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IL-2 and Antigen Dose Differentially Regulate Perforin- and FasL-Mediated Cytolytic Activity in Antigen Specific CD4⁺ T Cells

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Abstract

CD4 T cell effectors can promote survival against lethal influenza virus via perforin mediated cytolytic mechanisms, however; our understanding of how naïve CD4 cells differentiate into class II restricted killers remains obscure. To address this, TCR Tg CD4 cells were activated *in vitro* and examined for their ability to lyse target cells. We found that cytokine polarized CD4 T cell effectors displayed cytolytic activity with the hierarchy Th0>Th1>Th2. Further, IL-4 inhibited the generation of cytotoxic CD4 cells. LPS stimulated B cells and bone marrow derived dendritic cells (BMDC) both induced potent cytolytic activity; however, IL-6, TGF- β , IL-10, IL-12 or TNF- α were not required for inducing cytolytic activity in CD4 effectors. Antigen dose had a marked effect on cytotoxicity: low concentrations of peptide induced more potent cytolytic activity than relatively high concentrations. At low peptide concentration, exogenous IL-2 was necessary to drive granzyme B (GrB) expression and perforin mediated lysis. Thus, low antigen dose and early activation signals via IL-2 direct the CD4 T cell response toward effectors with perforin mediated cytolytic potential. These data have implications for the design of vaccines that may induce cytolytic CD4 cells *in vivo* and improve cell-mediated immunity to viral and bacterial infections.

Keywords

CD4 subsets; cytotoxicity; peptide dose; IL-2; perforin

Introduction

CD4 T cells are required to combat infectious diseases due to their ability to produce cytokines that directly affect pathogen replication in infected host cells and to indirectly affect innate and adaptive immune cells. The best-studied role of CD4 T cells is their ability to provide cognate and cytokine signals to induce B cell antibody class switching and plasma cell development with induction of high affinity antibodies [1]. CD4 cells are also necessary for inducing CD8 T cell responses to weak protein and minor histocompatibility antigens through cognate signals provided by CD40 ligation on DC [2;3;4], and are required in the priming phase for generation and maintenance of functional CD8 T cell memory responses [5;6]. Therefore, CD4 T cells have multifaceted roles in the generation of primary and secondary immune responses and

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current evidence suggests that naïve CD4 T cells can differentiate into multiple subsets that are functionally distinct and are cross-regulating [7;8;9].

CD4 T cells have been subdivided into two functionally distinct subsets, Th1 and Th2, based upon the cytokine profiles they produce [7;10]. When IL-4 is present early in the immune response, naïve CD4 cells differentiate to Th2 cells producing IL-4, IL-5 and IL-13 and are important in helminth infections and atopic disease [11]. The presence of IL-12 induces Th1 CD4 cells, which secrete IFN- γ and mediate cellular immune responses to bacterial and viral infections [7]. This functional dichotomy also exists at the level of transcription factors that regulate the development of both Th1 (via T-bet) [12] and Th2 (via GATA-3) [13] helper subsets.

While Th1 and Th2 populations have been the most studied to date, recent literature has uncovered more complexity in the generation of Th subsets. CD4 Tregs help to maintain peripheral tolerance to self-antigens and are important in modulating immune responses and down regulating Th1 and Th2 responses once pathogens are cleared. More recently, a new subset of Th cells, termed Th17 has been described that is important in regulating inflammation during infection [14]. It was first speculated that Th17 cells were a further differentiated subset of Th1 cells, however, the alternative hypothesis that Th17 cells developed as a separate and distinct subset from Th1 or Th2 cells was shown to be true [15] and the transcription factor ROR γ t was shown to be the key regulator of differentiation to the Th17 lineage [16]. Thus, CD4 T cell subsets have been identified that diverge from the canonical Th1 and Th2 developmental pathways and require different cytokine signals and transcription factors to differentiate.

Class II restricted CD4 T cells with cytolytic activity may represent another CD4 T cell subset, however; this additional feature of CD4 T cell function has not been widely studied. CD4 T cell clones generated *in vitro* were shown to develop cytolytic activity [17;18;19;20], however, the need for repeated antigen stimulation in these clones may have induced a phenotype with no real physiological role. Other studies demonstrated that cytotoxic CD4 cells develop in response to EBV transformed cells [21] mixed lymphocyte reactions [22] and are most easily identified in mice that lacked the conventional CD8 cytolytic compartment [23]; suggesting that these cells may arise when the normal CD8 cytotoxicity pathway is compromised. Our work describing the acquisition of perforin mediated cytolytic activity by influenza specific CD4 effectors *in vitro* [24] and *in vivo* as well as the identification of cytolytic CD4 cells in HIV [25], CMV [26] and in mice infected with LCMV [27] suggests that acquisition of lytic activity by CD4 cells is important in antigen specific, anti-viral responses in normal, immune competent hosts.

Although cytolytic CD4 cells have been described for decades, little is known regarding the factors that promote or regulate this activity in CD4 effectors. One hypothesis suggests that cytolytic CD4 effectors develop as a subset of antigen experienced cells and represent a terminally differentiated stage of development [28]. The use of TCR Tg CD4 cells specific for a single peptide allows us to differentiate naïve CD4 T cells under tightly controlled conditions and identify which exogenous factors are required to generate efficient/effective cytolytic activity in CD4 T cells *in vitro*. We demonstrate here that cytolytic CD4 cells develop during Th1 differentiation, but do not require IFN- γ to acquire this activity. Moreover, Th2 differentiation does not promote the cytolytic phenotype and IL-4 actively inhibits the ability of naïve CD4 cells to become cytolytic. APC were necessary to drive induction of cytolytic activity, however, blocking APC derived cytokines such as IL-6, TGF- β , IL-10, IL-12 and TNF- α did not inhibit the generation of cytolytic CD4 cells. IL-2 was identified as the only exogenous cytokine of those tested necessary for inducing CD4 effectors with high cytolytic activity. When peptide concentrations were low, IL-2 preferentially induced perforin mediated

cytolytic activity over FasL mediated lysis. Taken together, these data reveal a specific set of conditions for generating cytolytic CD4 T cells. These CD4 CTL cells may represent a novel subset of Th cells and may provide an additional layer of protection against intracellular infections.

