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Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor Expression, a Biomarker of Memory CD8$^+$ T Cell Immunity and Vaccine Efficacy

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Abstract

Interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) exhibit overlapping activities and are produced by activated T cells. In this study, the role of IL-3 and GM-CSF in CD8$^+$ T cells were assessed following HIV-1 prime-boost immunization. Data indicate that the expression of IL-3/GM-CSF by HIV-specific effector CD8$^+$ T cells is vaccine delivery route and time dependent, where purely systemic, intramuscular i.m./i.m. vaccination induce elevated levels of IL-3 in HIV-specific CD8$^+$ T cells compared to purely mucosal, intranasal i.n./i.n. immunization. Interestingly, the GM-CSF expression was optimal only following i.n./i.m. delivery. Data also revealed that peak IL-3 mRNA and protein expression in CD8$^+$ T cells were detected 16-20 h of $K^dGag_{197-205}$ peptide stimulation, whereas the expression kinetics of GM-CSF was similar to IFN-γ. Next the IL-3 and GM-CSF expression in HIV-specific CD8$^+$ T cells were assessed at acute, effector and memory stages of immunity using i.n./i.m. delivery of FPV-HIV/VV-HIV control vaccine compared to a novel IL-13Rα2 adjuvanted HIV-vaccine (FPV-HIV IL-13Rα2/VV-HIV IL-13Rα) that has shown to induce excellent high avidity CD8$^+$ T cells with greater protective immunity. The IL-13Rα2 adjuvanted vaccine induced greatly elevated HIV-specific memory CD8$^+$IL-3$^+$ and also CD8$^+$IL-3$^+$IFN-γ$^+$ T cells compared to the control vaccine, where the expression in memory phase was greater than effector T cells. Both the control and IL-13Rα2 adjuvanted vaccines, elicited elevated but similar numbers of antigen-specific GM-CSF$^+$ memory CD8$^+$ T cells. Data suggest that induction of both IL-3 and GM-CSF play a role in maintenance of antigen-specific memory CD8$^+$ T cells which is linked to better protective immunity. These results also demonstrate that route of delivery, time post vaccination, expression kinetics/ length of antigen exposure, should not be neglected when evaluating the vaccine efficacy. Also IL-3/GM-CSF expression by memory CD8$^+$ T cells could be a biomarker of protective immunity.

Keywords: IL-3/GM-CSF; HIV vaccines; Memory CD8$^+$ T cells; IL-13Rα2; Poxvirus prime-boost

Introduction

IL-3 is a hematopoietic growth factor that stimulates bone marrow progenitor cell proliferation and differentiation of granulocytes, monocytes, megakaryocytes and erythrocytes [1-3]. IL-3 exhibits overlapping activities with GM-CSF, which plays an important role in the activation and survival of granulocytes and macrophages [4-6]. The use of IL-3/GM-CSF in treatment of different bone marrow conditions or hematopoietic progenitor cell transplantation is well characterized [7-9]. IL-3 and GM-CSF are also detected in activated T cells during inflammation or immune responses to infections/ vaccination [10,11]. A recent study has shown that IL-3 can modulate the development of regulatory T cells and enhance the activity of anti-inflammatory cytokine IFN-γ in an arthritis model [12]. Also IL-3/IL-3R signalling has demonstrated to be critical for the recruitment of basophils to draining lymph nodes following helminth infection [13]. Moreover, the use of IL-3 and GM-CSF as molecular adjuvants to enhance immunity to vaccine antigens has been well documented [14-16].

Currently, majority of the vaccines (i.e. HIV, TB, Malaria) that have induced good immunity in animal models have failed to induce immunity in humans [17-20]. Therefore, more and more studies are indicating that following vaccination rather than only measuring IFN-γ expression, evaluation of poly-functional CD4/CD8 T cells can be a better predictor of vaccine efficacy [21-24]. Thus, it is thought that identifying novel biomarkers/ factors that can help determine the protective efficacy of a vaccine could be of great importance. Our previous studies have demonstrated that following systemic HIV-1 prime-boost vaccination, elevated IL-3 levels can be detected in antibody arrays following $K^dGag_{197-205}$ peptide stimulation and IL-3 levels being second highest compared to IFN-γ [11]. Therefore, in this study firstly we have evaluated the delivery route dependent expression profile of IL-3 and GM-CSF in $K^dGag_{197-205}$-specific CD8$^+$ T cells and the expression kinetics after different lengths of *in-vitro* peptide stimulation, following FPV-HIV/VV-HIV systemic and/or mucosal prime-boost immunization. Secondly, the IL-3 and GM-CSF expression profiles on HIV-specific CD8$^+$ T cells were evaluated at acute, effector and memory phases of immunity using two different vaccines i) a novel IL-13 inhibitor vaccine (FPV-HIV IL-13Rα2 /VV-HIV IL-13Rα2) that has shown to generates high avidity CD8$^+$ T cells with greater protective immunity [25] compared to the control FPV-HIV/VV-HIV vaccine.

