Title: IL-4 and IL-13 mediated down-regulation of CD8 expression levels can dampen anti-viral CD8⁺ T cell avidity following HIV-1 recombinant pox viral vaccination

Running title: IL-4/IL-13 regulation of CD8 can dampen CD8⁺ T cell quality

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¹Abbreviations

¹Abbreviations: interleukin (IL); T cell receptor (TCR); interferon (IFN); tumor necrosis factor (TNF); wild-type (WT); signal transducer and activator of transcription 6 (STAT6); peptide-major histocompatibility class I complexes (pMHC-I); intranasal (i.n.); intramuscular (i.m.); IL-13 receptor α 2 (IL-13R α 2); fowlpox virus encoding HIV-1 antigens (FPV-HIV); vaccinia virus encoding HIV-1 antigens (VV-HIV); FPV-HIV or VV-HIV encoding murine soluble IL-13R α 2 (IL-13 inhibitor vaccines); vaccinia virus Western Reserve strain (VV-WR); intraperitoneal (i.p.); red blood cell (RBC); Beckton Dickinson (BD); phosphate buffer saline (PBS); fetal calf serum (FCS); intracellular cytokine stain (ICS); mean fluorescent intensity (MFI); standard error of the mean (SEM); analysis of variance (ANOVA); type I interferons (IFN-I); lymphocytic choriomeningitis virus (LCMV).

Abstract

We have shown that mucosal HIV-1 recombinant pox viral vaccination can induce high avidity HIV-specific CD8⁺ T cells with reduced interleukin (IL)-4 and IL-13 expression compared to systemic vaccine delivery. In the current study how these cytokines act to regulate anti-viral CD8⁺ T cell avidity following HIV-1 recombinant pox viral prime-boost vaccination was investigated. Out of a panel of T cell avidity markers tested, only CD8 expression levels were found to be enhanced on K^dGag₁₉₇₋₂₀₅ (HIV)-specific CD8⁺ T cells obtained from IL-13^{-/-}, IL-4^{-/-} and signal transducer and activator of transcription of 6 (STAT6)^{-/-} mice compared to wild-type (WT) controls following vaccination. Elevated CD8 expression levels in this instance also correlated with polyfunctionality (interferon (IFN)- γ , tumour necrosis factor (TNF)- α and IL-2 production) and the avidity of HIV-specific CD8⁺ T cells. Furthermore, mucosal vaccination and vaccination with the novel adjuvanted IL-13 inhibitor (i.e. IL-13R α 2) vaccines significantly enhanced CD8 expression levels on HIV-specific CD8⁺ T cells, which correlated with avidity. Using anti-CD8 antibodies that blocked CD8 availability on CD8⁺ T cells, it was established that CD8 played an important role in increasing HIV-specific CD8⁺ T cell avidity and polyfunctionality in IL-4^{-/-}, IL-13^{-/-} and STAT6^{-/-} mice compared to WT controls following vaccination. Collectively, our data demonstrate that IL-4 and IL-13 dampen CD8 expression levels on anti-viral CD8⁺ T cells, which can down-regulate anti-viral CD8⁺ T cell avidity and polyfunctionality following HIV-1 recombinant pox viral vaccination. These findings can be exploited to design more efficacious vaccines not only against HIV-1, but many chronic infections where high avidity CD8⁺ T cells help protection.

Keywords: CD8⁺ T cell avidity/polyfunctionality; HIV-1 vaccines/vaccination; CD8 expression; IL-4/IL-13; mucosal vaccination

1. Introduction

Even though effective anti-retroviral drugs are currently available against HIV-1 [1], developing a vaccine still remains a major priority for slowing down the progression of HIV-1 incidences worldwide. Unfortunately, this has been a daunting task given the capacity of HIV-1 to maintain latency and mutate rapidly allowing it to evade the immune system [2]. Furthermore, many of the past HIV-1 vaccines that have elicited protective outcomes in animals have failed to do so in humans [3-8].

