ORIGINAL ARTICLE

Protection afforded by live attenuated SIV is associated with rapid killing kinetics of CTLs

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Abstract

Background Live attenuated SIV vaccines are highly efficacious, but how they mediate protection is poorly understood. A feature of the effectiveness of live attenuated vaccines is their ability to control high dose challenge viruses early, without a large peak of acute viraemia. We hypothesized that long-lived antigen exposure from live attenuated SIV may result in CD8+ cytotoxic T lymphocytes persistently capable of rapidly cytolytic potential.
Methods We employed a kinetic degranulation assay to study multiple tetramer+ SIV-specific CTL specificities before and after the SIV$_{mac251}$ challenge of pigtail macaques inoculated with a live attenuated SIV.
Results Live attenuated SIV-vaccinated animals rapidly controlled a subsequent challenge, with minimal viraemia after exposure. For over 9 months after the initial vaccination with live attenuated SIV we could detect both Gag- and Tat-specific CTLs that maintained a long-term capacity to rapidly degranulate (CD107a expression) and release granzyme B within 30 minutes of antigen exposure. This rapid cytolytic phenotype was maintained throughout the early period after challenge, despite the absence of a marked enhancement in CTL frequencies.
Conclusions Our results suggest that highly functional CTLs may contribute to the remarkable efficacy of live attenuated SIV vaccines. Studying the killing kinetics of CTLs induced by other, safer, HIV vaccines could facilitate a better understanding of the requirements for an effective HIV vaccine.

Introduction

Live attenuated SIV vaccines have consistently shown more efficient protection in macaque studies [10, 17, 33]. Although live attenuated HIV vaccines are currently perceived to be too dangerous for clinical trials [27], they provide an excellent model to study effective immunity to SIV in macaques [20]. A feature of the immunity induced by live attenuated SIV vaccines is how much more efficiently early viral replication is limited following SIV challenge compared to vector-based vaccination that also induce T cell immunity [10, 17, 20, 33]. Live attenuated SIV vaccine-mediated protection is reasonably robust even when very heterologous Env strains are used, and the protection is not readily transferred with passive antibodies [22]. These findings suggest additional effector arms of the immune system other than neutralizing antibodies at least in part mediating the protective effect.

Considerable evidence points to the partial effectiveness of CD8+ CTLs cells in controlling HIV-1 infection in humans and SIV infection in macaques [15, 22, 23, 29, 30]. However, a recent human phase IIb trial of an Adenovirus vector HIV vaccine failed to afford any protection from either infection or high viral loads despite inducing high levels of HIV-specific T cells capable of IFNγ secretion in the majority of vaccinated subjects [14].

Many studies are now focussing on the quality of CTLs as a mediator of the effectiveness of CTLs rather than simply the numbers of IFNγ-secreting cells. Growing evidence suggests the ability of CTLs to express multiple effector molecules ('polyfunctional') correlate with the effectiveness of CTLs [1, 7]. A potentially critical and understudied aspect of the utility of CTLs is their ability to rapidly kill antigen-expressing target cells. For CTLs to be maximally effective, infected targets need to be killed prior to generation of large numbers of new viruses. The particular proteins targeted by CTLs are also likely important. For example, Gag epitopes are targeted earlier in the virus life cycle since new virions are not required to be synthesized [28]. Env epitopes have been suggested to stimulate CTLs with poorer ability to control infection [19, 24].

Similarly, the speed with which the CTLs express effector cytokines and molecules which can lyse infected targets may also be a critical factor. Primed effector cells recently exposed to antigen typically lyse targets more quickly than memory CTLs that have not recently been exposed to antigen. This is a critical issue in current T-cell based HIV vaccine studies since most vector-based HIV vaccines do not persistently express vaccine antigens may result in memory CTLs with slow effector function upon initial virus exposure. Restricting the exponential growth of virus and early depletion of CD4+ T cells during acute infection via efficient CD8+ T cell killing of virus-infected cells may be required for maximally effective HIV vaccine strategies [25].

We recently developed a novel kinetic degranulation assay to study the rate with which tetramer+ SIV-specific CTLs express cytolytic markers upon antigen exposure [27]. We showed that poxvirus-based SIV vaccines induced Gag-specific CTLs with relatively slow abilities to exhibit a highly cytolytic phenotype. In contrast, a live attenuated SIV vaccine, at least early after inoculation, induced CTLs with a more rapid cytolytic phenotype [27]. Herein we evaluate whether the CTLs induced by live attenuated SIV retain a rapid phenotype for many months after inoculation and how this phenotype changes upon a SIV_{mac251} challenge.

