Expression of CD39 on Activated T Cells Impairs their Survival in Older Individuals

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SUMMARY

In an immune response, $CD4^+$ T cells expand into effector T cells and then contract to survive as long-lived memory cells. To identify age-associated defects in memory cell formation, we profiled activated $CD4^+$ T cells and found an increased induction of the ATPase CD39 with age. $CD39^+$ $CD4^+$ T cells resembled effector T cells with signs of metabolic stress and high susceptibility to undergo apoptosis. Pharmacological inhibition of ATPase activity dampened effector cell differentiation and improved survival, suggesting that CD39 activity influences T cell fate. Individuals carrying a low-expressing CD39 variant responded better to vaccination with an increase in vaccine-specific memory T cells. Increased inducibility of CD39 after activation may contribute to the impaired vaccine response with age.

Graphical abstract

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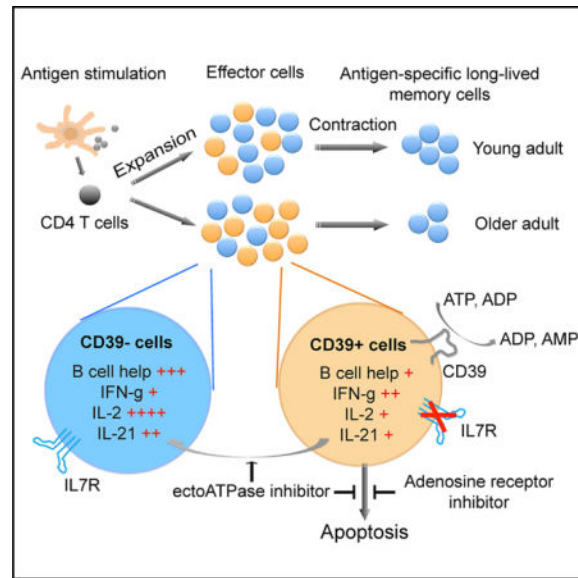
INTRODUCTION

Aging is associated with a decline in immune function, contributing to the increased susceptibility to infectious diseases and higher incidence of malignant disease (Goronzy and Weyand, 2013; Montecino-Rodriguez et al., 2013; Thompson et al., 2003; Weng, 2006). As a consequence of the impaired adaptive immune response, vaccinations are less efficacious. While naive T cell responses are particularly compromised (Petersen et al., 2013), memory T cell responses are also impaired as documented by the reduced efficacy of annual influenza vaccinations or the poor recall response to varicella zoster immunization (Dormitzer et al., 2011; Levin, 2012).

Most studies exploring mechanistic defects have focused on the early stages of an immune response (Goronzy and Weyand, 2013; Haynes and Swain, 2012; Zhang et al., 2014). In contrast to the mouse, age-associated changes in T cell repertoire composition are not sufficient to explain the failure in human immune competence with age. The number of naive T cells declines with age; however, at least for CD4$^+$ T cells, the decline is modest (Wertheimer et al., 2014), and the T cell receptor (TCR) repertoire continues to be sufficiently diverse in older adults to include T cell specificities to most if not all exogenous antigens (Qi et al., 2014). An increased threshold to respond to TCR triggering due to increased expression of the dual-specific phosphatase 6 is likely to compromise stimulation by low-affinity peptides for naive cells (Li et al., 2012).

Later phases of the T cell response have been less explored for age-associated defects. Subsequent to TCR stimulation, antigen-specific T cells exponentially expand and differentiate into effector cells. Most of these expanded cells undergo apoptosis; a few of them survive as long-lived memory cells (Kaech and Wherry, 2007; Williams and Bevan, 2007). CD8$^+$ T cells only require a short encounter with antigen to clonally expand and develop into effector cells (Kaech and Ahmed, 2001). The cell surface marker KLRG1 and the interleukin-7 (IL-7) receptor are useful to identify murine CD8$^+$ effector T cells that

survive and transition into memory cells (Kaech et al., 2003; Sarkar et al., 2008). CD4^{+} T cells require ongoing antigenic stimulation during clonal expansion. Transition into memory cells is dependent on the strength of the TCR signal, and only high-affinity T cells survive (van Leeuwen et al., 2009; Williams et al., 2008). The mechanisms regulating CD4^{+} effector cell clonal downsizing versus memory cell differentiation are undetermined. Ly6C has been suggested as phenotypic marker of CD4^{+} memory cell precursors in the murine effector cell population but does not exist in humans (Marshall et al., 2011).

The current study was designed to identify age-associated differences in the gene expression of human CD4^{+} effector cells that correlate with their propensity to undergo apoptosis or to survive as long-lived memory T cells. We identified the ecto-ATP/ADPase CD39 expressed on the subset of activated CD4^{+} T cells that is prone to apoptosis. Rather than a surrogate marker, we found the ATPase activity to be directly involved in T effector cell differentiation and apoptosis. CD39 was more frequently induced in T cell responses of old than young individuals. Increased expression of CD39, either due to age or to genetic polymorphism, may render individuals more susceptible to T cell apoptosis, resulting in the generation of a reduced number of long-lived memory T cells after vaccination.

RESULTS

Age-Associated Increase in CD39 Expression after T Cell Activation

To identify genetic programs that are distinct in CD4^{+} T cells of young and old individuals, we profiled transcripts in CD4^{+} memory T cells that were stimulated in vitro by dendritic cells (DCs) and the superantigen TSST-1 (GEO: GSE36476). We identified genes that were differentially expressed at 48 and 72 hr after stimulation and not in unstimulated T cells or early after activation (Yu et al., 2012). We hypothesized that these genes contribute to defective adaptive immune responses in older individuals by compromising effector function and generation of long-lived memory T cells. Ectonucleoside triphosphate diphosphohydrolase (ENTPD) 1, a cell membrane ATPase also referred to as CD39, was significantly overexpressed in the gene arrays of stimulated older CD4^{+} memory T cells. Variable expression in humans has been attributed to an A-to-G SNP at position rs_10748643 (Friedman et al., 2009). No significant age-associated differences in SNP distributions were found; ~30% of either the young or older population carried the low-expressing A/A genotype (Table S1). Flow cytometry confirmed that the A/A SNP correlated with low to absent CD39 cell surface expression on activated CD4^{+} T cells. In all subsequent comparisons between young and older T cells, individuals carrying the A/A SNP genotype were therefore excluded.

Gene expression array findings were confirmed by qPCR using CD4^{+} memory T cells from 20- to 35-year-old and 65- to 85-year-old individuals stimulated with immobilized anti-CD3/ CD28 antibodies (Abs). *ENTPD1* expression was low in unstimulated cells and was induced by stimulation. Induction on day 3 after stimulation was ~2-fold higher in the older population (Figure 1A). CD39 is inducible in central and effector memory CD4^{+} T cells, while end-differentiated TEMRA cells show lesser expression (Figure 1B). CD39 expression on activated naive CD4^{+} T cells is infrequent on day 4 (Figure 1B) but increases on subsequent days (data not shown). The age-associated difference in *ENTPD1* expression was

due to a higher frequency of CD39-expressing cells, which was seen for all T cell subsets. Figure 1C summarizes cytometric results of CD4^{+} memory T cells from 21 young and 23 older individuals stimulated with myeloid DCs from a young adult and SEB/TSST-1. A phenotypic characterization of CD39^{+} CD4^{+} T cells did not show any age-associated differences (Figure 1D). CD39^{+} T cells displayed a more activated phenotype with higher expression of CD25, HLA-DR, OX40, PD-1, and CTLA-4 compared to the CD39^{−}-T cell population. Expression of the IL-7 receptor was decreased (Figure 1E). KLRG1 was equally infrequent in CD39^{+} and CD39^{−} T cells (Figure 1F).

Functional Characterization of CD4^{+} T Cells Expressing CD39 after Activation

CD39 is expressed on T regulatory (Treg) CD4^{+} T cells, where it contributes to their regulatory activity by cleaving ATP and ADP and generating AMP that is further converted by CD73 to the immunosuppressive adenosine (Deaglio et al., 2007). In inflammatory lesions, Moncrieffe and colleagues distinguished two types of cells, CD39^{+} memory cells and CD39^{+} Tregs (Moncrieffe et al., 2010). We explored whether CD39^{+} CD4^{+} T cells generated in an immune response have Treg function. FOXP3 expression was not elevated in CD39^{+} activated T cells (Figure 2A). Moreover, expression of CD39 and CD73 was mutually exclusive (Figure 2B). In contrast to resting CD39^{+} Tregs that expressed low levels of CD26 (Figure 2D) (Dong et al., 1997; Mandapathil et al., 2010; Salgado et al., 2012), CD4^{+} T cells expressing CD39 after activation have increased CD26 expression (Figure 2C), suggesting that even if adenosine is generated, it is broken down to inosine. In functional assays, we could not demonstrate immunosuppressive activity. CD39^{+} T cells were generated by anti-CD3/CD28 stimulation of CD4^{+} T cells obtained from individuals differing in their CD39 SNP genotype (Figures 2E–2G). As expected, CD39 expression was infrequent in T cells with an A/A SNP and high in T cells carrying the A/G or G/G genotype. No positive correlation between the expression of CD39 and suppressive activity on proliferative responses was seen when the activated T cells were cocultured with fresh naive carboxyfluorescein succinimidyl ester (CSFE)-labeled CD4^{+} T cells activated with anti-CD3 Ab (Figure 2E). Moreover, inhibition of the ATPase activity by the ATP antagonist ARL in these coculture experiments did not increase the proliferative response. We also could not detect significant inhibitory activity on the polarization of naive T cells into Th1 or Th17 cells (Figure 2F) or interferon-γ (IFN-γ) production by effector T cells (Figure 2G).

