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Establishment of functional influenza virus-specific CD8$^+$ T cell memory pools after intramuscular immunization

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Running head: IM *versus* IP vaccination with live influenza virus (< 54 characters)

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ABSTRACT

The emergence of the avian-origin influenza H7N9 virus and its pandemic potential has highlighted the ever-present need to develop vaccination approaches to induce cross-protective immunity. In this study, we examined the establishment of cross-reactive $CD8^+$ T cell immunity in mice following immunization with live A/Puerto Rico/8/1934 (PR8; H1N1) influenza virus via two non-productive inoculation routes. We found that immunization via the intramuscular (IM) route established functional influenza-virus specific memory $CD8^+$ T cell pools capable of cross-reactive recall responses. Epitope-specific primary, memory and recall $CD8^+$ T-cell responses induced by the IM route, highly relevant to human influenza immunisations, were of comparable magnitude and quality to those elicited by the intraperitoneal (IP) priming, commonly used in mice. Furthermore, IM immunisation resulted in lower lung viral titres following heterologous challenge with A/Aichi/68 (X31; H3N2) compared to the IP route. Examining the ability of DCs from lymphoid organs to present viral antigen revealed that immune induction following IM immunization occurred in draining lymph nodes, while immunization via the IP route resulted in the priming of responses in distal lymphoid organs, indicative of a systemic distribution of antigen. No major differences in the pulmonary cytokine environment of immunized animals following X31 challenge were observed that could account for the improved heterologous protection induced by the IM route. However, while both routes induced similar levels of PR8-specific antibodies, higher levels of cross-reactive antibodies against X31 were induced following IM inoculation. Our data demonstrate how non-replicative routes of infection can induce efficient cross-reactive $CD8^+$ T cell responses and strong strain-specific antibody responses, with the additional benefit from IM priming of enhanced heterosubtypic antibody production.

INTRODUCTION

Infections with avian-origin influenza A/H7N9 virus resulted in ~30% fatality rate [1]. The threat of an impending pandemic outbreak caused by a novel strain for which prevalent immunity in the population is lacking [2,3], and the devastating consequences it might have on global population health and economies, highlights the need to develop new vaccines that induce universal cross-protective immunity. An important mediator of such immunity is the $CD8^+$ cytotoxic T lymphocyte (CTL) response, a major component directed against epitopes primarily located on the conserved internal viral proteins common to all influenza A strains, including those with pandemic potential. Broadly neutralising and non-neutralising antibodies, particularly those that can recognise conserved regions of the virus, have also been reported to facilitate effective heterologous viral clearance [4].

The importance of CTLs in control of human influenza has been demonstrated in studies that correlate the presence of these cells with better disease outcome [2,5,6], and broadly cross-reactive antibodies have been found in individuals responding to the pandemic H1N1-2009 virus [7]. Current inactivated influenza vaccines delivered by the intramuscular (IM) route have been reported to induce low levels of cross-reactive CTL responses, although at levels inefficient for heterologous protection [8]. These vaccines therefore rely on the induction of highly strain-specific neutralizing antibody directed predominantly against the hemagglutinin (HA) surface glycoprotein for their mechanism of action.

In the mouse, CTL response to influenza has been studied extensively [9]. A commonly used model to recapitulate the cross-protective properties of CTLs is to prime mice with a virulent influenza virus by the non-productive intraperitoneal (IP) route and then examine the amelioration of disease produced after exposure to a heterologous influenza virus infection by the productive IN route. In this model, protection against infection by serologically distinct viruses correlates with the induction and recall of CTL memory population within the animal [10-13].

The non-productive nature of the IP route is due to the absence of tissue-specific HA cleaving enzymes such as human airway trypsin-like proteases [14] and type II transmembrane serine proteases [15] required for the production of mature infectious virions and due to the lack of access to permissive cells. Nevertheless, the virus can undergo abortive replication in certain cell types such as macrophages and dendritic cells (DCs) in which the full complement of viral genome RNA and proteins are produced, despite an inability for these to be packaged into virions [16,17]. Viral HA, NA and M2 proteins expressed on the surface of these abortively infected cells [16] would provide an additional stimulus for B cells for the production of antibody above that of the input virus. In addition, DCs act as antigen-presenting cells for the efficient priming of T cells recognising endogenously-processed viral peptides. CTLs induced in this way are characterised by

memory responses lasting the lifetime of the mouse [18]. Although not a suitable route for human vaccination, examination of the responses induced via this non-productive provide insight into the requirements for establishing successful vaccine-induced broadly cross-reactive memory CTLs.