Methods

Animals, vaccination and SIV challenge

Juvenile *Macaca nemestrina* were free from HIV-1/SIV/simian retrovirus (SRV) infection and anaesthetized with ketamine (10 mg/kg intramuscular (i.m.))

prior to procedures. Experiments were approved by the University of Melbourne and CSIRO Livestock Industries Animal Experimentation and Ethics Committees.

We prospectively studied two *Mane-A*10+* pigtail macaques after live attenuated SIV vaccination. Results from early (up to 8 weeks) after the live attenuated SIV vaccination were recently published [27] and herein we present longer-term follow up after vaccination and the results after SIV_{mac251} challenge. The pigtail macaques were MHC typed for *Mane-A*10* using sequence-specific primer PCR and confirmed by Reference Strand-mediated Conformational Analysis as previously described [26, 31, 32]. SIV plasma viraemia was quantified by reverse-transcriptase real-time PCR on an Eppendorf realplex machine with a Taqman probe as described [8].

The two *Mane-A*10+* pigtail macaques were inoculated with a live attenuated SIV vaccination (LAV). The LAV inoculation used a previously reported nef/LTR-deleted SIV_{mac239} provirus ($SIV_{sbbcΔ3Δ5}$) modelled on the deleted HIV-1 strains present in a linked cohort of long-term slow progressors termed the Sydney Blood Bank Cohort [12, 17]. $SIV_{sbbcΔ3Δ5}$ expresses wild-type SIV Gag and contains an LTR deletion at both ends of the provirus to avoid recombination to wild-type [18]. The $SIV_{sbbcΔ3Δ5}$ was administered as proviral DNA, 75 μg inoculated half i.m. and half into an inguinal lymph node. Our previous studies have shown similar virus/inoculation systems to be a reliable vaccination method and, as with other studies of live nef-deleted SIV, efficacious in controlling acute viraemia following wild-type SIV_{mac251} challenge [17, 20]. The SIV_{mac251} challenge used a dose of 40 TCID50 intravenously, a method we have previously shown to reliably infect over 40 naïve pigtail macaques ([6, 27, 32] and unpublished data).

The tetramer degranulation assay

The tetramer degranulation assay [27] is essentially a combination of a CD8+ T cell tetramer staining assay and an intracellular cytokine staining assay [8, 31] similar to previous work [4]. In brief, 0.2 ml fresh whole blood was co-cultured with the anti-CD107a-APC monoclonal antibody (mAb) conjugate (Becton-Dickinson [BD, San Jose, CA], cat#624076) following restimulation with 1 μg/ml of the immunodominant CD8+ T cell epitope (KP9, SIV Gag164-172) or media alone. Whole blood restimulation was performed in the presence of 5 μg/ml Brefeldin A (Sigma-Aldrich), 5 μg/ml Monensin (Sigma-Aldrich), 1 μg/ml anti-CD28 (BD, cat#340975) and 1 μg/ml anti-CD49d

(BD, cat#340976). Parallel 0.2 ml whole blood samples were set up in 5 ml FACS tubes, either not incubated ($t = 0$, control) or incubated at 37°C with 5% CO_2 for variable times. At 0.5, 1, 3 or 5 h after incubation was initiated, the reactions were stopped by washing with ice cold PBS, followed by staining with an MHC class I specific tetramer (Mane-A*10/KP9-PE), anti-CD8-PerCP (BD, cat#347314) and anti-CD3-Pacific Blue (BD, cat#558124). Red blood cell lysis (BD, cat#349202) and cell permeablization (BD, cat#340973) preceded the staining with anti-IFNγ-Alexa Fluor 700 (BD, cat#557995), and anti-Granzyme B-FITC (Mabtech, Nacka, Sweden, clone GB11). The samples were fixed with 2% formaldehyde and acquired on a six colour LSRII flowcytometer (BD) within 24 hours of staining. The modification of the assay herein as compared to our previous publication [27] include (1) the addition of an anti-CD3 antibody, (2) a different IFNγ flourochrome conjugate and (3) in order to be able to collect enough tetramer events we started off with two identical tubes, each with 0.2 ml of whole blood which at the end of the assay were pooled together.