Materials and Methods

Mice

TCR Tg mice in which CD4 cells recognize a peptide 126–138 of HA protein from PR8 influenza were a generous gift from Dr. David Lo (Scripps Institute) and have been described in detail [29]. Perforin (Pfn^{-/-}) deficient mice on the BALB/c background were a generous gift from Dr. John Harty (University of Iowa) and were bred to HA specific CD4 TCR Tg mice at the Trudeau Institute Animal Breeding Facility. OVA 323–339 specific TCR Tg mice on the BALB/c background (DO11.10) and the C57BL/6 background (OT-11) were also used. All TCR Tg mice were maintained at Trudeau Institute. BALB/c By and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). All experimental animal procedures were carried out in accordance with the Trudeau Institute Animal Care and Use Committee guidelines.

Media and peptides

All cells were grown in RPMI 1640 media (Invitrogen, Carlsbad, CA) containing 2 mM L-glutamine, 100 IU penicillin, 100 µg/ml streptomycin (all from Invitrogen) 10 mM HEPES (Research Organics, Cleveland, Ohio), 50 µM 2-mercaptoethanol (Sigma, St. Louis, MO) and 8% fetal bovine serum (FBS) (Hyclone, Logan, UT). HA peptide 126–138, HNTNGVTAACSHE and OVA peptide 323–339, ISQAVHAAHAEINEAGR were synthesized by New England Peptide, Inc. (Gardner, MA).

Isolation of CD4 T cells and *in vitro* activation

CD4 effectors were generated *in vitro* as previously described [24], with modifications listed below. Briefly, naive CD4 cells were isolated from TCR Tg mice using a positive selection protocol with anti-CD4 beads (clone GK1.5) according to the manufacturer's instructions (Miltenyi Biotech, Auburn CA). B cell blasts were generated as previously described [30], pulsed with concentrations of peptide ranging from 5 µM to 0.005 µM and combined with naive CD4 cells at a 1:1 ratio to give a final concentration of 3×10^5 cells/ml. IL-2 was added to all cultures at 11 ng/ml and IL-12 at 2 ng/ml and anti-IL4 (clone 11B11) at 10 µg/ml were added to polarize CD4 cells to the Th1 phenotype characteristic of high IFN-γ secretion [24]. IL-4 at 15 ng/ml and anti-IFN-γ (clone XMG1.2) at 10 µg/ml were added to cultures to polarize to the Th2 phenotype [31]. After 2 d, an equal volume of complete media containing IL-2 at 11 ng/ml was added and cultures were incubated for an additional 2 d. In some experiments, naïve CD4 T cells were activated with 10 µg/ml anti-CD3 and 5 µg/ml anti-CD28. Anti-IL-6 (clone MP5 20F3), anti-TNF-α (clone XT3.11), anti-IL-12 (clone C17.8) purchased from BioXcell (West Lebanon, NH) and anti-TGF-β1 purchased from R&D systems (Minneapolis, MN) were added at 10 µg/ml in some experiments to block the effects of cytokines on cytolytic activity. To assay for activation and polarization, CD4 effectors at 1×10^6 cells/ml were restimulated with immobilized anti-CD3 (2C11) for 48 hours and supernatants were analyzed for cytokine secretion using Beadlyte® cytokine beadmates (Upstate Signaling, Lake Placid, NY) according to the manufacturer's instructions. Cytokines were detected using a Luminex 100 (Luminex Corp, Austin, TX). Standard curves were analyzed and unknowns calculated using Prism software (Graph Pad, San Diego, CA). Cells were incubated with allophycocyanin (APC) labeled anti-CD4 (clone RMA-4.4) and FITC labeled antibodies to Vβ8.3 (HA TCR Tg) Vα2, Vβ5 (OT-II) and KJ126 (DO11.10) for 30 mins in FACS buffer (PBS with 1% BSA, 0.1% Na azide) at 4° C to identify the purity of the resulting TCR Tg effector populations.

Phycoerythrin (PE) labeled antibodies to CD62L (MEL-14), CD43 (1B11), CD27 (LG.3A10), CD25 (3C7) and CD69 (H1.2F3) (all purchased from BD-Biosciences, San Jose, CA) were used to assess the activation state of the effector populations. Intracellular staining for GrB was done using PE labeled mouse anti-human GrB (GB11) and isotype matched controls, purchased from Caltag (Burlingame, CA). Cells were fixed in 1% paraformaldehyde, analyzed using a Becton Dickinson FACS Caliber and data processed using Flow Jo software (Treestar, San Carlos, CA).

In vitro cytotoxicity assay

Cytotoxicity was measured by the JAM assay [32] with minor modifications. CD4 effectors were washed and plated at decreasing concentrations in 96-well round bottom plates. H-2^d expressing B lymphoma cells (A20), labeled for 4 h with 4 μ Ci/ml ³H-thymidine (Amersham Biosciences, Piscataway, NJ) were added to effectors at a concentration of 1×10^4 cells/well. HA₁₂₆₋₁₃₈ peptide, or irrelevant peptide as a control, was added at a final concentration of 2 μ g/ml. After 4 h at 37°C, plates were harvested and incorporated ³H was measured using a Microbeta Trilux scintillation counter (Perkin Elmer, Shelton, CT). In some experiments, anti-FasL (clone MFL3) purchased from BD Biosciences at 10 μ g/ml was added to inhibit Fas mediated killing [21]. Percent specific lysis was calculated as (spontaneous CPM-experimental CPM)/spontaneous CPM * 100. LU are arbitrarily defined as the number of lymphocytes required to yield 30% lysis in a population of 10^6 cells.

