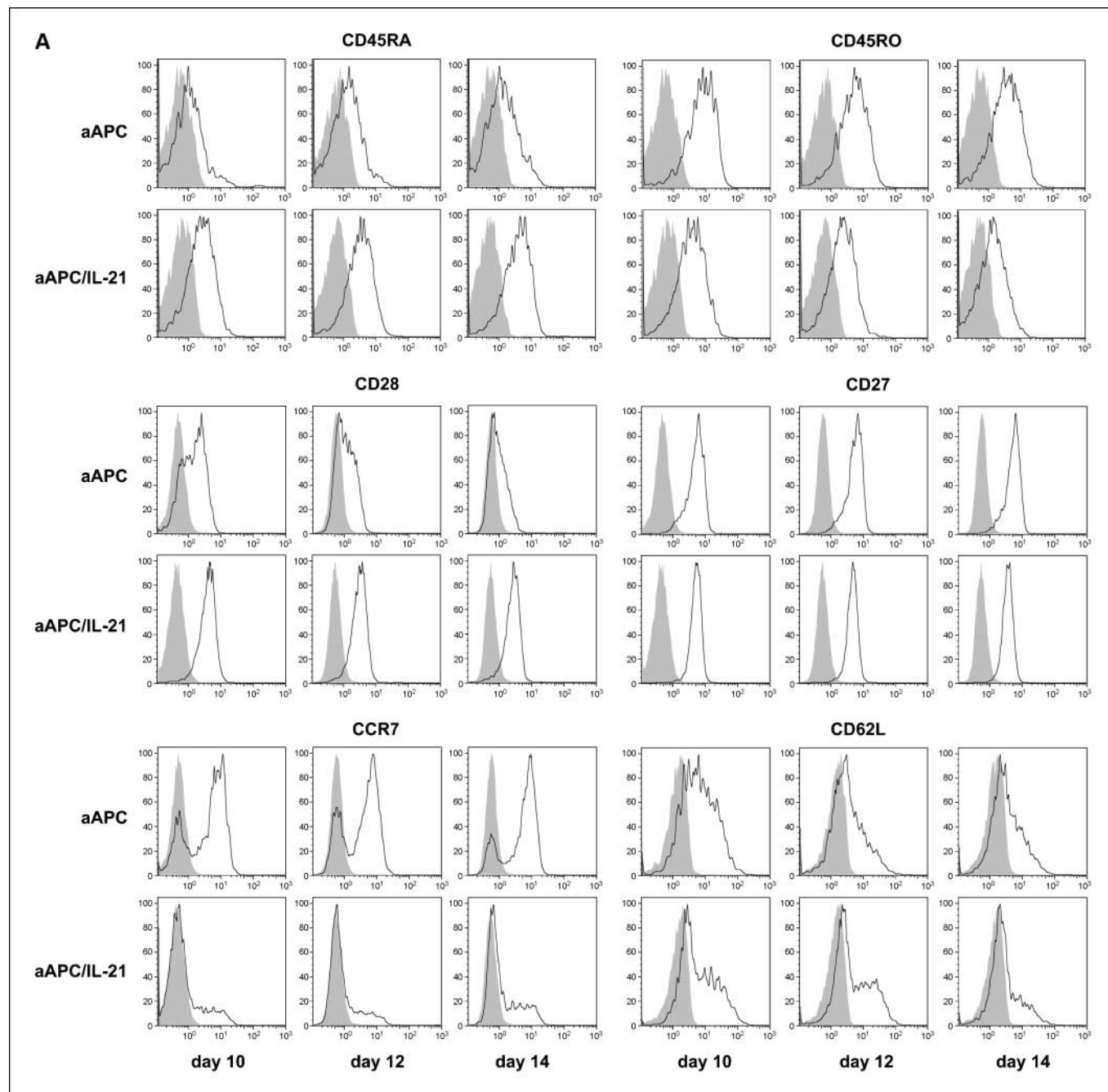


Fig. 4. Priming in the presence of IL-21 generates antigen-specific CD8⁺ T cells with distinct phenotype. Purified CD8⁺ T cells from three A2⁺ healthy donors were primed with either MART1 peptide-pulsed aAPC or aAPC/IL-21. Without any restimulation, each culture was supplemented with IL-2 (10 IU/mL) and IL-15 (10 ng/mL) every 3 d. After 10, 12, and 14 d, immunophenotype of MART1-specific T cells was analyzed by flow cytometry. **A**, a representative result of one donor out of three is presented. Open curves, staining for indicated molecules; shaded curves, isotype controls. Similar results were obtained using aAPC versus aAPC plus rIL-21.

repeatedly stimulated with either MART1 peptide-pulsed aAPC or aAPC/IL-21 on a weekly basis and cultures were supplemented with IL-2 and IL-15 between stimulations. After five stimulations, however, no large phenotypic difference was observed, except for a remnant of increased CD45RA positivity on MART1-specific CD8⁺ T cells chronically stimulated with aAPC/IL-21 (Fig. 4C). We previously observed that, in some donors, CD28 was retained following stimulation with aAPC (27). Similar results were observed with aAPC/IL-21. These data suggest that chronic stimulation of antigen-specific CD8⁺ T cells cancels the phenotypic differences induced by IL-21.

Repeated stimulation in the presence of IL-21 promotes the acquisition of IFN- γ secretion while preserving IL-2 secretion by antigen-specific CD8⁺ T cells. Chronically stimulated MART1-specific CD8⁺ T cells were generated as described above. CTL

cultures generated in the presence and absence of IL-21 both showed potent antigen-specific cytotoxicity as measured in a standard cytotoxicity assay (data not shown). Large differences between the percentage of multimer positive cells in the two CTL cultures prevent a reliable direct comparison of cytotoxicity on a per antigen-specific CTL basis (see Fig. 3A). Therefore, we compared the effector function of generated CTL by measuring peptide-specific intracellular release of IFN- γ and IL-2. As shown in Fig. 5, CTL generated in the presence of IL-21 possessed a larger population of cells, which secrete IFN- γ in an antigen-specific manner, suggesting that IL-21 may promote the acquisition of IFN- γ secretion by antigen-specific CD8⁺ T cells. Interestingly, IL-21-treated CTL were also capable of secreting IL-2 in an antigen-specific manner, which may be attributed to the unique function of IL-21, because it has been shown that the ability of IL-2 and IFN- γ secretion is exclusive in the