Since CD39 on activated CD4^{+} T cells did not confer regulatory function, we characterized the effector functions of these cells. Compared to CD39^{−} cells, expression of T-bet in CD39^{+} cells was higher, indicating that they have features of Th1 effector cells (Figure 3A). Cytokine profiling was performed after ionomycin/phorbol myristate acetate (PMA) restimulation on day 4. To control for possible differences in activation status, cells were gated for CD25 expression. Irrespective of age, CD39^{+} CD4^{+} T cells were prone to produce IFN-γ, IL-4, and IL-17, while IL-2 and IL-21 production was decreased. Representative scatterplots are shown in Figure 3B, and summary data are shown in Figure 3C. While neither CD39^{+} nor CD39^{−} cells expressed high levels of BCL6 under nonpolarizing culture conditions, polarization with transforming growth factor-β (TGF-β) and IL-23 induced BCL6 only in CD39^{−} T cells (Figure 3D). Only CD39^{−} CD4^{+} T cells were able to help B cell differentiation into plasmablasts in vitro. In these assays, CD4^{+} T cells were stimulated

with immobilized anti-CD3/CD28 Abs for 4 days to induce CD39 expression and then separated into CD39$^+$ and CD39$^−$ cells, each of which was cocultured with purified B cells from the same donor on immobilized anti-CD3/CD28 Abs. After 6 more days of culture, plasmablasts in the CD3$^−$ population were identified by staining for immunoglobulin D (IgD) and CD38 (Figure 3E). Results are summarized in Figure 3F. CD39$^+$ cells were deficient in promoting B cell differentiation, while CD39$^−$ activated T cells were as efficient as total CD4$^+$ T cells.

CD39$^+$ T Cells Are Metabolically Stressed

Activation of the metabolic master regulator AMPK and shifts in metabolic pathways are known to be important in CD8$^+$ effector T cells and their differentiation into long-lived memory cells (Rolf et al., 2013). We quantified Thr172AMPK phosphorylation in CD39$^+$ and CD39 T cells in cultures two to five days after activation with immobilized anti-CD3/CD28 Abs. CD39$^+$ T cells displayed significantly increased Thr172 phosphorylation of AMPK (Figure 4A). Flow cytometric data were confirmed by western blotting of CD39$^+$ and CD39$^−$ T cell subsets purified on day 4 after stimulation (Figure 4A, right). Consistent with the increased AMPK phosphorylation, cytoplasmic ATP levels in CD39$^+$ T cells were markedly reduced (Figure 4B). To exclude that ongoing CD39 ATPase activity after cell lysis artificially lowered ATP measurements, cytoplasm and membrane fractions from CD39$^+$ and CD39$^−$ T cells were isolated and ATP concentrations were determined in mixtures of the different fractions. No evidence of ongoing ATPase activity in any of these lysates was found consistent with the enzyme activity strictly dependent on C- and N-terminal membrane insertion.

ATP production in activated memory cells comes from the glycolytic pathway with variable contribution from oxidative phosphorylation. Expression of the rate-limiting glucose transporter GLUT1 was slightly higher in CD39$^+$ CD4$^+$ T cells, excluding that the increased metabolic stress was due to lower glucose influx (Figure 4C). In contrast, mitochondrial function was compromised in CD39$^+$ T cells. Staining with the JC-1 dye showed reduced aggregation in mitochondria of CD39$^+$ T cells (Figure 4D).

AMPK has a multitude of molecular targets that aim at maintaining the energy balance of a cell. We have recently described an increased expression of DUSP4 and EGR1, the transcription factor that controls DUSP4 expression, as consequences of increased AMPK phosphorylation in older CD4$^+$ T cells. As shown in Figure 4E, CD39$^+$ CD4$^+$ T cells exhibited increased transcription of EGR1 and DUSP4. Nuclear DUSP4 expression inhibits T cells' ability to help B cell differentiation and to proliferate and induces cellular senescence (Tresini et al., 2007; Yu et al., 2012). One other target of pAMPK is p53 that, when phosphorylated at Ser15, regulates p21 expression (Jones et al., 2005). Phosphorylation of p53 at Ser15 was increased in CD39$^+$ CD4 T cells at days 4 and 5 after stimulation (Figure 4F). In parallel, p21 expression at both mRNA and protein levels was upregulated (Figure 4G). Upregulation of *TIGAR* transcripts, another well-established target of Ser15 phosphorylated p53 (Bensaad et al., 2006), provided further evidence for the activation of this pathway (Figure 4H). Pharmacological inhibition of AMPK activity reduced p53 phosphorylation and p21 in CD39$^+$ cells (Figure 4I) and protected them from

undergoing apoptosis (Figure 4J; p = 0.0003). Based on the increased expression of p21, CD39⁻ T cells should have a competitive advantage over CD39⁺ cells. We purified CD39⁺ and CD39⁻ T cells on day 5 after stimulation and labeled them with CFSE and Claret, respectively. The two cell types were then recombined and restimulated with DCs and SEB/TSST-1 in the absence or presence of IL-2 and IL-7. Cells deriving from CD39⁻ T cells were enriched in the cultures, even more so when proliferation was augmented by the addition of IL-2 and IL-7, consistent with partial cell-cycle arrest or increased apoptosis in CD39⁺ T cells that could not be overcome by the cytokines (Figure 4K).

CD39⁺ T Cells Have Increased Susceptibility to Undergo Apoptosis

After T cell activation and clonal expansion, most effector T cells do not survive; only a few cells escape clonal contraction and transit into long-lived memory cells. To determine whether CD39 identifies cells that are prone to undergo apoptosis, CD4⁺ T cell cultures were stained with Annexin V on day 4 after activation. Close to 40% of CD39⁺ T cells in the cultures were apoptotic compared to less than 10% of CD39⁻ T cells (Figure 5A; p = 0.006). Decreased survival of CD39⁺ T cells was also demonstrated when CD39⁻ T cells were stained with CSFE and cocultured with CD39⁺ cells without further restimulation or addition of cytokines. Progenies of CD39⁻ T cells slowly accumulated in the cultures over the next 5 days from a 1:1 to a 1.5:1 ratio (Figure 5B). Since cells only negligibly proliferated under these conditions, results are consistent with reduced survival of CD39⁺ T cells.

BCL2 expression as determined by flow cytometry and by qPCR was not different in the CD39⁺ and CD39⁻ T cell subsets (Figure 5C). Moreover, we did not see a difference in BIM, which has been implicated in contraction of effector T cells (van Leeuwen et al., 2009; Williams et al., 2008) (Figure 5D). Since we found evidence for Ser15-phosphorylated p53 in CD39⁺ T cells (Figure 4F), we analyzed the expression of pro-apoptotic genes that are controlled by p53. We did not find increased expression of PUMA (Figure 5E). However, expression of BAX was increased at the transcript as well as the protein level (Figure 5F). Moreover, we found evidence of BAX activation in CD39⁺ T cells as evidenced by the presence of BAX oligomers in CD39⁺, but not CD39⁻ T cells (Figure 5G).

ATPase Activity of ENTPD1 Contributes to the Functional Profile of CD39⁺ T Cells

We have shown so far that CD39 is a phenotypic marker of effector cells that are metabolically stressed and more prone to undergo apoptosis. ATPase activity of ENTPD1 is strictly extracellular, but it can modify signals from ATP and its derivatives through purinergic receptors. To determine whether the ATPase activity contributes to the characteristic profiles of CD39⁺ cells, we activated CD4⁺ T cells and cultured them in the presence or absence of an ATPase inhibitor, ARL. Cells were restimulated on day 4 with ionomycin and PMA and assayed for the production of cytokines. ARL increased the production of IL-2 and IL-21 while reducing IFN-γ production in CD39⁺ cells (Figures 6A and 6B). In contrast, ARL only had a slight and nonsignificant effect on the frequencies of cytokine-producing cells in CD39⁻ cells, presumably indirectly by blocking CD39 on CD39-positive cells present in the same cultures. Moreover, *ENTPD1* transfection of CD4⁺ T cells from an A/A donor and subsequent activation selected for IFN-γ and against IL-2-

producing cells (Figure 6C). ARL also partially restored the ability of CD39$^+$ T cells to provide help for B cell differentiation (Figure 6D). These data support the notion that the ATPase activity of CD39 reinforces effector cell differentiation.