The induction of high avidity anti-viral CD8⁺ T cells responses is thought to be critical for effective protective immunity against viruses including HIV-1 [9, 10]. Various molecules that facilitate cell adhesion (e.g. CD2 and CD11a), cognate peptide-major histocompatibility class I complexes (pMHC-I) recognition (i.e. densities of CD8 and T cell receptor (TCR) interaction) and intracellular signaling such as lck have been known to regulate T cell avidity [11-14]. High avidity anti-viral CD8⁺ T cells can recognize and respond to lower densities of cognate pMHC-I on virus-infected cells compared to low avidity anti-viral CD8⁺ T cells [15]. This allows high avidity HIV-specific CD8⁺ T cells to minimise or prevent the spread of this virus from early exposed genito-rectal mucosae to the gut where the greatest CD4⁺ T cell depletion occur during HIV-1 infection [2, 10]. High avidity anti-viral CD8⁺ T cells are also polyfunctional (i.e. produce multiple anti-viral/survival cytokines such as IFN- γ , TNF- α and IL-2) [16-17]. Numerous studies have demonstrated that elite controllers of HIV-1 infection maintain more high avidity and polyfunctional CD8⁺ T cells than non-controllers [18-20]. Therefore, development of vaccines that can induce high avidity HIV-specific CD8⁺ T cells could be extremely beneficial for optimising protective outcomes against HIV-1.

Despite the knowledge that high avidity anti-viral CD8⁺ T cells are important for efficient virus control, the mechanisms responsible for enhancing the avidity of anti-viral CD8⁺ T cells following vaccination is not well understood. Previous studies have shown that IL-4 and IL-13 can modulate anti-viral efficacy of CD8⁺ T cells and control of virus infection [21-24]. We have shown that HIV-specific CD8⁺ T cells that develop following mucosal (intranasal (i.n.)/i.n. or i.n./intramuscular (i.m.)) compared to purely systemic (i.m./i.m.) HIV-1 recombinant pox viral prime-boost vaccination have reduced expression of IL-4/IL-13, but are higher in avidity [25]. In subsequent studies, we have shown following pox viral infection and HIV-1 recombinant pox viral prime-boost vaccination that IL-4, IL-13 and STAT6 can dampen the avidity and polyfunctionality of anti-viral CD8⁺ T cells [26-27]. Based on this knowledge we have now developed a novel HIV-1 recombinant pox viral IL-13 receptor α 2 (IL-13R α 2) adjuvanted vaccine that can be used to transiently inhibit IL-13 activity at the vaccination site [24]. This vaccine was shown to enhance the avidity/polyfunctionality of HIV-specific CD8⁺ T cells as well as the protective efficacy [24]. Given that pox virus vectors are commonly used in vaccination studies, here we have addressed how IL-4 and IL-13 can dampen the avidity of anti-viral CD8⁺ T cells following HIV-1 recombinant pox viral vaccination with the hope of developing more effective HIV-1 vaccines that translate into future phase I clinical trials.

2. Materials and methods

2.1 Mice

Pathogen-free 6-8 weeks old female WT, IL-4^{-/-}, IL-13^{-/-}, and STAT6^{-/-} BALB/c mice were all purchased from the Australian Phenomics Facility, the Australian National University. All the mice were handled and maintained under the Australian National University Animal Experimentation and Ethics Committee approved guidelines and protocol number A2011/018.

2.2 Recombinant HIV-1 prime-boost vaccination and vaccinia virus infection

The recombinant fowlpox virus vectors encoding HIV-1 AE clade Gag, Pol and Env (FPV-HIV) and recombinant vaccinia virus encoding HIV-1 AE clade Gag and Pol (VV-HIV) used in the current study were exactly as those used previously in Ranasinghe *et al* [28]. Recombinant FPV-HIV and VV-HIV co-expressing the murine soluble IL-13R α 2 (IL-13 inhibitor vaccines) were constructed exactly as describe in [24].

Vaccinia virus Western Reserve (VV-WR) strain and infection with this virus was exactly as described in Wijesundara *et al* [26]. Briefly, BALB/c mice were infected for 7 days intraperitoneally (i.p.) prior to isolation of splenocytes for flow cytometry analysis. For all recombinant HIV-1 prime-boost vaccinations except where indicated, pathogen free 6-8 weeks old BALB/c mice were primed i.n. with 1×10^7 PFU of FPV-HIV followed by i.m. booster vaccination with 1×10^7 PFU of VV-HIV 14 days apart as described in Ranasinghe *et al* [28]. T cell responses were evaluated 14 days post booster vaccination.