Isolation of bone marrow and generation of BMDC

BMDC were isolated as described [33] with some modifications. Briefly, bone marrow was flushed from the femurs of BALB/c or C57BL/6 mice using a 3 ml syringe with 26g needle. Cells were washed, collected and resuspended in complete medium at a concentration of 2×10^6 cells/ml. Five ml of cell suspension was cultured in 6-well plates in the presence of GM-CSF at 5 ng/ml for a total of 7–8 days. BMDC cultures were fed on days 3, 5 and 7 by removing half of the media and replacing with fresh media containing 5 ng/ml GM-CSF. In some experiments, BMDC were activated with 1 μ g/ml LPS for 18–20 hours before isolation and use as APC to activate CD4 cells.

Statistical analyses

Statistical analyses were performed by Prism 4.0 software (GraphPad, San Diego CA) using Student's t-tests for parametric data, or one-way ANOVA.

Results

Cytolytic activity develops during Th1 differentiation, but does not require IFN- γ

Our laboratory has previously demonstrated that CD4 TCR Tg cells specific for HA peptide of influenza PR8 acquire perforin mediated cytolytic activity important for B cell independent protection against lethal doses of influenza H1N1 infection [24]. In past studies, CD4 T cell effectors polarized to the Th1 phenotype acquired cytolytic activity, however, it was not known whether cytolytic activity developed concurrently with Th1 development, or whether IFN- γ was required for this process. To test this, the cytotoxic activity of HA specific WT or IFN- γ ^{-/-} CD4 TCR Tg cells generated under Th1 polarizing conditions were compared. At 2, 3 and 4 days, cells were harvested and assayed for levels of GrB, cytolytic activity and cytokine secretion. Cytotoxicity is measurable as early as day 2 in culture and corresponds with low levels of GrB expression (Fig. 1A, B). Cytolytic activity and GrB expression increases with time in culture in both WT and IFN- γ ^{-/-} CD4 effectors and is high by d3 and d4 of culture. FACS analysis of activation markers such as CD43 and CD25 mirrors GrB expression and indicates that CD4 effectors become more activated the longer in culture with highest levels

of CD43 and CD25 at d4 (data not shown). Importantly, cytolytic activity was induced in Th1 cells independently of IFN- γ secretion, suggesting these effector functions are not linked [34]. Figure 1C shows that CD4 cells can produce cytokines as early as 2d post activation and cytokine secretion peaks at d3 post activation, however, full polarization appears to require at least 4 days in culture, as WT cells still retain the ability to secrete IL-5, IL-13 and IL-17 at 2 and 3 days after activation, but lose this ability by 4d post culture initiation. These data suggest that naïve CD4 cells can differentiate into cytolytic CD4 effectors that lyse class II⁺ targets, but this activity does not require IFN- γ and may be initiated prior to full Th1 polarization.

Cytolytic activity correlates with increased GrB expression and Th1 cytokines and is inhibited by IL-4

Our previous experiments used Th1 polarized CD4 effectors due to the importance of Th1 cells in influenza infection [35;36]. It has been demonstrated previously that Th1 influenza specific CD4 clones [17] and effectors [24] can lyse class II⁺ targets, while influenza specific Th2 clones can not [17]. However, both Th1 and Th2 polarized clones reactive to EBV could lyse transformed B cells [21], therefore, the capacity of different CD4 subsets to differentiate into CD4 killers remains controversial. We wanted to re-evaluate whether cytolytic activity was unique to the Th1 subset or if Th2 cells generated by a primary stimulation with antigen and cytokines could also acquire a lytic phenotype. Furthermore, lytic activity was apparent as early as d2 after culture initiation and more robust in the absence of IFN- γ , suggesting that CD4 effectors that have not been fully polarized may acquire lytic activity. To test the effect of cytokine polarization on cytotoxicity, HA specific naïve CD4 T cells were polarized to Th1, Th2 or incubated with IL-2 only (Th0) for 4 days and assessed for the ability to lyse target cells. Th1 polarized cells have a high level of cytolytic activity (Fig. 2A), while Th2 cells do not lyse targets very efficiently. Surprisingly, Th0 effectors showed a statistically significantly higher level of cytolytic activity compared to Th1 or Th2 polarized cells (Fig. 2B). Th0 cells demonstrated a 2-fold increase in cytolytic activity compared to Th1 cells and were 20-fold higher than Th2 cells. Polarized Th1 and Th2 CD4 effectors secrete IFN- γ and TNF- α or IL-4, IL-5, IL-13 and IL-10 respectively indicating that they are adequately polarized (Fig. 2C). Th0 effectors resemble Th1 cells based on the cytokine profiles they produce (Fig. 2C). One striking feature is that Th0 effectors retain the ability to produce IL-2 whereas polarized effectors no longer secrete this cytokine (Fig. 2C and [37]). To determine if increased cytolytic activity correlated with activation, Th1, Th2 or Th0 CD4 effectors were incubated with antibodies to activation markers such as CD43, CD62L, CD25, CD27 and CD69 (data not shown) or proteins important for target cell lysis such as GrB (Fig. 2D). Levels of intracellular GrB positively correlated with cytotoxic activity, in that, Th0 cells had the highest level of GrB expression and the highest lytic unit (LU) per population. CD107a expression also correlates with cytotoxicity in human CD4 cytolytic cells [26], however, we could not detect this marker on murine CD4 cytolytic cells after restimulation as has been previously described [38]. Th0 cells had the lowest CD25 and highest CD62L expression (data not shown) indicating that high cytolytic activity may not necessarily correlate with full activation and/or differentiation. Since Th0 and Th1 effectors could synthesize Th1 type cytokines and had demonstrable levels of cytolytic activity, we reasoned that IL-4 may be inhibiting the development of cytolytic activity. To test this, CD4 effectors were generated in the presence of IL-2 alone and increasing concentrations of IL-4. IL-4 at 3 and 30 ng/ml can inhibit cytolytic activity in Th0 effectors (Fig. 2E); however, IL-4 added at 0.3 ng/ml did not have an effect on cytotoxicity. These data suggest that IL-4 inhibit development of cytolytic CD4 T cells in a dose dependent manner. To rule out the contribution of IFN- γ and IL-4 produced by Th0 effectors, neutralizing antibodies to both cytokines were added during culture generation, but were not required for the induction of cytolytic CD4 cells (data not shown). These data indicate that antigenic stimulation in the presence of IL-2 is sufficient to induce cytolytic activity in CD4 T cells and this activity correlates with increases in GrB expression and a Th1-like cytokine profile, but