Methods

Immunization of mice and preparation of lymphocytes

Pathogen free 6-8 weeks old female BALB/c mice were obtained

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from Australian National University (ANU) animal breeding facility or from Animal Resource Centre (ARC), Perth, Western Australia, maintained and used in accordance with ANU animal experimentation and ethics approved guidelines. The two vaccines used in this study were FPV-HIV/VV-HIV and FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2. These recombinant viruses were constructed as described previously [25,26-28].

BALB/c mice (5 per group) were primed with FPV-HIV and boosted with VV-HIV or primed with FPV-HIV IL-13Rα2 (1×10^7 pfu) and boosted with VV-HIV IL-13Rα2, using combined mucosal/systemic (intranasal (i.n.)/intramuscular (i.m.)), purely systemic immunization (i.m./i.m.) or purely mucosal immunization (i.n./i.n.) regimes. The recombinant viruses were given intranasal to each mouse in a final volume of 20 µl (10 µl per nostril) whereas intramuscular immunizations were carried out in a 100 µl (50 µl per leg (quadriceps muscle)) volume. Before each immunization the virus was diluted in sterile PBS and sonicated to obtain a homogenous viral suspension. Mice were sacrificed at different time intervals (3 days, 14 days or 8 weeks respectively) post-boost immunization and systemic T cell responses were measured in splenocytes cell suspensions prepared in complete RPMI.

Tetramer staining

Allophycocyanin (APC) conjugated H-2K^{d}Gag AMQMLKETI MHC class I restricted tetramer was synthesized at the Bio-Molecular Resource Facility, JCSMR. The tetramer staining was performed as described previously [11,29,30]. Briefly, 2×10^6 splenocytes were stained with Fluorescein isothiocynate (FITC) conjugated anti-CD8 antibody (BD Pharmingen) and APC- conjugated tetramer in FACS buffer, and cells were incubated at room temperature (RT), in the dark for 40 min. Samples were run on a four-color FACS Calibur Flow Cytometer (Becton Dickinson) until 100,000 events were acquired and were then analyzed on FACS Calibur flow cytometer (Becton-Dickinson) using Cell Quest Pro analysis software.

Intracellular Cytokine Staining (ICS) of IFN-γ, IL-3 and GM-CSF

2×10^6 splenocytes were stimulated for 16 hours in the presence of H-2K^d ^{197}AMQMLKETI^{205}, 9 mer gag peptide and then cells were further incubated for 4 hours in presence of brefeldin A, as described previously [11,29,30]. To study kinetics of cytokine expression, for some experiments cells were stimulated initially for 3-4 hours in presence of peptide and then for further 2 hours in presence of brefeldin A. Following stimulation cells were surface stained with anti-CD8 APC (BD Pharmingen) in FACS buffer for 30 min at 4°C, fixed and permeabilised before staining with anti-mouse IFN-γ FITC (eBiosciences) and anti-IL-3 PE (Biolegend) or with anti- GM-CSF FITC (eBiosciences) and anti-IL-3 PE. Samples were run on a four-color FACS Calibur Flow Cytometer (Becton Dickinson) until 100,000 gated events were acquired. Data were analyzed using Cell Quest Pro analysis software. During analysis unstimulated cells from each sample were used as the background control and where appropriate these values were subtracted from each sample before plotting the data.

Enzyme Linked Immunosorbent Assay (ELISA)

Mouse IL-3 ELISA Max™ Deluxe kit (Biolegend) was used to determine the "amount" of IL-3 produced by HIV-specific CD8$^+$ T cells. Firstly the IL-3 standards were prepared by serial dilutions of assay diluent two-fold to obtain 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, 7.8 pg/ml, 3.9 pg/ml, 2.0 pg/ml concentrations. For experimental samples, 2×10^6 cells were stimulated in the presence of ^{197}AMQMLKETI^{205}, 9 mer gag peptide for 16 hrs, samples were centrifuged and supernatants were collected. To perform ELISA, 1:200 diluted mouse IL-3 specific monoclonal capture antibody was coated on to 96-well ELISA plates overnight at 4°C. Plates were washed with wash buffer, blocked with 200 µl 1x assay diluents and incubated for 1 hr at RT on a plate shaker. Following incubation, plates were washed and standards and experimental samples (100 µl/well) were loaded in duplicates and were incubated at RT for 2 hrs with gentle shaking, followed by 100 µl/well of pre-titrated Biotinylated detection antibody (1:200 in 1x assay diluent) for1 hr at RT, 100 µl of Avidin-HRP solution (1:1000) for 30 min at RT and finally 100 µl of freshly prepared TMB substrate solution for 15 min in the dark. In between reactions plates were washed 3-4 times with PBS-Tween. The reaction was stopped by adding 100 µl of stop solution to each well. Absorbance was then read at 450 nm and 570 nm using micro-plate reader with Softmax Pro software.