2.3 Flow cytometry

Monoclonal antibodies against mouse antigens CD2 (clone RM2-5), CD11a (clone 2D7), TCR β (clone H57-597), IFN- γ (clone XMG1.2), H-2K^d (clone SF1-1.1) and CD8 β .2 (clone 53-5.8) were all purchased from BioLegend with the exception of CD8 α (clone 53-6.7), Ick (clone MOL 171) and TNF- α (clone MP6-XT22), which were purchased from Becton Dickinson (BD) Biosciences. These antibodies were used as purified (not conjugated) or conjugated with fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, allophycocyanin or pacific blue. Peridinin chlorophyll protein conjugated monoclonal antibody against mouse IL-2 (clone JES6-5H4) was purchased from eBioscience. All cell surface and intracellular staining procedures with these antibodies were performed as described previously [26]. Stained samples were analysed using the BD FACScalibur or BD LSR II prior to analysis using the FlowJo Tree Star software (version 8.8.7). For each sample $2 \times 10^5 - 1 \times 10^6$ events were acquired.

2.4 Tetramer staining and dissociation assays

Allophycocyanin conjugated K^dA52₇₅₋₈₃ or K^dGag₁₉₇₋₂₀₅ tetramers were synthesized by the Biomolecular Resource Facility, the John Curtin School of Medical Research. Tetramer staining was done using red blood cell (RBC)-depleted single cell suspensions from the spleens of vaccinated mice exactly as that described in Ranasinghe *et al* [25]. For blocking CD8 engagement with K^dGag₁₉₇₋₂₀₅ tetramer, cells (2×10^6 cells/25 μ l phosphate buffer saline (PBS)) were incubated with varying concentrations of purified anti-mouse CD8 β .2 for 30 minutes at 4 °C prior to tetramer staining. When tetramer dissociation was performed, tetramer stained cells were incubated for 60 minutes at 37

°C + 5% CO₂ in the presence of 25 µg/ml of anti-mouse H-2K^d (2 x 10⁶ cells/40 µl PBS) to prevent tetramer re-binding to cells. After tetramer staining and/or dissociation, cells were washed twice using PBS + 1 % fetal calf serum (FCS) prior to conducting a cell surface stain.

2.5 Peptide stimulation for intracellular cytokine staining (ICS)

H-2K^d restricted Gag₁₉₇₋₂₀₅ (AMQMLKETI) peptides were synthesized at the Biomolecular Resource Facility, the John Curtin School of Medical Research. K^dGag₁₉₇₋₂₀₅ peptide stimulation of splenocytes was performed similar to that described previously [26]. Briefly, 4 x 10⁶ RBC-depleted splenocytes resuspended in 200 µl of RPMI + 10% FCS were incubated for 1 hour at 37 °C + 5% CO₂ in the presence or absence of 0.1 µg/ml of K^dGag₁₉₇₋₂₀₅ peptide. Subsequently, each culture was incubated with 1x brefeldin A (eBioscience) for further 4 hours at 37 °C + 5% CO₂ prior to cell surface and intracellular staining. When CD8 blocking was performed, cells were incubated with 1 µg/ml of purified anti-mouse CD8β.2 prior to and during K^dGag₁₉₇₋₂₀₅ peptide stimulation.

Statistical analysis

Mean fluorescent intensity (MFI) values and flow cytometry plots were obtained following analysis of data using FlowJo Tree Star software (version 8.8.7). The data plotted in all the graphs represent the mean and the error bars depict the standard error of the mean (SEM). Statistical significance of the data and the p values were calculated using the Graph InStat software (version 3.10). In all statistical significance analysis, a student's t-test or one-way analysis of variance (ANOVA) was used. The p values are denoted as follows: ns - p > 0.05, * - p < 0.05, ** - p < 0.01, and *** - p < 0.001.

3. Results

3.1 IL-4 and IL-13 selectively dampen CD8 expression levels on HIV-specific CD8⁺ T cells

We initially examined whether IL-4 and IL-13 regulated the expression of T cell associated molecules, which could dampen the avidity of anti-viral CD8⁺ T cells induced following HIV-1 recombinant pox viral prime-boost vaccination. For this purpose, the expression of a representative panel of T cell avidity markers (i.e. CD8, lck, TCR β , CD2 and CD11a) were measured on K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells that were induced following i.n. FPV-HIV/i.m. VV-HIV vaccination of WT, IL-13^{-/-}, IL-4^{-/-} and STAT6^{-/-} BALB/c mice (Fig. 1A and 1B).

