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Lower GrB+CD62L^{high} CD8 T_{CM} effector lymphocyte response to influenza virus in older adults is associated with increased CD28^{null} CD8 T lymphocytes

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Abstract

Older adults who are at risk of developing influenza illness, have a low level of influenza virus-stimulated cytotoxic T lymphocyte (CTL) activity as measured by an assay of granzyme B (GrB). The purpose of this study was to determine whether aging affected memory CTL populations identified by GrB expression in influenza virus-stimulated peripheral blood mononuclear cells (PBMC). The expression and activity of GrB increased with virus stimulation over five days of culture. Virus-specific CD8 effector T cells with the phenotype, GrB+CD62L^{high} CD8 T_{CM}, were found to be the source of the early CTL response to influenza virus. Comparing the CD8 T cell response in 5-day PBMC cultures of 161 adult subjects, the response of GrB+CD62L^{high} CD8 T_{CM} lymphocytes in older individuals was significantly lower than in younger adults after viral stimulation ($p<0.001$). The increase in the proportion of CD28^{null}CD8 T cells in fresh PBMC negatively correlated with the proportion GrB+CD62L^{high} CD8 T_{CM} lymphocytes in virus-stimulated PBMC. Thus, the increase in CD28^{null}CD8 T cells with age may contribute to the limited CTL response to influenza vaccination and diminished protection in older adults.

Keywords

T cell; Influenza; Granzyme B; Cytotoxicity

1. Introduction

The diminished protection offered by influenza vaccination in older adults is well-recognized (Glezen et al., 2000; Thompson et al., 2004). The reduced protection is believed to be due to poor stimulation of cellular immunity (Powers et al., 1993; McElhaney et al., 2006) and risk for influenza illness in vaccinated older adults is associated with low levels of granzyme (GrB), a key cytolytic mediator of the CTL response to influenza (McElhaney et al., 2001; 2006; Schmidt et al., 2004). This current study focused on age-related changes in the virus-specific

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memory CTL (CD8+) response to influenza that may explain the increased risk of influenza illness in older adults.

Antigen-specific memory T cells have the capacity to mediate, accelerate and provide a vigorous response to secondary viral challenge. Memory T cells can be resolved into two major subsets based on their expression of lymph node homing receptors (CD62L and CCR7), referred to as central memory (CD62L^{high}CCR7+) (T_{CM}) and effector (CD62L^{low}CCR7^{-}) memory (T_{EM}) T cells (Sallusto et al., 1999; Lefrancois et al., 2002). T_{CM} cells are predominantly found in lymphoid tissues, whereas T_{EM} cells are found in both lymphoid and peripheral tissues. Analysis of the response to systemic lymphocytic choriomeningitis virus (LCMV) infection in the mouse showed that CD8 T_{CM} cells respond more vigorously to secondary challenge than T_{EM} cells (Wherry et al., 2003). The results of Sendai virus infection in mouse lung showed that T_{EM} cells generated a recall response that was at least as strong as that mediated by T_{CM} cells (Roberts et al., 2004). The relationship between T_{CM} and T_{EM} is a recent hotly debated topic. Early studies indicated that the T_{CM} precursors had the capacity to become fully functional effector T_{EM} cells after secondary challenge (Wherry et al., 2003; Roberts et al., 2005) Some studies suggested that a $T_{EM} \rightarrow T_{CM}$ transition was possible, both during the acute response and in long-term memory (Bouneaud et al., 2005; Roberts et al., 2005). Recently, the diverse T cell receptor profiles detected with influenza tetramers suggested that the CD62L^{high} T_{CM} cells constitute a relatively stable pool that is not maintained by conversion from the CD62L^{low} population (Kedzierska et al., 2006). Others suggest that the T_{EM} and T_{CM} subsets segregate immediately into different lineages from the time of primary antigen challenge, but the conversion between CD62L^{high} and CD62L^{low} T cells was never directly demonstrated (Marzo et al., 2005). CD62L expression alone is not sufficient to define distinct CTL functional subpopulations of memory CD8 T cell subsets (Jackson et al., 2005). If the T_{CM} and T_{EM} are distinct cell lineages, their functions, especially for T_{CM}, remains undetermined. These studies were based on mouse models and there was very little information about how these memory T cell subsets contribute to the response to influenza virus in people (Schwaiger et al., 2003).

The proliferation and differentiation of effector T cells requires effective CTL activation and the co-stimulatory molecule, CD28. CD28 signal transduction serves primarily as an amplifier of the TCR signal. Recent studies showed that CD28^{null} T cells accumulate with advancing age and loss of CD28 occurs more rapidly among CD8 T cells relative to CD4 T cells (Effros et al., 2005). Low antibody response to influenza vaccination in older adults is correlated with high frequencies of CD28^{null} T cells (Goronzy et al., 2001; Saurwein-Teissl et al., 2002). The lack of CD28 potentially diminishes T cell responses to influenza vaccination and suggests that CD28 plays a critical role in the subsequent response to infection (Lumsden et al., 2000).

Current cytolytic assays do not distinguish effector memory CTL from other memory cells. Previous results have shown that the level of GrB is correlated with cytotoxicity in ^{51}Cr-release assays of virus-stimulated human PBMC and is a very sensitive measure of functional CTL (McElhaney et al., 1996; Ewen et al., 2003). Therefore, GrB may be an accurate marker of the effector response to influenza virus challenge in CD8 T cells using flow cytometric methods (Rong et al., 2004).

This paper provides evidence that human CD8 T_{CM} lymphocytes are the source of influenza-specific CD8 effector cells identified by the expression of GrB mRNA, the related enzymatic activity, and its association with degranulation of CD8 T cells in response to influenza virus. Further, it was postulated that, there is an age-related decline in the influenza-specific memory T cell response to influenza. Human PBMC stimulated in vitro with live influenza virus was used as the model to characterize the interaction between CD8 T cells (CTL) and influenza virus, and age-related changes in this interaction. An age-related difference in the CTL response

to influenza virus and the effect of influenza vaccination on CD8 T cell subsets, particularly in GrB+CD62L^{high} CD8 T_{CM} cells, has been identified. Further, T_{CM} are stimulated at an early stage of influenza virus stimulation. GrB+CD62L^{high} CD8 T lymphocytes appear within 20 hours of virus stimulation and contribute to the expansion of the effector CD8 T cell subset in PBMC cultures. In older adults, the increase in the proportion of CD28^{null} CD8 T cells may limit the CTL response to influenza and influenza vaccination.

