IL-21 Can Supplement Suboptimal Lck-Independent MAPK Activation in a STAT-3–Dependent Manner in Human CD8+ T Cells

Osamu Imataki, Sascha Ansén, Makito Tanaka, Marcus O. Butler, Alla Berezovskaya, Matthew I. Milstein, Kiyotaka Kuzushima, Lee M. Nadler and Naoto Hirano

J Immunol published online 11 January 2012
http://www.jimmunol.org/content/early/2012/01/11/jimmunol.1003446

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/01/11/jimmunol.1003446.DC1

Why The JI?
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication
*average

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
IL-21 Can Supplement Suboptimal Lck-Independent MAPK Activation in a STAT-3–Dependent Manner in Human CD8+ T Cells

Osamu Imataki,*†,‡,1 Sascha Ansén,*†,‡,1,2 Makito Tanaka,*†,‡ Marcus O. Butler,*†,‡ Alla Berezovskaya,* Matthew I. Milstein,* Kiyotaka Kuzushima,§ Lee M. Nadler,*†,‡ and Naoto Hirano*†,‡,1,2

Although both MHC class II/CD8α double-knockout and CD8β null mice show a defect in the development of MHC class I-restricted CD8+ T cells in the thymus, they possess low numbers of high-avidity peripheral CTL with limited clonality and are able to contain acute and chronic infections. These in vivo data suggest that the CD8 coreceptor is not absolutely necessary for the generation of Ag-specific CTL. Lack of CD8 association causes partial TCR signaling because of the absence of CD8/Lck recruitment to the proximity of the MHC/TCR complex, resulting in suboptimal MAPK activation. Therefore, there should exist a signaling mechanism that can supplement partial TCR activation caused by the lack of CD8 association. In this human study, we have shown that CD8-independent stimulation of Ag-specific CTL previously primed in the presence of CD8 coligation, either in vivo or in vitro, induced severely impaired in vitro proliferation. When naive CD8+ T cells were primed in the absence of CD8 binding and subsequently restimulated in the presence of CD8 coligation, the proliferation of Ag-specific CTL was also severely hampered. However, when CD8-independent T cell priming and restimulation were supplemented with IL-21, Ag-specific CD8+ CTL expanded in two of six individuals tested. We found that IL-21 rescued partial MAPK activation in a STAT3- but not STAT1-dependent manner. These results suggest that IL-21 is critical for the expansion of postthymic peripheral Ag-specific CTL in humans. However, STAT3-mediated IL-21 signaling can supplement partial TCR signaling caused by the lack of CD8 association.

The Journal of Immunology, 2012, 188: 000–000.

T he coreceptor CD8 molecule is part of the Ag recognition complex on CD8+ CTL and plays a critical role in CD8+ T cell selection in the thymus (1). CD8 coligation is crucial in the productive activation of MHC class I-restricted Ag-specific CD8+ CTL. Many studies have demonstrated that CD8 molecules improve the efficiency of Ag recognition by CD8+ CTL by enhancing extracellular interactions between MHC/peptide molecules and Ag-presenting cells. Moreover, CD8 coreceptor engagement is required for the full activation of peripheral Ag-specific CTL. However, although the CD8 coreceptor binds to MHC/peptide multimers that do not bind CD8 molecules (10–12), several groups have demonstrated that high-affinity pMHC/TCR interactions can overcome a lack of CD8 coreceptor binding for full activation of naive T cells (6–8). Sewell and colleagues (9) reported that CD8 coreceptor dependence is inversely correlated to pMHC/TCR affinity. Furthermore, they have demonstrated that CTL with high functional avidity can be selectively identified by MHC/peptide multimers that do not bind CD8 molecules (10–12). These in vitro results indicate that high-affinity CTL may not require CD8 coreceptor engagement for their full activation.

CD8 molecules may also not be absolutely required in vivo. Whereas CD8α knockout (KO) mice have a strong bias toward CD4+ T cells and have difficulties inducing CTL responses, these mice were able to contain acute and chronic viral infections (13–15). In MHC class II−/− deficient CD8α−/−–double-KO mice, peripheral double-negative CTL were fully functional, producing protective CTL responses upon acute viral infection (16). CD8β KO mice have 3- to 5-fold lower numbers of mature CD8α+ T cells in the periphery, and, yet, they mount normal primary cytotoxic CD8 responses upon acute viral infection (17, 18). Furthermore, they are able to generate potent secondary and memory CD8+ T cell responses. Importantly, in both MHC class II−/− deficient CD8α−/−–double-KO mice and CD8β KO mice, peripheral CTL were largely CD8 independent and highly avid (13, 16, 17). In humans, a homozygous missense mutation in the CD8α gene has been reported in a family with three affected members (19). All three individuals had a total absence of CD8+ T cells and an increase in circulating CD4+ CD8− T cells with...
a CTL phenotype. Despite this, only one, a 25-y-old man, suffered from nonsevere repeated respiratory bacterial infections, and his two sisters were entirely asymptomatic. These in vivo observations demonstrate that it is possible to develop CTL responses in the absence of the CD8 coreceptor that are qualitatively sufficient to target Ags.

The significance of common γ-chain receptor cytokines (e.g., IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) in T cell biology has been well established (20, 21). All of these cytokines transmit STAT-mediated signaling via the common γ-chain receptor in T cells (22, 23). Importantly, it has been demonstrated that cross-talk between TCR and STAT signaling occurs in CD8+ T cells (24, 25). For example, partial or weak TCR-initiated signaling via the Ras/MAPK pathway can be complemented by IL-2–induced STAT activation (25, 26). This mechanism enables adaptive cytokines to supplement suboptimal TCR signaling or to modulate optimal TCR signaling. Importantly, even though these cytokines bind to and signal through the common γ-chain receptor to support T cell proliferation and differentiation, each cytokine has a distinctive role in T cell growth, lineage control/determination, differentiation, function, and death (20–23). Previously, we and others have shown that both IL-15 and IL-21 can enhance CTL effector functions and induce the proliferation and maturation of CTL with high TCR avidity (27–30).