does not correlate with activation or differentiation state as measured by cell surface marker expression. Furthermore, IL-4 prevents naïve CD4 T cells from developing into cytolytic effectors.

APC induce peptide specific cytotoxicity that is not dependent on IL-10, IL-6, TNF- α , TGF- β or IL-12

Since cytokines appeared to influence the generation of cytotoxic CD4 T cells, we wanted to test the requirement for APC and APC derived cytokines in the generation of cytolytic CD4 cells. CD4 effectors were generated by culture with BMDC, A20 B cell lymphoma cells, or immobilized anti-CD3 and anti-CD28. To determine if different APC populations could induce cytolytic activity in different Th subsets, naïve CD4 cells were polarized to Th1, Th2 or non polarized to Th0 phenotypes. CD4 effectors generated using APC such as B cell blasts (Fig 2A), BMDC or A20 cells (Fig 3A) showed the same hierarchy of cytolytic activity: Th0>Th1>Th2. Surprisingly, immobilized anti-CD3 and anti-CD28 did not induce peptide specific cytolytic activity, but induced non specific killing in Th1 polarized effectors, and no evidence of killing in Th0 effectors. This suggests interactions with APC are required to promote the cytolytic phenotype in CD4 T cells and substitution of this signal with anti-CD3 and anti-CD28 is not sufficient to generate antigen specific CD4 killers. These data are in contrast to Tc1 or Tc2 polarized populations of class I restricted CD8 T cells that can lyse peptide coated targets equally well and can be induced with anti-CD3 and anti-CD28 [34;39; 40].

Since APC, but not anti-CD3 and anti-CD28 can induce cytolytic activity in naïve CD4 cells, we reasoned that APC derived soluble factors would be important in generating cytolytic CD4 cells. B cell blasts and BMDC stimulated with LPS secrete IL-10, IL-6, TNF- α , MIP-1 β , RANTES and KC (Fig. 3B). To test if IL-10 was important for cytolytic CD4 generation, IL-10 deficient BALB/c mice were used as a source of B cell blasts. In addition, TCR Tg mice deficient in IL-10 production were also used to rule out any contribution by T cell derived IL-10 in inducing the cytolytic phenotype. Figure 3C, left panel demonstrates that IL-10 is not required for the acquisition of cytolytic activity in CD4 effectors. Cytokines such as IL-6, TNF- α and IL-1 have been implicated in enhancing the CD4 T cell response to antigen [41;42], especially during suboptimal responses [43]; however, blocking IL-6 or TNF- α had no effect on the generation of cytolytic CD4 cells (Fig. 3C, middle panel). Since APC derived IL-12 or TGF- β and IL-6 can differentiate naïve CD4 cells to Th1 or Th17 cells respectively, we also blocked these cytokines with neutralizing antibodies and tested for the ability of CD4 cells to acquire the cytolytic phenotype. Figure 3C, right panel, demonstrates that TGF- β alone or in combination with IL-6, or IL-12 alone is not required for inducing cytolytic CD4 cells. Therefore, APC are necessary for differentiation to the cytolytic phenotype; however, IL-6, TGF- β , TNF- α , IL-10, or IL-12 are not required for the acquisition of lytic activity in CD4 T cells.

Perforin and FasL mediated cytolytic activity inversely correlate with antigen dose

Peptide dose has profound effects on the generation of polarized Th subsets such that high/intermediate peptide density induces a Th1 profile while lower peptide doses skew responses to Th2 polarization [37;44]. Therefore, we wanted to determine whether decreasing peptide dose would have an effect on cytolytic activity in CD4 T cells. In addition, we used 2 additional strains of TCR Tg mice to confirm that cytolytic activity in CD4 cells was not unique to a particular TCR specificity or merely an artifact of *in vitro* culture conditions. H-2^d restricted TCR Tg CD4 cells that recognize a peptide in influenza HA (Fig 4A) or Ova (data not shown) or H-2^b restricted TCR Tg CD4 cells that recognize Ova peptide 323–339 (Fig 4B) are able to differentiate into cytolytic CD4 effectors. Peptide concentrations as low as 5 nM generated effectors with the highest level of cytolytic activity. Our previous work demonstrated that the