2. Materials and Methods

2.1. Human subjects and procedures

Between June 2005 and November 2006, 29 younger adults (mean age, 29 years older; range, 22–40 years older) and 130 older adults (mean age, 74 years older; range, 60–94 years older) were recruited for this study through written, informed consent. The Institutional Review Board of the University Connecticut Health Center approved the protocol and informed consent document. All subjects had received the 2004–05 influenza vaccine in the previous year. In a subset of the study cohort, 20 younger (mean age, 31 years older; range, 22–40 years older) and 144 older (mean age, 75 years older; range, 60–94 years older) adults were vaccinated with the 2005–06 licensed influenza vaccine (Aventis Pasture Inc.) containing 15 μg each of A/California/7/2004 (H3N2)-like, A/New Caledonia/20/99 (H1N1)-like, and B/Shanghai/361/2002-like antigens. Younger adults defined as age 20–40 years older and without any underlying chronic conditions, were enrolled in the study as controls for the 'normal' response to influenza vaccination. Older adult subjects were defined as age 60 years and older and characterized according to underlying medical diagnoses, medications and performance on the Six-Minute Walk Test (SMWT) (Bittner et al., 1993). Venous blood samples were collected in the summer of 2005, or just prior to and four weeks following vaccination in the fall of 2005. Volunteers who refused vaccination or had not been vaccinated in the previous year, had an egg allergy, had a previous severe reaction to the vaccine, or had an acute illness in the 2-week period before vaccination were excluded.

2.2. Preparation of human peripheral blood mononuclear cells (PBMC) and stimulation with virus

PMBC were prepared from heparinized whole blood (35cc) by Histopaque-1077 (Sigma-Aldrich) gradient purification, treated with RBC Lysis Solution (Bio-Rad) to remove red blood cells, washed, resuspended in AIM-V media (Gibco Laboratories, Grand Island, NY), and diluted to a concentration of 2×10^6/ml. One ml of suspended cells was added into each well of a 48-well multiplate (Nalge Nunc), stimulated with influenza virus A/Wyoming/03/2003 [A/H3N2] (or other influenza strains as noted) for 20 hours or 5 days using a multiplicity of infection (MOI) = 2 (4×10^6/ml $TCID_{50}$/ml) and incubated in a humidified atmosphere at 37° C in 5% CO_2.

2.3. RNA preparation and RT-PCR

The level of expression l of GrB and Perf (perforin) mRNA was compared in fresh and virus-stimulated human PBMC using GAPDH as a positive control. Human GrB primers were 5′-AAGACGACTTCGTGCT-3′ and 5′-CAGATTCGCACTTTCGATC-3′. Human Perf primers were 5′-ACGCAAATTCGCAAACT-3′ and 5′-GGATTAGCGTGTAAACCC-3′. Primers to the human housekeeping gene, GAPDH, were 5′-GTCGGAGTCAACGGAT-3′ and 5′-CCACGACGTACTCAGC-3′. Total RNA from PBMC samples were prepared with Qiagen RNeasy Mini Kit. 1μg total RNA per sample was used for the reverse transcriptions (Bio-Rad iScript cDNA Synthesis Kit), followed by 35 cycles of PCR (Clontech Advantage 2 Kit), and detection on 1.5% agarose gel stained with ethidium bromide. Real-Time PCR was carried out in a Bio-Rad iCycler with the Bio-Rad SYBR Green PCR kit.

2.4. Detection of intracellular GrB activity

Human PBMC (1.5×10^6 cells/ml in AIM-V media) were stimulated with virus (MOI=2) for 20 hours and 5 days and PBMC lysates were prepared and analyzed by the GrB assay using the Ac-IEPD-pNA (paranitroanilide) substrate according to previously described methods (McElhaney et al., 2006). GrB activity was standardized in the assay using an YT cell lysate and calculated as A_{405} per mg. protein in the BCA Protein Assay Kit (Pierce).

2.5. Antibodies and flow cytometry

CD28 expression on CD8 T-cells was measured on fresh PBMC. All other fluorescent antibody staining for flow cytometry was performed on virus-stimulated PBMC in culture. Antibodies were purchased from BD Pharmingen including: anti-CD8-Percp, anti-Perf-PE, anti-CD62L-APC, anti-CD28-PE, anti-CD107b-FITC and anti-CD69-APC-Cy7. Anti-CD3-PE-Cy7 was purchased from eBioscience. Fv17 single chain anti-GrB antibody (scFv GrB) (Rong et al., 2004) was labeled with FITC (Sigma) or APC (Dojindo Molecular Technologies, Inc.). Influenza APC-Pentamer (HLA-A0201/GILGFVFTL) was purchased from ProImmune. Cells were prepared for flow cytometry as previously described (McElhaney et al., 2006). Briefly, cells ($0.5–1\times10^6$) were incubated with surface Abs, washed with colder 0.2%BSA/PBS before and after fixing with 2% paraformaldehyde and then resuspended in colder permeabilization buffer (0.3% saponin, 5% normal human serum PBS). Following scFv GrB intracellular staining, cells were washed with 0.1% saponin and 0.2%BSA/PBS, resuspended in 0.2%BSA/PBS, transferred to FACS tubes for data acquisition on the BD LSR II. 30,000 events per each sample were counted and analyzed using Flow Jo software (Tree Star).