Based on these observations, we have hypothesized that CD8 coreceptor-independent T cell stimulation in the presence of complementary adaptive cytokines will preferentially stimulate high-avidity Ag-specific CTL. To investigate the role of CD8 binding during T cell stimulation, previous studies, to the best of our knowledge, invariably used T cell clones or TCR transgenic T cells that had been isolated after originally being primed in vivo binding during T cell stimulation, previous studies, to the best of our knowledge, invariably used T cell clones or TCR transgenic T cells that had been isolated after originally being primed in vivo in the presence of full CD8 coligation. Therefore, it has yet to be determined how the lack of CD8 engagement during priming and/or subsequent restimulation will affect the generation of Ag-specific CTL. Previously, we reported the generation of K562-based artificial APC (aAPC) by transducing HLA-A2, CD80, and CD83 (28, 31). This aAPC can prime naive CD8+ T cells in vitro and generate high-avidity Ag-specific CD8+ CTL with a central memory–effector memory phenotype (28). Using our aAPC-based system, we have addressed this hypothesis.

Materials and Methods

Cells
Peripheral blood samples were obtained from healthy donors following institutional review board approval. All donors were identified to be positive for HLA-A*0201 (A2) by high resolution HLA DNA typing (American Red Cross). Mononuclear cells were obtained by density gradient centrifugation (Lymphoprep; Nycomed Pharma). CD8+ T cells were purified by CD8 Microbeads (Miltenyi Biotec). K562 is an erythroleukemic cell line defective for HLA expression. T2 is a HLA-A2–positive T cell leukemia/B-lymphoblastoid cell line hybrid. Jurkat is a T cell leukemic cell line. J.CaM1.6 is a Jurkat mutant, which lacks the expression of Lck. Jurkat/IL-21R and J.CaM1.6/IL-21R were retrovirally transduced with CD8+ a.a. and CD83, and its derivative, which constitutively secretes IL-21, was reported previously (4). The generation of aAPC expressing HLA-A2, CD80, and CD83, and its derivative, which constitutively secretes IL-21, was reported previously (27, 28, 35). Briefly, purified CD8+ lymphocytes were plated at 2 × 106 cells/well in RPMI 1640 supplemented with 10% FCS and gentamicin (Invitrogen).

Transfectants
CD8α and CD8β cDNAs were cloned by RT-PCR from total RNA isolated from normal PBMC according to the published sequence. Sequences were verified by the Molecular Biology Core at the Dana-Farber Cancer Institute. K562 was transduced with CD8α to establish K562/CD8α cells using a retrovirus system, as reported previously (31). Briefly, a packaging cell line, 293GFP, was transfected with PMX/CD8α retrovirus vector using TransIT-293 (Takara Bio), and virus supernatant was harvested. One million K562 cells were infected with the virus supernatant in the presence of 8 μg/ml polybren. CD8α-positive K562/CD8α cells were collected using biotin-conjugated anti-CD8α mAb and anti-biotin beads, according to the manufacturer’s instruction (Miltenyi Biotec). K562/CD8α cells were further retrovirally transduced with CD8β to generate K562/CD8αβ. K562/CD8αβ cells were purified using biotin-conjugated anti-CD8β mAb and anti-biotin beads, as described above. Mutated HLA-A2 cDNA bearing two amino acid substitutions at positions 227 and 228 (D227K/T228A) that abrogate the interaction with A2 was reported previously (4). The generation of aAPC expressing HLA-A2, CD80, and CD83, and its derivative, which constitutively secretes IL-21, was reported previously (27, 31). Mut-aAPC secreting IL-21 express mutated HLA-A2 in lieu of wild-type (wt) A2 and were generated similarly using a retrovirus system. Wt- and mut-aAPC secreting IL-21 secreted similar amounts of IL-21 (0.33 ± 0.06 and 0.25 ± 0.07 μg/ml, respectively) per 106 cells over 24 h. The cell line, membranous form of OKT3 (mOKT3)–aAPC, expresses a membranous form of anti-CD3 mAb (clone OKT3), CD80, and CD83 on K562 to allow polyclonal expansion of CD3+ T cells regardless of Ag specificity and HLA restriction (32). CDNAs encoding a membranous form of the H and L chains of anti-CD3 mAb were molecularly cloned from mouse hybridoma cells (clone OKT3). After retroviral transduction with drug resistance genes and subsequent drug selection, anti-CD3 mAb-expressing cells were isolated by magnetic bead-guided sorting using PE-conjugated goat anti-mouse Ig polyclonal Ab (Jackson ImmunoResearch Laboratories) and anti-PE MicroBeads (Miltenyi Biotec). J.CaM1.6 is a Jurkat mutant, which lacks the expression of Lck. Jurkat/IL-21R and J.CaM1.6/IL-21R were retrovirally generated, as described elsewhere (27).

HLA/peptide multimer staining
Wt HLA-A2 and mutated a2/peptide multimers were produced and used to stain cells, as described previously (31, 33).

Flow cytometry analysis
PE- and PC5-conjugated anti-CD8α (clone B9.11) and anti-CD8β (clone 2ST5.87H) mAbs were purchased from Beckman Coulter and used to stain CD8α homodimers and CD8β heterodimers, respectively. To determine the phenotype of MART1–specific T cells, T cells were first stained with HLA-A2/peptide multimers, as previously described (31, 33). Multimer-positive T cells were co-stained with the following mAbs: FITC-conjugated anti–CCR7 (clone 150503; R&D Systems), Tri–Color–conjugated anti–CD45RA (clone MEM-56, Invitrogen), Tri–Color–conjugated anti–CD45RO (clone UCHL1; Invitrogen), and PC5–conjugated anti–CD52L (clone DREG56; Beckman Coulter) mAbs.