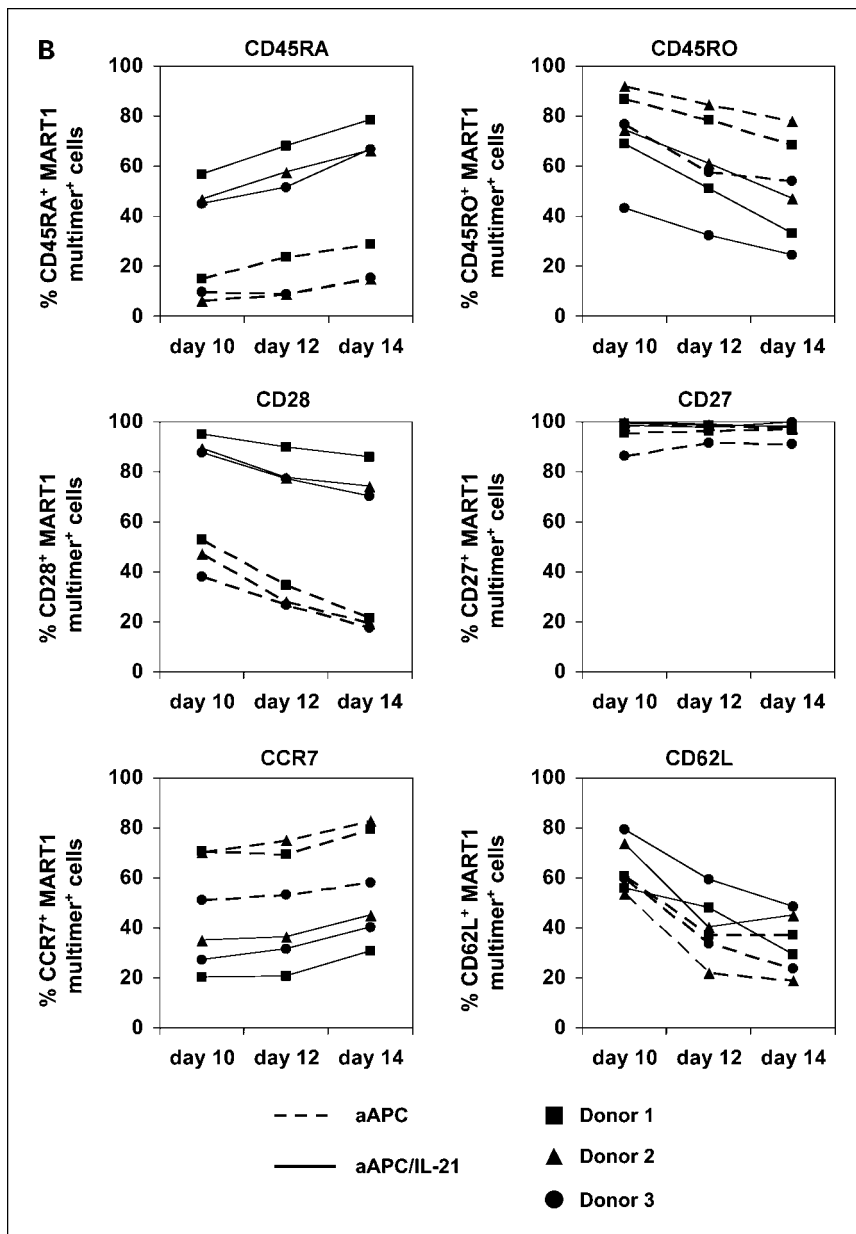


Fig. 4 Continued. B, a summary of the phenotypic analysis of MART1 multimer positive T cells from all 3 donors on all 3 d studied is shown.

maturation/differentiation ontogeny of CD8⁺ T cells (31). It should be noted that even after taking into account the higher multimer positivity of IL-21-treated CTL, IL-21 treatment produced MART1 CTL with a higher capacity to secrete IFN- γ on a per cell basis. These results suggest that, although the phenotype of CTL chronically stimulated with aAPC or aAPC/IL-21 was similar, the effector function of these two MART1-specific CD8⁺ T cells were markedly distinct.

IL-21 facilitates the generation of antitumor CD8⁺ CTL with low precursor CTL (pCTL) frequency and/or growth potential. Survivin is an inhibitor of apoptosis protein, which is overexpressed in many types of malignant cells and has been shown to be immunogenic in studies both *in vivo* and *in vitro*. Therefore, it has been considered to be a promising target for T-cell immunotherapy for cancer (32). Although many groups have reported the successful *ex vivo* generation of survivin-specific human CD8⁺ T cell lines, the frequency of survivin-specific T cells included in these lines has not always been high, indicating a low pCTL frequency and/or growth potential (33–35). In fact, previously reported anti-survivin CTL lines exhibited only modest effector function even at high effector:target ratios (33, 34).