ATPase Activity of ENTPD1 Contributes to Apoptosis and Attrition of Expanded Effector Cells

Frequencies of apoptotic cells decreased when cultures were performed in the presence of the ENTPD1 inhibitors ARL (Figure 7A; p = 0.0004) and POM-1 (Figure 7B; p < 0.0001), suggesting that the ATPase activity contributes to the apoptotic susceptibility of CD39$^+$ T cells. A2A receptor blocking showed that at least in part the induction of apoptosis was mediated by adenosine (Figure 7C; p = 0.0002). In addition to proximity of adenosine generation, increased A2AR expression on CD39$^+$ cells may contribute to their higher sensitivity to undergo apoptosis (Figure 7D). Moreover, inhibition of CD39 activity also reduced pAMPK in CD39$^+$ cells (Figure 7E; p = 0.03), while addition of adenosine to CD39$^-$ T cells induced AMPK phosphorylation (Figure 7F). To provide further evidence, CD4$^+$ T cells were transfected with a construct encoding GFP and CD39 and examined for the frequencies of apoptotic cells 48 hr after transfection. GFP$^+$ cells transfected with the control plasmid had frequencies of ~20% Annexin V$^+$ cells, most of which lacked staining for 7-AAD. In contrast, ~50% of CD39$^+$ GFP$^+$ cells were Annexin V$^+$, half of which costained with 7-AAD (Figure 7G).

Since ENTPD1 ATPase activity contributed to the apoptotic susceptibility, we reasoned that individuals lacking CD39 expression may have lesser T cell contraction in a T cell response and therefore benefit more from vaccination. Frequencies of IFN-γ–producing virus-specific T cells were quantified before and four weeks after vaccination by ELISpot in 28 healthy individuals older than 50 years who had received immunization with the live varicella zoster virus (VZV) vaccine Zostavax. Cytometric analysis of VZV-specific CD4$^+$ T cells with an HLA-DRB1*1501 tetramer including the VZV IE63-derived peptide QRAIERYAGAETAEY after vaccination showed CD39 expression on a subset of cells (Figure 7H). Individuals with the A SNP that is associated with lower CD39 expression tended to have a higher fold increase in VZV-specific T cells than individuals carrying a G genotype (p = 0.1 by trend test; Figure 7I). Similar results were obtained in an influenza vaccination cohort of 74 adults older than 60 years, 42 of whom had serological evidence of prior cytomegalovirus (CMV) infection. After adjusting for CMV status, a significant influence of the CD39 SNP genotype was seen for the frequencies of virus-specific T cells after vaccination with the A/Victoria (AA versus AG, p = 0.019; AA versus GG, p = 0.016) and B/Wisconsin strains (AA versus GG, p = 0.004, trend test for the association of A with fold increase in antigen-specific T cell frequencies, p = 0.008; Figure 7J). No difference was seen for the vaccination with the California strain. Cumulatively, these data suggest that the *ENTPD1 G* genotype is associated with a lesser gain in long-lived memory T cells after vaccination. These data document a negative function of CD39 on T memory formation in vivo that, although not excluding CD39-mediated Treg activity, is consistent with the proposed model of increased clonal contraction of CD39-expressing effector T cells.

In addition to vaccine responses, the CD39 SNP status also has an impact on the T cell memory to chronic viral responses. A subset of CMV carriers has an expanded subset of end-differentiated effector cells known as TEMRA that are considered to reflect chronic stimulation due to viral reactivation. CD4$^+$ TEMRA T cells are lower in CMV-positive individuals with the A versus the G genotype (Figure 7K), while no association was seen in CMV-negative individuals (p = 0.8).

DISCUSSION

After a period of rapid expansion upon antigen stimulation, most of the responding T cells die from apoptosis and do not transition into long-lived memory cells. The factors that determine differentiation and survival are of central importance for successful memory formation after infection and vaccination. While progress has been made in mouse models to identify memory cell precursors, the process remains poorly defined in humans. Here, we describe that the expression of CD39 on activated CD4$^+$ T cells identifies effector cells that are prone to culling due to activation of the AMPK-p53 pathway. CD39 transfection experiments identified a direct role of CD39 in influencing effector cell differentiation and T cell attrition. Pharmacological inhibition experiments employing several distinct inhibitors support the notion that its ATP/ADPase activity is involved in these effects. Since CD39 is more readily induced in older individuals, increased attrition of effector T cells may contribute to the impaired vaccine responses with age.

CD39 is an ecto-ATPase that cleaves ATP to ADP and AMP. By cleaving ATP, CD39 influences purinergic signals through ionotropic P2X and G-protein-coupled P2Y receptors (Boeynaems et al., 2005). AMP generated by CD39 can be further cleaved by CD73. The concerted activity of CD39 and CD73 generates adenosine, which is considered an important pathway for Tregs to exert their immunosuppressive function (Deaglio et al., 2007; Ohta and Sitkovsky, 2014). Although we found evidence of adenosine production, we did not find regulatory activities in T cells gaining CD39 expression after activation, possibly due to increased expression of CD26 that binds adenosine deaminase in humans, thereby facilitating the deamination to inosine (Dong et al., 1997; Mandapathil et al., 2010; Salgado et al., 2012). Several investigators have shown considerable heterogeneity within the CD39$^+$ T cell subset. Although a transcriptional signature of FOXP3$^+$ Tregs includes *ENTPD1* transcripts, expression levels do not correlate with suppressive activity (Ferraro et al., 2014). CD39$^+$ CD4$^+$ memory T cells that do not express FOXP3 and lack Treg function but produce effector cytokines have been described in the setting of kidney transplantation and chronic synovitis in vivo (Dwyer et al., 2010; Moncrieffe et al., 2010). The profile of these ex vivo cells is similar to the cells described here.

The reasons why CD39 is only induced in a subset of T cells and why these cells are enriched with age are unknown. Functionally, CD39$^+$ differ from CD39$^-$ CD4$^+$ T cell in that they are highly differentiated effector T cells with increased expression of T-bet, higher production of effector cytokines and lower production of IL-2. Since IL-4, IL-17 and IFN-γ are equally increased, CD39 expression is not lineage specific except that CD39$^+$ T cells produce less IL-21 and are unable to provide help for B cell differentiation.

Importantly, expression of CD39 appears not only to be a subset-specific marker, but its ATPase activity appears to be directly involved in effector cell differentiation. ATPase inhibition during T cell differentiation improved the ability to produce IL-2 and IL-21 and restored the ability to help B cells. CD39 silencing experiments were inconclusive due to the high apoptosis susceptibility of these effector cells, but we used two chemically different ATPase inhibitors and off-target effects are therefore unlikely to account for the effects. Moreover, we observed a clear difference of ARL inhibition on CD39+ and CD39− T cells. CD39 may exert its effect on T cell differentiation by preventing a signal by ATP through a P2X receptor. P2X7 is widely expressed on T cells and requires high ATP concentrations to induce a Ca^{2+} influx. P2X7-mediated autocrine activity of ATP during TCR stimulation has been shown to mediate a costimulatory signal (Schenk et al., 2008; Yip et al., 2009). Activated T cells also transcribe many P2Y receptors; P2Y1, P2Y12, and P2Y13 preferentially sense ADP and could transmit a signal in response to the CD39-generated ADP. Finally, AMP generated by CD39 is further cleaved to adenosine, likely by CD73-containing exosomes in the culture (Clayton et al., 2011).

CD39+ CD4+ effector T cells are highly susceptible to undergo apoptosis, suggesting that CD39 identifies effector cells that are subject to contraction and do not develop into long-lived memory cells. Given the central role of T cell memory formation in adaptive immunity, defining the mechanisms how T cells survive the contraction phase and develop into memory cells has been of keen interest. Elegant studies in murine infectious disease models identified KLRG1 in CD8+ T cells as a phenotypic marker distinguishing apoptosis-prone effector T cells from cells that differentiate into memory cells (Sarkar et al., 2008). KLRG1 is absent on most CD4+ T cells, including CD39+ cells (Figure 1F). Marshall and colleagues proposed high expression of Ly6C as marking CD4+ effector T cells that do not survive (Marshall et al., 2011). A human gene corresponding to Ly6C does not exist. However, murine KLRG1high CD8+, murine Ly6Chigh CD4+, and human CD39+ CD4+ T cells are similar in that they are highly differentiated effector cells with high expression of T-bet. In mouse CD8+ T cells, T-bet has a direct influence on survival by repressing transcription of the IL-7 receptor (Intlekofer et al., 2007). Similarly, CD39+ CD4+ T cells lacked IL-7 receptor expression (Figure 1E). In murine studies, central memory cell differentiation is supported by BCL6 and ICOS-mediated interaction with B cells (Pepper et al., 2011). Analogously, human CD39+ T cells are not able to provide help for B cell differentiation (Figures 3E and 3F).

In contrast to clonal contraction in the mouse (van Leeuwen et al., 2009; Williams et al., 2008), the increased apoptosis susceptibility of CD39+ T cells was not related to an imbalance between BCL2 and BIM. CD39+ T cells activate the p53 pathway to enter senescence or to undergo apoptosis (Figures 4 and 5). Downstream of p53, *p21* transcription is induced, causing cell-cycle arrest. In parallel, expression and activation of BAX is increased, both controlled by p53 (Chipuk et al., 2004; Miyashita and Reed, 1995). In contrast, expression of PUMA, also regulated by p53, was not different between CD39− and CD39+ T cells.