Cytokine antibody array

Cytokine antibody arrays were performed according to manufactures instructions (Ray Biotech Inc., USA). Briefly, 2×10^6 splenocytes were cultured in complete RPMI for 16-20 h in the presence of ^{197}AMQMLKETI^{205}, 9 mer gag peptide. Following stimulation, supernatants were collected and arrays were performed as described previously [11,31]. Protein expression signal intensities were calculated as a percentage absorbance, normalized against the positive controls on the membrane using Multi Gauge V3.0 software density linear calibration analysis (A–B/mm^2; where A is the average absorbance of the cytokine, B is the average background absorbance, mm is the average area).

mRNA analysis

CD8$^+$ T cells were negatively isolated and stimulated with ^{197}AMQMLKETI^{205}, 9 mer gag peptide for different time intervals, mRNA was prepared and cDNA was synthesized and real-time PCR was performed. The mRNA analysis was performed exactly as described in Ranasinghe et al. [30].

Statistical analysis

The *p* values were calculated by performing two-tailed unpaired Student's *t*-test using GraphPad Prism. For ELISA data, Prism nonlinear regression analysis was used to calculate the cytokine concentrations from a standard curve. Other than where stated the experiments were repeated at least three times.

Results

IFN-γ and IL-3 expression kinetics following K^{d}Gag$_{197\text{-}205}$ peptide stimulation

Our studies have revealed that following i.n./i.m. prime-boost immunization, cytokine mRNA and protein expression profiles in HIV-specific CD8$^+$ T cells can differ considerably according to the length of peptide stimulation (Figures 1A and 1B). Consistent with other findings the pattern of both IL-2 and TNF-α mRNA expression was found to be similar and the highest expression was detected after 3-4 hrs of K^{d}Gag$_{197\text{-}205}$ peptide stimulation (Figure 1A). In contrast, the highest IFN-γ, IL-3 and GM-CSF mRNA expression levels were detected after 15 hr of peptide encounter, no IL-3 mRNA was detected after 3-4 h of K^{d}Gag$_{197\text{-}205}$ peptide stimulation (Figure 1A). When the kinetics of IL-3 protein expression was assessed in effector CD8$^+$ T