majority of cytotoxicity exhibited by CD4 cells was perforin mediated, however, Pfn^{-/-} cells acquired cytolytic activity that was completely blocked by antibody to FasL [24]. HA specific Pfn^{-/-} CD4 cells were used to determine if peptide dose could regulate FasL mediated lysis and effectors generated in the presence of 5 nM HNT peptide demonstrated the highest level of FasL mediated cytotoxicity (Fig 4C). Cytolytic activity was again calculated as LU per 10⁶ cells over a number of experiments and Figure 4D shows that peptide at 5 nM induces cytolytic CD4 cells with the greatest level of lytic activity (45 LU), this was statistically significant compared to 0.5 and 5 μ M peptide ($p < .01$ and $.001$ respectively) and ranged from 4-fold to 20 fold higher. LU of CD4 effectors generated with 50 nM peptide (35 LU) were also statistically higher than LU of cells generated with 0.5 and 5 μ M peptide ($p < .001$). Interestingly, expansion of CD4 effectors correlated with peptide dose but inversely correlated with LU (Fig 4D, right panel). As expected, CD4 cells generated with higher amounts of peptide (5–.05 μ M) still show a Th1 type cytokine pattern with high amounts of IFN- γ and TNF- α . However, lowered peptide doses also increased IL-5, IL-10 and IL-13 production. Finally, at 5 nM peptide, CD4 effectors express lower levels of IFN- γ , higher levels of Th2 cytokines and importantly, retain the ability to produce IL-2 (Fig. 4E). Although CD4 effectors generated with 5 nM peptide produce more Th2 type cytokines than effectors generated with higher amounts of peptide, these levels do not reach those produced by Th2 polarized effectors (compare Fig. 2C with 4E), suggesting that lower peptide concentrations induce cells that are not fully polarized.

Exogenous IL-2 is necessary to induce perforin mediated cytotoxicity and GrB expression at low doses of peptide

CD4 effectors that demonstrated the highest level of cytolytic activity also retained the ability to secrete IL-2 (Fig 2C and 4E). This led us to hypothesize that IL-2 may be important in driving the generation of CD4 effectors with high cytolytic potential. To determine if IL-2 has an effect on the generation of cytolytic activity, CFSE labeled HA specific CD4 cells were cultured with increasing concentrations of HNT peptide in the presence or absence of IL-2. Figure 5A shows FACS plots of GrB expression and proliferation by loss of CFSE in gated CD4 effectors and indicates that GrB levels are highly regulated by IL-2, especially at low peptide concentrations. Furthermore, GrB expression and proliferation appear to be differentially regulated in that there is a substantial population of cells that have lost CFSE and proliferated, but have not upregulated GrB (Fig. 5A). CD4 effectors generated with 50 and 5 nM peptide in the presence of IL-2 exhibit high cytolytic activity while CD4 effectors generated without IL-2 (open symbols) display intermediate to low cytolytic activity (Fig 5B). In addition, IL-2 is absolutely required to generate cytolytic CD4 effectors when peptide concentrations are in the nanomolar range (compare closed triangles and open triangles). The fact that GrB expression was higher in cells generated in the presence of IL-2 led us to hypothesize that the cytolytic activity observed in the absence of IL-2 may be due to Fas:FasL interactions. Figure 5C confirms earlier data demonstrating that cytolytic activity is decreased in the absence of IL-2. The addition of anti-FasL antibody in the lytic assay demonstrates that killing mediated by CD4 effectors cultured in the absence of IL-2 is predominantly Fas:FasL mediated, while effectors grown in the presence of IL-2 show predominantly perforin mediated killing. Pfn^{-/-} effectors demonstrated the same level of cytolytic activity with or without the addition of exogenous IL-2, and this was completely blocked by the addition of anti-FasL antibody in the killing assay. Therefore, IL-2 is a potent inducer of perforin mediated cytolytic activity in CD4 effectors; however, FasL mediated killing can be induced in the absence of exogenous IL-2.

Discussion

There have been many reports describing the cytolytic potential of class II restricted CD4 cell clones [17;18;19;20], however since clones may not reflect the response of a primary

stimulation, we systematically tested the factors that could drive the generation of cytolytic CD4 cells during a single, primary encounter with antigen. Peptide dose had a marked effect on the level of cytolytic activity in that CD4 effectors generated with low peptide doses (5nM) demonstrated the highest cytolytic activity. Furthermore, IL-2 was indispensable for perforin mediated, but not FasL mediated cytolytic activity especially at low concentrations of peptide. These data suggest that acquisition of cytolytic activity may be an early event in CD4 T cell differentiation and can be accomplished with physiological antigen concentrations. In addition, although cytolytic activity correlated with Th1 conditions, the observation that non-polarized CD4 effectors acquired strong CTL activity suggests that this cell type may represent yet another CD4 T cell subset. Defining the requirements for generating class II restricted cytolytic CD4 cells may facilitate the design of novel vaccine strategies that can promote the “killer” phenotype and provide multiple levels of protection against viral infections.

CD4 CTL are not equivalent to CD8 CTL

Relatively little is known about the factors that induce or regulate cytolytic activity in CD4 T cells, therefore, we began by testing a variety of culture conditions, APC populations and antigen specific TCRs. Our finding that Th2 polarization inhibited the development of cytolytic activity is in agreement with previously published reports that Th2 clones were not cytolytic [17;45]. In the current report, we have extended these findings and demonstrated that addition of IL-4 to primary cultures blocked the development of CD4 CTL in a dose dependent manner (Fig 2). These results are in contrast to data obtained using TCR Tg CD8 cells in which both Tc1 and Tc2 populations exhibited the same level of cytotoxic activity [34;39;40], however, another group has demonstrated that Tc2 cells showed decreased cytotoxicity compare to Tc1 [46]. Another finding that separates cytolytic CD4 cells from conventional CD8 killers is that CD4 cells require the presence of APC for full differentiation to the cytolytic phenotype. Bypassing the recognition of the TCR using anti-CD3 and providing co-stimulatory signals using anti-CD28 did not induce antigen specific cytolytic CD4 T cells, even in the presence of Th1 polarizing cytokines (Fig 3). This is again in contrast to published reports in which CD8 cells activated with anti-CD3 and anti-CD28 demonstrated high, peptide specific lysis [34; 40]. Further, CD4 CTL are slightly less effective than CD8 CTL when generated *in vitro* using identical culture conditions, but demonstrate similar lytic ability when adoptively transferred and assayed *in vivo* [24].