Using survivin as a model antigen with a low pCTL frequency, we attempted to generate survivin-specific CD8⁺ CTL from two HLA-A2 positive healthy donors using synthetic Sur9 (ELTLGFLKL) peptide. Purified HLA-A2 positive CD8⁺ T cells were stimulated with Sur9 peptide-pulsed aAPC/IL-21 at weekly intervals and cultures were supplemented with IL-2 and IL-15 twice a week. Because HLA-A2/Sur9 peptide multimer was not available due to difficulties in the manufacturing process, we used an IFN- γ ELISPOT to enumerate the frequency of Sur9-specific CD8⁺ T cells in the culture. After four stimulations, as shown in Fig. 6A, 72 \pm 8 and 36 \pm 6 cells per 1,000 cells in donors 1 and 2, respectively, secreted IFN- γ in an antigen-specific manner. Survivin-derived Sur9 peptide-specific CTL specifically cytolized T2 cells pulsed with Sur9 peptide but not T2 cells pulsed with the control peptide, Pol, at an effector target ratio as low as 1:1 (Fig. 6B). To show that the Sur9 specific CTL lines generated have high functional T-cell receptor avidity, the standard cytotoxicity of hematologic cancer cells from different lineages was tested (Fig. 6C). HLA-A2 positive and HLA-A2 transduced Survivin⁺ cancer cells but not HLA-A2 negative or mock transduced Survivin⁺ cells were recognized by the CTL at effector:target ratios as low as 3:1. These