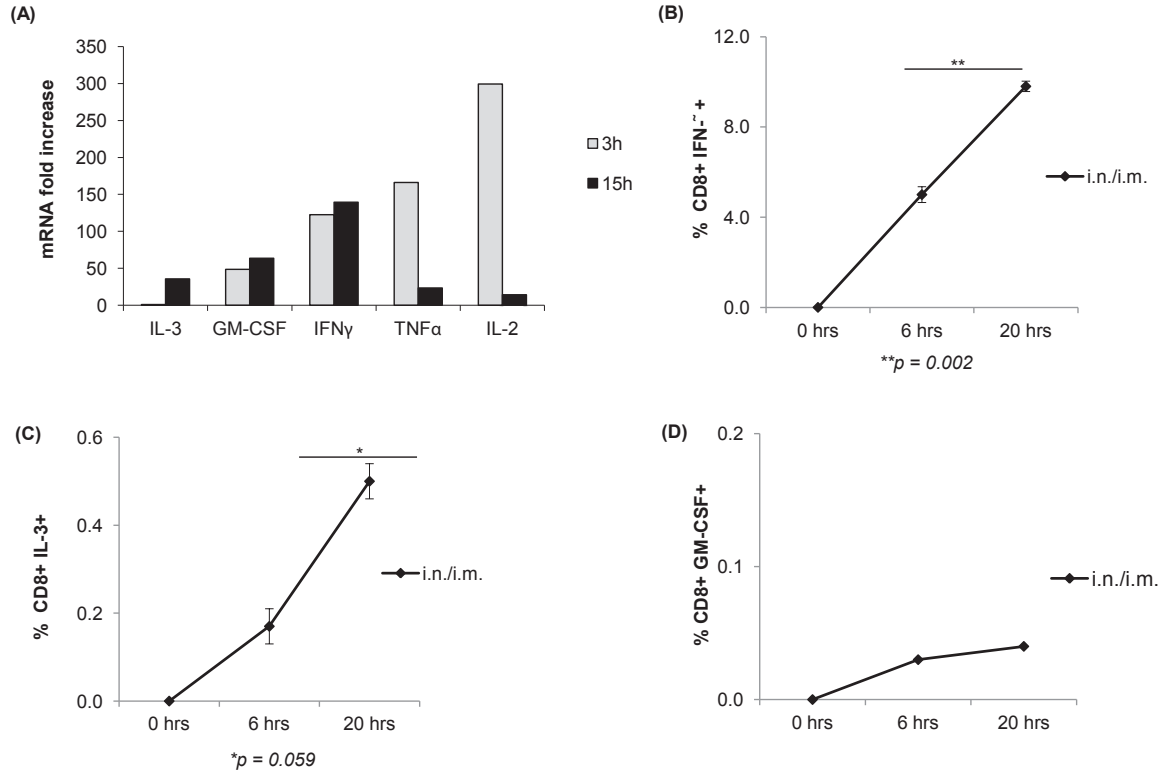


Figure 1: Cytokine expression kinetics in $K^dGag_{197-205}$-specific CD8+ T cells.
(A) IL-3, GM-CSF, IFN-γ, TNF-α and IL-2 mRNA fold increase in $K^dGag_{197-205}$ peptide stimulated and unstimulated cells were evaluated at 3 hrs and 15 hrs as indicated in methods. (B-D) Two weeks following i.n./i.m. FPV-HIV/VV-HIV prime-boost immunization, splenocytes from BALB/c mice (n=4) were stimulated with $K^dGag_{197-205}$ peptide for 6 hrs and 20 hrs. The kinetics of (B) IFN-γ and (C) IL-3 and (D) GM-CSF protein production was determined by ICS at different time intervals (0 hr, 6 hr and 20 hr). Data represent mean values and ± standard error of the mean. Data were representative of two experiments and p values were calculated using student unpaired t-test.

cells using intracellular cytokine staining, the data confirmed that the highest HIV-specific $CD8^+IL\text{-}3^+$ were detected following 20 h of peptide stimulation (Figure 1C). Unlike at the mRNA expression profile, the protein expression kinetics by HIV-specific $CD8^+$ T cells was similar between the three cytokine groups at 6 and 20 h post $K^dGag_{197-205}$-specific peptide stimulation (Figures 1B-1D). However, compared to IL-3 very low levels of GM-CSF protein was detected in HIV-specific effector $CD8^+$ T cells (Figure 1D).

Enumeration of effector $K^dGag_{197-205}$-specific $CD8^+IL\text{-}3^+$ T cells following systemic immunization

Following FPV-HIV/VV-HIV prime-boost immunization, firstly the $K^dGag_{197-205}$-specific $CD8^+$ T cell responses generated following systemic and mucosal immunization regimes were evaluated. Consistent with our previous findings [26,27] comparison to purely mucosal (i.n./i.n.) and purely systemic immunization regime (i.m./i.m.), combination mucosal and systemic (i.n./i.m.) immunization regime induced the highest percentage of $K^dGag_{197-205}$-specific $CD8^+$ T cells as measured by tetramer staining (Figure 2A). The IL-3 cytokine profile in $K^dGag_{197-205}$-specific effector $CD8^+$ T cells were then assessed and compared to IFN-γ following 20 h of $K^dGag_{197-205}$ peptide stimulation. Interestingly, both IFN-γ expression and IL-3 production by HIV-specific systemic $CD8^+$ T cells were found to be vaccine delivery route dependent, showing a hierarchical expression profile of i.n./i.m. ≥ i.m./i.m. > i.n./i.n (Figures 2B and 2C).

To further evaluate vaccine delivery route dependent IL-3 protein production in $K^dGag_{197-205}$–specific $CD8^+$ T cells, the quantity of IL-3 produced in $K^dGag_{197-205}$ peptide stimulated cell supernatants were evaluated using a mouse IL-3 ELISA. The data shows that the purely systemic immunization (i.m./i.m.) regime induced the highest concentration of IL-3 (324.5 pg/ml) compared to the other two strategies (Figures 3A and 3B). The concentrations of IL-3 measured by IL-3 ELISA were in the order of i.m./i.m. > i.n./i.m > i.n./i.n (Figures 3A and 3B). This immunization regime dependent IL-3 expression was further substantiated by the cytokine antibody array analysis where elevated IL-3 levels were associated with systemic immunization (Figure 3C). At 14 days post booster immunization, compared to IL-3 the number of effector $K^dGag_{197-205}$-specific $CD8^+$ T cells expressing GM-CSF was very low. However out of the three groups tested, i.n./i.m. immunized group showed the highest GM-CSF expression compared to the other two regimes. Interestingly, antibody array data further substantiated these findings eliciting a GM-CSF protein expression profile in the order of i.n./i.m > i.n./i.n. > i.m./i.m (Figure 3D). Taken together the above findings, data indicated out of the three delivery routes tested, only i.n./.m. delivery could induced the expression of both IL-3 and GM-CSF by HIV-specific $CD8^+$ T cells.