Cell surface molecules on transfectants were stained with FITC-conjugated anti-HLA-A2 (clone BB7.2; BD Biosciences), PE-conjugated anti–CD80 (clone L307.4; BD Biosciences), and PE-conjugated anti–CD83 (clone HB15e; Invitrogen). IL-21R was indirectly stained with anti–IL-21R mAb and Alexa Fluor 488-conjugated anti–rat IgG1 (Invitrogen). Cytotoxic T cells were stained with Alexa Fluor 488-conjugated anti–CD8 PE-conjugated anti–CD11b (clone M1/70; BD Biosciences), and PerCP-Cy5.5–conjugated anti–CD45R0 (clone 5B11; Invitrogen) mAbs. Cell surface molecules on transfectants were stained with FITC-conjugated anti–CD80 (clone BB7.2; BD Biosciences), PE-conjugated anti–CD80 (clone L307.4; BD Biosciences), and PE-conjugated anti–CD83 (clone HB15e; Invitrogen). IL-21R was indirectly stained with anti–IL-21R mAb and PE- and PC5-conjugated goat anti-mouse Ig polyclonal Ab (Jackson ImmunoResearch Laboratories).

Intracellular phosphorylated MAPK, STAT1, and STAT3 were stained with Alexa Fluor 488-conjugated anti–phospho-p44/42 MAPK (ERK1/2) (Th202/Ty202, clone E10; Cell Signaling Technology), PE-conjugated anti–phospho-STAT1 (Tyr701) (clone 4A4; BD Biosciences), and PerCP-Cy5.5–conjugated anti–phospho-STAT3 (Tyr705, clone 4B12; BD Biosciences). Intracellular phosphorylated MAPK, STAT1, and STAT3 were stained with Alexa Fluor 488-conjugated anti–phospho-p44/42 MAPK (ERK1/2) (Th202/Ty202, clone E10; Cell Signaling Technology), PE-conjugated anti–phospho-STAT1 (clone HB15e; Invitrogen), Tri–Color–conjugated anti–CD45RO (clone UCHL1; Invitrogen), and PE-conjugated anti–CD52L (clone MEM-56; Beckman Coulter) mAbs.

Cell surface molecules on transfectants were stained with FITC-conjugated anti-HLA-A2 (clone BB7.2; BD Biosciences), PE-conjugated anti–CD80 (clone L307.4; BD Biosciences), and PE-conjugated anti–CD83 (clone HB15e; Invitrogen). IL-21R was indirectly stained with anti–IL-21R mAb and PE- and PC5-conjugated goat anti-mouse Ig polyclonal Ab (Jackson ImmunoResearch Laboratories).

Production of HLA-A*0201–restricted peptide-specific CD8+ T cells
Peptide-specific cytotoxic CD8+ T cells were generated using aAPC, as described previously (27, 28, 31, 35). Briefly, purified CD8+ lymphocytes were plated at 2 × 106 cells/well in RPMI 1640 supplemented with 10% FCS and 1% human AB serum. The stimulator APC were pulsed with 1 μM of either HLA-A2*–specific or HLA-A2*–specific peptide (32, 33). Briefly, resting or stimulated T cells were counted, and restimulated every week. T cell analysis was performed 1 d prior to or on the day of restimulation. HLA-A2–restricted HIV pol476 peptide (32, 33) was used as a control.
Cell division tracing assay

CellTrace Violet (Invitrogen) was added to one million T cells in 1 ml serum-free media at a final concentration of 5 μM and incubated for 20 min at 37°C. Labeled cells were stimulated with wt- or mu-aAPC pulsed with MP18s or MART127 peptide, as described above. Three to five days later, stimulated T cells were stained with PE-conjugated multimere and PC5-conjugated anti-CD8α and analyzed by flow cytometry.

ELISPOT analysis

IFN-γ ELISPOT assay was conducted, as described elsewhere (27, 28, 31, 35, 36). Briefly, polyvinylidene difluoride plates (Millipore) were coated with capture mAb (clone 1D1K; MABTECH). T cells (1 × 10^6 per well) were incubated with 2 × 10^4 per well of indicated APC in the presence of peptide for 20–24 h at 37°C. Plates were washed and incubated with biotin-conjugated streptavidin was used to develop IFN-γ spots. Functional avidity was tested using T cells pulsed with graded concentrations of MART127 peptide as stimulators in an IFN-γ ELISPOT assay. Where indicated, mOKT3-aAPC (2 × 10^4 per well) or a combination of 1.5 μg/ml anti-CD3 and 2 μg/ml anti-CD28 mAbs (Fitzgerald Industries International) was used as a stimulator in the absence or presence of 100 ng/ml rIL-21 (Peprotech). To inhibit STAT1, STAT3, and MAPK, graded concentrations of fludarabine (Sigma-Aldrich), S3I-201 (Merck4Biosciences), and/or PD98059 (Merck4Biosciences), respectively, were added to the cultures, as indicated.

Cytotoxicity assay

Cytotoxicity assay was conducted, as described previously (28, 31). Briefly, 5 × 10^3 T2 cells pulsed with graded concentrations of MART127 peptide were mixed with 5 × 10^4 MART1 CTL for 4 h at 37°C in a 96-well round-bottom plate. Percent specific lysis was calculated (experimental result – spontaneous release)/[maximum release – spontaneous release)] × 100%.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2004/2008 for Mac and GraphPad Prism 5.0d. A p value <0.05 was considered significant. To determine whether two groups were statistically different for a given variable, analysis was performed using the Student t test (two sided) or by a repeated measures ANOVA, followed by Tukey’s posthoc multiple comparisons test.