Apoptosis susceptibility was directly related to the expression of CD39. Transfecting CD39− T cells with CD39 conferred increased cell death (Figure 7G). Conversely, inhibition of its

ATPase activity improved survival of CD39^{+} T cells (Figures 7A and 7B). We could partially block apoptosis by inhibiting the stimulation of the A2A receptor by adenosine (Figure 7C), presumably generated from further degradation of AMP by exosomes that are produced by CD73-expressing cells present in the culture system (Clayton et al., 2011). The high expression of the A2AR receptor on CD39^{+} T cells may favor suicide over fratricide (Figure 7D). Possibly more importantly, extracellular adenosine, taken up by adenosine transporters, has been shown to induce AMPK activation (Aymerich et al., 2006; Nieminen et al., 2013). Indeed, we found that adenosine induces AMPK phosphorylation in CD39^{−} T cells (Figure 7F). In addition to activating p53, AMPK regulates transcription of stress-inducible genes by directly promoting histone H2B phosphorylation (Bungard et al., 2010). These direct transcriptional activities of AMPK do not include PUMA, which may explain why PUMA was not different between CD39^{−} and CD39^{+} T cells.

Generation of extracellular adenosine may not be the only mechanism that accounted for the increased AMPK phosphorylation. Increased anabolic activity associated with T cell proliferation causes metabolic stress and activates AMPK. We found expression of GLUT1 is even higher in CD39^{+} T cells than in CD39^{−} T cells, indicating that the glycolytic pathway is intact. However, CD39^{+} T cells had low cytoplasmic concentrations of ATP. Since mitochondrial function was impaired in CD39^{+} T cells, they may be unable to produce sufficient ATP by oxidative phosphorylation and therefore initiate the apoptotic pathways. CD39 may be directly involved, since a similarly reduced cytoplasmic concentration of ATP has also been recently described for CD39^{+} Tregs (Zhao et al., 2010).

In contrast to our model for human CD4^{+} effector T cells, where pAMPK induces cell-cycle arrest and apoptosis, activation of AMPK or inhibition of mTORC1 has been shown to support the effector to memory cell transition stage for murine CD8^{+} T cells (Araki et al., 2009; Rolf et al., 2013). Differences in the requirements of CD4^{+} and CD8^{+} T cells for mTORC1 activity are supported by the finding that mTORC1 inhibition induces anergy or Tregs and does not improve memory in CD4^{+} T cells (Powell and Delgoffe, 2010; Xie et al., 2012). Moreover, extent and context of AMPK activation that determine whether the outcome is apoptosis, cell-cycle inhibition, or survival may be different.

Our in vitro studies provide evidence for the model that CD39 is not only a biomarker but also that its ATPase activity is mechanistically involved in effector cell differentiation and controls the clonal contraction seen in the transition from effector to long-lived memory cells. This interpretation is supported by our in vivo findings that individuals defective in CD39 expression had a higher increase in antigen-specific memory T cells after vaccination with VZV and two out of the three influenza strains. In this model, the increased expression of CD39 seen with CD4^{+} T cells from older individuals could contribute to their reduced ability to respond to vaccination. CD39 inhibitors, currently under development in oncology, could be explored to improve memory cell generation. Also, A2A receptor antagonists may be effective, since at least part of the CD39 effect is mediated by A2A stimulation by adenosine.

EXPERIMENTAL PROCEDURES

Detailed description of the methods used for cell isolation and culture, flow cytometry, ELISpot, qPCR, immunoblotting, and statistical analysis can be found in Supplemental Experimental Procedures.

Study Population

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy individuals 20–85 years of age. 28 individuals older than 50 years were vaccinated with the VZV vaccine Zostavax. 74 individuals older than 60 years and with known CMV serology were enrolled in a vaccine study with the trivalent influenza vaccine Fluzone in the 2012/2013 season. Individuals were genotyped for the CD39 rs_10748643 SNP by PCR with 0.25 μl specific forward/reverse primers and probes (allele 1: VIC/MGB-NFQ; allele 2: FAM/MGB-NFQ; Applied Biosystems) in quintuplicates in 384-well plates using the ABI 7900HT system. The study was approved by the Stanford institutional review board, and participants gave informed consent. CD39$^+$ T cells were generated by in vitro stimulation of CD4 subsets as described in Supplemental Experimental Procedures.

Pharmacological Assays

To inhibit CD39 ATPase activity, 100–400 μM of the nondiffusible ATPase inhibitor ARL 67156 trisodium salt (Tocris) was added to cultures on day 1 after anti-CD3/CD28 Abs activation. Functional assays (cytokine production, T/B cell coculture), apoptosis assays, and flow cytometry for pAMPK on CD39$^+$ T cells were performed on day 4. Alternatively, ATPase activity was inhibited in day 4 activated CD4$^+$ T cells with 10 or 50 μM of the ENTPD inhibitor POM-1 (TOCRIS) for 6 hr before assaying apoptosis.

To block adenosine stimulation of the A2A receptor, SCH 442416 (Tocris) was added at a concentration of 10 μM on day 1 after initial stimulation.

To inhibit pAMPK activity, 2 μM Compound C (Sigma-Aldrich) or control DMSO was added to CD4$^+$ T cell cultures on day 1 after stimulation with anti-CD3/CD28 beads. pAMPK, p-p53 (ser15), and p21 expression in CD39$^+$ T cells was determined by western blotting; apoptosis was determined by flow cytometry.

Adenosine (0.5, 1.0, or 1.5 mM; Sigma-Aldrich) was added to CD4$^+$ T cell cultures on day 3 after stimulation. Phosphorylation of AMPK in purified treated and untreated CD39$^-$ CD4 T cells was examined by western blotting on day 4.

CD39 Transfection

CD4$^+$ T cells of the low-expressor A/A SNP genotype were transfected with 3 μg CD39-pEGFP-C1 or 3 μg pEGFP-C1 construct (as a negative control) using the Lonza 4D-Nucleofector system and Amaxa P3 Primary cell 4D-Nucleofector kit (Lonza). 48 hr after transfection, cells were stained with APC-labeled Annexin V and 7-AAD to assess apoptosis rates in GFP-expressing cells. Alternatively, CD4$^+$ T cells were stimulated first with anti-CD3/28 overnight, then transfected followed by additional 4 days of stimulation. Cells were

then restimulated with ionomycin/PMA and intracellular cytokines in GFP^{+} cells were determined by cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- CD39 identifies human effector CD4 T cells that are prone to apoptosis

- CD39 is more readily induced in CD4 T cell responses of older individuals

- The ATPase activity of CD39 regulates effector cell differentiation and apoptosis

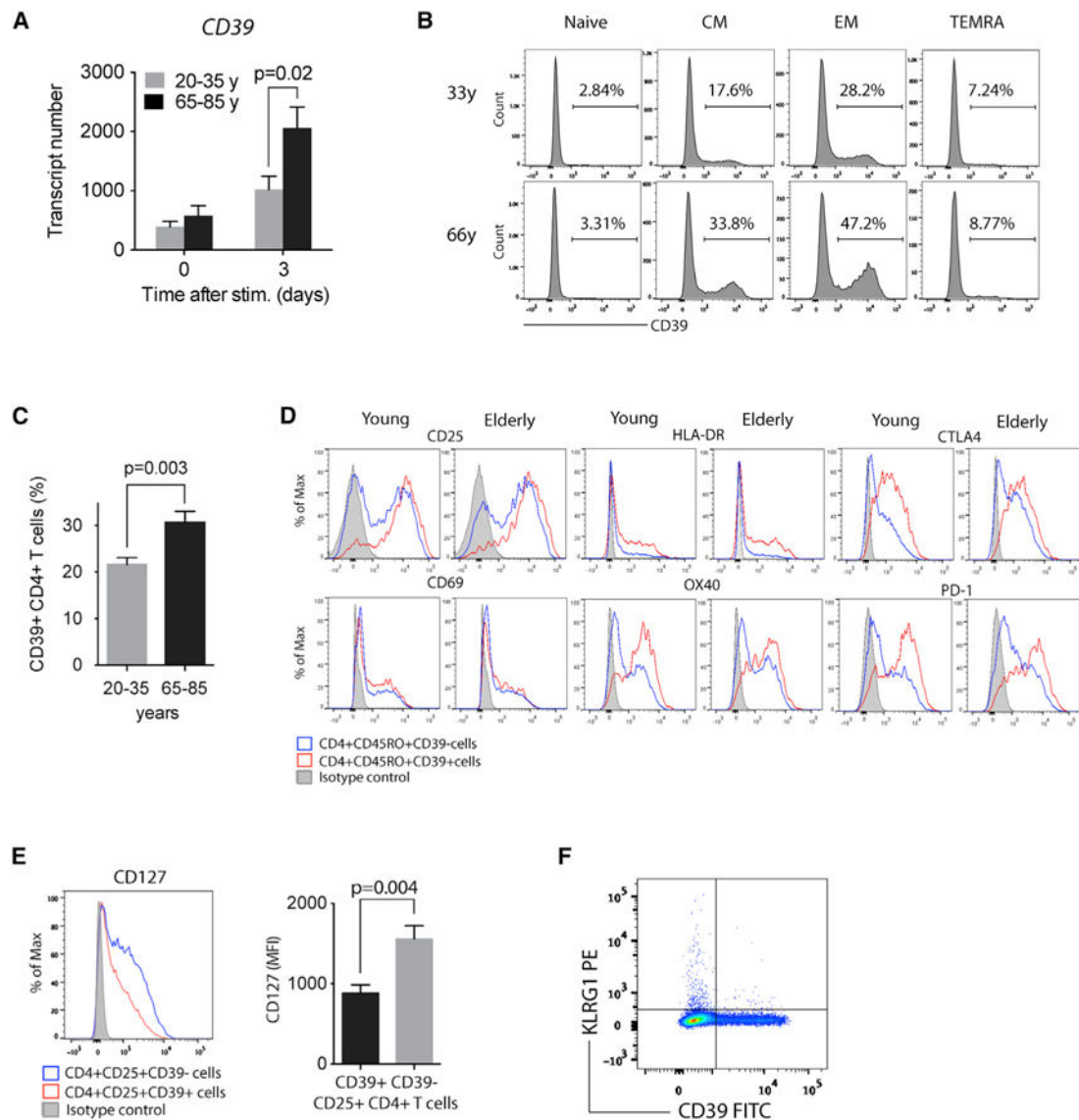- CD39 expression inversely correlates with T memory cell generation after vaccination