Evaluation of immune responses at acute, effector and memory stages of immunity

IL-3 and GM-CSF cytokine expression by HIV-specific $CD8^+$ T cells were then evaluated over time, following a novel IL-13Rα2

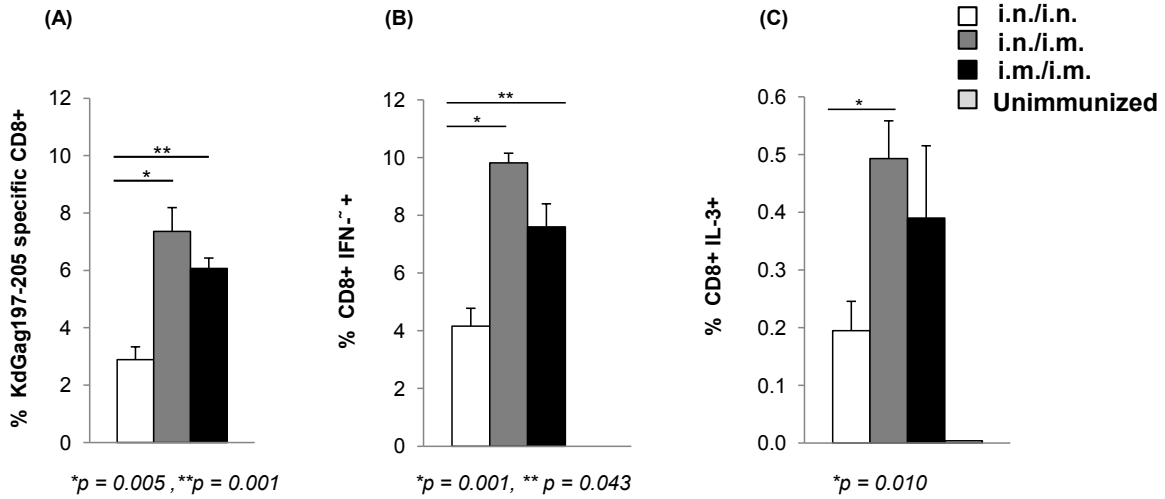
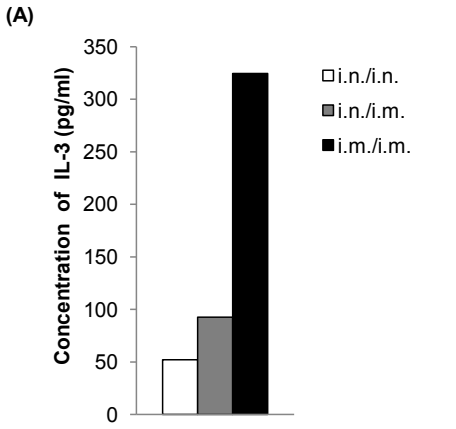


Figure 2: Immunization regime dependent $K^dGag_{197-205}$-specific $CD8^+$ T cell responses, following FPV-HIV/VV-HIV prime-boost immunization. BALB/c mice (n=4 per group) were immunized i.n./i.n. (white bar), i.n./i.m. (grey bar) and i.m./i.m. (black bar) with FPV-HIV/VV-HIV, unimmunized mice were kept as a control. At 14 days post-boost immunization, (A) percentages of $K^dGag_{197-205}$-specific $CD8^+$ T cells were measured using tetramer staining as indicated in methods. (B) IFN-γ and (C) IL-3 expression by splenic $CD8^+$ T cells were measured by intracellular cytokine staining. Splenocytes were stimulated with $K^dGag_{197-205}$ peptide, unstimulated cells from each sample were used as background controls and this value was subtracted from each sample before plotting graph. Data are representative three experiments. The data represent mean plus standard error of mean and p values were calculated using student unpaired t-test.



(B)

Vaccine delivery route	Absorbance (nm)	IL-3 Concentration (pg/ml)
i.n./i.n	0.419	52.032
i.n./i.m.	0.632	92.636
i.m./i.m.	1.552	324.52

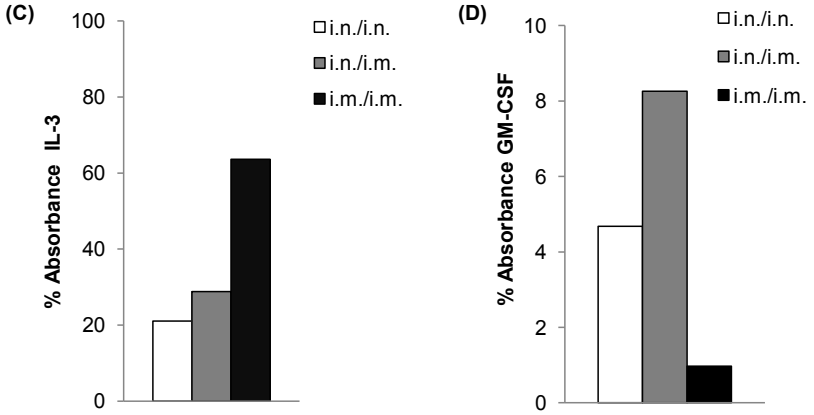


Figure 3: Immunization regime dependent IL-3 and GM-CSF production by effector $CD8^+$ T cells. Two weeks following i.n./i.n., i.n./i.m, and i.m./i.m. prime-boost immunization, 2×10^6 cells from pooled spleens (n=4 per group) were cultured overnight in complete RPMI, in the presence of $K^dGag_{197-205}$ peptide. Supernatants were then collected and assessed for IL-3 cytokine production, (A and B) represent the amount of IL-3 produced by HIV-specific $CD8^+$ T cells as measured by IL-3 ELISA. Data represents pooled mean value for each immunization regime. The graphs (C and D) indicate IL-3 and GM-CSF percentage absorbance respectively, measured by antibody arrays as described in methods.