Following HIV-1 recombinant prime boost vaccination, only CD8 α and CD8 β levels were found to be consistently enhanced on K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells obtained from gene knockout mice relative to the WT controls (Fig. 1A and 1B). Although some reports have suggested that high avidity anti-viral CD8⁺ T cells can enhance the expression ratios of CD8 β :CD8 α [29], these ratios were similar on K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells from all vaccinated animals (Fig. 1C). IL-4 and IL-13 mediated reduction of CD8 densities was also observed on K^dA52R₇₅₋₈₃-specific CD8⁺ T following VV-WR infection suggesting that this was phenomenon was not specific to K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells (Fig. 1D). Our previous studies have demonstrated that the avidity of K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells can be regulated depending on the route of vaccine delivery (i.e. avidity: i.n./i.n. > i.n./i.m. > i.m./i.m.) [25]. The avidity hierarchy from this published report correlated with the CD8 expression levels on K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells (i.e. CD8 expression: i.n./i.n. > i.n./i.m. > i.m./i.m.) in the current study (Fig. 1E). Furthermore, we have recently shown that vaccines that transiently inhibit IL-

13 activity at the vaccination site can induce high avidity CD8⁺ T cells with better protective efficacy (i.e. avidity and protection: IL-13Rα2 adjuvanted IL-13 inhibitor vaccine > control vaccine) [24]. In the current study, compared to the control vaccination (i.n. FPV-HIV/i.m. VV-HIV), IL-13 inhibitor vaccination strategy (i.n. FPV-HIV-IL-13Rα2/i.m. VV-HIV-IL-13Rα2) also facilitated the development of K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells with elevated CD8 expression levels (Fig. 1F). Collectively, our data demonstrated that IL-4 and/or IL-13 can down-regulate CD8 expression levels, which correlated with reduced avidity on anti-viral CD8⁺ T cells.

3.2 Regulation of CD8 expression levels is a feature of CD8⁺ T cell polyfunctionality

In order to further understand the involvement of CD8 in modulating CD8⁺ T cell avidity following HIV-1 vaccination, we next examined how the expression levels of CD8 were regulated on different polyfunctional subsets of HIV-specific CD8⁺ T cells. Highly polyfunctional anti-viral CD8⁺ T cells have been reported to produce IFN-γ, TNF-α and IL-2 compared to poorly polyfunctional anti-viral CD8⁺ T cells, which predominantly produce IFN-γ [30]. Therefore, we next measured the CD8 expression levels on IFN-γ⁺ TNF-α⁺ IL-2⁺ (highly polyfunctional), IFN-γ⁺ TNF-α⁺ IL-2⁻ (moderately polyfunctional) and IFN-γ⁺ TNF-α⁻ IL-2⁻ (poorly polyfunctional) K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells following i.n. FPV-HIV/i.m. VV-HIV vaccination (Fig. 2). IFN-γ was monitored (Fig. 2B and 2C) as a positive control given that highly polyfunctional T cells have been reported to produce more IFN-γ on a per cell basis compared to poorly polyfunctional T cells [31]. Greatly elevated CD8α and CD8β levels were detected in highly polyfunctional compared to poorly/moderately polyfunctional K^dGag₁₉₇₋₂₀₅ specific CD8⁺ T cells (Fig. 2B and 2C). Overall, increasing

CD8 expression levels appeared to be a feature of anti-viral CD8⁺ T cells that were highly polyfunctional following HIV-1 recombinant pox viral prime-boost vaccination.

3.3 HIV-specific CD8⁺ T cells require CD8 for optimal cognate pMHC-I engagement

The studies presented thus far suggest that regulation of CD8 on anti-viral (i.e. K^dGag₁₉₇₋₂₀₅-specific) CD8⁺ T cells correlate with enhancement in T cell avidity and polyfunctionality. However, CD8 null tetramer studies have shown that TCR engagement alone on effector CD8⁺ T cells could be sufficient for maintaining a high avidity interaction with cognate pMHC-I [32]. Therefore, we next examined whether the availability of CD8 on K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells was important in maintaining a high avidity interaction with cognate pMHC-I.

In order to achieve the above, splenocytes obtained from i.n. FPV-HIV/i.m. VV-HIV vaccinated mice were incubated with increasing concentrations of purified anti-CD8β.2 to limit the availability of CD8 molecules prior to K^dGag₁₉₇₋₂₀₅ tetramer staining (Fig. 3). Data demonstrated a dose dependent reduction of K^dGag₁₉₇₋₂₀₅ tetramer engagement with increasing concentrations of anti-CD8β.2 (Fig. 3). Therefore, our data indicated that CD8 on K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells was crucial for maintaining a high avidity interaction with cognate pMHC-I molecules.