2.6. Virus rechallenge

Virus-stimulated PBMC were rechallenged with live virus to evaluate the cytolytic response of GrB+ CD8 T cells by flow cytometry, using cell-surface expression of CD107b as an indicator of degranulation (Betts et al., 2003). PBMC (2×10^6/ml) were stimulated with influenza virus A/Wyoming/03/2003 [A/H3N2] (MOI=2) for 5 days. The cells were collected, washed once with PBS, resuspended in 1ml AIM-V medium containing 1μg/ml anti-CD107b antibody and separated into two wells. A/Wyoming/03/2003 [A/H3N2] (MOI=2) was added to one well and the other well was used as a control. Culture plates were incubated in a humidified atmosphere at 37°C in 5% CO_2 for 4 hours, followed surface staining of CD3 and CD8 and intracellular staining of GrB for flow cytometry.

2.7. Statistics

The mean and standard error was used to describe the samples. Analyses were performed using SAS 9.1 (SAS Institute Inc.) and significant differences between the younger and older adults, virus strains, were assessed using ANOVA. Significant differences between pre-vaccination and 4-week post-vaccination samples were examined using the paired *t* test. The Pearson correlation coefficient was used to assess the correlation between different CD8 T cell subsets. All tests are two-sided and were reported as significant at the 95% level of confidence. All PBMC sample testing was blinded to the source of PBMC. Information on study participants was compiled only after all experiments were completed and the data were prepared for analysis.

3. Experimental results

3.1. Influenza virus stimulation increased the expression and activity of GrB in human PBMC

To evaluate the CTL response to influenza virus in PBMC cultures, the expression of GrB mRNA and the enzymatic activity of GrB were measured. RT-PCR results showed that the expression of GrB mRNA increased from a very weak band in fresh PBMC and gradually

increasing over 6 hours, 20 hours and 5 days of virus stimulation. Under the same conditions, Perf showed a relatively high level of mRNA expression in both fresh and stimulated PBMC (Fig. 1A). The results of Real-Time RT-PCR confirmed that the expression of GrB mRNA in PBMC increased with virus stimulation (Fig. 1B). The enzymatic activity of GrB in lysates of virus-stimulated PBMC was measured by Ac-IEPD-pNA cleavage after 20 hours and 5 days in culture. The activity of GrB in fresh PBMC lysates was below the limits of detection in the assay. GrB levels showed a 3-fold increase in activity from 20 hours to 5 days in culture; similar levels of GrB activity were obtained with two different virus strains, A/Panama/2007/99 [A/H3N2] and A/New Caledonia/20/99 [A/H1N1] (Fig. 1C). The contrasting profiles of GrB and Perf mRNA expression were supported by flow cytometric studies showing that fresh PBMC contained a significant proportion of Perf+ CD8 T cells with no GrB while both Perf+GrB− and Perf+GrB+ CD8+T cells were observed in 5-day virus stimulated PBMC (Fig. 1D). These results suggested that GrB compared to Perf, is a more sensitive marker of CTL activation. Given that IEPD cleavage by GrB corresponds to cytolytic activity in virus-stimulated PBMC (Ewen et al., 2003), the GrB assay is a sensitive and simple assay to detect the immune response to influenza virus under both *ex vivo* (20 hours) and *in vitro* (5 day) conditions.

3.2. GrB+CD62L^{high} CD8 T_{CM} cells were the source of the early effector CTL response to influenza

Next the phenotypes of CD8 T cells induced by virus stimulation were examined through cell surface and intracellular staining. L-selectin (CD62L) is a cell surface marker of lymph node homing and memory T cells (Stamenkovic et al., 1995). GrB was chosen as the marker of a cytotoxic effector response, as compared to Perf, it was a more specific marker of CTL activation. Fluorescent anti-CD3 antibody was used to gate T lymphocytes from other cells. PBMC were stained for CD69 (early activation marker), CD62L, CD3, CD8 and GrB. After 20 hours of virus stimulation, most of the GrB+ CD8 T cells were CD69+ (Fig. 2A) ; unstimulated CD8 T cells did not express CD69 or GrB. In virus-stimulated PBMC, GrB +CD62L^{high} CD8 T cells were shown to be the main responding memory CD8 T cell subset as early as 4 hours through 17 hours of stimulation (Fig. 2B). At day 5, both CD62L$^{\boldsymbol{high}}$ and CD62L$^{\boldsymbol{low}}$ subsets of GrB+ CD8 T cells were observed while only a sparse population of GrB +CD62L$^{\boldsymbol{low}}$ CD8 T cells was observed in unstimulated PBMC (Fig. 2C). Compared to the response at 17 hours, there were a higher number of CD62L−GrB− CD8 T cells at 5 days. This observation may result from the response to virus infection, CD62L is lost during the proliferative response (Sallusto et al., 1999) and GrB is removed from the cell with degranulation. All GrB+ CD8 T cells were also Perf+ and displayed a “blast” like morphology with an increase in the forward and side-scatter profile related to the increase in cell size and cytoplasmic granularity, respectively. An ELISpot assay showed the responding cell clusters in PBMC cultures to be rich in IFN-γ after virus stimulation (Lindemann et al., 2006). The GrB +CD62L$^{\boldsymbol{high/low}}$ CD8 T cells expressed higher IFN-γ, were CD28+ and CD27+/−, and 0.2–0.5% of CD8 T cells in HLA-A2+ individuals were influenza tetramer+ (data not shown). Since virus-stimulated CD62L$^{\boldsymbol{high}}$ CD8 T_{CM} cells contained Perf and GrB and expressed the activation marker, CD69, the phenotype of CD8 T_{CM} cells was consistent with activated virus-specific CTL effectors. It was concluded that the GrB+CD62L^{high} CD8 T_{CM} cells are the early source of virus-specific CTL effectors.