adjuvanated prime-boost immunization strategy (i.n. FPV-HIV IL-13Rα2/i.m. VV-HIV IL-13Rα2) that has been shown to induce high avidity CD8+ T cells in comparison with the i.n. FPV-HIV/i.m. VV-HIV immunization strategy tested above [25]. Consistent with our previous finding [25], results revealed that FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 immunization induced significantly elevated effector and memory $K^dGag_{197-205}$-tetramer reactive CD8+ T cells and also CD8+IFN-γ+ T cells *(*p=0.0013 and **p=0.0001 respectively)* when compared to the control FPV-HIV/VV-HIV immunization (Figures 4A and 4B). In general the $K^dGag_{197-205}$-specific CD8+ T cell numbers were highest at the effector stage of immunity compared to the memory stage. Strikingly, even though the peak IFN-γ expression was detected at effector stage of immunity, the peak IL-3 expression was detected at memory stage of immunity (Figure 4C) effector stage *vs.* memory stage, *#p=0.038*. Moreover, mice that received IL-13Rα2 adjuvanated vaccine elicited enhanced CD8+IL-3+ T cells numbers, compared to the

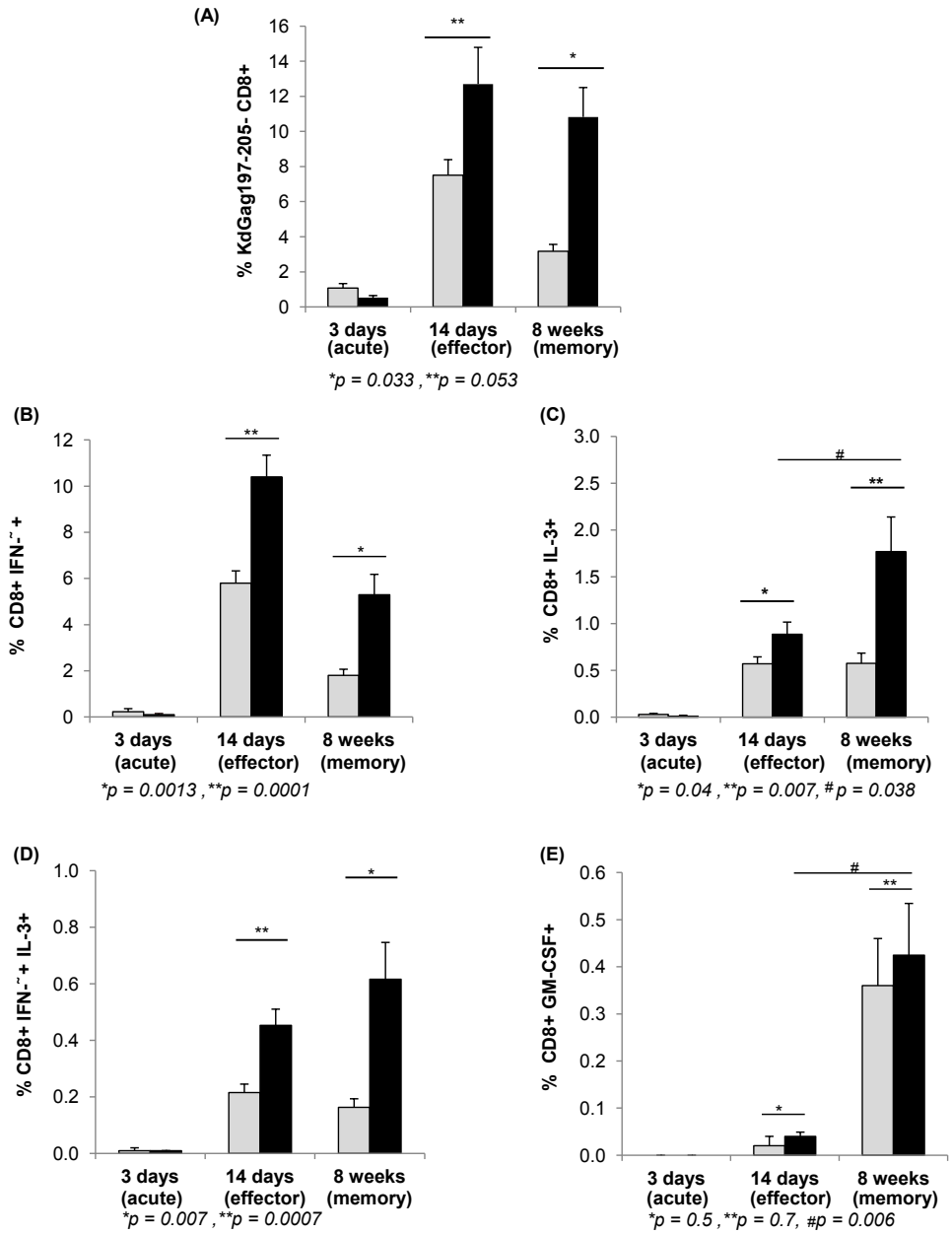


Figure 4: $K^dGag_{197-205}$-specific CD8+ T cell responses at acute, effector and memory stage of immunity.
BALB/c mice (n=5 per group) were immunized i.n./i.m. with FPV-HIV/VV-HIV (grey bar) and FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (black bar). At 3 days (acute stage), 14 days (effector stage) and 8 weeks (memory stage) post-boost immunization, (A) percentages of $K^dGag_{197-205}$ - positive CD8+ T cells were measured by tetramer staining following FACS analysis. Following post-boost immunization, splenocytes were stimulated with $K^dGag_{197-205}$ peptide for 20 hrs and the percentages of $K^dGag_{197-205}$-specific (B) CD8+IFN-γ+ (C) CD8+ IL-3+ (D) CD8+IFN-γ+IL-3+ and (E) CD8+GM-CSF+ T cells were measured by intracellular cytokine staining. Unstimulated cells from each sample were used as a background control and this value was subtracted from each sample before plotting the data. These experiments were repeated over three times and in each graph the data represent mean of three experiments (n=15/group) plus standard error of the mean. p values were calculated using two-tailed unpaired student's t-test.

control FPV-HIV/VV-HIV immunization *(**p=0.007)* (Figure 4C). Furthermore, majority of IL-3 producing effector and memory $CD8^+$ T cells were also IFN-γ positive (Figure 4D). When the expression of GM-CSF by $K^dGag_{197-205}$-specific $CD8^+$ T cells was evaluated, following both immunization strategies the percentages of $CD8^+$GM-CSF^+ T cells were low at the acute and effector stages of immunity, however, by 8 weeks post-booster immunization significantly enhanced $CD8^+$GM-CSF^+ T cells were detected *(#p=0.006)* (Figure 4E).