A second non-productive route for influenza infection is the intramuscular (IM) route. Harris et al. [19] showed that pre-vaccination of mice with live-virus via the IM route resulted in an increase in the CTL number after heterosubtypic challenge. The pre-vaccinated mice showed a 3-log reduction in lung viral load on day (d) 7 post-challenge compared to PBS pre-treated mice, indicating faster viral clearance. Given that the circumstances associated with where and how T cells are primed following influenza infection is key to determining the quality, magnitude and fate of the resultant T cell- and B cell-memory responses [12,13,20,21], we compared the IP route with the second non-productive route, IM, for the ability to prime effector, memory and recall CTLs and to induce cross-protective immunity. We sought to understand whether dispersed antigen distributed by inoculation of live-virus into the peritoneal cavity, or that restricted to the muscle and surrounding tissue, favours greater cross-reactive immune induction that can act to reduce viral load in the lung environment. We provide evidence that priming via the IM route results in better cross-strain protection against heterologous viral challenge compared to the IP route and examine the role that cross-reactive CTLs and antibody play in reducing viral loads.

MATERIALS & METHODS

Infection of mice. C57BL/6 and OT-1 [22] mice were bred and housed at Department of Microbiology and Immunology, University of Melbourne. Experiments were approved and conducted under guidelines set by the University of Melbourne Animal Ethics Committee. Mice were anaesthetised by inhalation of methoxyflurane and infected with $1.5x10^7$ plaque forming units (PFU) of A/Puerto Rico/8/1934 (PR8; H1N1) influenza virus via the IP (in 500µl of PBS) or IM route (40µl into each quadriceps muscle). Mice were challenged intranasally 6 weeks later with 1×10^4 PFU of X31 (H3N2) virus in 30µl PBS. Lungs were harvested for viral load in a Madin-Darby canine kidney (MDCK) plaque assay [23].

Ex vivo antigen presentation by dendritic cells (DCs). Lymph node (LN) and splenic DCs were isolated as described (Vremec 2008). Naïve $CD8^+$ T cells from LNs of OT-I mice were negative selected using MAbs against CD11b (M1/70), F4/80, Ter-119, Gr-1 (RB6), MHC class II (M5/114), and CD4 (GK1.5) or CD8 (YTS169.4). CFSE (carboxyfluorescein succinimidyl ester)-labelled OT-1 cells (5×10^4) [21] were cultured for 60hrs with 5×10^3 purified DCs. Proliferation was determined by CFSE.

In vivo T cell proliferation assay. CFSE-labelled OT-1 cells (1×10^6) were transferred intravenously into C57BL/6 mice. One day later, mice were inoculated with recombinant PR8 influenza virus containing the $K^bOVA_{257-264}$ epitope (amino acid sequence SIINFEKL) within the stalk of the NA protein (PR8-OVA) [22]. Lymph nodes were obtained on d3. CFSE levels on $CD8^+CD45.1^+$ cells were assessed by flow cytometry.

Influenza virus-specific CTL responses. Cells were recovered from spleen and the site of infection (bronchoalveolar lavage; BAL). Lymphocytes were stimulated with peptides representing the $H2D^b$-restricted $NP_{366-374}$ (ASNENMTEM) or $PA_{224-233}$ (SSLENFRAYV) epitopes in a intracellular cytokine secretion (ICS) assay [15]. Cells were also incubated with $D^bNP_{366-374}$ or $D^bPA_{224-233}$ tetramers conjugated to PE or allophycocyanin [15]. Results were analysed using FlowJo software (Treestar, USA).

Influenza virus-specific antibody. Sera were prepared from blood taken 4 weeks after infection with PR8 virus via IM or IP route. ELISA were performed in 96-well plates coated with 5µg/ml of split virus vaccine-derived from PR8 (CSL Limited, Australia) or recombinant HA protein derived from X31 (provided by Stephen Kent) for 18–20 hours. Serial dilutions of sera were incubated overnight. Bound antibody was detected using HRP-conjugated rabbit anti-mouse IgG Abs (Dako, Denmark) in conjunction with enzyme substrate (0.2mM 2,2′-azino-bis 3-ethylbenzthiazoline-sulfonic acid). Ab titers are expressed as the reciprocal of the highest dilution of serum required to achieve an OD of 0.2. The sera were treated with receptor-destroying enzyme (RDE) and examined in hemagglutination-inhibition assays against PR8 and X31. Assays were performed using 1% chicken red blood cells [25].

Statistical Analyses. Analysis of variance and *p* values in this study were obtained using one-way ANOVA non-parametric analyses and Tukey's post-hoc range tests or students t-test (GraphPad Software, La Jolla, California USA).

RESULTS

Live-virus delivered by the IM route leads to DC-mediated antigen presentation to CTLs at fewer lymphoid sites than live-virus delivered by the IP route.

As the route of inoculation can affect the site of early CTLs priming [26], and the subsequent immune response, we examined the ability of DCs at different lymphoid sites to process and present viral antigen to CTLs after IM and IP delivery of live-virus. DCs were isolated from cervical, mediastinal, mesenteric, spleen, inguinal and lumber lymph nodes (LNs) following IM or IP inoculation with the recombinant influenza virus PR8-OVA containing the $K^bOVA_{257-264}$ epitope from ovalbumin in the influenza NA stalk [22,24]. The LN cells were co-cultured with CFSE-labelled $CD8^+$ T cells from OT-1 transgenic mice (OT-1 T cells) expressing the Vα2-TCR specific for the OVA_{257} peptide presented by $H2k^b$ (Fig.1A). Analysis of CFSE levels of OT-1 cells on d3 revealed that only DCs isolated from inguinal and lumber LNs of IM inoculated mice could induce high levels of T cell proliferation (Fig.1B), compared to those obtained from distal LNs (cervical, mediastinal and mesenteric LNs) or the spleen. Conversely, DCs isolated from the inguinal, lumber, mesenteric and mediastinal LNs and the spleen of IP inoculated mice were proficient at stimulating T cell proliferation, suggesting that the primary sites of immune induction following inoculation via the IM route is localised to the draining LNs, while inoculation via the IP route results in the systemic distribution of antigen to distal sites.