3.3. GrB+ CD8 T cells (including GrB+CD62L^{high} cells) can degranulate and contribute to the response to influenza virus infection

The accumulation of granular membrane proteins (CD107a and CD107b) on the cell surface of responding antigen-specific T cells provides a positive marker of CTL degranulation. CD107a and CD107b appear on the cell surface as early as 30 minutes following stimulation of CD8 T cells in primary cell cultures, and reached a maximum by 4 hours (Betts et al., 2003). CD107b on the cell surface and intracellular GrB were dually expressed in the CD8 T

cell subset (data not shown), providing further evidence that GrB+ CD8 T cells are a true effector population. To characterize the behavior of GrB+CD8 T cells, including CD62L^{high} cells, during influenza virus infection, PBMC were stimulated with influenza virus for 5 days, washed, and rechallenged with live virus. Virus-stimulated PBMC that were rechallenged were compared to control virus-stimulated PBMC that were not rechallenged, for the cell surface expression of CD107b after a further 4 hours of culture. Compared to the controls, virus rechallenged PBMC showed a further shift in CD107b expression, from CD107b− to CD107 + GrB+ CD8 T cells suggesting that rechallenge results in degranulation in additional virus-specific CD8 T cells. In contrast, GrB− CD8 T cells showed no expression of CD107b on the cell surface in controls or rechallenged PBMC, highlighting the specificity of the response in GrB+ CD8 T cells (Fig. 3A, 3B). In prevaccination samples, virus rechallenge resulted in a decrease in the proportion of CD107b+GrB+ CD8 T cells in both younger (N=17) and older adults, but the difference was significant only in older adults potentially due to the larger numbers in this group (N=109; paired t-test, $p<0.01$). Vaccination was associated with an increase in CD107b+GrB+ CD8 T cells in both controls and rechallenged PBMC but was not statistically significant (Fig. 3C). In older adults, vaccination was associated with a significant increase in the proportion of GrB+ CD8 T cells that became CD107b+ when virus-stimulated PBMC were rechallenged (Fig. 3D). Trends in the younger adult group may not have achieved statistical significance due the smaller group size and reflecting a Type II error. The results showed that all GrB+ CD8 T cells (including CD62L^{high} and CD62L^{low} cells) could degranulate and contribute to the response to influenza virus, and that the percentages of GrB +CD107b+ CD8 T cells in the older group were significantly higher compared to the younger group ($p<0.01$) (Fig. 3C, 3D). These results may be due to the significantly higher proportion of CD8 T cells in PBMC isolates from younger compared to older adults.

3.4. Older adults lack CD8 T lymphocytes and GrB+ CD62L^{high} CD8 T_{CM} lymphocyte responses

To determine whether there was an age-related change in the CD8 T_{CM} cell response to live virus, younger and older adults were compared for the percentage of GrB+CD62L^{high} CD8 T_{CM} cells in 5-day virus-stimulated PBMC. Fig. 4A shows that there were overall fewer CD8 T cells and a lower proportion of GrB+CD62L^{high} CD8 T_{CM} cells following virus stimulation in older compared to younger adults. Further analysis showed there was approximately a one-third reduction in the proportion of CD8 T cells in fresh PBMC (Fig. 4B) and a similar reduction in the percentage of CD8 T cells that were GrB+CD62L^{high} following virus stimulation in older (N=128) compared to younger adults (N=17; $p<0.001$) (Fig. 4C). These results suggest that there is an age-related decline in the central memory CTL response to influenza virus stimulation.

Older adults showed a significant increase from pre- to post-vaccination in the mean percentage of CD8 T cells that were GrB+CD62L^{high} (Fig. 4C). In contrast, there was no significant change in the overall GrB+CD62L$^{high/low}$ subset (18.8% pre-vaccination, 17.9% post-vaccination; data not shown) Younger adults showed a similar trend but the increase did not reach statistical significance for GrB+CD62L^{high} CD8 T cells (Fig. 4C). These results suggest that influenza vaccination enhances GrB+CD62L^{high} CD8 T_{CM} cell response to influenza virus.

3.5. The proportion of CD28^{null} CD8 T cells in fresh PBMC negatively correlated with the proportion of GrB+CD62L^{high} CD8 T_{CM} cells

CD28 plays a critical role in the response to influenza infection. Recent studies have shown that CD28^{null} T cells accumulate with advancing age, particularly in the CD8+ subset (Goronzy et al., 2001). Consistent with these results, the proportion of CD8 T cells that were CD28^{null} in fresh PBMC was higher in the older (37.9%, SE=1.7% of total CD8 T cells) compared to the younger adult group (31.0%. SE=3.8% of total CD8 T cells) (older, N=128; younger, N=19;

p<0.05). Due to the important co-stimulatory role of CD28, the relationship between CD28 ^{null}CD8 T cells in fresh PBMC and GrB+CD62L^{high} CD8 T cells in virus-stimulated PBMC in older adults was examined. Prior to vaccination, a significant negative correlation was observed between the percentage of CD8 T cells that were CD28^{null} in fresh PBMC and the proportion that were GrB+ CD62L^{high} in virus-stimulated PBMC (R=−0.22, p<0.001). A similar, but stronger correlation was observed post-vaccination for CD28 ^{null} CD8 T cells and GrB+ CD62L^{high} CD8 T_{CM} cells (R=−0.38, p<0.001) (Fig. 5). This relationship was not observed in GrB+CD62L^{low} CD8 T_{EM} cells or total CD8 T cells. These results suggest that the increased proportion of CD28^{null} CD8 T cells and overall reduction of CD8 T cells with aging contributes to a diminished the reservoir of CTL (CD8+) that can respond to influenza vaccination.

4. Discussion

Previous studies have shown that older adults who develop influenza illness in spite of vaccination, have low levels of GrB in virus-stimulated PBMC (McElhaney et al., 2001; 2006). The present study was designed to explore a potential mechanism for the poor response to influenza vaccination in older adults. Initial experiments showed that there was a progressive increase in GrB mRNA and a parallel increase in GrB activity over five days of stimulation with influenza virus. Virtually all GrB+ CTL expressed the activation marker, CD69+, at 20 hours and had an effector phenotype, GrB+CD62L^{high} or GrB+CD62L^{low} after 5 days in culture. In contrast, Perf mRNA and protein were expressed in both fresh and virus-stimulated PBMC. Thus, GrB was chosen for the subsequent experiments as a more responsive marker to influenza virus stimulation.