In addition to studying the SIV Gag KP9 CTL epitope, we also studied two novel SIV Tat epitopes KSA10 (KKAKANTSSA) and KVA10 (KKETVEK-AVA) that we recently mapped and restricted to *Mane-A*10* (Rosemarie Mason, Stephen Kent, unpublished data). We folded the recombinant Mane-A*10 MHC class I protein around this peptide to construct the KSA10/Mane-A*10 and the KVA10/Mane-A*10 tetramers to define these T cell populations.

Results

Rapid killing kinetics of SIV-specific CTLs maintained 9 months after live attenuated SIV vaccination

We previously showed that viral-vector-based vaccines induced high levels of KP9-specific CTLs expressing IFNγ in pigtail macaques, but that these CTLs had a slow capacity to degranulate (express CD107a and lose Granzyme B) upon antigen stimulation. In contrast, live attenuated SIV vaccination using a nef/LTR-deleted provirus induced CTLs with a rapid ability to degranulate. However, our earlier work was confined to the first 8 weeks after live attenuated SIV inoculation, and may have been confounded by higher levels of antigen after inoculation.

The durability of the rapidly cytolytic CTLs induced by live attenuated SIV inoculation was therefore evaluated by examining the cytolytic phenotype of the KP9-specific CTLs 41 weeks after the initial vaccination

(Fig. 1A). Again, we found the CTLs consistently began to express CD107a, a marker of degranulation, within 30 minutes after KP9 peptide exposure *in vitro*, indicative of a rapidly cytolytic phenotype.

It was possible that SIV Gag KP9-specific CTLs may not accurately reflect other CTL specificities, particularly given observations about the relative utility of Gag-specific CTLs compared to other CTLs specific for other antigens observed in both humans and macaque models. We recently defined two CD8+ CTL epitopes in the Tat protein (KSA10 and KVA10) in Mane-A*10+ pigtail macaques and were able to restrict these epitopes to Mane-A*10 and construct KSA10/Mane-A*10 and KVA10/Mane-A*10 tetramers. This enabled us to accurately gate on Tat-specific CTLs to define their cytolytic phenotype. The animal with the highest Tat-specific CTL population was animal 8305 to the KSA10 Tat epitope and we were able to show that the Tat-specific CTLs also had a rapidly cytolytic phenotype (Fig. 1A). The rapid kinetics of CTL degranulation after live attenutated SIV vaccination is not confined to Gag-specific CTLs and may be a more general phenomenon.

Efficacy of nef/LTR-deleted live attenuated SIV

Fourty-one weeks after the initial live attenuated SIV inoculation, both macaques were challenged intravenously with pathogenic SIV_{mac251}. The challenge was performed over 6 months after SIV RNA was last detected in plasma after vaccination. No quantifiable viraemia was detected after challenge (Fig. 2A). During the early weeks after challenge, several plasma samples had very low levels of SIV RNA detected ($< 2.7 \log_{10}$ copies/ml) but our in house assay does not reliably quantitated levels this low. No significant CD4+ T cell depletion was detected (Fig. 2B). The protection afforded by live attenuated SIV was starkly contrasting to that we observed with live-vector-based vaccinations, where a peak of viraemia usually exceeding 5 $\log_{10}$ copies/ml and dip in peripheral CD4+ T cells is general observed at week 2 after challenge, even in animals that go on to later control viraemia [27].