Although APC appeared to be required for inducing cytolytic CD4 cells, IL-12, IL-10, TNF- α , TGF- β , or IL-6 that can be produced by APC populations, were not necessary for differentiation of CD4 CTL (Figure 3). It has been shown that IL-10 was partially required for inducing perforin and GrB in human IFN- γ /IL-10 producing cells that showed cytolytic activity [47] and could enhance cytolytic function in activated CD8 cells [48;49]; therefore, we reasoned that cytolytic activity may be abrogated in the absence of IL-10. In contrast, IL-10 can dampen Th responses by inhibiting APC function or inhibiting proliferation and cytokine production of Th1 cells [50], therefore, blocking IL-10 may enhance CD4 cytotoxicity. It was surprising to us that the total absence of this cytokine had no effect on the development of CD4 CTL, either enhancing or inhibiting cytolytic activity. Both BMDC and B cell blasts produced TNF- α and IL-6, pleiotropic cytokines known to enhance the expansion and migration of CD4 T cells [42] and the induction and differentiation of cytotoxic T cells [51;52]. In addition, the cytokine TGF- β is required for driving the differentiation of the Th17 subset, with DC-derived IL-6 acting as a co-factor [53;54]. However, blocking TGF- β , TNF- α or IL-6 alone, or blocking TGF- β and IL-6, during CD4 effector generation had no effect on cytolytic activity (Figure 3) and suggests that cytolytic CD4 cells may represent a unique subset of T “helper” cells. It appears that APC are required for generation of cytolytic CD4 cells; however, blocking CD40/CD40L interactions using DCs or T cells from knockout mice had no effect on the generation of cytolytic activity in CD4 effectors (data not shown). It has been suggested that CD28/CD80

signals are important for generation of human CD4 CTL [55] and experiments are underway to determine the involvement of direct APC/T cell interaction in the development of CD4 CTL.

Cytolytic CD4 cells, a new subset, or heterogeneous effector functions?

Previous studies demonstrated that Th1, but not Th2 influenza specific clones [17] and Th1 poliovirus specific clones [20] could acquire cytolytic activity. However, cytolytic CD4 clones that recognized EBV antigens could secrete both Th1 and Th2 cytokines and were characterized as Th0 [21]. One caveat with these early experiments is that by using clones rather than primary naïve cells, cytolytic activity could only be *correlated* with a particular phenotype, instead of determining which cytokines could drive the differentiation of cytolytic CD4 cells. By utilizing TCR Tg CD4 cells we were able to activate naïve cells without polarization to a Th0 phenotype characterized by the production of IL-2, IL-4 and IFN- γ ; and these Th0 effectors had the highest level of cytolytic activity, regardless of the type of APC used (Fig. 2 and 3). Although Th1 polarization correlated with cytolytic activity, IFN- γ was not required for generation of CD4 CTL and IL-4 inhibited the cytolytic phenotype. Furthermore, antibodies to IL-12, IL-4 and IFN- γ did not block cytolytic activity suggesting that the canonical Th1 and Th2 cytokines were not involved in driving cytotoxicity in CD4 cells. Therefore, CD4 helper T cells demonstrated a spectrum of killing potential that may or may not correlate with their ability to provide help and led us to speculate that cytolytic Th cells may represent a unique subset of CD4 T helper cells.

Since Th0 CD4 effectors exhibited the highest level of cytotoxicity, an alternative hypothesis would suggest that a cell that is less differentiated would acquire cytolytic activity before full differentiation into Th1 effectors. Figure 1 seems to support this, as cytolytic activity is high by d3 in culture, while full polarization measured by cytokine synthesis does not occur until d4 in culture. Perhaps generation of Th0 CD4 effectors induces a multifunctional Th cell that can secrete many cytokines as well as lyse target cells. Th1 cells simultaneously secreting IL-2, IFN- γ and TNF- α have been identified in L. major infection and the vaccines that could elicit these multifunctional cells correlated with conferring a higher level of protection than vaccines that induced Th1 cells secreting only one or two cytokines [56]. McElhaney's group has also correlated T cells expressing GrB, IFN- γ and IL-10 with decreased risk of developing influenza after vaccination, especially in elderly populations [57]. It appears that multifunctional CD4 cells are important prognosticators of protection against infectious diseases [58]. It remains to be determined whether single cells within the population of CD4 CTL we have described here can secrete multiple cytokines, or whether subsets of cells secrete IFN- γ or IL-2 or TNF- α . It is not known yet whether Th17 cells could acquire cytolytic activity, but it has been demonstrated that Treg cells could utilize GrB as a mechanism of suppression [59], and IL-10 producing human Treg populations could express both perforin and GrB [47]. Nevertheless, more work is needed to determine whether cytolytic CD4 cells represent a new Th subset, or are merely Th1 and Treg cells with multiple effector functions.