Discussion

Currently, increasing number of studies are suggesting that following vaccination rather than only focusing on IFN-γ measurements, evaluating multi-functional T cells would offer better insights into the quality and protective efficacy of a vaccine [32,33]. Although often overlooked, kinetics of cytokine expression is dependent upon the length, strength, dose and/or type of antigen/mitogen encounter [34,35]. Our previous IL-2 and IFN-γ ELISpot data following immunization have also demonstrated that upon $K^dGag_{197-205}$ peptide stimulation the protein expression profiles of these two cytokines can be distinctly different and are time dependent [11]. Current data further substantiate these findings eliciting that following immunizations, the cytokine expression by $K^dGag_{197-205}$-specific $CD8^+$ T cells is heavily dependent upon the duration of *in vitro* peptide/protein stimulation. In HIV-specific $CD8^+$ T cells the peak IL-3 mRNA and protein expressions were detected at 20 hours of $K^dGag_{197-205}$ peptide stimulation. Interestingly, even though the IL-3 mRNA expression kinetic differed from GM-CSF and IFN-γ, the intracellular protein expression kinetics between the three cytokines were very similar, peaking at 20 h. The results here suggest that unlike GM-CSF or IFN-γ in HIV-specific $CD8^+$ T cells the IL-3 gene expression may mainly be regulated at the level of transcription, with an increase in the rate of transcription following T cell activation in the presence of peptide. Previous studies have shown that transcription factor c-Rel is required by T lymphocytes for the production of IL-3 and GM-CSF [36]. These c-Rel knockout mice studies have demonstrated that absence of c-Rel transcription factor results in impaired production of IL-3 and GM-CSF by T cells following mitogen stimulation [37]. NF-κB/Rel transcription factors are also known to regulate IFN-γ, IL-2, TNF-α activity by T cells [38]. Interestingly, Symeonidou and co- workers have recently shown that different parasitic infections can alter the patterns and kinetics of NF-κB activity and thus can modulate subsequent immune outcomes [39]. The above findings clearly highlight that when evaluating immunity against different vaccines, especially when assessing multi-functionality, it is important to first establish the expression kinetics (mRNA and/or protein) of cytokines, depending upon the immune environment even the closely related cytokines can render uniquely different expression profiles.