Figure 1. CD4+ T Cells from Older Individuals Are Poised to Express the Ecto-ATPase CD39 upon Activation

(A) *CD39* transcripts were quantified by qPCR in CD4+ memory T cells before and after 3-day stimulation with immobilized anti-CD3/CD28 Abs (n = 17). The results are expressed as mean ± SEM of transcript numbers after normalization to 18sRNA.

(B) Subsets of CD4+ T cells were stimulated for 4 days, and CD39 expression was determined by cytometry. The example shown is representative of five young and five older adults. CD39 expression in naive T cells further increased on day 6 after stimulation.

(C) CD4+ memory T cells from twenty-one 20- to 35-year-old and twenty-three 65- to 85-year-old adults were stimulated with DCs and SEB/TSST-1 and analyzed for the expression of CD39 on CD25+ CD4+ T cells by flow cytometry on day 4. Results are given as mean ± SEM.

(D) On day 5 after CD3/CD28 stimulation, expression of indicated activation markers were analyzed by flow cytometry on gated CD39+ (red line) and CD39− T cells (blue line).

Shaded area represents isotype control. Histograms of T cells from a young and an elderly individual are representative of two experiments.

(E) Expression of CD127 (IL-7R) on gated CD39$^+$ and CD39$^-$ T cells was assessed by flow cytometry. Results are shown as a representative histogram (left) and mean ± SEM fluorescence intensity (MFI) of four experiments.

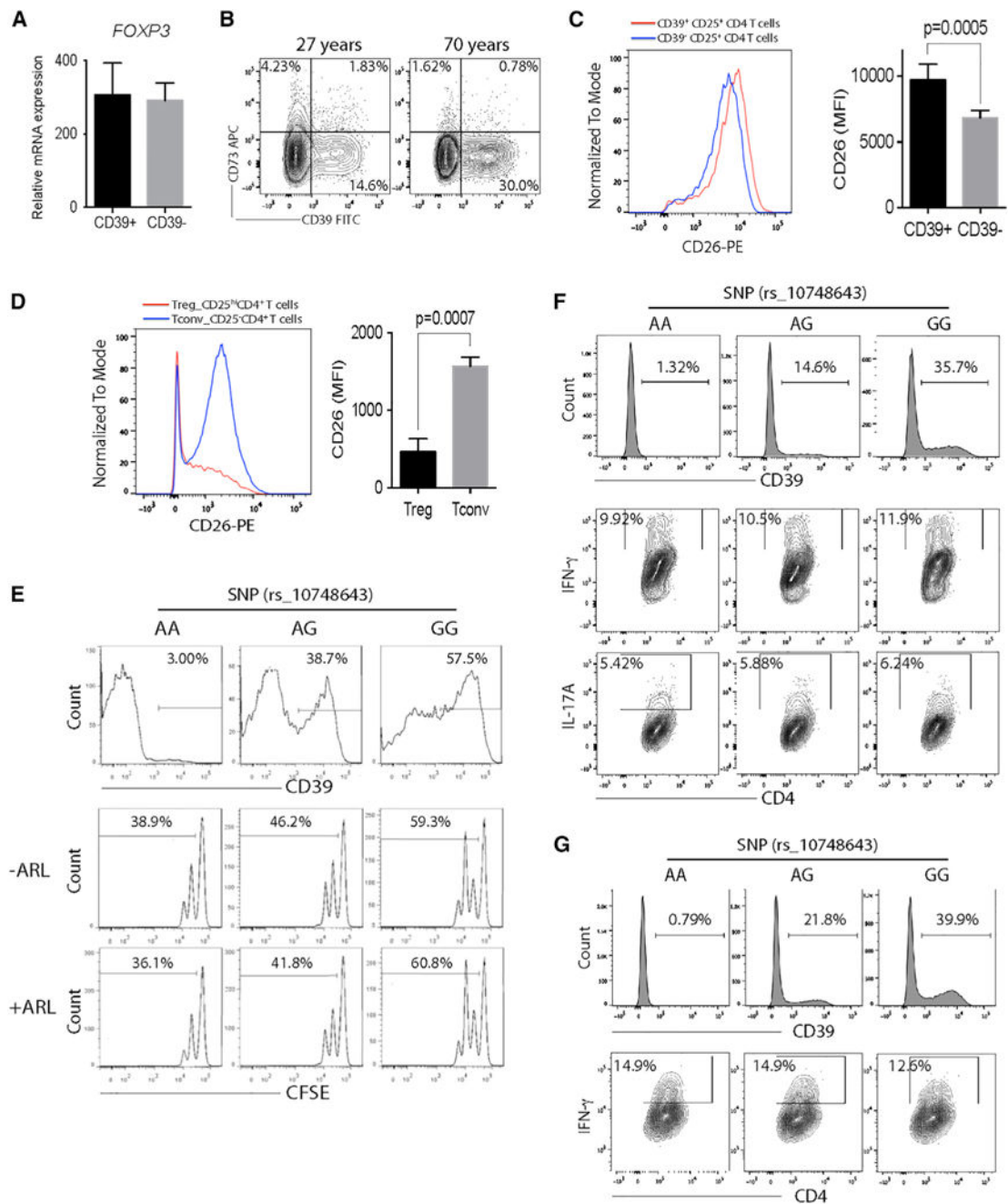(F) Scatterplot of KLRG1 expression is representative of three experiments.

See also Table S1.

Figure 2. CD39 Expressed on Activated CD4$^+$ T Cells Does Not Confer Regulatory Activity

(A) CD39$^+$ and CD39$^-$ CD4$^+$ T cell subsets were separated after 5 days of stimulation with anti-CD3/CD28 Dynabeads. *FOXP3* transcripts were quantified by qPCR and are shown as mean ± SEM of seven experiments relative to *18 s rRNA* transcripts.

(B and C) Anti-CD3/CD28-activated CD4$^+$ T cells were assayed for the expression of CD73 (B) and CD26 (C) on CD39$^+$ and CD39$^-$ cells by flow cytometry.

(D) CD26 expression on resting CD4$^+$ Treg cells is shown for comparison.

(E–G) CD4$^+$ T cells from individuals with the different *CD39* SNP genotypes representing low, intermediate, and high CD39 expressers were stimulated by CD3/CD28 cross-linking.