Results

Mutated A2 molecules cannot bind either CD8αα or CD8αβ

Most peripheral CD8^+ TCRββ T cells carry CD8αβ heterodimers on their cell surface. In contrast, CD8αα homodimers are found only on NK cells, TCRγδ T cells, and intraepithelial lymphocytes (37). Whereas CD8αα homodimers bind directly to MHC molecules, CD8ββ homodimers do not have detectable MHC-binding activity. However, CD8β chain possesses amino acids in its Ig domain that directly contact MHC class I molecules in the context of CD8αβ heterodimers, thereby strengthening the binding of MHC/peptide to TCR (38–40). It has been reported that mutated HLA-A2 (mut A2) molecules carrying two amino acid substitutions (D227K/T228A) do not bind CD8αα homodimers (4, 41). To test whether mut A2 is also unable to bind CD8αβ heterodimers, K562 were transduced with CD8αα alone or in conjunction with CD8β to establish K562/CD8αα and K562/CD8αβ, respectively (Fig. 1A). K562/CD8αα and K562/CD8αβ were incubated with wt HLA-A2/MART1 tetramer or mut A2/MART1 tetramer. Unlike wt tetramer, mut A2 tetramer was unable to bind both K562/CD8αα and K562/CD8αβ (Fig. 1B). These results suggest that mut A2 molecules (D227K/T228A) cannot bind either CD8αα or CD8αβ.

Previously, we reported the generation of K562-derived HLA-A2 artificial APC (wt A2-aAPC), which expresses wt HLA-A2 as a sole HLA allele in conjunction with CD80 and CD83 (31). Wt A2-aAPC can prime naive CD8^+ T cells isolated from HLA-A2^+ donors and expand highly avid HLA-A2–restricted Ag-specific CTL with a central–effector memory phenotype (28, 35, 36).

FIGURE 1. HLA-A2 molecules with impaired CD8-binding ability are incapable of binding CD8αα and CD8ββ. A, K562 can ectopically express CD8αα homodimers and CD8ββ heterodimers on the cell surface. K562 was retrovirally transduced with CD8αα (K562/CD8αα) and subsequently with CD8ββ (K562/CD8ββ). Transfectants were stained with anti-CD8α and anti-CD8β mAbs and subjected to flow cytometric analysis. Similar experiments were repeated three times. B, K562/CD8αα and K562/CD8ββ were incubated with CD8αβ mAb and with wt or mut A2/MART127 tetramers in which CD8 binding is abolished. Similar experiments were repeated three times. C, Established HLA-A2–restricted MART127-specific CTL were stained with control (ctrl), wt or mut tetramer and subsequently with anti-CD8α mAb. Representative tetramer-staining data of one CTL line of nine are shown.

Several groups have reported that CTL with high functional avidity can be selectively identified with mut A2 tetramers (10–12, 42). When MART127-specific CTL generated using wt A2-aAPC were stained with wt and mut A2 tetramers, mut A2 tetramer was able to stain MART1 CTL, although at a lower percentage than wt A2 tetramer (Fig. 1C). These results suggest that mut A2/peptide molecules can recognize cognate TCR expressed on a subpopulation of CTL, possibly with high avidity, in the absence of CD8 association.

CD8-independent TCR engagement is defective in inducing the proliferation of Ag-specific CTL previously primed in the presence of CD8 engagement

We have replaced wt A2 molecules on A2-aAPC with mut A2 molecules to generate mut A2-aAPC (Supplemental Fig. 1). Using wt and mut A2-aAPC, we investigated the requirement of the CD8 coreceptor association for the expansion of Ag-specific human CD8^+ T cells. Most HLA-A2^+ healthy donors possess memory CTL against MP158 that have been primed in vivo in the presence of CD8 coligation. CD8^+ T cells freshly purified from A2+ donors were stimulated once per week using wt- or mut-A2-aAPC pulsed with MP158 peptide. After three stimulations, Ag specificity was measured by tetramer staining. The percentage of MP158-specific T cells was significantly lower for CTL stimulated with mut-aAPC compared with wt-aAPC (Fig. 2A, left). Cell division tracing assay revealed that MP158-specific CTL stimulated with mut-aAPC did...
not proliferate as robustly as those stimulated with wt-aAPC (Fig. 2A, center). Accordingly, the total number of generated MP158-specific CTL was significantly lower after stimulation with mut-aAPC compared with wt-aAPC (Fig. 2A, right).

Previously, we and others demonstrated that MART127-specific CD8+ precursor cells in HLA-A2+ healthy donors are immunologically and phenotypically naive (31, 43, 44). Naive CD8+ T cells isolated from A2+ healthy donors were initially primed with MART127 peptide-pulsed wt-aAPC in the presence of CD8 coligation. Primed CD8+ T cells were split and subsequently restimulated three times using either MART127 peptide-pulsed wt- or mut-aAPC. The percentage of MART127 tetramer-positive CTL was significantly lower after restimulation with mut-aAPC compared with wt-aAPC (Fig. 2B, center). Data are representative of six donors. The number of peptide-specific T cells was determined by calculating the product of the total number of T cells and the percentage of tetramer-staining cells (right). The percentage tetramer positivity and the total number of expanded MART127-specific CD8+ T cells are statistically compared using paired, two-sided Student t tests.