We have previously shown that the route of poxvirus prime-boost immunization (systemic *vs.* mucosal delivery) can influence the magnitude of HIV-specific IFN-γ^+CD8^+ T cells (i.n./i.m. ≥ i.m./i.m. > i.n./i.n.) and also the cytokine expression profile [11,30,31]. In this study, compared to purely mucosal i.n./i.n. immunization regime the i.n./i.m. and i.m./i.m. immunization regimes showed elevated IL-3 expression by HIV-specific $CD8^+$ T cells. Similarly, administration of *Leishmania major* antigens by different routes of delivery, has been associated with differential regulation and expression of IL-3 [40]. Thus, expression of IL-3 by T cells appears to be linked to the mode of antigen uptake/presentation by different antigen presenting cell subsets in the systemic or the mucosal compartments. It is well established that following mucosal immunization, mode of antigen uptake and presentation by microfold (M) cells and dendritic cells (DC) in the nasal-associated lymphoid tissue (NALT), is uniquely different to non mucosal tissues [41]. In the current study the optimal IL-3^+ production by HIV-specific $CD8^+$ T cells was driven mainly by systemic antigen encounter. Interestingly, we have shown that i.n. FPV HIV/i.m. VV HIV prime-boost immunization can induce high avidity mucosal and systemic $CD8^+$ T cells with better protective immunity [11,30]. Current findings demonstrate that i.n./i.m. strategy can also induce not only IL-3 but also GM-CSF expression by HIV-specific memory $CD8^+$ T cells. Thus, our results indicate that most likely whilst i.n. delivery promote the induction of initially high avidity $CD8^+$ T cell subsets [25], the i.m. delivery may promote the expansion and maintenance of a healthy memory T cell populations via the induction of IL-3 and GM-CSF. The latter is consistent with other findings where the IL-3/GM-CSF locus has shown to undergo progressive stepwise activation, throughout the course of T cell differentiation [32]. These studies have shown that the human IL-3/GM-CSF locus is epigenetically silent in immature thymocytes and is progressively activated during T cell development [42]. Our data further substantiate that immune outcomes and cytokine expression profiles by antigen-specific $CD8^+$ T cells can differ according to the route of vaccine delivery [11,29,30,43-45].

Consistent with our previous finding, the IL-13Rα2 adjuvanted vaccine induced significantly elevated effector and memory $CD8^+IFN\text{-}\gamma^+$ T cells compared to the control vaccination [25]. Moreover, the IL-13Rα2 adjuvanted vaccine induced elevated proportions of $K^dGag_{197-205}$-specific $CD8^+IL\text{-}3^+$ T cells at the memory stage of immunity where, memory stage > effector stage > acute stage. Elevated numbers of HIV-specific IFN-γ^+IL-3^+ double positive memory $CD8^+$ T cells were also detected in this group. Our previous findings have shown that this IL-13Rα2 adjuvanted vaccine can induce greater protection following intranasal influenza-$K^dGag_{197-205}$ challenge [25], while other studies have shown that elevated IFN-γ and IL-3 can be associated with protective immunity [46]. These results therefore, suggest that following FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 prime-boost immunization, IL-3 cytokine expression is most likely linked to maintenance of $CD8^+$ memory T cells and play a role in protective immunity. Recently, GM-CSF has been identified as a major $CD8^+$ T cell derived licensing factor for DC activation [47]. In our study, although there were no significant differences in the numbers of HIV-specific $CD8^+GM\text{-}CSF^+$ T cells between the control or the IL-13Rα2 adjuvanted vaccination, elevated numbers were detected at memory stage compared to acute or effector stage of immunity. As GM-CSF and IL-3 share overlapping functions, we postulate that i) IL-3/GM-CSF produced by memory $K^dGag_{197-205}$-specific $CD8^+$ T cells most likely recruit DC's where they interact with $CD8^+$ T cells and promote the production of pro-inflammatory cytokines and ii) then as a positive feedback guide memory $CD8^+$ T cell differentiation. It has been shown that in comparison to low levels of GM-CSF produced by naïve $CD8^+$ T cells, elevated GM-CSF production by antigen-specific memory $CD8^+$ T cells has the ability to stimulate DC IL-12p70 production, which drives the development of Th1 responses and protective immunity [48]. Similarly, several other studies have shown that cytokines such as IL-7, IL-15 play a fundamental role in memory T cell maintenance [49].

Collectively, our data indicate that the IL-3 expression by effector $K^dGag_{197-205}$-specific $CD8^+$ T cells is vaccine delivery route dependent (systemic > mucosal) and maximal expression of both IL-3 and GM-CSF cytokines are found after 20 h *in-vitro* peptide encounter, suggesting that these parameters should be taken into consideration when evaluating vaccine-specific immunity. Also the IL-13Rα2 adjuvanted HIV i.n./i.m. immunization (that has previously shown to induce high avidity $CD8^+$

T cells with better protective immunity), elicited significantly elevated IL-3 and GM-CSF levels in memory $K^dGag_{197-205}$-specific $CD8^+$ T cells, suggest that these cytokines play an important role in maintenance of an effective memory $CD8^+$ T cell population. Thus, we propose that IL-3 and GM-CSF expression by memory $CD8^+$ T cells has the potential to be used as a biomarker of effective protective immunity.

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Author Contribution

S.T. conducted all the studies, data analysis and preparation of the manuscript. R.J.J. designed and constructed the IL-13Rα2 adjuvanted vaccine and critical evaluation of the manuscript. C.R. conceived the study, designed the immunological experiments and helped with data analysis/writing of the manuscript.

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