Histograms in the upper row show the degree of CD39 expression depending on the SNP genotype. On day 4, activated CD4$^+$ T cells were added to fresh CD4$^+$ T cells and immobilized anti-CD3/CD28 Abs. Regulatory activity on proliferation was assessed by CSFE dilution in the absence or presence of 200 μM of the ATPase inhibitor ARL (E). Alternatively, activated T cells were assessed for their ability to inhibit polarization into Th1 or Th17 cells (F) or suppress cytokine production of effector cells (G). Each dataset is representative of two experiments.
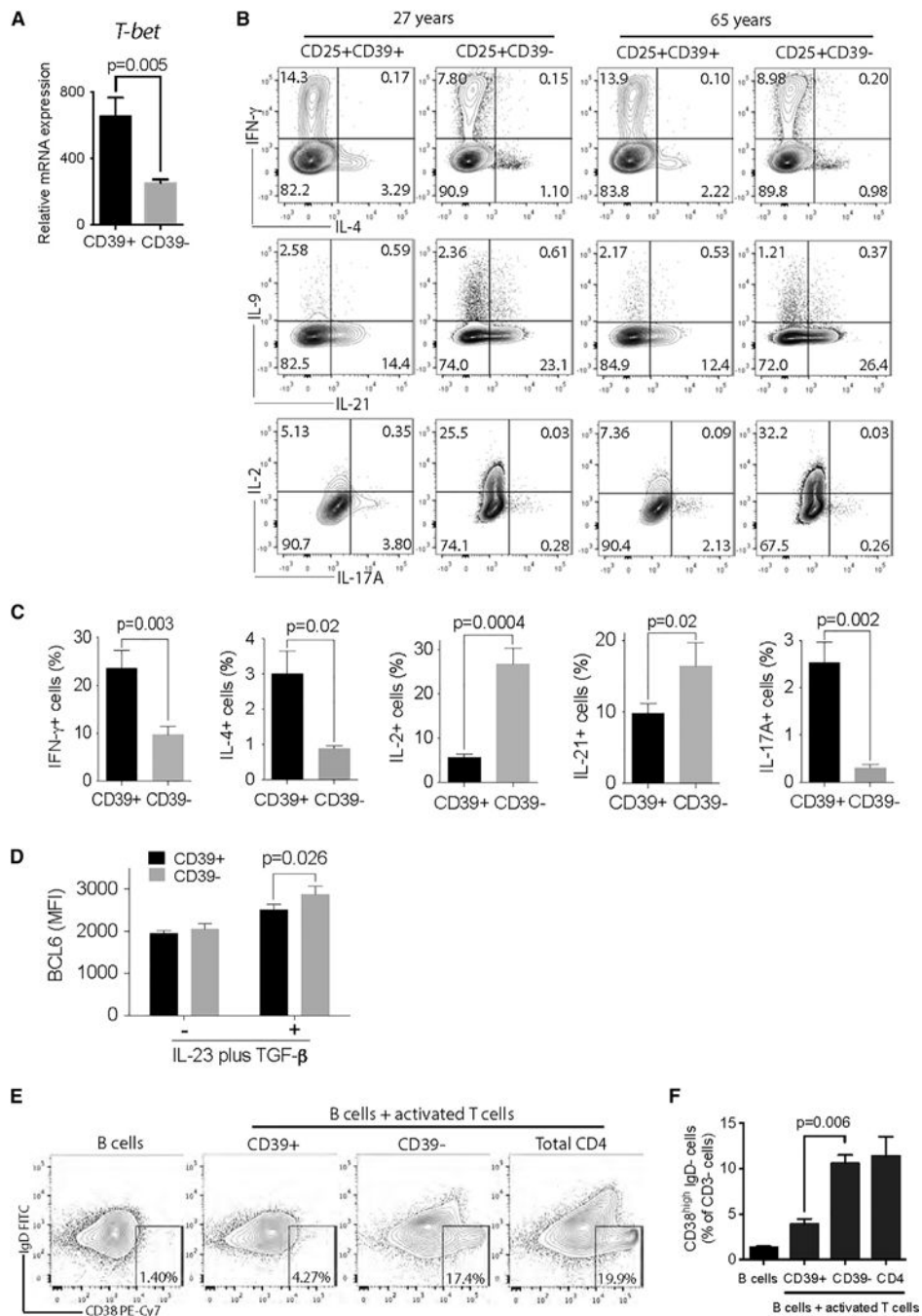
Figure 3. Functional Characterization of CD4+ T cells Expressing CD39 after Activation
(A) *T-bet* transcripts were quantified as described in Figure 2A for *FOXP3*.
(B and C) CD4+ T cells, stimulated with anti-CD3/CD28 Dynabeads for 4 days, were restimulated with 2.5 ng/ml PMA and 500 ng/ml ionomycin for an additional 2 hr. Contour plots of intracellular cytokine expression in gated CD25+CD39+ and CD25+CD39− cells from a 27- and a 65-year-old individual (B) and mean ± SEM of six to seven experiments (C) are shown.

(D) CD39$^+$ and CD39$^-$ cells were generated under nonpolarizing and TFH-polarizing conditions (n = 4) and compared for the expression of BCL6.

(E and F) CD39$^+$ and CD39$^-$ cells were isolated from activated CD4$^+$ T cells, mitomycin treated, and cocultured with autologous B cells and immobilized anti-CD3/CD28 Abs for 6 additional days. Contour plots of CD38 and IgD expression on CD3$^-$ cells are shown (E). Frequencies of IgD$^-$CD38^{high} plasmablasts from four experiments are shown as mean ± SEM (F).
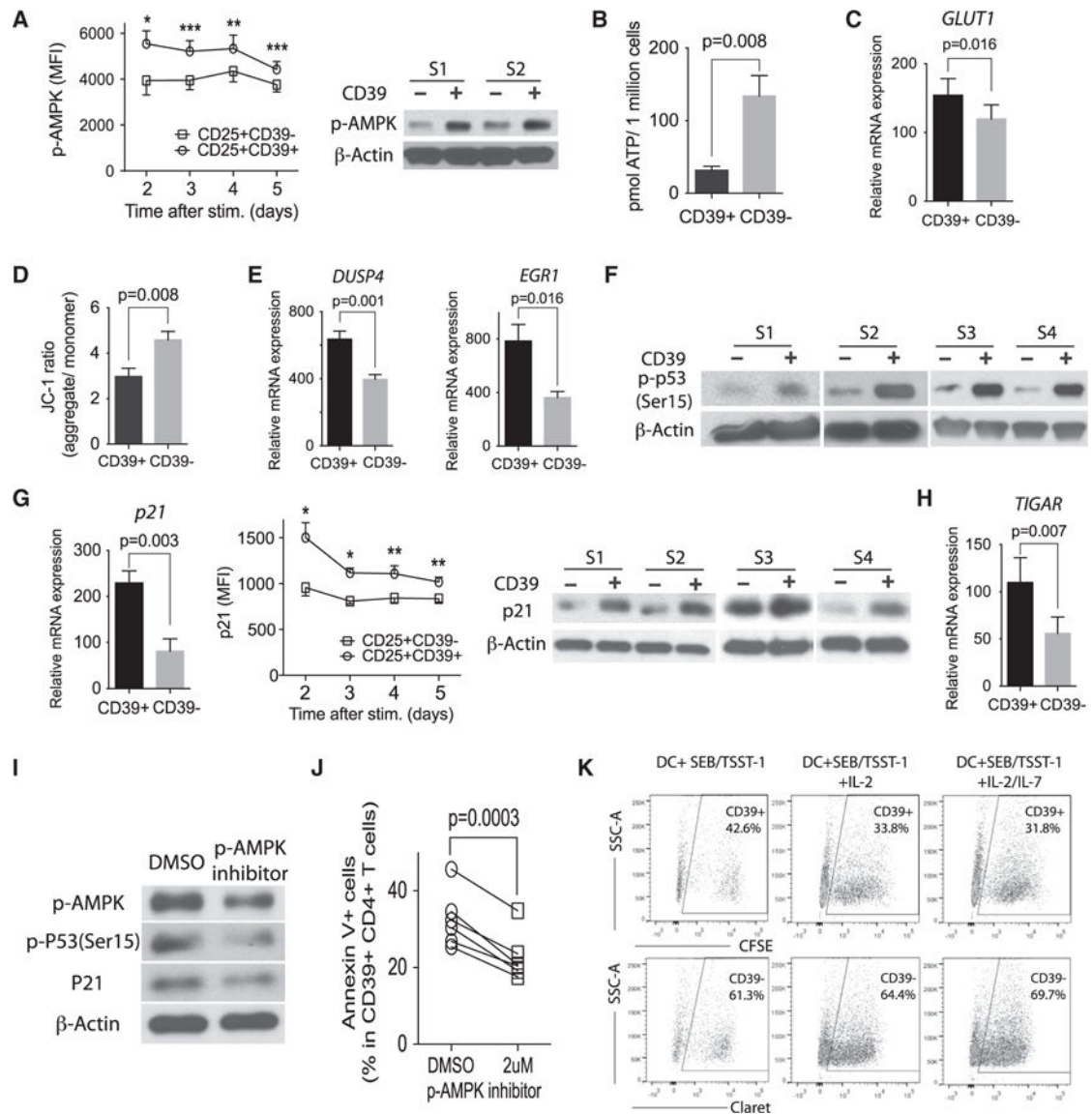
Figure 4. Activated CD4⁺ T Cells Expressing CD39 Are Metabolically Stressed

(A) CD4⁺ T cells were analyzed for AMPK phosphorylation on days 2–5 after anti-CD3/CD28 Abs stimulation. Results from flow cytometry of six experiments are shown for gated CD25⁺CD39⁺ and CD25⁺CD39⁻ T cells as mean ± SEM geometric mean fluorescence intensity normalized to forward scatter (left). Western blots of isolated T cell subsets from a young and an older adult on day 4 after stimulation (right) are representative of two independent experiments.

(B) Intracellular ATP concentrations in lysates of purified CD39⁺ and CD39⁻ cells from activated CD4⁺ T cells were determined using a luciferase kit (n = 8).

(C) *GLUT1* mRNA expression was quantified by qPCR in CD39⁺ and CD39⁻ activated T cells from seven individuals.

(D) Mitochondrial membrane potentials in day 5 activated CD4⁺ T cells were assessed. Results are given as the mean ± SEM JC-1 aggregate: monomer ratio (n = 8).