There is much debate about how CD62L^{high} T_{CM} and CD62L^{low} T_{EM} subsets relate to each other (Wherry et al., 2003; Roberts et al., 2005; Bouneaud et al., 2005; Kedzierska et al., 2006). Earlier studies suggested that the proliferation capacity of the CTL response resided in the CD62L^{high} T_{CM} subset and with multiple rounds of replication. CD62L expression declined and generated CD62L^{low} T_{EM} cells with low proliferation capacity (Lanzavecchia and Sallusto, 2002). More recent studies showed that the TCM and TEM are distinct cell lineages (Jackson et al., 2005). However, previous studies did not distinguish true “effectors” from other memory cells. Using GrB intracellular staining as a marker of CTL effectors, GrB+CD62L$^{\boldsymbol{high}}$ CD8 T_{CM} cells accumulated within 20 hours of influenza virus stimulation with virtually no GrB +CD62L$^{\boldsymbol{low}}$ CD8 T_{EM} cells suggesting that GrB+CD62L$^{\boldsymbol{high}}$ CD8 T_{CM} cells provides the early effector response. By five days in culture, there was considerable expansion of both these GrB + CD8 T cell subsets and a reduction in GrB−CD62L$^{\boldsymbol{high}}$ and CD62L$^{\boldsymbol{low}}$ subsets in both stimulated and unstimulated PBMC. These results would support the earlier findings in the mouse, that CD8 T_{CM} cells are the proliferating memory T cell population that gives rise to GrB+CD62L$^{\boldsymbol{low}}$ CD8 T_{EM} cells. However, the co-expression of GrB and CD107b on both CD62L$^{\boldsymbol{high}}$ CD8 T_{CM} and GrB+CD62L$^{\boldsymbol{low}}$ CD8 T_{EM} cells provides evidence for degranulation and suggests that both subsets contribute to CTL effector function through the later stages of virus stimulation. While the mean percentage of GrB+ CD8 T cells the express CD107b is relatively low (~2%), it should be highlighted that this in the context of virus-infected targets that vastly outnumber the CD8 effector cells in PBMC cultures. This contrasts with usual cytolytic assays of stimulated human PBMC with effector: target ratios of 25–50: 1 to produce 20–30% mean specific lysis (Powers el al., 1993). Thus, a small but statistically significant increase in the mean percentage of GrB+ CD8 T cells expressing CD107b should translate to clinically meaningful differences in CTL activity. Since the expression of CD62L alone is not sufficient to define distinct CTL functional subpopulations of memory CD8 T cell subsets (Jackson et al., 2005), these findings would support the combined use of intracellular GrB and CD62L as markers for delineating functional subsets of memory CD8 T cells.

It has been shown that CD62L^{high} CD8 T_{CM} cells respond more vigorously to secondary challenge than CD62L^{low} T_{EM} cells in terms of their expansion and capacity to clear virus (Wherry et al., 2003). In contrast, it was recently reported that CD62L^{low} T_{EM} response was at least equal to, or greater than, T_{CM} (CD62L^{high}CCR7+) cells in Sendai virus rechallenge (Roberts et al., 2004). The evidence presented in this paper support the earlier studies showing that GrB+CD62L^{high} CD8 T_{CM} cells were the major responders to influenza stimulation especially in the early phase of infection. Differences in the results in human PBMC reported herein, from the recent published studies in the mouse, may be due to the effects of local draining lymph nodes, the spleen and non-lymphoid peripheral tissues on CTL responses in the mouse. There may also be some inherent differences in how influenza stimulates human PBMC, and how Sendai virus interacts with the immune system in mouse models.

This study also addressed the cellular immune mechanisms that may explain the age-related increase in susceptibility to influenza illness and diminished vaccine efficacy. The results showed that the lower proportion of CD8 T cells in total PBMC and higher proportion of CD28^{null} CD8 cells of CD8 in older compared to younger adults may limit the CTL response to influenza vaccination and protection from influenza illness. Since CD28 is critical for the initial expansion of CD8 T cells during influenza infection (Effros et al., 2005; Goronzy et al., 2001), a reduction in the proliferative response to influenza would be anticipated with increasing numbers of CD28^{null} CD8 T cells. The present study showed that there was a negative correlation between the proportion of CD28^{null} CD8 T cells and the rapidly proliferating GrB+CD62L^{high} CD8 T_{CM} cell subset responding to influenza virus, which suggests that the increased proportion of CD28^{null} CD8 T cells interferes with the CD8 T_{CM} response to virus infection. Also consistent with the hypothesis that CD28^{null} CD8 cells interfere with the memory CTL proliferative response to influenza, there was no similar correlation observed between CD28^{null} CD8 cells and the non-proliferating GrB+CD62L^{low} CD8 T_{EM} cell subset in virus-stimulated PBMC. The proportion of CD28^{null} cells has previously been associated with poorer antibody responses to vaccination in older adults (Saurwein-Teissl et al., 2002). Interestingly in the current study, an increase in the negative correlation between CD28^{null} CD8 and GrB+CD62L^{high} CD8 T_{CM} subsets was observed following vaccination suggesting that CD28^{null} CD8 T cells may also impact on the response to influenza vaccination. Taken together, these results suggest that CD28^{null} cells have a pervasive effect on the immune response to influenza vaccination, and that high levels of CD28^{null} Cd8 T cells may be a predictor of poor protection from the current split-virus vaccines in older adults. This may be a target for future vaccine development to provide a stronger stimulus to the CTL response and decrease the inhibitory effect of CD28^{null} CD8 T cells.

In summary, GrB was shown to be an accurate marker of virus-specific stimulation in human CTL and that GrB+ CD62L^{high} CD8 T_{CM} cells were identified as the early effector population responding to influenza virus. In older adults, the response to influenza virus in this CD8 T_{CM} subset declined with an increasing proportion of CD8 T cells with a senescent CD28^{null} phenotype.