CTL response to challenge inoculum

A dramatic anamnestic rise in CTL numbers is usually observed after SIV or SHIV challenge of macaques vaccinated with DNA and live-vector vaccines. The CTLs do not generally reach a maximal level until just after the peak of viraemia and many have argued that the delay in CTL activation is 'too late'. We studied in detail the CTL numbers in response to the SIV_{mac251} challenge
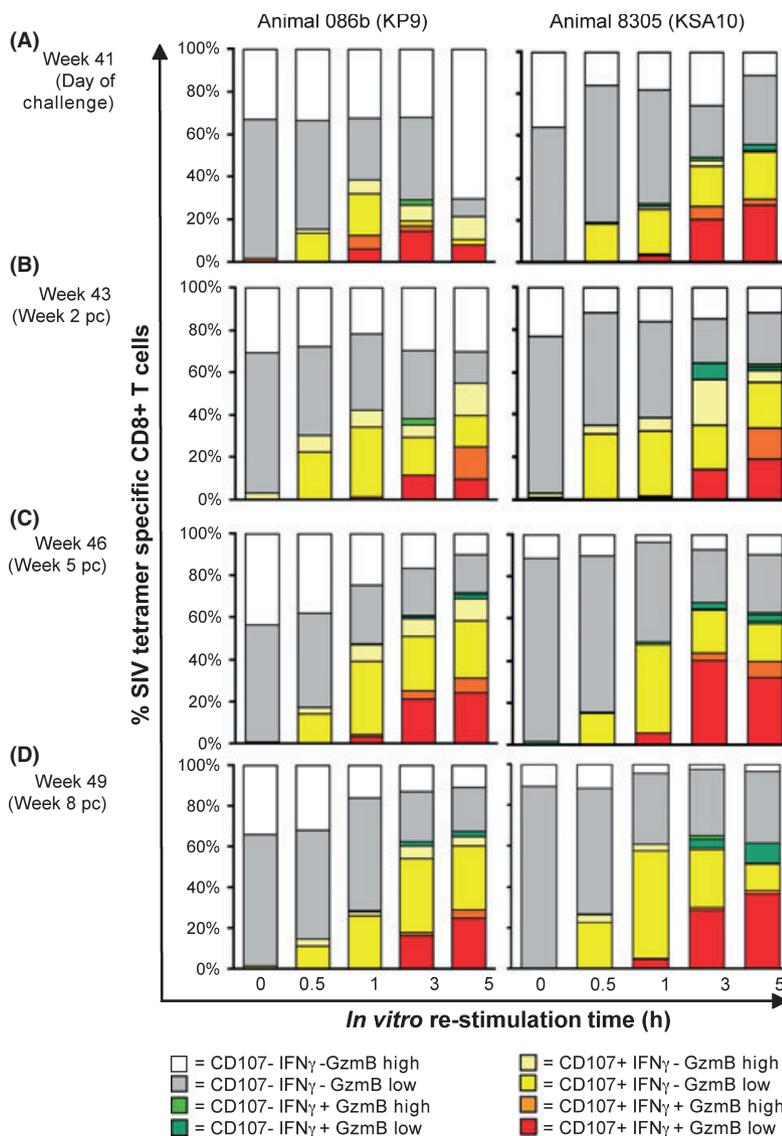
Fig. 1 Degranulation profiles of CD3+CD8+SIVtetramer+ T cells induced by Live attenuated vaccination and following SIVmac251 challenge. The tetramer positive T cells are divided into eight different degranulation/activation phenotypes as illustrated by differently coloured bars. Measuring the degranulation at different time-points of *in vitro* peptide restimulation generates both the degree of degranulation/activation and the kinetics with which this occurs. (A) Still 41 weeks after inoculation of a live attenuated vaccine we can measure rapid degranulation profiles in SIV Gag KP9 tetramer+ T cells from animal 086b (left) and in SIV Tat KSA10 tetramer+ T cells from animal 8305 (right). The same day (week 41) both animals were challenged I.V. with a pathogenic SIVmac251 inoculum. The degranulation assay was performed 2 (B), 5 (C) and 8 (D) weeks after the viral challenge. Both animals maintain rapid degranulation kinetics, but notably a large fraction of the KP9+ tetramer positive cells appear to have predominantly poorly loaded cytolytic granule, as shown by low levels of intracellular Granzyme B *ex vivo* (white bars, day of the challenge time 0).

in our study. Surprisingly, we found no dramatic early expansion in tetramer+ CTLs either to Gag KP9 or the 2 Tat epitopes KVA10 and KSA10 out to 2 months after challenge (Fig. 3A and B). We were technically able to monitor two CTL frequencies in the same tube using tetramers with differing fluorochromes (Fig. 3C). Our results suggest the possibility that the CTLs induced by live attenuated SIV vaccination may have been functioning efficiently at the time of challenge ('early'), resulting in a limited expansion of viraemia and no requirement for high-level expansion.

Killing kinetics post challenge

We hypothesized that exposure to the SIVmac251 challenge might change the activation state and degranula-

tion kinetics of SIV-specific CTLs even in the absence of an expanded number of CTLs. We therefore studied the degranulation kinetics at 2, 5, and 8 weeks after challenge for both the KP9 Gag and KSA10 Tat epitopes (Fig. 1B–D). No marked alteration in degranulation kinetics was observed after challenge to either epitope. This maintenance of a rapid degranulation profile is also illustrated in Fig. 4 for both animals to KP9 Gag where the % of cells expressing the degranulation marker CD107a after 1 hour of *in vitro* stimulation is shown. The marked difference in degranulation kinetics between live attenuated SIV vaccination and prime–boost viral-vector vaccination prior to challenge is shown (Fig. 4). Although CTLs from the viral-vector vaccination 'catch-up' in terms of degranulation kinetics after challenge, expansion of the challenge