CD4 effectors preferentially use perforin to lyse target cells

It has been well documented that CD4 cells can utilize FasL to dampen immune responses and lyse class II expressing targets such as B cell lymphomas [60;61;62]. In contrast, cytolytic CD8 T cells and NK cells preferentially use perforin and the granule exocytosis pathway to mediate target cell lysis [63]. In the current report, we demonstrate that non polarized Th0 CD4 effectors use perforin as the major mechanism to kill A20 targets (Fig. 4). However, in the absence of perforin, CD4 effectors can compensate by using a FasL mediated killing pathway and this pathway is also regulated by Th1 or Th0 polarization and antigen dose (Figure 4,5). Perforin dependent cytotoxicity by CD4 cells, in the absence of CD8 cells has been reported by Williams and Engelhardt [22], and our unpublished data demonstrates that perforin mediated cytolytic activity by CD4 cells also develops *in vivo*, even in the presence of a strong CD8 response

(Brown, in preparation). Taken together, these data suggest that perforin and the granule exocytosis pathway is predominant in CD4 cells, and the FasL pathway is compensatory.

IL-2 may be the master regulator of perforin mediated cytolytic activity in CD4 cells, since CD4 effectors could still use FasL in the absence of exogenous IL-2, while the perforin mediated pathway and up regulation of GrB was inhibited (Figure 5). It has been demonstrated that IL-2 can lead to a direct induction of perforin and GrB gene expression in CD8 cells, independent of its effect on survival and cell proliferation [64] and regulates perforin gene expression through Stat5 [65]. The tools to assess this directly in CD4 cells (i.e. Ag specific bcl-2 tg mice) are not yet available; however, the observation that FasL mediated lytic activity was unaffected in the absence of IL-2 strongly suggests that IL-2 drives perforin and GrB mediated lysis by CD4 effectors. It has been shown that both resting and activated human CD8 CTL clones can express perforin and lyse virally infected targets, however, only activated, but not resting, human CD4 CTL express high levels of perforin mRNA and lyse virally infected autologous targets [66]. This suggests that the upstream signaling pathways that control perforin gene expression and subsequent cytolytic effector function may be differentially regulated in CD8 and CD4 CTL. Furthermore, signaling through the IL-2 receptor on CD8 T cell clones regulates perforin expression through stat5 phosphorylation. It remains to be determined whether IL-2 signaling through stat5 is responsible for increases in perforin gene expression in CD4 T cells similar to CD8 cells. In addition, the requirement for exogenous IL-2 suggests that a certain level of expression of IL-2R must be achieved, or a certain threshold of receptors must be occupied in order to induce signaling and thus, perforin up regulation.

Generation of cytolytic CD4 cells is exquisitely sensitive to peptide dose

It has been suggested that peptide dose or TCR receptor occupancy levels can dictate the differentiation of naïve CD4 cells into Th1 or Th2 polarized subsets, with low dose priming antigen leading to Th2 subsets and high or intermediate dose leading to Th1 polarization [44], and very high peptide levels (10–100 μ M) inducing the Th2 phenotype [67]. Previous work from our lab confirmed the hypothesis that high dose antigen (5–10 μ M) combined with exogenous IL-2 led to Th1 cytokine secretion, while low dose antigen (10 nM) induced a Th0 pattern (IFN- γ , IL-4 and IL-2) of cytokine secretion [37]. Figure 4 demonstrates that at low peptide dose (5 nM), the effectors with the best cytolytic activity (Fig 4A), also showed a Th0 pattern of cytokine secretion with detectable levels of IFN- γ , IL-4 and IL-2 (Fig 4F). Nevertheless, in our system, low peptide concentrations appear to induce a more uncommitted Th cell with high cytolytic activity, rather than a Th2 cell as described by Bottomly and colleagues [44].

Peptide concentration has also been shown to have a marked effect on CD8 CTL generation. CD8 cells cultured with 0.1 nM peptide showed greater lysis than cells cultured with 100 μ M peptide, and are termed high avidity CTL, since they can recognize limiting amounts of peptide/MHC complexes on APC [68]. These high avidity CTL were much more efficient at viral clearance *in vivo*, compared to the low avidity lines, even though both cells could lyse virally infected targets *in vitro* [69]. Generating CTL lines from bulk cultures after immunization would suggest that clones with high affinity TCR gave rise to the high avidity CTL and would not be applicable to our studies since all CD4 cells from TCR Tg mice should express the same affinity TCR. However, studies using CD8 TCR Tg mice demonstrated that high avidity CTL could be generated with low dose peptide and low avidity cells generated with high dose peptide, even from precursors that bore the same affinity TCR [70]. These data are analogous to our results shown in Figure 4 in which very low peptide concentrations led to CD4 effectors with high cytolytic activity and begs the question whether these so called high avidity CD4 CTL would also be more effective at clearing virus than effectors generated with higher peptide concentration. Clearly peptide concentration has a marked effect on the generation of CD4

CTL and is an important factor in the consideration of vaccine formulations that can induce the best protective response against viral infections.

Implications for Vaccine Design

The acquisition of perforin mediated cytolytic activity by CD4 effectors *in vitro* [24] and *in vivo* (Brown, in preparation) [25;27] suggests that CD4 cytotoxicity is a significant component of the anti-viral response. Our own unpublished data demonstrates that CD4 CTL can traffic to the lung during influenza infection and those cells lyse peptide pulsed targets in a perforin dependent manner, directly *ex vivo* (Brown, in preparation). Furthermore, we have detected class II expression on lung epithelial cells and CD4 effectors in close contact, during experimental influenza infection (Brown, in preparation). Therefore, defining the factors that induce cytolytic CD4 cells, as we have begun with this report, will provide important information for the design of effective vaccines to combat infectious diseases. It is becoming more apparent that the quality of the T cell response, and not necessarily the magnitude of that response, is a key indicator of the ability to confer protection against numerous infections and tumors [57;58;68]. Therefore, vaccine formulations that account for antigen dose, availability of IL-2 and plasticity in the CD4 T cell response will undoubtedly be successful in generating multifunctional T cells that can confer greater protection.