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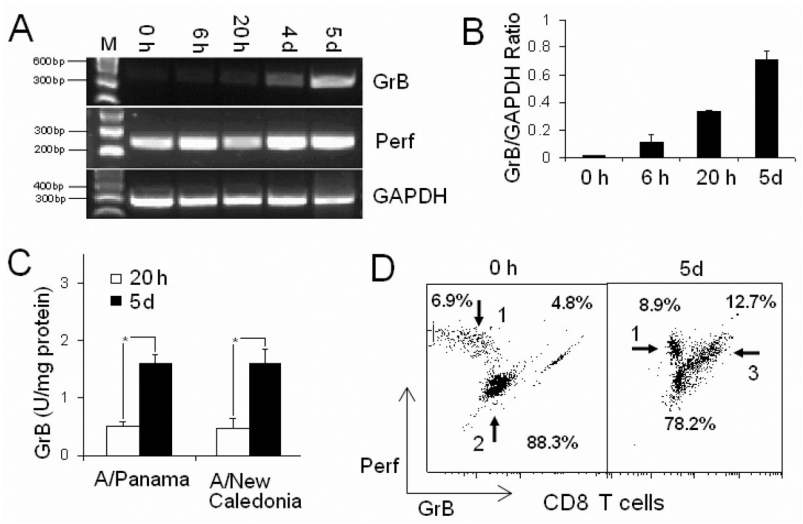

Fig. 1.

Influenza virus stimulation increased the expression of GrB mRNA and the activity of GrB in human PBMC. *A*, RT-PCR electrophoresis of GrB, Perf and the housekeeping gene GAPDH in human PBMC is shown. Human PBMC were fresh (0h) or stimulated with A/Panama/2007/99 [A/H3N2] for 6 hours (6h), 20 hours (20h), 4 days (4d) or 5 days (5d). The expression of GrB mRNA increased with influenza virus stimulation while the level of Perf mRNA expression remained high at all time points under the same conditions. *B*, Real-Time PCR confirmed the increase of GrB mRNA expression with virus stimulation. Mean ratio for the GrB/GAPDH copy number in PBMC stimulated under the same conditions, showed a significant increase over time (N=3, ANOVA, $p<0.001$). Error bars represent standard error. *C*, Mean GrB activity showed a significant 3-folder increase from 20 hours to 5 days in PBMC stimulated with influenza virus strains, A/Panama/2007/99 [A/H3N2] and A/New Caledonia/20/99 [A/H1N1](N=5, ANOVA, $p<0.001$), with no significant difference between the two virus strains. Error bars represent standard error. *D*, The proportion of Perf+ and Perf+GrB+ CD8 T cells increased with virus stimulation. Compared to fresh PBMC (0h), CD8 T cells in PBMC stimulated with A/Panama/2007/99 [A/H3N2] for 5 days (5d) contained a higher proportion of both Perf+GrB− (arrow 1) and Perf+GrB+ (arrow 3) CD8 T cells and a reciprocal lower proportion of $Perf^{low}GrB-$ (arrow 2).

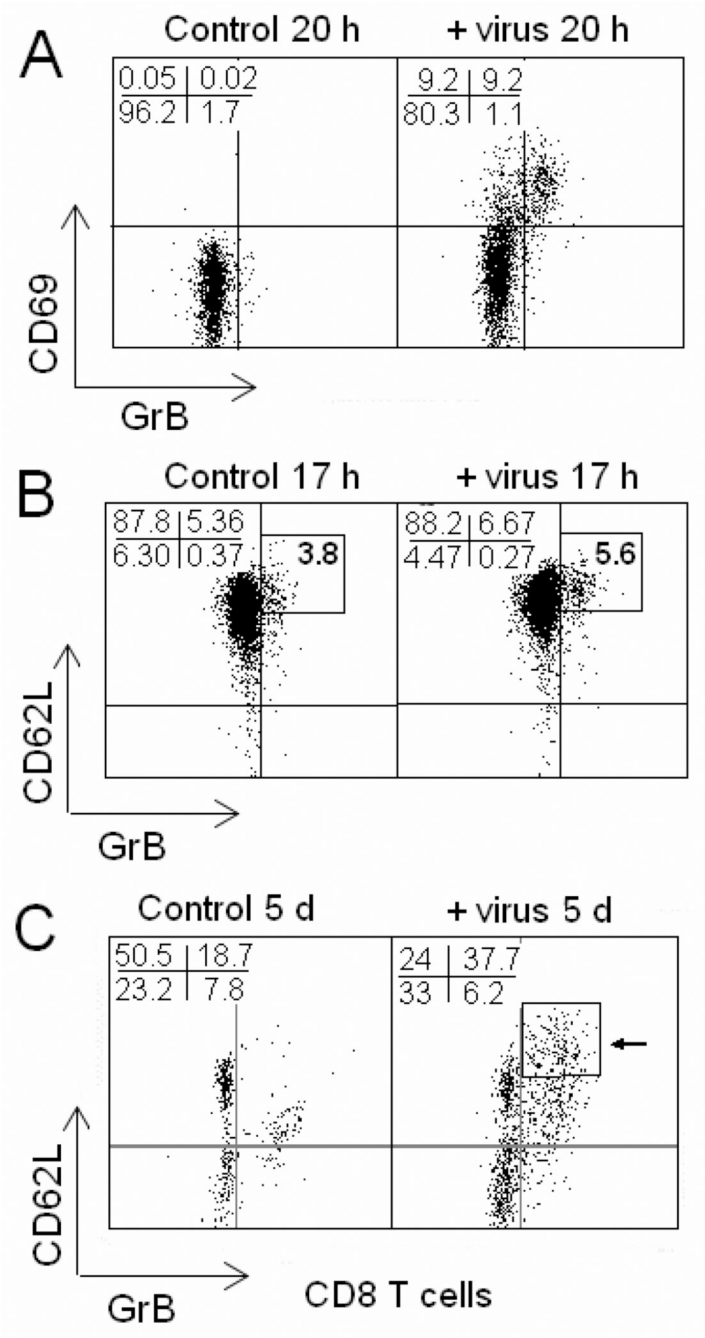

Fig. 2.