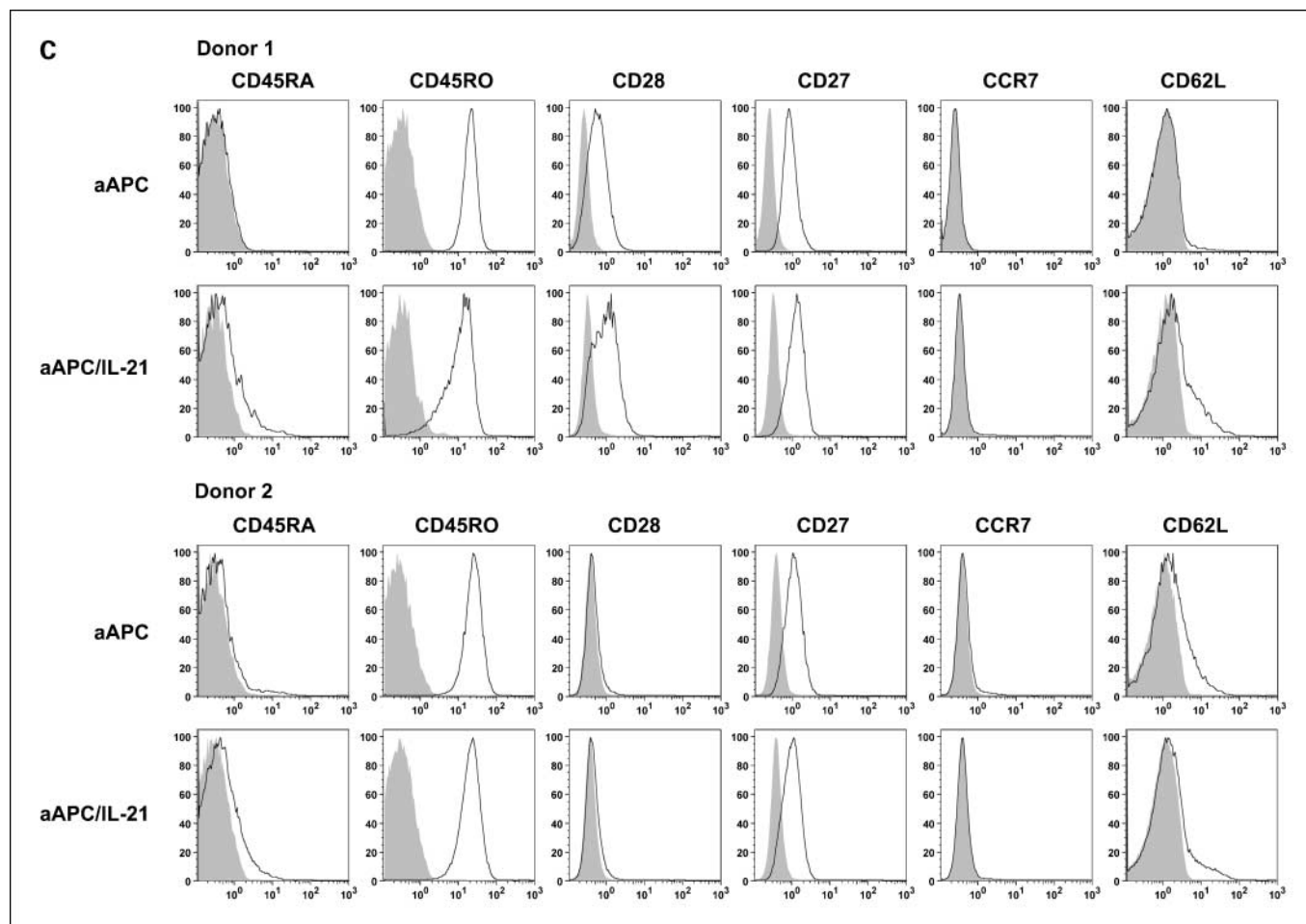


Fig. 4 Continued. C, the effect of chronic stimulation in the presence of IL-21 on the phenotype of antigen-specific T cells was also evaluated. Purified CD8⁺ T cells from two different healthy donors were stimulated once a week with either MART1 peptide-pulsed aAPC or aAPC/IL-21. Between stimulations, T cells were supplemented with IL-2 (10 IU/mL) and IL-15 (10 ng/mL). After three and five stimulations, MART1 multimer positive T cells were found to have a similar phenotype. Results after five stimulations are shown. Open curves, staining for indicated molecules; shaded curves, isotype controls. Two independent experiments were done with similar results.

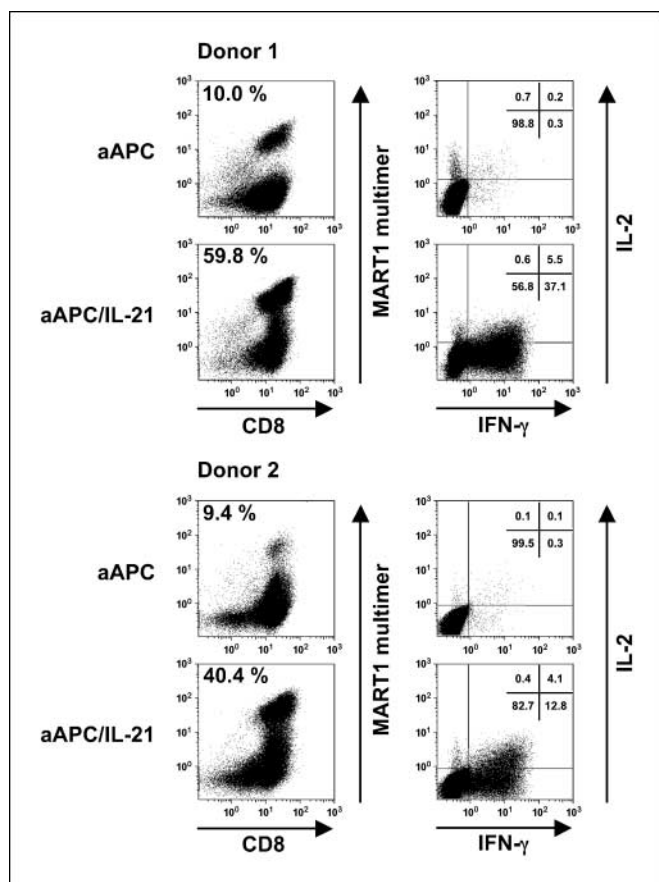


Fig. 5. IL-21 promotes the acquisition of IFN- γ secretion while preserving IL-2 secretion by antigen-specific CD8 $^+$ T cells. Purified CD8 $^+$ T cells from A2 $^+$ healthy donors were primed and restimulated with either aAPC or aAPC/IL-21 pulsed with MART1 peptide on a weekly basis. Between stimulations, T cells were supplemented with IL-2 (10 IU/mL) and IL-15 (10 ng/mL). After a total of four stimulations, T cells were analyzed for antigen specificity by HLA/peptide multimer staining and antigen-specific effector function. The panels in the left column depict MART1 multimer staining and the percentages in the upper left corner indicate MART1 multimer $^+$ cells within CD8 $^+$ T cells. The panels in the right column show the antigen-specific intracellular release of IL-2 and IFN- γ on gated CD8 $^+$ T cells of the corresponding T-cell cultures. The percentages of cells present in each quadrant are shown. The results of two different donors out of three are shown.

results indicate that IL-21 may facilitate the generation of antitumor CD8 $^+$ CTL with low pCTL frequency and/or growth potential.