3.4 IL-4 and IL-13 mediated CD8 density regulation on HIV-specific CD8⁺ T cells modulate T cell avidity/polyfunctionality

We next examined whether IL-4 and IL-13 mediated reduction of CD8 densities (Fig. 1) could also dampen the avidity of HIV-specific CD8⁺ T cells from WT controls compared to IL-13^{-/-}, IL-4^{-/-} and STAT6^{-/-} BALB/c mice. For this purpose, splenocytes

from i.n. FPV-HIV/i.m. VV-HIV vaccinated mice were incubated with or without anti-CD8 β .2 prior to conducting an ICS for measuring polyfunctionality or tetramer dissociation assay for measuring avidity. The rationale here was that if reduction of CD8 densities on HIV-specific CD8⁺ T cells was important then blocking CD8 availability for tetramer engagement and cytokine production should abrogate avidity differences observed between IL-13^{-/-}, IL-4^{-/-} and STAT6^{-/-} BALB/c mice and WT controls.

In the absence of CD8 blocking (mock) greater proportion of IFN- γ producing K^dGag₁₉₇₋₂₀₅-specific CD8⁺ T cells were found to express TNF- α and IL-2 in IL-13^{-/-}, IL-4^{-/-} and STAT6^{-/-} BALB/c mice compared to BALB/c WT mice (Fig. 4A and 4C). The absolute numbers of IFN- γ ⁺ TNF- α ⁺ IL-2⁺ K^dGag₁₉₇₋₂₀₅ specific CD8⁺ T cells were also enhanced in the gene knockout mice (Fig. 4B). Furthermore, in the absence of CD8 blocking the amount of tetramer dissociation that occurred on WT K^dGag₁₉₇₋₂₀₅ specific CD8⁺ T cells was greater than that of the gene knockout mice following vaccination (Fig. 4D). Collectively these findings are consistent with previous reports, which have shown that greater proportion of high avidity anti-viral CD8⁺ T cells express TNF- α and IL-2 in addition to IFN- γ compared to low avidity anti-viral CD8⁺ T cells [33].

Significant reduction in the proportion of IFN- γ producing CD8⁺ T cells that also expressed TNF- α and IL-2 was observed in all vaccinated animals when CD8 availability for splenocytes was blocked during K^dGag₁₉₇₋₂₀₅ peptide stimulation (Fig. 4C). CD8 blocking of splenocytes from all vaccinated mice also reduced the amount of K^dGag₁₉₇₋₂₀₅ tetramer engagement by CD8⁺ T cells during tetramer dissociation (Fig. 4D). Furthermore, statistically significant enhancement in K^dGag₁₉₇₋₂₀₅ tetramer

dissociation of CD8⁺ T cells from WT mice was not observed relative to the gene knockout mice when CD8 was blocked on splenocytes during tetramer dissociation (Fig. 4D). Similarly, statistically significant enhancement in the proportions of IFN- γ ⁺ K^dGag₁₉₇₋₂₀₅ CD8⁺ T cells that produced TNF- α and IL-2 was not observed in vaccinated gene knockout mice relative to WT mice when CD8 was blocked on splenocytes during K^dGag₁₉₇₋₂₀₅ peptide stimulation (Fig. 4D). Thus, up-regulation of CD8 expression levels on K^dGag₁₉₇₋₂₀₅ CD8⁺ T cells from gene knockout mice appeared to be important in enhancing avidity and polyfunctionality.

4. Discussion

Due to the disappointing outcomes of numerous HIV-1 vaccine trials conducted thus far, development of more efficacious HIV-1 vaccines is a necessity for slowing the global progression of HIV-1. This requires the fundamental understanding of mechanisms that generate favourable immune outcomes following vaccination. In previous studies, we have shown that following HIV-1 recombinant pox viral prime-boost vaccination, IL-4 and IL-13 can dampen the avidity of HIV-specific CD8⁺ T cells [24, 27]. In this study, we report that IL-4 and IL-13 can dampen CD8 expression levels on anti-viral CD8⁺ T cells following HIV-1 recombinant pox viral prime-boost vaccination. Furthermore, we also report that reduced expression levels of CD8 was a feature of low avidity anti-viral CD8⁺ T cells that developed in various pox-viral prime-boost vaccination settings.