Influenza virus stimulates an increase in GrB+ CD8 T cell frequency, especially GrB+ CD62L^{high} CD8 T cells. Human PBMC stimulated with or without A/Wyoming/03/2003 [A/H3N2] for 20 hours or 5 days. *A*, GrB+ CD8 T cells appeared after stimulation with virus for 20 hours. All GrB+ CD8 T cells expressed the early activation marker, CD69. *B*, GrB+ CD8 T cells were CD62L^{high} after stimulation of PBMC with A/Wyoming/03/2003 [A/H3N2] for 17 hours. *C*, GrB+ CD8 T cells increased after stimulation with influenza virus for 5 days, particularly in the CD62L^{high} (arrow) CD62L^{low} subsets. Greater than 90% of GrB+ CD8 T cells were CD62L$^{high/low}$. Numbers represent the percentages of total CD3+CD8 T cells.

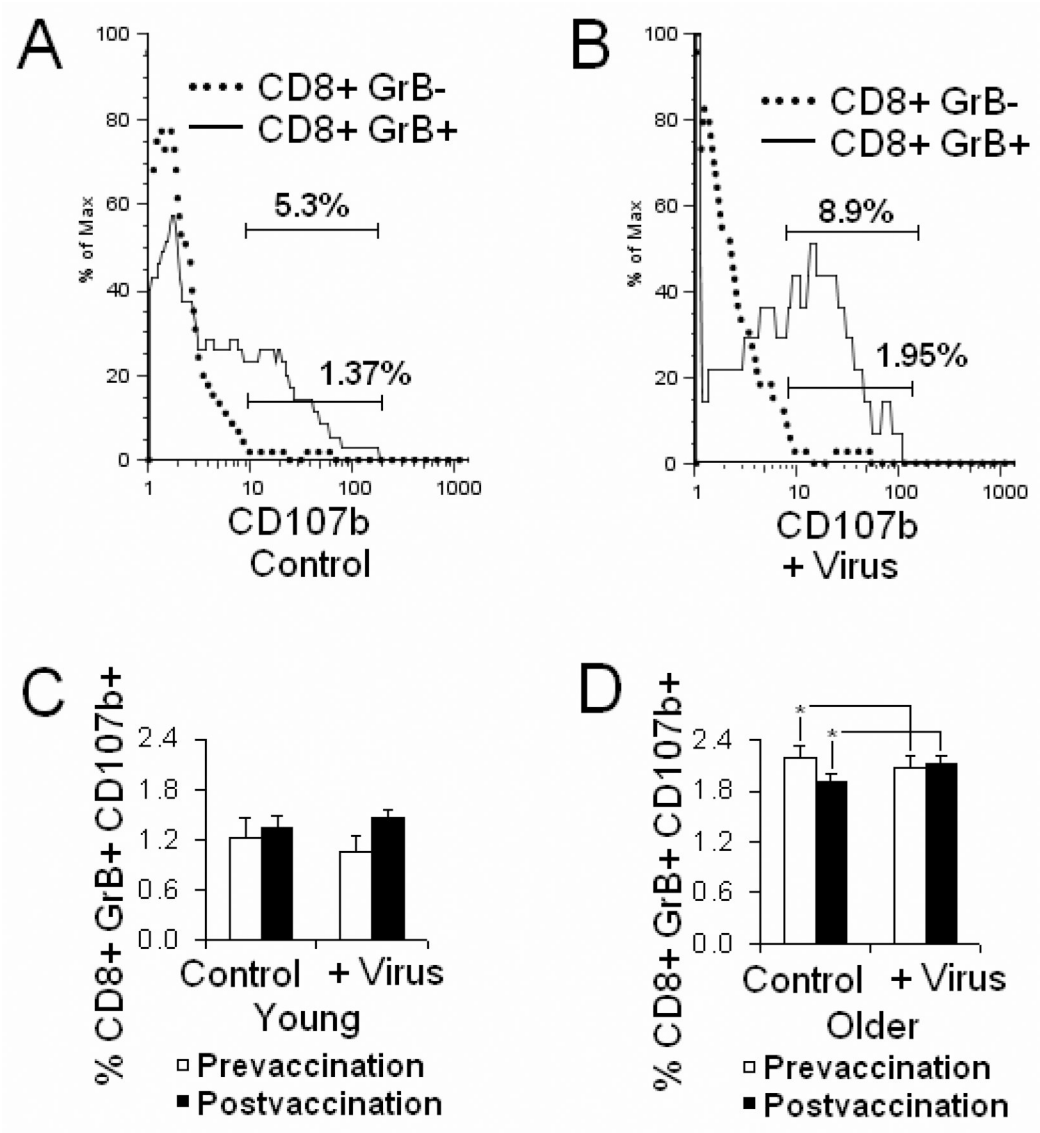

Fig. 3.

GrB+ CD8 T cells degranulate in response to influenza virus and the proportion of degranulating GrB+ CD8 T cells is higher in older compared to younger adults. Human PBMC stimulated for 5 days with influenza virus, A/Wyoming/03/2003 [A/H3N2], were rechallenged with the same virus in parallel (+ virus), the controls that were not rechallenged. The proportion of cells with cytolytic activity was detected by CD107b expressed on the cell surface in the process of degranulation. *A,* The stimulated control (not rechallenged), showed that only GrB +CD8 T cells accumulated CD107b on the cell surface while most of GrB− subset were CD107b−. Numbers indicate the percentages of CD107b+ cells in total CD8 T cells. *B,* GrB− CD8 T cells remained CD107b− with virus rechallenge while the GrB+ subset continued to accumulate and becomes largely CD107b+. Numbers indicate the percentages of CD107b+ cells in total CD8 T cells. *C,* In younger adults, virus rechallenge did not significantly change the mean percentage of CD107b+ in the GrB+CD8+ subset and this response did not change following vaccination. There was no significant increase in the mean proportion of CD107b +GrB+CD8 T cells. *D,* In older adults prior to vaccination, virus rechallenge was associated with a decrease in the mean percentage of CD107b+GrB+CD8+ in the total CD8 T cell subset.