(A)

Plasma viral load

(B)
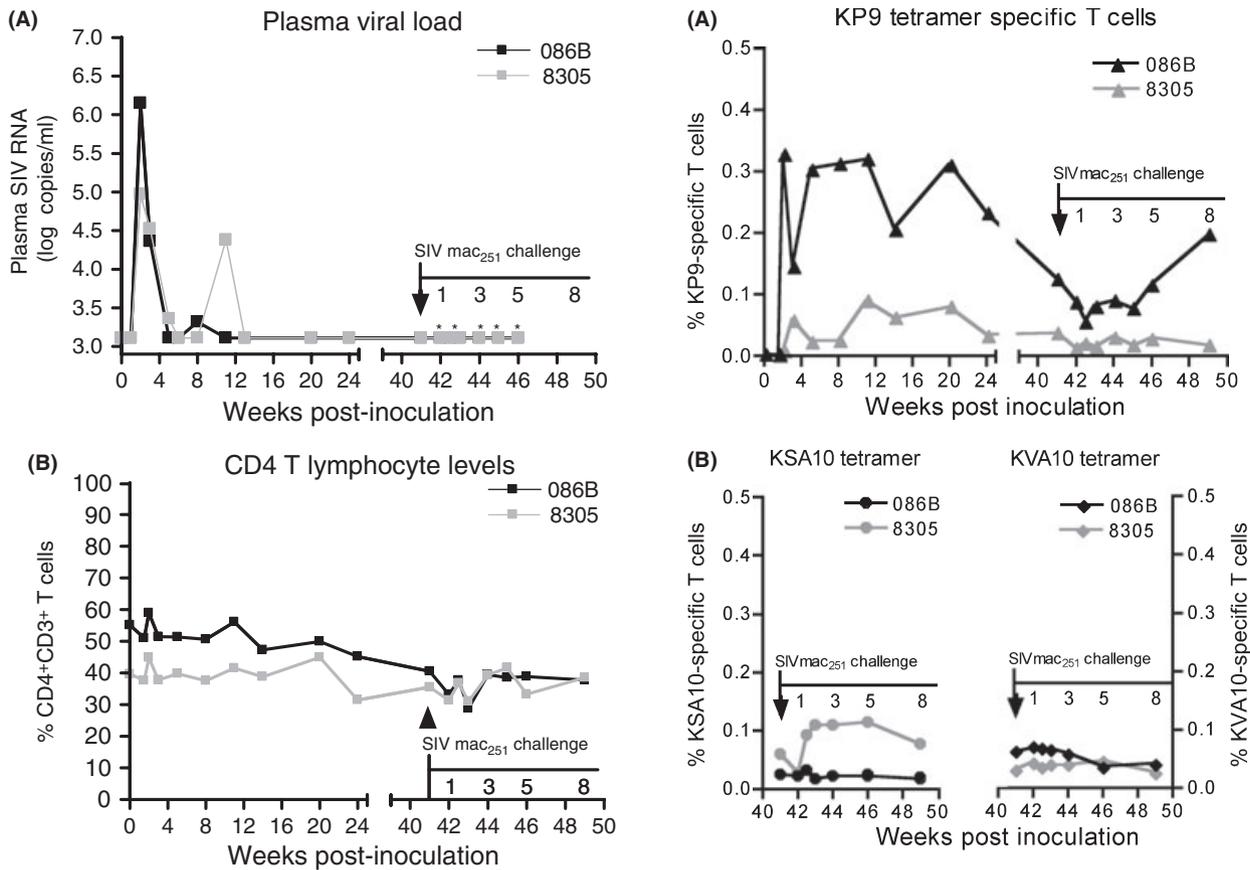
CD4 T lymphocyte levels

Fig. 2 Plasma viral load and CD4 counts. (A) The SIV viral load is shown in two pigtail macaques (086b and 8305) from the day of inoculation with live attenuated virus (week 0) through to the day of challenge with SIVmac$_{251}$ (week 41) and up to eight weeks following challenge (week 49).*Virus was detected at <3.1 log$_{10}$ copies/ml at week 4 after challenge for animal 086b and weeks 0, 1, 3, 5 after challenge for animal 8305. (B) CD4 counts over time in the same two animals. The CD4 count is displayed as the % of CD3$^+$CD4$^+$ T cells divided by all CD3$^+$ T cells multiplied by 100.

virus has already occurred by 2 weeks after exposure [27].