C, Wt- and mut-aAPC can present pulsed A2-restricted peptides with a similar efficiency. IFN-γ ELISPOT was conducted where CD8+ T cells freshly isolated from A2+ healthy donors were stimulated using wt- or mut-aAPC pulsed with 0.1 μg/ml MP158 peptide. Established MART127-specific CTL were also subjected to IFN-γ ELISPOT analysis using wt- or mut-aAPC pulsed with 10 μg/ml MART127 peptide as a stimulator. The peptide concentrations used are the same as those used in A and B. For each peptide, similar results were obtained from three different donors. Representative data for each peptide are demonstrated. Error bars show SD.
can indeed engage TCR with similar efficiency, we tested whether MP126-specific memory CTL effector functions induced by wt- and mut-aAPC are comparable. An IFN-γ ELISPOT assay using freshly isolated CD8+ T cells detected similar frequencies of IFN-γ-secreting CD8+ T cells when stimulated with MP126 peptide-pulsed wt or mut A2-aAPC (Fig. 2C, left). Similar results were obtained with previously established MART127-specific CTL (Fig. 2C, right). These results suggest that, under the experimental conditions tested, both wt and mut A2 molecules on our aAPC are able to engage cognate TCR with similar efficiency.

**Priming in the absence of CD8 coligation is defective in inducing the subsequent expansion of Ag-specific CTL upon restimulation**

To study whether priming in the absence of CD8 coengagement is sufficient to enable subsequent Ag-specific proliferation upon restimulation, naive CD8+ T cells isolated from A2+ healthy donors were split into three populations and primed with either mut-aAPC pulsed with either irrelevant HIV pol476 or MART127 peptide or with wt-aAPC pulsed with MART127 peptide. Following priming, these three CD8+ T cell cultures were all restimulated three times in a weekly manner with MART127 peptide-pulsed wt-aAPC in the presence of CD8 coligation. The percentage of MART127 tetramer-positive CTL was significantly lower after priming with MART127-pulsed mut-aAPC compared with MART1-pulsed wt-aAPC in all individuals tested (Fig. 3). Furthermore, the total number of generated MART127-specific CTL was also significantly lower when mut-aAPC was used compared with wt-aAPC (data not shown). Importantly, CD8-independent priming with mut-aAPC pulsed with MART127 or HIV pol476 was both significantly inferior in subsequent expansion of MART1-specific CTL. This suggests that priming in the absence of CD8 signaling does not induce a sufficient survival or stimulatory signal for naive CD8+ T cells to subsequently proliferate as Ag-specific CD8+ T cells.

**IL-21 can supplement suboptimal CD8-independent TCR stimulation**

The data presented in Fig. 2 demonstrate that CD8-independent restimulation is defective in expanding Ag-specific CD8+ T cells. We show in Fig. 3 that priming of naive CD8+ T cells in the absence of CD8 coengagement is insufficient to induce the full expansion of Ag-specific CD8+ T cells upon subsequent restimulation. These results strongly suggest that, if naive CD8+ T cells are primed and subsequently restimulated in the complete absence of CD8 coligation, Ag-specific CD8+ T cells do not grow. To confirm this, naive CD8+ T cells purified from healthy A2+ donors were both primed and restimulated with either wt- or mut-aAPC pulsed with MART127 peptide. Between stimulations, the experiments were treated with IL-2 and IL-15. As shown in Fig. 4A, priming and restimulation with MART127 peptide-pulsed mut-aAPC in the total absence of CD8 coligation failed to induce an expansion of MART127-specific CTL. In contrast, CTL stimulated with MART127 peptide-pulsed wt-aAPC in the presence of CD8 engagement successfully generated MART127-specific CTL. It should be noted that, in our laboratory, we have successfully generated MART127-specific CTL using wt-aAPC from >100 A2+ healthy donors without any failure under the experimental conditions employed (27, 28, 31, 35, 36). These results clearly demonstrate that priming and subsequent restimulation of naive CD8+ T cells in the complete absence of CD8 coligation cannot expand MART127-specific CTL.

As shown above, CD8-independent priming and restimulation with mut-aAPC in the presence of IL-2 and IL-15 were unable to expand MART1-specific CD8+ T cells. Because the total loss of CD8 molecules is not lethal in humans, however, a rescue signal should be present that complements partial T cell stimulation caused by the absence of CD8 coligation (19). It has been demonstrated that STAT signaling mediated by common γ-chain receptor cytokines can supplement partial or weak TCR signaling (24, 25). Because our T cell cultures already included both IL-2 and IL-15, it was unlikely that IL-2 and/or IL-15 would rescue suboptimal TCR signaling delivered by mut-aAPC. We and others previously reported that IL-21 possesses unique immunologic properties that are not shared by either IL-2 or IL-15 (22, 23, 27, 29). This prompted us to test whether IL-21 would be able to supplement the defective CD8-independent TCR signaling in T cells stimulated by mut-aAPC. Purified CD8+ T cells from A2+ donors were stimulated using MART127 peptide-pulsed wt- or mut-aAPC constitutively secreting IL-21. As shown in Fig. 4B, CD8-independent priming and restimulation using mut-aAPC-producing IL-21 successfully expanded MART127-specific CTL in two of the

**FIGURE 3.** CD8-independent TCR stimulation is defective in priming naive T cells and the proliferation of Ag-specific CTL. Naive CD8+ CTL cannot be primed in the absence of CD8 coligation. Purified naive CD8+ T cells from six healthy donors were initially stimulated with one of three aAPC: mut-aAPC pulsed with 10 μg/ml HIV pol476 or MART127 peptide or with wt-aAPC pulsed with MART127 peptide. Following this attempted priming, all CD8+ T cell cultures were repeatedly restimulated with wt-aAPC pulsed with 10 μg/ml MART127 in a weekly manner. Between stimulations, the CTL cultures were given 10 IU/ml IL-2 and 10 ng/ml IL-15 every 3 d. After a total of four stimulations, MART127 specificity of generated CTL was analyzed by wt tetramer staining. Representative tetramer-staining data from three donors of six are depicted (left). The percentage of tetramer-positive MART127-specific CD8+ T cells from all six donors is statistically compared (right). Note that data points, which are shown for all six donors, partially overlap. Statistics were determined by repeated measures ANOVA, followed by Tukey’s posthoc multiple comparison test.
six donors studied (donors 4D and 4F). Priming and restimulation using mut-aAPC that does not secrete IL-21 did not expand any MART127-specific CTL at all (Fig. 4A, data not shown). Under the experimental conditions employed, we did not observe a decrease in total number of T cells or MART127-specific CTL by the addition of IL-21 to T cell cultures (Fig. 4A, 4B) (27). Furthermore, no consistent difference in surface phenotype was observed between MART127-specific CTL expanded using IL-21–secreting wt- and mut-aAPC (Supplemental Fig. 2).