(E) CD39$^+$ and CD39$^-$ cells isolated from activated CD4$^+$ T cells were analyzed for *DUSP4* and *EGR1* expression by qPCR (n = 7).

(F) Ser15-phosphorylated p53 in CD39$^+$ and CD39$^-$ cells was determined by western blot. Results for four adults, S1–S4, are representative of six individuals.

(G) *p21* transcripts were quantified by qPCR (n = 7, left). Expression of p21 proteins in CD39$^-$ and CD39$^+$ T cells were compared by flow cytometry on days 2–5 (n = 4, middle) and by western blot on day 4 (n = 7, right).

(H) *TIGAR* mRNA expression was quantified by qPCR (n = 7).

(I and J) CD4$^+$ T cells were activated in the presence of the AMPK inhibitor Compound C. Western blots show the expression of pAMPK, p-p53, and p21 (I). The effect of pAMPK inhibition on apoptosis rates is shown in (J).

(K) CD39$^+$ and CD39$^-$ cells were purified from activated CD4$^+$ T cells and stained with CFSE and Claret, respectively. Cells were mixed at a 1:1 ratio and restimulated with DCs and SEB/TSST-1 with or without IL-2 and IL-7 for another 6 days. Results are representative of two experiments.
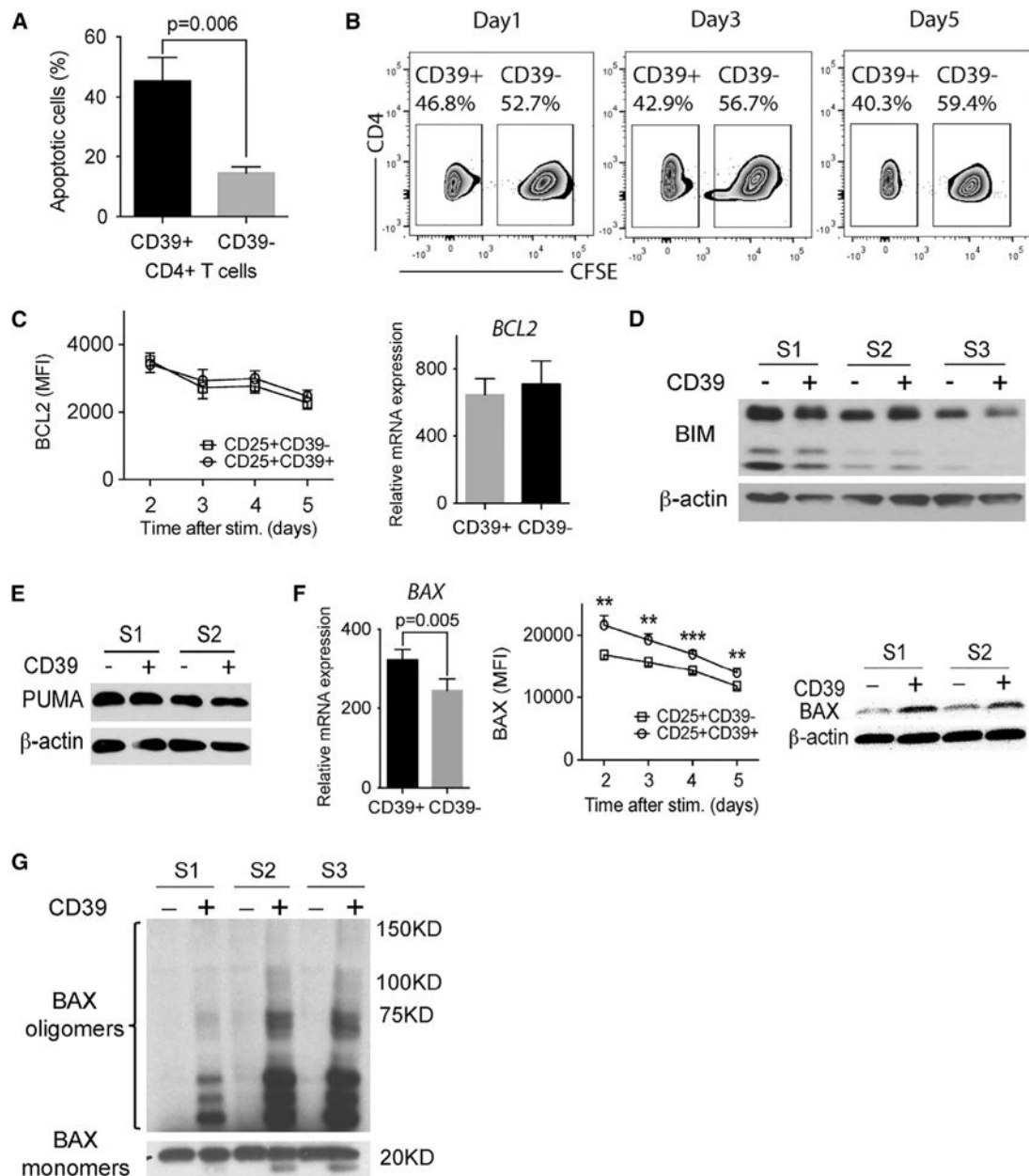
*p < 0.05, **p   0.01, ***p   0.001

Figure 5. Activated CD4$^+$ T Cells Expressing CD39 Are Susceptible to Undergo Apoptosis

(A) Activated CD4$^+$ T cells from eight individuals were assessed for the frequencies of apoptotic cells on day 4 after anti-CD3/CD28 stimulation. The percentages of Annexin V$^+$ apoptotic cells within the populations of CD25$^+$ CD39$^+$ and CD25$^+$ CD39$^-$ T cells are shown as mean ± SEM.

(B) CD39$^-$ T cells were purified from activated CD4$^+$ T cells, stained with CFSE, and recombined with CD39$^+$ T cells at a 1:1 ratio. Relative survival of the two subsets was monitored over the next 5 days by flow cytometry.

(C) BCL2 expression in CD25$^+$ CD39$^+$ and CD25$^+$ CD39$^-$ T cells was determined by flow cytometry on days 2–5 after anti-CD3/CD28 stimulation. Results are shown as mean ± SEM

geometric MFI normalized to forward scatter (n = 6). *BCL2* transcripts were quantified in purified CD39+ and CD39 T cells by qPCR (right, n = 6).

(D and E) Expression of BIM (D) and PUMA (E) was determined by western blot.

(F) *BAX* transcripts were quantified by qPCR (left panel, mean ± SEM, n = 13). BAX protein was detected by flow cytometry (middle) (mean ± SEM geometric MFI normalized to forward scatter, n = 7, **p 0.01, ***p 0.001) and western blotting (right).

(G) BAX oligomerization in CD39+ and CD39− T cells purified from activated CD4+ T cells from a young, middle-aged, and older adult was assessed by western blotting under nonreducing condition. One experiment representative of three is shown.
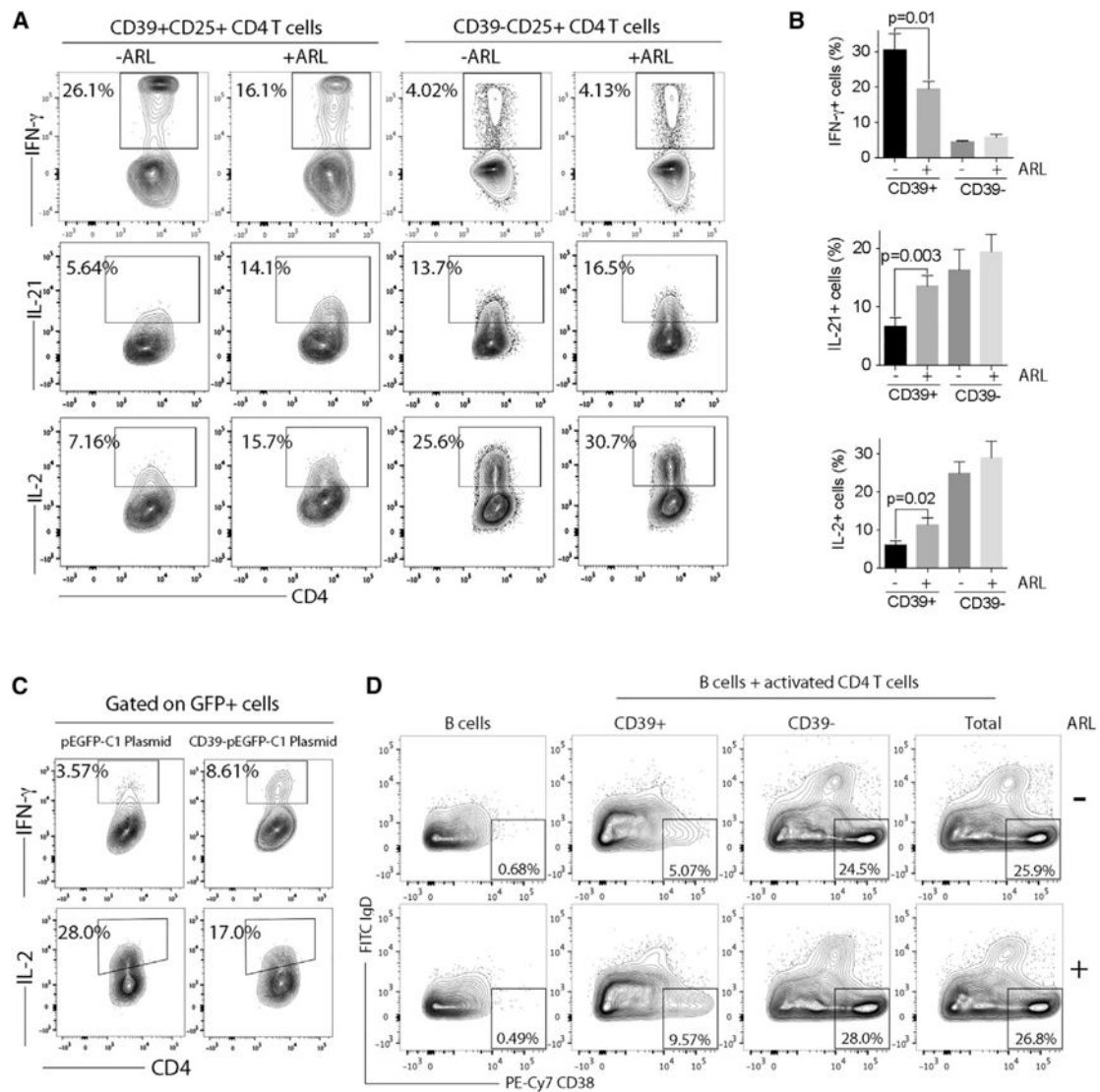
Figure 6. ATPase Activity of ENTPD1 Contributes to the Functional Profile of Activated CD4$^+$ T Cells Expressing CD39