The kinetic degranulation assay can be employed without a tetramer reagent

It would be useful if our kinetic degranulation assay could be utilized without prior knowledge of specific minimal epitopes and the use of tailor-made tetramers. In order to address this, we sampled an SIV-infected animal (5612) with a high frequency (~5%) of KP9/ *Mane-A*10 tetramer CD8+ T cells in the blood and performed the assay and analysed the tetramer positive cells and alternatively gated on all CD8+ T cells, irregardless if positive for the tetramer or not (Fig. 5).

(A)

KP9 tetramer specific T cells

(B)

KSA10 tetramer   KVA10 tetramer
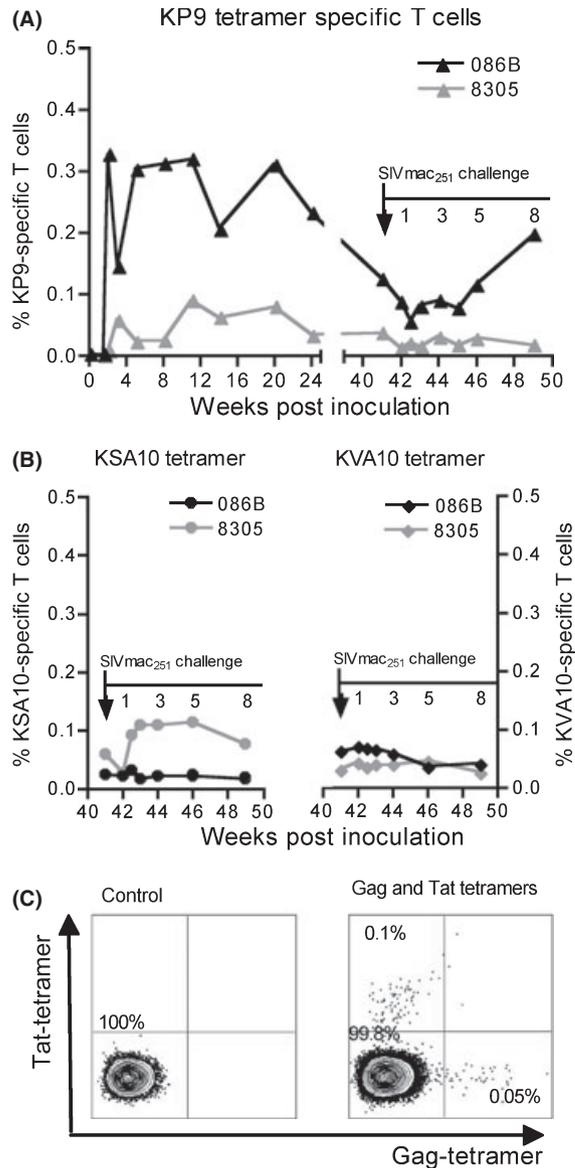

(C)

Fig. 3 SIV Gag- and Tat-specific CD8 T cell frequencies measured in two vaccinated/challenged animals over time. (A) The SIV gag KP9 tetramer frequency measured after live attenuated vaccine inoculation (weeks 0–41) and after a subsequent SIVmac$_{251}$ challenge (weeks 41–49). The KP9 tetramer frequency is measured as CD3+CD8+KP9+ T lymphocytes divided by total CD3+CD8+ T lymphocytes multiplied by 100. (B) The SIV Tat KSA10 and Tat KVA10 tetramer frequencies were measure after challenge (week 41–49). All tetramer values shown are above tetramer FMO control staining (not shown). (C) Two different tetramers can be labelled with different flourochromes allowing for identification of two different SIV T cell clones in one single sample. This opens up the possibility to follow the degranulation/activation profiles of two or more populations in one single tube.