Our data suggested that IL-4 and IL-13 selectively regulated the expression levels of CD8, but not other T cell avidity markers such as TCR, CD2, CD11a and lck on anti-viral CD8⁺ T cells following recombinant HIV-1 pox viral prime-boost vaccination. To our knowledge, this is the first report to show the involvement of IL-13 in regulating CD8 expression levels on anti-viral CD8⁺ T cells. Previously, Xiao *et al* [34] have shown that CD8 densities are down-regulated on anti-viral CD8⁺ T cells following cognate antigen encounter during pox virus infections. In Wijesundara *et al* [26], we have shown that naïve CD8⁺ T cells in the absence of IL-4 and IL-13 signalling (i.e. in IL-4^{-/-}, IL-13^{-/-} and STAT6^{-/-} mice) express lower levels of IL-4 receptor α making them less responsive to these cytokines during T cell priming. Therefore, we propose that IL-4/IL-13 signalling through activation of STAT6 promotes greater down-regulation of CD8 densities on naïve CD8⁺ T cells following cognate antigen encounter

during pox virus infection and vaccination. In agreement with this proposed mechanism, previous reports showing that IL-4 exposure during naïve T cell priming can down-regulate CD8 expression levels to reduce the anti-viral capacity of effector CD8⁺ T cells [35-36]. Interestingly, type I interferons (IFN-I) have also been shown to reduce CD8 expression levels and avidity of anti-viral CD8⁺ T cells [34]. Therefore, it would be of interest to also investigate whether IFN-I cooperate synergistically with IL-4 and/or IL-13 in down-regulating CD8 expression levels on anti-viral CD8⁺ T cells following pox virus infection or vaccination.

Belyakov *et al* [37-38] have shown that mucosal vaccination strategies are efficient in inducing high avidity anti-viral CD8⁺ T cells particularly at tissues closer to the sites of immunization. Our initial studies have shown that mucosal i.n./i.m. HIV-1 recombinant pox viral vaccination promotes the development of high avidity HIV-specific CD8⁺ T cells that produce low levels of IL-4 and IL-13 at both mucosal and systemic compartments [25]. In the current study mucosal HIV-1 recombinant pox viral vaccination strategies were found to enhance CD8 expression levels on HIV-specific CD8⁺ T cells, which correlated with T cell avidity. Several other studies, including ours have shown that mucosal vaccination strategies can induce robust CD8⁺ T cell responses that can protect macaques against simian immunodeficiency virus challenge [39-41]. Our recent work [24] and the current study collectively show that mucosal vaccination strategies that temporarily inhibit IL-13 function (i.e. IL-13R α 2 adjuvanted vaccines) can enhance CD8 densities and avidity of HIV-specific CD8⁺ T cells with better protective outcomes. These findings are consistent with Ahlers *et al* [23] study showing that immunization of mice with peptide vaccine constructs in conjunction with granulocyte macrophage colony stimulating factor and IL-13R α 2-Fc antibodies can

enhance the cytotoxic activity of HIV-specific CD8⁺ T cells and protection. Our recent studies demonstrated that the excellent quality HIV-specific CD8⁺ T cells generated following mucosal vaccination could be further improved when IL-4 and IL-13 functions are inhibited at the mucosae [24]. Interestingly, Isakov *et al* [42] have shown that IL-15 plays an important role in enhancing avidity of anti-viral CD8⁺ T cells following mucosal immunization, which is consistent with Oh *et al* [43] where IL-15 was shown to enhance CD8 densities and avidity of anti-viral CD8⁺ T cells. Therefore, development of vaccines that prevent significant down-regulation of CD8 densities on antigen-specific CD8⁺ T cells offer exciting prospects for developing efficacious vaccines against intracellular mucosal pathogens such as HIV-1.

The establishment of polyfunctional anti-viral CD8⁺ T cells have been shown to correlate with resistance to HIV-1 acquisition in elite controllers of HIV-1 infection [10, 18-19, 44]. Even though polyfunctionality correlates with avidity of anti-viral CD8⁺ T cells [10, 33], regulation of molecules that can affect both T cell avidity and polyfunctionality is not well understood. In the current study, enhancement in CD8 expression levels highly correlated with enhancement in avidity and polyfunctionality of HIV-specific CD8⁺ T cells following pox viral vaccination. Furthermore, blocking CD8 availability significantly reduced the polyfunctional capacity and avidity of HIV-specific CD8⁺ T cells. Similar findings have been obtained on polyclonal influenza-specific CD8⁺ T cells following influenza virus infection of mice [33]. Thus, monitoring CD8 expression levels could be used as a predictor of both avidity and polyfunctional capacity of anti-viral CD8⁺ T cells. The caveat here is that IFN- γ , TNF- α and IL-2 production by lymphocytic choriomeningitis virus (LCMV)-specific monoclonal P14 transgenic CD8⁺ T cells can occur independently of CD8, especially during later stages

of LCMV infection [14, 45]. Therefore, it is likely that according to different viruses and/or the infection state (i.e. acute or chronic) multiple redundant mechanisms may be involved in regulating polyfunctionality of anti-viral CD8⁺ T cells. These mechanisms may include regulation of lipid raft formation [46] and molecules such as TCR [47], IL-4 receptor α [26], lck [14] and IL-15 receptor α [43] on CD8⁺ T cells.