(A and B) CD4$^+$ T cells were activated by CD3/CD28 stimulation, ARL (0, 400 μM) was added on day 1, and cytokine expression was determined after PMA/ionomycin restimulation on day 4. Results are shown as representative contour plots (A) and mean ± SEM of four or five experiments (B).

(C) Overnight-stimulated CD4$^+$ T cells from an individual with the CD39 A/A SNP were transfected with a CD39-pEGFP-C1 or a pEGFP-C1 control construct and stimulated with anti-CD3/CD28 Abs. Cytokine production after ionomycin/PMA restimulation on day 5 after stimulation is shown as contour plots; data are representative of two experiments.

(D) CD4$^+$ T cells were activated and ARL (0, 200 μM) was added on day 1 of stimulation. The ability of CD39$^+$ and CD39$^-$ T cells to provide help for B cells to differentiate into plasmablast was assessed as described in Figure 3E and determined by flow cytometry. Data are representative of two experiments.
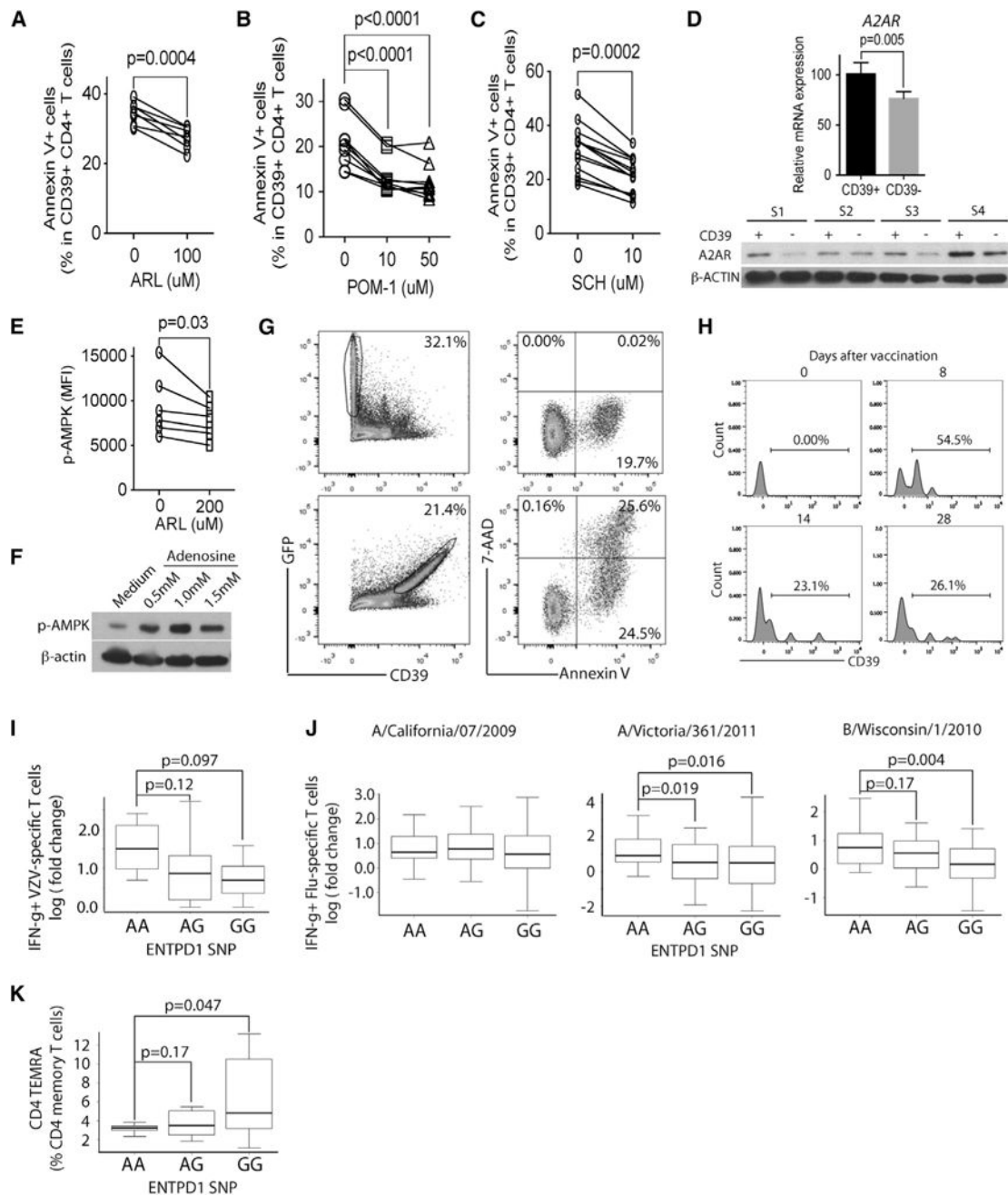
Figure 7. ATPase Activity of ENTPD1 Contributes to the Susceptibility of Activated CD4+ T Cells to Undergo Apoptosis and Clonal Contraction

(A and B) CD4+ T cells were activated by anti-CD3/CD28 Abs; the ATPase inhibitors ARL (A) or POM-1 (B) were added on day 1 for 3 days or day 4 for 6 hr, and percentages of apoptotic cells were determined by staining for Annexin V on day 4.

(C) Binding of adenosine to the A2A receptor was blocked by adding 10 μM SCH on day 1 after activation. Apoptotic rate was determined on day 4.

(D) Anti-CD3/CD28 activated CD4+ T cells were assessed for the expression of the adenosine receptor A2AR. Flow cytometric results (mean ± SEM from seven individuals, top) and western blots from four individuals (bottom) are shown.

(E and F) Phosphorylation of AMPK was assessed for CD39$^+$ T cells cultured with the ATPase inhibitor ARL (E) and in CD39$^-$ T cells cultured with increasing concentrations of adenosine (F).

(G) CD4$^+$ T cells from an individual with the CD39 A/A SNP were transfected with a CD39-pEGFP-C1 or a pEGFP-C1 control construct. 48 hr after transfection, apoptotic cells in gated pEGFP$^+$ cells were determined by staining with 7-AAD and Annexin V. Scatterplots are representative of two experiments.

(H–J) Healthy individuals were vaccinated with the varicella zoster virus (VZV) vaccine Zostavax. A subset of CD4$^+$ T cells specific for a VZV IE63 peptide in the context of HLA-DRB1*1501, as defined by major histocompatibility complex class II tetramer staining, expressed CD39 after vaccination (H). Vaccinated individuals (n = 28) were genotyped for the *ENTPD1* SNP at rs_10748643. Frequencies of IFN-γ-secreting T cells after stimulation with VZV IE63 peptide pool were quantified by ELISpot before and 4 weeks after vaccination. Fold change in frequencies shown as box plots correlated with the A genotype (trend test p = 0.1) (I).

(J) Healthy individuals older than 60 years immunized with trivalent influenza vaccine (n = 74) were genotyped for the *ENTPD1* SNP. Results of vaccine responses are shown as box plots of fold increase in influenza-specific T cells secreting IFN-γ. The A genotype correlated with the increase in antigen-specific T cells for the Victoria (trend test p = 0.04) and Wisconsin strains (trend test p = 0.008).

(K) Frequencies of TEMRA CD4$^+$ T cells were determined in healthy individuals older than 60 years with a positive CMV serology for longer than 15 years (n = 34), indicating chronic infection, and correlated with *ENTPD1* SNP genotypes.