There was a comparable activation/degranulation profile on all CD8+ T cells independently of tetramer staining (Fig. 5C top vs. bottom panel). It is worth
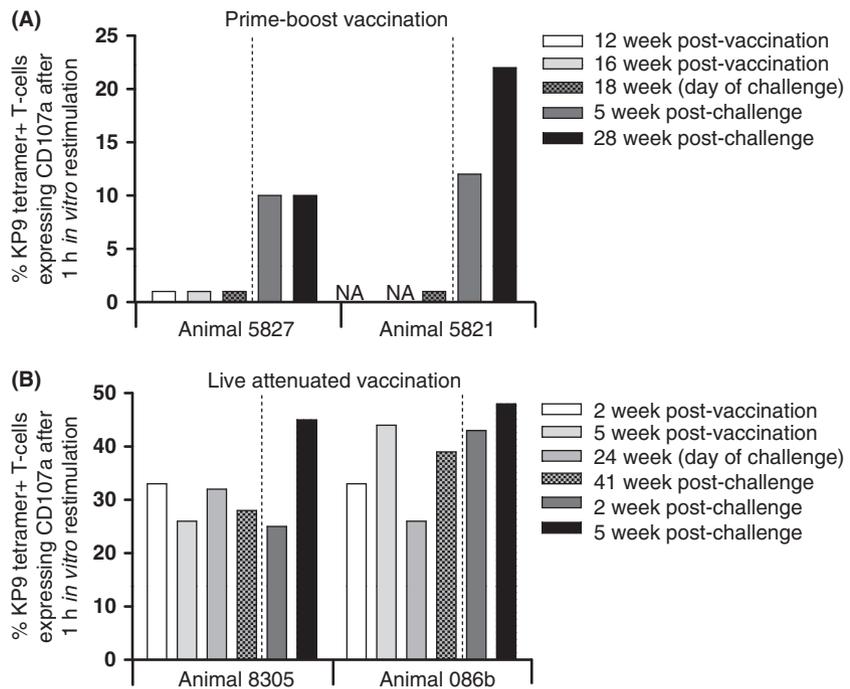
Fig. 4 Live attenuated vaccination induces virus specific CD8+ T cells that degranulate rapidly. (A) Prime–boost immunization using vaccinia and fowlpox virus vectors expressing SIV gag induced KP9-specific CD8+ T cells that take ≥3 hours to degranulate [17]. (B) Live attenuated vaccination induces CD8+ T cells that express CD107a very rapidly (within 1 hour), a phenotype that persist for at least 41 weeks post vaccination and after challenge.
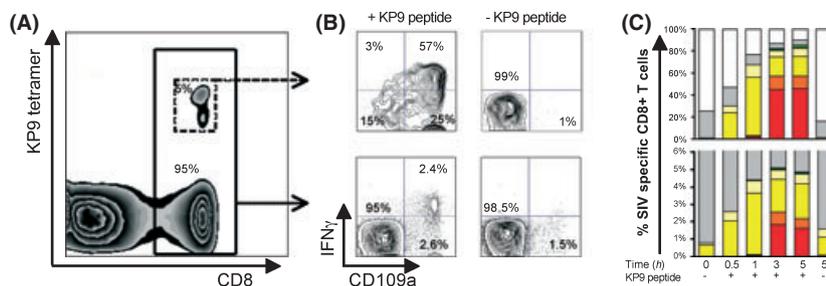

Fig. 5. The degranulation assay can be used without the SIV tetramer. Fresh blood is obtained from a *Mane-A*10+* SIV-infected pigtail macaque (5612) with a known high population of KP9-specfic CD8 T cells and stimulated with KP9 peptide as in the methods. (A) The gating strategy of either the 5% tetramer+ CD8+ T cells only (top dotted box) or all 100% CD8+ T cells (lower larger dotted box). Cells shown are all derived from a CD3+ lymphocyte gate. (B) The upper panel shows the established IFNγ vs. CD107a profile after peptide restimulation for 5 hours with (left) or without KP9 peptide (right). The two lower panels show the profile if the tetramer staining is ignored and all CD8+ T cells are included. Approximately 5% of all CD8+ T cells responded with IFNγ/CD107a in the peptide restimulated (bottom left) panel with background stimulation of 1.5% CD107a in the unstimulated sample (bottom right panel). (C) Since the tetramer allows gating on all SIV-specific cells the percentage of response cells will be calculated out of all SIV-specific cells, 100% over time (top panel). A similar profile is obtained when gating on all CD8+ T cells, regardless of tetramer staining (bottom panel). Color coding is as for Fig. 1.

pointing out a few limitations when use of the assay without tetramer staining. The fraction of SIV-specific cells expressing Granzyme B *ex vivo* (0 hour) is lost if the tetramer is not used since the SIV-specific cells are not identified at time 0. Further, the frequency of SIV-specific cells need to be relatively large to readily distinguish the responding antigen-specific cells above the background level (e.g. CD107 gives background on bulk CD8+ T cells, Fig. 5B and C). Note that when a tetramer gate is employed, the 'signal-to-noise' ratio between peptide/no peptide becomes much larger than

if the tetramer is not used. Despite these limitations, our observations open up the use of this kinetic degranulation assay to studying bulk CD8+ T cell populations.