Most vaccination strategies against HIV-1 to date have mainly focused in the induction of either neutralizing antibodies or CD8⁺ T cell responses against HIV-1. However, the induction of heterosubtypic immunity (both neutralizing antibody and CD8⁺ T cell responses) is expected to be beneficial in generating optimal protective immunity against HIV-1 [2]. We have now shown that HIV-1 recombinant pox viral vaccination strategies that inhibit IL-13 function can enhance the quality of anti-viral CD8⁺ T cell immunity most likely through up-regulation of CD8 expression levels on anti-viral CD8⁺ T cells [24]. We anticipate that such strategies could also enhance the quality of antibody immunity, as enhanced IgE production has been reported to correlate with enhanced pathogenesis of HIV-1 infections and cytokines such as IL-4 and IL-13 are known to mediate isotype switching of antibodies to IgE [48-50].

In conclusion, we have demonstrated that IL-4/IL-13 can mediate the down-regulation of CD8 expression levels and dampen the avidity/polyfunctionality of anti-viral CD8⁺ T cells following vaccination. We have also shown that HIV-1 recombinant pox viral mucosal vaccination strategies and recombinant pox viral vaccines that can transiently inhibit IL-13 activity at the vaccination site can enhance CD8 expression levels and avidity of anti-viral CD8⁺ T cells. We propose that in the absence of IL-4 and IL-13 signalling, anti-viral CD8⁺ T cells express higher amounts of CD8 allowing these cells

to build high avidity interactions with cognate pMHC-I complexes presented on virus infected cells. This should facilitate high avidity CD8⁺ T cells to maintain more durable interactions with virus-infected cells allowing them to exert effector functions efficiently. Thus, we believe that vaccine strategies that inhibit IL-4/IL-13 activity offer great promise for future vaccines against many chronic infections including HIV-1 that require high avidity CD8⁺ T cells for protective immunity.

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Figure captions

Fig. 1. IL-4 and IL-13 dampen CD8 densities on anti-viral CD8⁺ T cells following recombinant HIV-1 vaccination and VV-WR infection of mice. (A) Representative histogram plots showing expression of the indicated T cell avidity markers on K^dGag₁₉₇₋₂₀₅ tetramer specific CD8⁺ splenocytes obtained from WT, IL-13^{-/-}, IL-4^{-/-} and STAT6^{-/-} BALB/c mice prime-boost vaccinated i.n FPV-HIV/ i.m.VV-HIV 14 days apart. (B and C) MFI representing the expression of the indicated markers (B) or CD8β:CD8α expression ratio (C) calculated using MFI values on K^dGag₁₉₇₋₂₀₅ tetramer specific CD8⁺ splenocytes from mice (n = 4) prime-boost vaccinated in (A). (D) MFI representing the expression of CD8α on K^dA52₇₅₋₈₃ tetramer specific CD8⁺ splenocytes from mice (n = 6) of indicated genetic background infected i.p. for 7 days with VV-WR. Statistical significance of the data from B-D is shown relative to BALB/c WT mice using one-way ANOVA (Dunnett's multiple comparison post-hoc test). (E) MFI representing CD8α expression on K^dGag₁₉₇₋₂₀₅ tetramer specific CD8⁺ splenocytes from BALB/c WT mice (n = 4) prime-boost vaccinated i.n./i.n., i.n./i.m. or i.m./i.m. 14 days apart with FPV-HIV/VV-HIV. Statistical significance of the data was calculated using one-way ANOVA (Tukey's multiple comparison post-hoc test). (F) MFI representing CD8α expression on K^dGag₁₉₇₋₂₀₅ tetramer specific CD8⁺ splenocytes from BALB/c WT mice (n = 4) prime-boost vaccinated i.n./i.m. with FPV-HIV/VV-HIV (control) or FPV-HIV-IL-13Rα2/VV-HIV-IL-13Rα2 (IL-13 inhibitor vaccine). Statistical significance of the data was calculated using a student's unpaired t-test. Data presented in this entire figure is representative of at least two independent experiments and the error bars depict the SEM. ns – p > 0.05; * - p < 0.05; ** - p < 0.01; *** - p < 0.001.