Discussion

Functional IL-21R is expressed by most immune cells such as B cells, T cells, natural killer cells, and dendritic cells. The requirement of the unique IL-21R chain for signal transduction suggests that IL-21 may possess novel immunologic functions (18–20). *In vivo* studies using IL-21 and IL-21R null mice showed that IL-21:IL-21R signaling critically regulates the proliferation and function of T, B, and natural killer cells (22, 23, 36). In these studies, however, the specific functional role of IL-21 and IL-21R on dendritic cells has not been fully illuminated. Murine studies *in vitro* have previously shown that IL-21 inhibits the up-regulation of class II, CD80, and CD86 expression by dendritic cells in response to maturation signals

(24). Our study confirms and extends these results as we have shown for the first time in humans that IL-21 has a similar immunoinhibitory effect on human dendritic cell immunogenicity and expression of costimulatory molecules. Our results also suggest, however, that the IL-21 effect on human dendritic cells is more selective than in the mouse given the preserved expression of HLA-DR.

A number of reports have been published that IL-21 can enhance the generation of highly avid CD8 $^+$ T cells with potent effector function (21, 23, 37, 38). However, IL-21R is constitutively and widely expressed on most APC such as dendritic cells, B cells, monocytes and macrophages that are commonly used for stimulation of responder T cells. Therefore, it has remained elusive whether IL-21 acts directly on responding CD8 $^+$ T cells or by enhancing the stimulatory effects of APC. To address this controversy, we generated a novel IL-21R negative aAPC that constitutively secretes IL-21 and tested whether IL-21 can act directly on antigen-specific CD8 $^+$ T cells. Because aAPC and aAPC/IL-21 lack the expression of IL-21R, it is highly unlikely that IL-21 modulates the immunogenicity of these APC through the delivery of signals in an autocrine or paracrine fashion. Using an experimental system composed of only two cell components, i.e., aAPC/IL-21 and purified CD8 $^+$ T cells, we have unequivocally shown that IL-21 directly delivers its stimulatory signal to antigen-specific CD8 $^+$ T cells. We have also shown that IL-21 during priming mediates consistent phenotypic changes during CD8 $^+$ T-cell maturation and differentiation. Findings in variance with previously published reports on the phenotype of T cells generated using IL-21 are likely due to the effect of IL-21 on cocultured IL-21R positive APC that are absent in our cultures. Moreover, aAPC/IL-21 enabled the expansion of antitumor CTL with low pCTL frequency. Because IL-2 and IL-15 are included as a default in our T-cell culture, it is clear that the observed effects are unique to IL-21 secreted from aAPC/IL-21 and cannot be replaced by IL-2 and/or IL-15. The fact that rIL-21 can be used with aAPC to recapitulate the presented immunologic functions of aAPC/IL-21 further corroborates our findings (data not shown).