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Abbreviations

GrB	granzyme B
PR8	influenza A/Puerto Rico/8/34
Pfn	perforin

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Figure 1. Cytolytic activity develops during Th1 differentiation, but does not require $\text{IFN-}\gamma$
 TCR Tg CD4 cells from WT or $\text{IFN-}\gamma^{-/-}$ mice were cultured for 2, 3 or 4 days in the presence of polarizing conditions as described in the Materials and Methods. A) 4 hr *in vitro* cytolytic assay (JAM assay) comparing lytic activity of WT (left panel) or $\text{IFN-}\gamma^{-/-}$ (right panel) CD4 effectors to peptide pulsed (or unpulsed) A20 target cells. GrB levels were detected by FACS (D). Cytokine secretion was analyzed from WT (C) or $\text{IFN-}\gamma^{-/-}$ (D) supernatants using a Beadlyte multiplex cytokine assay after 2, 3, or 4d in culture and restimulation with anti-CD3 for an additional 36 hr. Shown is the amount of cytokine in ng/ml per 1×10^6 input cells. Shown is a representative experiment of three performed.

Figure 2. Cytolytic activity correlates with increased GrB expression, Th1 cytokines and is inhibited by IL-4

TCR Tg CD4 cells were polarized to Th1 or Th2 or given IL-2 only for 4 days in culture and assayed for cytolytic activity by JAM assay (A). B) LU were calculated as described in the Materials and Methods for five separate experiments. * denotes significant differences by one way ANOVA analyses ($p = .001$) C) Th1, Th2 or IL-2 only effectors were restimulated with plate bound anti-CD3 for 48 hr and the supernatants analyzed for cytokines using a Beadlyte multiplex cytokine assay. Shown is the amount of cytokine in ng/ml per 1×10^6 input cells. D) Th1, Th2 or IL-2 only 4 day effectors were stained for GrB and analyzed using FlowJo software. E) TCR Tg CD4 cells were incubated with peptide pulsed B cell blasts in the presence

of IL-2 only or increasing concentrations of IL-4 for 4 days. Cells were then assayed for cytolytic activity as described. Th2 cells are shown as a negative control. This experiment was repeated twice with similar results.

Figure 3. APC induce peptide specific cytotoxicity that is not dependent on IL-10, IL-6, TNF- α , TGF- β or IL-12

TCR Tg CD4 cells were polarized to Th1 or Th2 effectors or cultured with IL-2 alone as described in the Materials and Methods. A) Th1, Th2 or IL-2 only effectors were stimulated with BMDC, A20 B cell lymphoma or anti-CD3 and anti-CD28 coated plates and assayed for cytolytic activity against peptide pulsed (closed symbols) or unpulsed (open symbols) target cells. A representative experiment of three performed is shown. B) B cell blasts or BMDC were stimulated with LPS for 48 h or 24 h respectively and the supernatants analyzed for cytokines using a Beadlyte multiplex cytokine assay. Shown is the amount of cytokine in pg/ml. C) Peptide pulsed WT B cells or B cells from IL-10^{-/-} mice were incubated with WT T cells or

IL-10^{-/-} T cells for 4 days in the presence of IL-2 (left panel). TCR Tg CD4 cells were incubated with peptide pulsed B cell blasts in the presence of IL-2 only or isotype control antibody, anti-IL-6 or anti-TNF- α at 10 μ g/ml for 4 days (middle panel) or anti-IL-12, anti-TGF- β or anti-TGF- β and anti-IL-6 at 10 μ g/ml for 4 days (right panel). Cells were then assayed for cytolytic activity as described. Knock-out and blocking experiments were repeated with similar results.

Figure 4. Perforin and FasL mediated cytolytic activity inversely correlate with antigen dose
 CD4 cells were isolated from HNT (A), OT-II (B) or HNT.Pfn^{-/-} (C) TCR Tg mice and stimulated with B cell blasts pulsed with various concentrations of peptide for 4 days. The same number of live cells in each group was plated in the JAM assay to account for differences in expansion. Cells were then assayed for cytolytic activity as described. D) LU (left panel) were calculated as described in the Materials and Methods from four to ten separate experiments. Fold expansion was also calculated at the end of the 4 day culture period and is shown in the right panel. *Means are statistically different by 1-way ANOVA. E) Supernatants from CD4 effectors (shown in A) incubated with plate-bound anti-CD3 for 48h were assayed for cytokines using a luminex bead array.

Figure 5. Exogenous IL-2 is necessary to induce perforin mediated cytotoxicity and GrB expression at low doses of peptide

CD4 cells were isolated from TCR Tg mice, labeled with CFSE and stimulated with B cell blasts pulsed with various concentrations of peptide in the presence or absence of IL-2 for 4 days. A) GrB expression and proliferation by loss of CFSE expression was analyzed by flow cytometry. Resulting histograms were gated on CD4 T cells in the culture. B) Cytolytic activity of CD4 effectors grown in the presence of IL-2 (closed symbols) or absence of IL-2 (open symbols) was determined by JAM assay. The same number of live cells in each group was plated in the JAM assay to account for differences in expansion. C) CD4 cells were isolated from WT or Pfn^{-/-} TCR Tg mice and stimulated with B cell blasts pulsed with 50 nM peptide

in the presence (squares) or absence (circles) of IL-2. Cells were then assayed for cytolytic activity in the presence (open symbols) or absence (closed symbols) of anti-FasL antibody. D) GrB expression in gated CD4 cells cultured in the presence or absence of IL-2 was assayed by flow cytometry. Shown is a representative experiment of three performed.