Discussion

Live attenuated SIV vaccination stimulates SIV-specific CD8+ T cell with an ability to rapidly express cytolytic markers after antigen exposure *in vitro*. This phenotype is maintained for at least 9 months after initial

vaccination, long after viraemia has ceased to be detectable in peripheral blood. The durable kinetics of activated T cell immunity is consistent with prolonged efficacy of live attenuated SIV vaccines [20]. The maintenance of the rapid killing profile may either be intrinsic to live attenuated vaccines or as a result of low-level persistence of antigen. Future studies of models of other live attenuated vaccines (e.g. measles) or vaccines with prolonged antigen exposure (e.g. herpes virus vectors [16]) may help define the mechanisms of inducing highly functional T cell immunity.

Highly efficient control of a high-dose intravenous SIV_{mac251} challenge was achieved with our DNA-delivered nef/LTR-deleted live attenuated SIV vaccine in the animals studied, with virtually no acute viraemia or depletion of CD4+ T cells detected. Indeed, there was minimal expansion of the SIV Gag KP9-specific CTLs after challenge (0.04–0.20% after challenge). This is starkly contrasting to our, and others, experience with DNA and live-vector vaccines, where anamnestic SIV-specific tetramer responses exceeding 10% of all CD8+ T cells are commonly observed after virus challenge, albeit with a 2 week delay [3, 5, 9, 11]. Further, peak viraemia can be large after SIV or SHIV challenge of macaques immunized with vector-based vaccines and at least a transient reduction in peripheral and mucosal CD4+ T cells is usually observed [3, 5, 8, 11, 27]. We recently showed that the delayed activation and expansion of KP9-specific CTLs is associated with failure to control reversion of a KP9-escape mutant SHIV [13], strongly supporting the concept that highly functional CTLs at the time of challenge provide a more robust impact on controlling the rapid migration and expansion of virus within the first 2 weeks after challenge.

We cannot exclude that other immune effector capabilities are playing a role in the protective immunity mediated by the live attenuated vaccination we observed. For example, we found it curious that the 'loading' of intracellular Granzyme B within the SIV-specific CTLs was relatively low both before and after challenge (12–42%, white bars at time 0 in Fig. 1) despite rapidly upregulating the degranulation marker CD107a after antigen stimulation. This was in contrast to previous observations early after challenge of animals immunized with vector-based vaccines, where, although there was a delay until the CTLs became rapidly cytolytic, the vast majority of the cells (>70%) finally expressed high levels of Granzyme B [27]. Our results suggest the possibility that the live attenuated SIV vaccination stimulates CTLs loaded with other cytolytic effector molecules (e.g. Perforin, Granzyme A, Granzyme K), or that the CTLs have already

released Granzyme B in tissues prior to migration into the circulation. Further studies analysing a larger range of effector molecules in both lymphatic tissues and blood are suggested by this work.

In this report, we also expand the technical capabilities of our kinetic cytolysis assay. We show that the assay can be adapted to non-Gag CTL epitopes, such as the novel KSA10 Tat epitope also restricted by *Mane-A*10*. This now provides an ability to study the differential effectiveness of CTLs to non-Gag proteins. This is a critical topical issue since others and we have questioned the utility of CTLs to non-Gag proteins [19, 24, 28]. Further, we also show that the kinetic cytolysis assay can be used on large SIV-specific T cell responses without the requirement to gate on tetramer+ T cells. This significantly expands the utility of this assay since it can be performed on outbred subjects with unknown MHC haplotype presenting undefined SIV or HIV epitopes. It should be cautioned, however, that in the setting of very low-level T cell responses the ability to rigorously define the epitope-specific CTLs becomes more difficult (Fig. 5).

In conclusion, we show that effective live attenuated SIV vaccination is associated with durably rapid killing kinetics of both Gag- and Tat-specific CTLs. The rapid killing phenotype was maintained throughout the SIV_{mac251} challenge period in the absence of significant viraemia. Our results suggest a potential mechanism associated with the remarkable protection from virus challenge by live attenuated SIV. Analyses of the killing kinetics of safer alternate vaccine approaches are warranted.

Acknowledgments

Conflicts of interest

The author has no conflicts of interest.

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