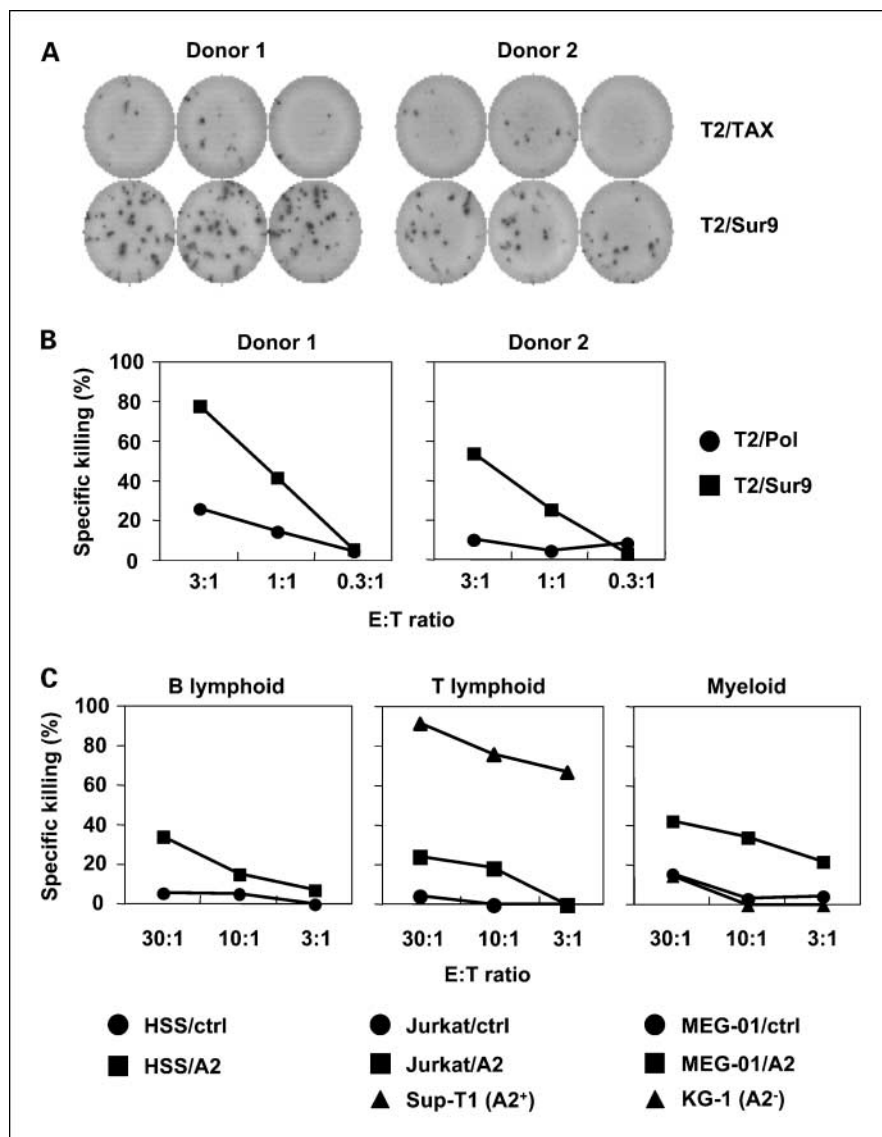
There are at least two proposed models that explain the molecular mechanisms of CD4 $^+$ T-cell help for CD8 $^+$ T cells. The first model suggests that helper CD4 $^+$ T cells and CD8 $^+$ CTL recognize their cognate antigens simultaneously on the same APC and that cytokines produced by the activated helper CD4 $^+$ T cells act in a paracrine fashion to facilitate the CD8 $^+$ CTL response (39, 40). Alternatively, it has been suggested that dendritic cells are activated, or “licensed,” by interacting with antigen-specific CD4 $^+$ helper T cells through CD40-CD40L interactions resulting in the ability of activated dendritic cells to prime antigen-specific CD8 $^+$ T cells (41–43). IL-21 is located at a unique position in the generation of antigen-specific cellular responses because IL-21 is predominantly secreted by activated CD4 $^+$ T cells and because IL-21 possesses opposing effects on dendritic cells and CD8 $^+$ T cells. While CD4 $^+$ T cells may “license” dendritic cells through CD40 ligation, they can also limit this activation through IL-21 secretion. On the other hand, however, CD4 $^+$ T cells may directly provide “help” to primed CD8 $^+$ T cells through the secretion of IL-21, as well as IL-2 and IL-15. Thus, IL-21 can control CD8 $^+$ T-cell responses by positively regulating CD8 $^+$ T cells but also simultaneously limiting this response through the negative regulation of dendritic cells.