We next compared the functional avidity of the CTL generated using IL-21–secreting wt- and mut-aAPC. An IFN-γ ELISPOT assay demonstrated that the functional avidity of MART127-specific CTL generated by IL-21–secreting mut-aAPC was 4.0 × 10⁻³ μg/ml peptide concentration inducing 50% of the maximum response (MC₅₀), which was higher than that of MART127-specific CTL generated by IL-21–secreting wt-aAPC (2.5 × 10⁻² μg/ml) (Fig. 4C). We also conducted a Cr⁵¹ release assay using MART127-specific CTL generated from a different donor using IL-21–secreting wt- and mut-aAPC. MART127-specific CTL generated using IL-21–secreting mut-aAPC possessed similar or slightly higher functional avidity (3.0 × 10⁻⁴ μg/ml) than MART127-specific CTL generated using IL-21–secreting wt-aAPC (7.0 × 10⁻₅ μg/ml) (Fig. 4D). These results suggest that IL-21 signaling can supplement CD8-independent suboptimal T cell stimulation by mut-aAPC and enable the expansion of MART127-specific CTL.

**IL-21 can complement suboptimal CD8-independent MAPK signaling in a STAT3-dependent manner**

IL-21 delivers its intracellular signal mainly via STAT1 and STAT3 (21). To gain insights into the mechanism of IL-21–mediated rescue of CD8-independent T cell stimulation, we examined phosphorylation of MAPK, STAT1, and STAT3 in MART1-specific CD8⁺ T cells stimulated by IL-21–secreting wt- and mut-
aAPC. When stimulated by IL-21–secreting wt-aAPC, MART127-specific CTL generated with IL-21–secreting wt-aAPC demonstrated some MAPK phosphorylation. IL-21–secreting mut-aAPC induced minimal MAPK phosphorylation of MART127-specific CTL generated with IL-21–secreting wt-aAPC. This suggests that stimulation by mut-aAPC in the absence of CD8 coligation is not sufficient to evoke MAPK phosphorylation, probably because of the lack of Lck activation, which is a downstream event of CD8 coligation (1). Neither STAT1 nor STAT3 phosphorylation was observed under the experimental conditions used (Fig. 5A, left). In contrast, MART127-specific CTL generated with IL-21–secreting mut-aAPC demonstrated phosphorylation of STAT3 but not MAPK or STAT1 when stimulated with either IL-21–secreting wt- or mut-aAPC (Fig. 5A, right). This suggests that, in MART127-specific CTL generated with IL-21–secreting mut-aAPC in the absence of CD8 coligation, STAT3–mediated IL-21 signaling predominates over MAPK-mediated TCR signaling. Note that MART127-specific CTL were stimulated by cell-based aAPC in an Ag-specific manner. Therefore, the observed phosphorylation levels were not very high because the induced stimulations were not maximal and were directed against only the MART1-specific TCR subset.

Using specific inhibitors in an IFN-γ ELISPOT assay, we also studied how IL-21 signaling supplements CD8-independent T cell signaling during the effector phase (Fig. 5B). IFN-γ secretion by both MART127-specific CTL lines generated using IL-21–secreting wt- and mut-aAPC was completely abrogated by STAT3 inhibition alone, indicating a critical role of STAT3 in IFN-γ secretion. The inhibition of MAPK alone severely hampered the IFN-γ secretion by MART127-specific CTL lines generated using IL-21–secreting wt-aAPC. Although inhibition of MAPK alone had minimal impact on MART127-specific CTL generated using IL-21–secreting mut-aAPC, the effect of STAT3 inhibition was markedly augmented by the coinhibition of MAPK. The effect of STAT1 inhibition was modest, at best, in MART127-specific CTL lines generated using IL-21–secreting wt-aAPC and was not observed in those generated using IL-21–secreting mut-aAPC. These results suggest that IL-21 can complement suboptimal CD8-independent MAPK signaling via a STAT3-dependent manner.

**IL-21 can rescue suboptimal T cell stimulation via a STAT3-dependent manner in the absence of Lck, a downstream effector molecule of CD8 coligation**

CD8 is associated with the tyrosine kinase, Lck. When CD8 binds pMHC, Lck phosphorylates components of the TCR/CD3 signaling complex and enhances signal transduction (1). Therefore, lack of CD8 binding to pMHC results in partial TCR signaling (4, 46, 47). In fact, Lck KO mice had a profound defect in thymocyte development. Using Jurkat and Jurkat-derived Lck-null J.CaM1.6 cells, we studied whether IL-21–mediated STAT signaling can rescue partial TCR signaling incurred by the absence of Lck. To stimulate Jurkat and J.CaM1.6 transfectants, we used mOKT3-aAPC, on which HLA-A2 was substituted with a membraneous form of anti-CD3 mAb. Jurkat and J.CaM1.6, both devoid of IL-21R expression, were engineered to constitutively express IL-21R (Supplemental Fig. 3). When stimulated by mOKT3-aAPC, control Jurkat and J.CaM1.6 minimally responded to IL-21 (Fig. 6). However, both IL-21R–transduced Jurkat and J.CaM1.6 minimally responded to IL-21 (Fig. 6). Although the magnitude of the responses to IL-21 was less, especially in IL-21R–transduced J.CaM1.6 cells, similar results were obtained when anti-CD3/CD28 mAbs were used as stimulators. It should be noted that, unlike anti-CD3/CD28 mAbs, mOKT3-aAPC delivers not only signals 1 and 2, but also other signals mediated by immunostimulatory...
molecules, such as CD54, CD58, and CD83 (28, 31). These results suggest that IL-21 can complement suboptimal TCR signaling in the absence of Lck.