However, at 4-weeks post-vaccination in older adults, virus rechallenge resulted in a significant increase in the mean percentage of CD107b+GrB+CD8 subset of total CD8 T cells (N=109, $p<0.01$). Error bars represent standard error of the mean.

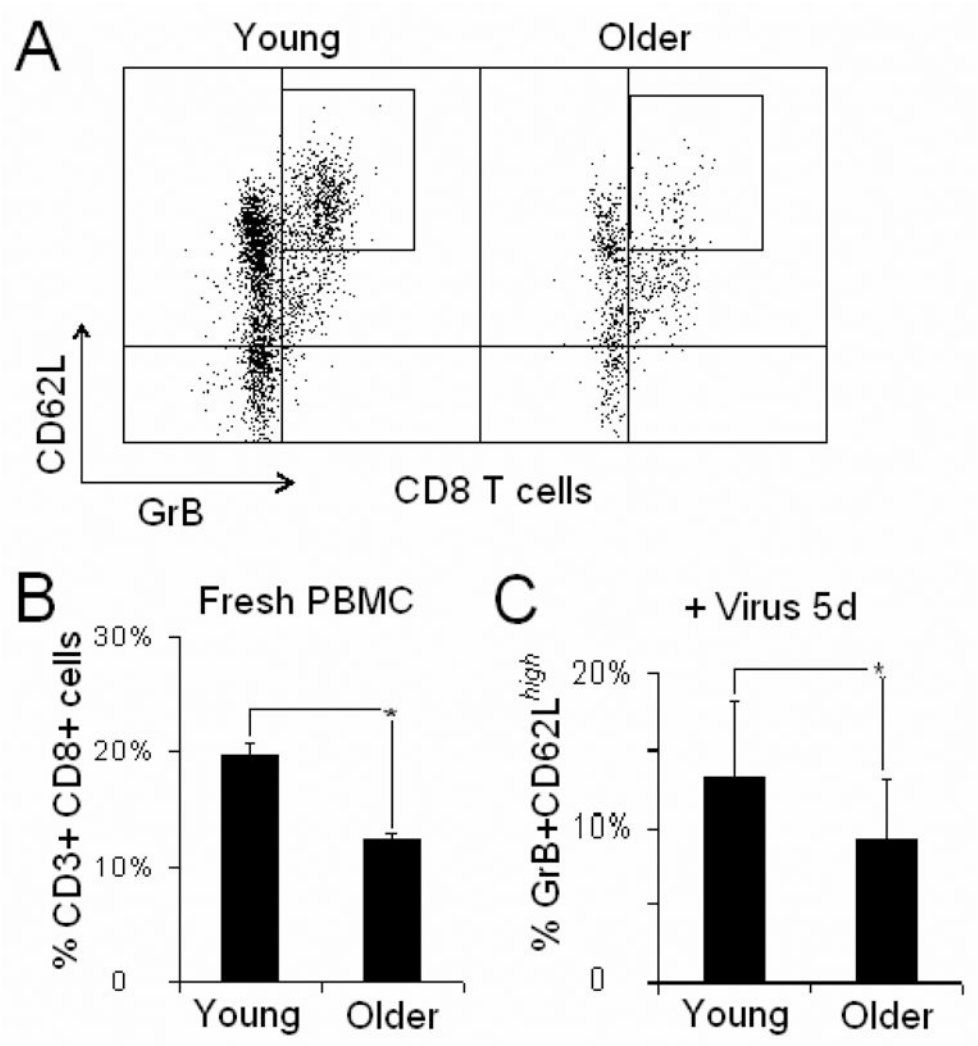

Fig. 4.

There were lower CD8 T cells within PBMC isolates and a reduced proportion of GrB +CD62L^{high} cells within CD8 T cells following virus stimulation in older adults compared to younger adults. *A*, Representative dot graphs of CD8 T cells from a younger and an older adult PBMC stimulated with A/Wyoming/03/2003 [A/H3N2] for 5 days showing an overall reduction in the proportion of the GrB+CD62L^{high} CD8 T cell subset in the older adult CD8 T cells. *B*, The mean percentage of CD8 T cells in fresh PBMC, and *C*, the mean percentage of the GrB+CD62L^{high} subset of the total CD8 T cell population in virus-stimulated PBMC, was significantly higher in younger (N=17) compared to older adults (N=119, $p<0.001$ for both comparisons). Error bars represent standard error.

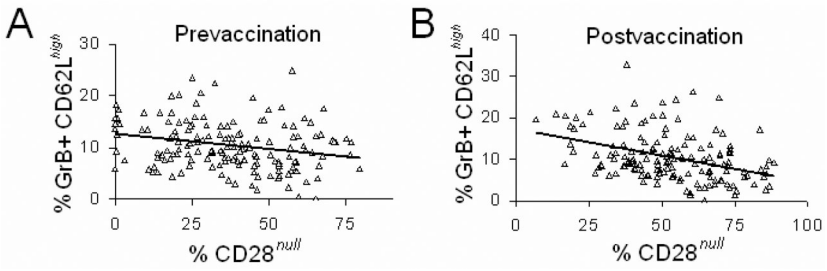

Fig. 5.

The frequency of CD28^{null} cells in fresh PBMC was negatively correlated with GrB +CD62L^{high} CD8 T cells in virus-stimulated PBMC from older adults. *A*, Prior to vaccination, there was a negative correlation between the percentage of CD8 T cells that were CD28^{null} in fresh PBMC, and the percentage of CD8 T cells that were GrB+CD62L^{high} in PBMC stimulated with A/Wyoming/03/2003, [A/H3N2] for 5 days (N=149, R=−0.22, p<0.001). *B*, Four weeks after vaccination, a stronger negative correlation between percentage of CD8 T cells that were CD28^{null} in fresh PBMC and GrB+CD62L^{high} in virus-stimulated PBMC (N= 149, R=−0.38, p<0.001).