By circumventing the immunosuppressive effects of IL-21, our IL-21R-deficient aAPC might serve as a unique platform to generate large numbers of tumor-specific CTL. We have previously shown that these T cells can be maintained *in vitro* for remarkably long periods of time, up to 1.5 years in some cases. Also, as shown in Fig. 4B and C, CD8⁺ CTL generated with our IL-21R negative aAPC express consistently high levels of CD27. Studies conducted with *ex vivo* expanded tumor-infiltrating lymphocyte samples that were administered to melanoma patients have indicated that transferred T cells that persist for two months display an effector memory phenotype with a CD27⁺ CD28⁺ CD62L⁻ CCR7⁻ profile (44). The size of the pool of CD27⁺ CD8⁺ T cells in bulk tumor-infiltrating lymphocytes was highly associated with the ability of these tumor-infiltrating lymphocytes to mediate tumor regression following adoptive transfer (45). We have successfully produced a clinical grade aAPC, aAPC³³, and are embarking on a "first into human" clinical trial where advanced melanoma patients will be infused with autologous MART1-specific CD8⁺ CTL. It would be intriguing to compare CTL lines generated in

the presence or absence of IL-21 with regard to their ability to persist and home to tumor sites, because IL-21 up-regulates the expression of CD28 and down-regulates CCR7 expression as shown in Fig. 4A and B.

The biological activity of IL-21 on CTL and natural killer cells led to the testing of this cytokine in clinical trials in metastatic melanoma, renal cell carcinoma, and non-Hodgkin's lymphoma either alone or in combination with other approved drugs. It has been reported that IL-21 administration to humans is reasonably well tolerated and has shown preliminary evidence of antitumor activity in patients with melanoma and renal cancer (46, 47). However, our results clearly suggest that IL-21 administration may result in unexpected immunosuppressive effects by inhibiting the maturation of dendritic cells, which is critical for the optimal priming of naive T cells and the generation of antitumor effector T cells. Even after priming, IL-21 exposure may result in the generation of dendritic cells with impaired maturity which may anergize or tolerize existing effector T cells and attenuate their antitumor response. However, we also clearly

Fig. 6. IL-21 can enhance the generation of antitumor CD8⁺ T cells with low pCTL frequency and/or growth potential. Survivin-specific CTL were generated using aAPC/IL-21. Purified CD8⁺ T cells from A2⁺ healthy donors were stimulated on a weekly basis with aAPC/IL-21 pulsed with Sur9 peptide. Between stimulations T cells were supplemented with IL-2 (10 IU/mL) and IL-15 (10 ng/mL). After four stimulations, survivin-specific effector functions were evaluated by IFN- γ ELISPOT and standard ⁵¹Cr release assay. **A**, Survivin-specific IFN- γ secretion was studied by IFN- γ ELISPOT. CD8⁺ T cells were incubated with T2 cells pulsed with either Sur9 or human T-cell lymphotropic virus, type I-derived TAX control peptide. **B**, cytotoxicity assay was done using radiolabeled T2 cells pulsed with Sur9 peptide (■) or HIV-derived Pol peptide (●). **C**, Sur9 peptide-specific CD8⁺ T cells generated by aAPC/IL-21 possessed high TCR avidity and recognized hematologic tumor cells in an HLA-A2-restricted manner. Cytotoxicity assay was done using radiolabeled hematologic cancer cells from different lineages. HLA-A2 negative cell lines were engineered to express HLA-A2 as previously described (26). The cell lines used as targets were HSS (HS-Sultan, myeloma cell line); Sup-T1 (T lymphoblastic lymphoma cell line); Jurkat (T-cell leukemia cell line), MEG-01 (megakaryoblastic cell line), and KG-1 (acute myelogenous leukemia cell line).



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show that IL-21 can unequivocally enhance the expansion of CTL with potent effector function. The strategic use of IL-21, therefore, could be a powerful tool for enhancing immune responses. Therefore, it is imperative that the use of IL-21 in the clinic is guided by these underlying principles, and that treatment strategies are designed that exploit these dichotomous effects to therapeutic advantage.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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