Intracellular staining revealed that IL-21 induced robust phosphorylation of STAT3 upon stimulation with mOKT3-aAPC (Fig. 7A). As expected, MAPK phosphorylation was minimal in either of J.CaM1.6 transfectants probably because of the lack of Lck activation. No STAT1 phosphorylation was observed in any of the cell lines. Because polyclonal potent T cell stimulation was provided by anti-CD3 mAb-expressing mOKT3-aAPC, the level of phosphorylation in these cell lines was higher than that in primary T cells stimulated with peptide-pulsed aAPC in Fig. 5A.

To further study the functional role of STAT3 in Lck-independent IL-21 signaling, J.CaM1.6/Ctrl and IL-21R–transduced J.CaM1.6/IL-21R cells were stimulated by aAPC plus IL-21 in the presence of specific inhibitors (Fig. 7B). In both cell lines, IFN-γ secretion was completely abrogated by STAT3 inhibition, indicating a critical role of STAT3 in IFN-γ secretion in the absence of Lck. Whereas the MAPK inhibitor blocked IFN-γ secretion by >80% in J.CaM1.6/Ctrl, it did so by <50% in J.CaM1.6/IL-21R cells. This suggests that, in cells lacking Lck, IL-21–induced enhancement of T cell responses is mediated by two pathways, as follows: one both STAT3 and MAPK dependent, and the other STAT3 dependent but MAPK independent. The combination of suboptimal doses of both the STAT3 inhibitor with the MAPK inhibitor completely nullified the IFN-γ secretion, demonstrating an additive effect between STAT3 and MAPK inhibitors. In contrast, STAT1 only partially inhibited the T cell responses in J.CaM1.6/IL-21R cells (Fig. 7C). The addition of the

**FIGURE 6.** IL-21 signaling can rescue partial T cell responses caused by the lack of Lck. Constitutive expression of IL-21R can enhance IL-21–dependent T cell responses in the absence of Lck. Jurkat cell line (10⁴ cells/well) and its Lck-null derivative, J.CaM1.6 (10⁵ cells/well), stably expressing mock or IL-21R, were stimulated with mOKT3-aAPC (aAPC) in the presence or absence of IL-21 in an IFN-γ ELISPOT. aAPC denotes a K562-derived APC expressing a membranous form of anti-CD3 mAb, CD80, and CD83. Unpaired, two-sided Student t test was used for two-sample comparisons. Similar experiments were repeated three times.

**FIGURE 7.** In the absence of Lck, IL-21 signaling enhances T cell responses in a STAT3- but not STAT1-dependent manner. A. IL-21 induces robust phosphorylation of STAT3 in the absence of Lck. Jurkat cell line and its Lck-null derivative, J.CaM1.6, stably expressing mock or IL-21R were stimulated with mOKT3-aAPC (aAPC) in the presence or absence of IL-21. The phosphorylation level of MAPK (pMAPK), STAT1 (pSTAT1), and STAT3 (pSTAT3) was determined by intracellular staining using specific mAbs for phosphorylated molecules in a flow cytometry analysis. Isotype mAb staining was used as a control. Similar experiments were repeated three times. B. IFN-γ secretion induced by aAPC plus IL-21 in the absence of Lck is STAT3 dependent. Lck-null J.CaM1.6/Ctrl and J.CaM1.6/IL-21R were stimulated with mOKT3-aAPC and IL-21 in the presence of graded concentrations of inhibitors against MAPK (PD98059) and/or STAT3 (S31-201). Similar experiments were repeated three times. C. J.CaM1.6/IL-21R cells were stimulated with mOKT3-aAPC and IL-21 in the presence of graded concentrations of inhibitors against MAPK (PD98059) and/or STAT1 (Fludarabine). IFN-γ secretion was analyzed by an ELISPOT assay, as depicted in Fig. 6. Similar experiments were repeated three times.
STAT1 inhibitor to the MAPK inhibitor did not demonstrate an additive effect. These results suggest that STAT3– but not STAT1–mediated IL-21 signaling can rescue the suboptimal MAPK activity caused by the lack of Lck.

Discussion

It is well accepted that biological responses of CTL such as proliferation, cytokine secretion, and cytotoxicity require different levels of T cell activation, as determined by the level of TCR occupancy and signal intensity elicited by TCR engagement (45, 48). In general, proliferation requires the strongest activation signals, whereas cytotoxicity requires the least. To determine whether CD8 coligation is necessary for immunologically functional CTL activation, most previous in vitro studies have employed measurements of effector functions, which are more sensitive biological responses than proliferation. Furthermore, to the best of our knowledge, TCR or T cell clones used in the previous studies were virtually all derived from T cells originally primed in the presence of CD8 coligation. Therefore, little is known regarding the absolute requirement of CD8 coreceptor engagement for priming of postthymic naive peripheral CD8$^+$ T cells and for their proliferation as Ag-specific CTL. We previously reported that wt A2-expressing aAPC (K562 transduced with wt HLA-A2, CD80, and CD83) can prime naive CD8$^+$ T cells and expand Ag-specific CD8$^+$ CTL (27, 28, 31, 35, 36). Using a modified aAPC-expressing mutant A2 (D227K/T228A), which has abrogated CD8 binding, we addressed this question in vitro in humans.