Fig. 2. Enhancement of CD8 densities correlate with enhancement in cytokine polyfunctionality of HIV-specific CD8⁺ T cells from recombinant HIV-1 prime-boost vaccinated mice. RBC-depleted splenocytes from i.n. FPV-HIV/i.m. VV-HIV (14 days apart) prime-boost vaccinated BALB/c WT mice (n = 4) were stimulated with 0.1 µg/ml of K^dGag₁₉₇₋₂₀₅ peptide and analysed for expression of CD8α, CD8β and IFN-γ on cytokine producing CD8⁺ T cells. A, Representative dot plots from a peptide unstimulated or stimulated culture where IFN-γ⁺ TNF-α⁻ IL-2⁻, IFN-γ⁺ TNF-α⁺ IL-2⁻ and IFN-γ⁺ TNF-α⁺ IL-2⁺ CD8⁺ T cells were gated for analysis. (B) Representative histogram plots showing the expression of CD8α, CD8β and IFN-γ on different polyfunctional populations of K^dGag₁₉₇₋₂₀₅ specific CD8⁺ T cells. (C) MFI values representing the expression of the indicated proteins on different polyfunctional populations of K^dGag₁₉₇₋₂₀₅ specific CD8⁺ T cells. Each line is representative of an individual mouse from a total of four mice examined. Data presented in this figure is representative of at least two independent experiments. A paired t-test was used to determine the statistical significance of the data. ns – p > 0.05; * - p < 0.05; ** - p < 0.01; *** - p < 0.001.

Fig. 3. CD8 is required by K^dGag₁₉₇₋₂₀₅ specific CD8⁺ T cells to optimally engage with K^dGag₁₉₇₋₂₀₅ tetramer. Splenocytes obtained from i.n. FPV-HIV/i.m. VV-HIV (14 days apart) prime-boost vaccinated BALB/c WT mice (n = 4) were incubated with varying concentrations of purified anti-CD8β.2 prior to staining with K^dGag₁₉₇₋₂₀₅ tetramer. Representative histogram plot (A) or mean (n = 4) percentage of maximum K^dGag₁₉₇₋₂₀₅ tetramer binding (B) on K^dGag₁₉₇₋₂₀₅ tetramer specific CD8⁺ splenocytes is shown. The numbers in the histogram plot represents the concentration (μg/ml) of anti-CD8β.2 used for CD8 blocking. The data is representative of three independent experiments and the error bars depict the SEM.

Fig. 4. IL-4 and IL-13 can regulate CD8 expression levels and modulate T cell quality of K^dGag₁₉₇₋₂₀₅ specific CD8⁺ T cells following recombinant HIV-1 prime-boost vaccination. WT, IL-13^{-/-}, IL-4^{-/-} and STAT6^{-/-} BALB/c mice (n = 5-8) were i.n. FPV-HIV/i.m. VV-HIV (14 days apart) prime-boost vaccinated. Splenocytes from these mice with (1 µg/ml anti-CD8β.2) or without (mock) CD8 blocking were stimulated using 0.1 µg/ml of K^dGag₁₉₇₋₂₀₅ peptide for ICS or stained using K^dGag₁₉₇₋₂₀₅ tetramer for tetramer dissociation. (A) Representative dot plots showing TNF-α and IL-2 production on gated IFN-γ⁺ CD8⁺ splenocytes from vaccinated mice that were stimulated with K^dGag₁₉₇₋₂₀₅ peptide in the absence of CD8 blocking. (B) Number of IFN-γ⁺ TNF-α⁺ IL-2⁺ K^dGag₁₉₇₋₂₀₅ specific CD8⁺ T cells that developed in vaccinated mice (n = 5). (C) The proportion of IFN-γ⁺ CD8⁺ splenocytes that also produced TNF-α and IL-2 in the absence or presence of CD8 blocking of splenocytes during K^dGag₁₉₇₋₂₀₅ peptide stimulation. (D) Percentage of maximum K^dGag₁₉₇₋₂₀₅ tetramer binding of CD8⁺ T cells following tetramer dissociation for 60 minutes in the absence or presence of CD8 blocking (n = 8). The data in this figure is representative of 2-3 independent experiments and the error bars depict the SEM. Statistical significance of the data was calculated using a one-way ANOVA relative to WT mice (Dunnett's multiple comparison post-hoc test). ns- p > 0.05; * - p < 0.05; ** - p < 0.01.

Figure 1

Figure 2

Figure 3

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B

Figure 4