We have found that in the absence of CD8 coligation, it is virtually impossible to prime and expand Ag-specific CTL in vitro, even in the presence of IL-2 and IL-15. However, the addition of IL-21 to T cell cultures enables the priming and subsequent expansion of Ag-specific CTL in some individuals. Obviously, in this study, it was imperative to use a CD8$^+$ T cell epitope against which generating CTL from naive T cells using wt-aAPC is always successful. Otherwise, failure to prime naive CD8$^+$ T cells and/or to subsequently expand Ag-specific CD8$^+$ T cells could be simply attributed to a low number of precursor CTL (pCTL). This study mainly used the MART127 antigenic peptide because we and others have intensively studied this HLA-A2–restricted immunogenic CD8$^+$ CTL epitope (49–51). MART127 is unique because it is one of the Ags for which the greatest number of pCTL is detectable (52, 53). pCTL are detectable by tetramer (<0.05%) in nearly half of A2$^+$ healthy donors and are immunologically and detectable (52, 53). pCTL are detectable by tetramer (<0.05%) in nearly half of A2$^+$ healthy donors and are immunologically and phenotypically naive (43, 44). Using K562-based wt-aAPC, we have successfully established MART127-specific CTL from >100 HLA-A*A*0201$^+$ individuals tested without failure in our laboratory. Our results demonstrate that the role of CD8 coreceptor coengagement is critical even for MART127, which is by far the easiest Ag for growing CD8$^+$ CTL. Therefore, the generation of CD8$^+$ T cell responses against other Ags, whose pCTL inevitably undergo central selection, resulting in low numbers and avidity, is also most likely highly dependent on engagement of the CD8 coreceptor.

Wt-aAPC can prime naive CD8$^+$ T cells in vitro and is capable of expanding MART127-specific CTL in a similar manner to dendritic cells (DC) (28, 31, 35, 36). However, we cannot definitively state whether our findings in this study can be applied to professional APC such as DC. In humans, it remains technically challenging to completely eradicate endogenous HLA expression and express only one allele of mutated HLA like mut-aAPC. An alternative method to confirm our findings with mature DC might be the use of blocking mAbs against CD8. However, it has been reported that anti-CD8 mAbs possess multiple effects on the interaction between MHC and TCR that cannot be explained by a simple disruption of the pMHC/CD8 interaction (54, 55). Furthermore, all anti-CD8 mAbs tested induce tyrosine phosphorylation of T cell signaling molecules (54, 55). Therefore, at this moment, we are unable to confirm our findings using K562-based aAPC with mature DC in humans.

CTL that possess high functional avidity are known to be optimal for the clearance of pathogens and tumors in vivo. Therefore, induction of such CTL is critical for the success of cellular immunotherapeutics. In vivo, several factors have been suggested that can modulate functional avidity: TCR affinity (10–12, 56), expression level of costimulatory molecules (57, 58), IL-12 and IL-15 (30, 59), and the level of CD8 expression (39, 60). Less is known about the parameters that determine the functional avidity of CTL in vitro. Previous studies demonstrated that the functional avidity of the CTL lines generated in vitro was inversely correlated with the peptide densities presented by stimulating APC (61). Based on this, some groups have selectively generated CTL with high functional avidity by stimulating with APC pulsed with low doses of peptide (60, 62). In addition, it has been reported that IL-21 can selectively expand highly avid CTL in vitro (63). In this context, our finding that CD8-independent stimulation supplemented by IL-21 treatment can grow CTL is intriguing. Low doses of peptide will decrease the magnitude of TCR engagement/CD3 signaling by lowering the number of TCR/CD3 complexes engaged, but will not likely affect the quality of the signal. In contrast, CD8-independent T cell stimulation will not change the quantity of TCR/CD3 complexes engaged. Instead, it will change the quality of signaling by specifically abrogating the downstream effects of CD8 molecules. It is yet to be determined whether a change in quantity or quality is better for generating CTL grafts for clinically effective adoptive immunotherapy. It should be noted that high functional avidity is just one of the requirements of CTL for adoptive transfer. Adoptively transferred CTL must be able to expand, persist, and traffic to the target cells in vivo.

Humans with CD8 deficiency have been reported (19). Among three family members with complete absence of CD8$^+$ T cells, one suffered from mild chronic infections, whereas the other two appeared healthy. These results suggest that there might be a mechanism to counterbalance the loss of CD8 expression in vivo. In this context, our finding that IL-21 can supplement partial T cell stimulation caused by the lack of CD8 coligation in vitro is intriguing. Many investigators, including us, previously reported a unique function of IL-21 that can modulate the quantity and quality of CD8$^+$ CTL (21, 64). IL-21 is predominantly secreted by activated CD4$^+$ T cells, but not by CD8$^+$ T cells. Furthermore, recent in vivo experiments using mouse models showed that IL-21 can be a mediator of CD4$^+$ T cell help to CD8$^+$ T cells (65–67). It is conceivable that, in these patients with total CD8 deficiency, IL-21 secreted by CD4$^+$ T cells may play a critical role to correct the low number of CTL by enhancing the CTL's function and ability to eliminate pathogens.

There were some donors from whom we were not able to expand MART127-specific CTL using mut-aAPC and IL-21. We studied >30 healthy individuals for the expression level of IL-21R on T cells upon activation (data not shown). Although none of their resting T cells expressed IL-21R, there was great diversity in the induced expression level of IL-21R among the individuals. We speculated that, in those patients who were unable to expand MART127-specific CTL using mut-aAPC and IL-21, the expression level of IL-21R on MART127 pCTL was not sufficient to transmit IL-21–mediated supplementary signaling. Unfortunately, T cell activation required for the induction of IL-21R expression downregulates the expression of TCR and CD8. Therefore,
IL-21 RESCUES PARTIAL MAPK ACTIVATION VIA STAT3 IN T CELLS