To confirm these observations *in vivo*, we adoptively transferred CFSE-labelled OT-1 cells into wild-type naïve mice 1 day prior to IP or IM inoculation with PR8-OVA virus. OT-1 cell expansion in LNs and spleen were analysed on d3 (Fig.1C). Consistent with *in vitro* observations, IP inoculation was more effective than IM inoculation at inducing systemic responses, as characterised by the presence of significantly higher numbers of OT-1 cells in the cervical, mediastinal, mesenteric LNs and spleen (Fig.1D), while significant expansion of T cells occurred in the inguinal and lumber LNs draining the inoculation site after IM immunization. This trend was also apparent when we analysed numbers of OT-1 cells that had undergone division in these lymphoid organs (Fig 1E) and to some degree was also reflected in the total $CD8^+$ T cell numbers (Supplementary Fig 1).

Inoculation via the IM route results in better heterologous viral clearance than via the IP route.

Considering that inoculation of live-virus by the IP route resulted in more systemic presentation of antigen for the priming of T cells, including in the lung-associated MLN, it is plausible that this may result in greater induction of cross-protective immunity. To determine the capacity of live-virus delivered by non-productive routes to impact on a heterologous challenge infection, mice were infected with PR8 virus (H1N1) via the IM or IP routes and then challenged by the productive

IN route with X31 virus (H3N2) 6 weeks later. Analysis of lung viral titres in IM and IP immunized mice on d3 were not significantly different (data not shown) but on d6 showed that although immunization via both routes resulted in significantly lower viral titres compared to PBS-controls, viral titres in mice inoculated via the IM route were ~10-100-fold lower (Fig.2). By d8 however, virus was effectively cleared in all mice, as only minimal lung viral titres detected. This indicates that priming with live-virus via the IM route induces immune responses that are more effective at clearing secondary heterologous infection than priming via the IP route.

Magnitude of effector, memory and recall CTLs or the breadth of cytokine production does not account for differences in heterologous viral clearance.

To determine whether the observed differences in viral clearance were due to differences in cross-reactive CTL responses, we explored whether the localized priming by the IM route resulted in CTLs of different quality, longevity or ability to be recalled to the lung, compared to those primed by more systemic exposure to live-virus. We enumerated the frequencies of viral antigen-specific CTLs induced at d10 and d30 after inoculation with live PR8 virus by IM or IP routes. Tetramer analysis revealed that the frequencies of $D^bNP_{366}^+$CTLs and $D^bPA_{224}^+$CTLs in both IP- or IM-inoculated animals were similar at the peak of the effector phase (d10) and after contraction of the response on d30 (Fig.3A). After secondary challenge with X31, an increase in the percentage of antigen-specific CTL populations was apparent, but no differences in cell number in either the spleen or BAL were observed. The absence of any significant differences in responses was also reflected when cell numbers were taken into account (Fig.3B).

To examine the quality of responses induced by different immunisation routes, the ability of CTLs to produce cytokines was examined in an ICS. Similar to Fig.3B, inoculation of all animals resulted in the induction of comparable frequencies of IFN-γ-producing D^bNP_{366}- or D^bPA_{224}-specific CTLs in both primary and secondary responses, irrespective of inoculation route (Fig.3C). Moreover, similarities in T cell polyfunctionality were observed as highlighted by their ability to secrete both TNF-α and IL-2. Thus, although there were differences in the anatomical site of T cell priming by different routes, this did not lead to any disparity in the quantity or quality of the subsequent primary, early memory and recall T cell responses.

Similar protection against excessive cytokine responses after challenge is observed with IM and IP inoculation.

While cytokines play an important role in orchestrating influenza innate immunity and shaping adaptive immune responses, cytokine/chemokine milieu induced by infection may also lead to uncontrolled inflammation, increased pathogenicity and disease severity [27]. To determine

whether different inoculation routes could affect the pulmonary cytokine environment induced by heterologous challenge and thereby contribute to the reduced lung viral titres observed for the IM route, mice were inoculated with live PR8 virus via the IP or IM route and then challenged intranasally with X31 virus six-weeks later. At d6 and d8 post-challenge, lung homogenates were analysed for cytokines/chemokines (Fig.4). Compared to PBS-inoculated animals, IFN-, IL-6 and MCP-1 levels on d6 were significantly reduced in mice previously exposed to live-virus by the non-productive routes but no significant differences between the routes were observed. These differences had largely disappeared by d8 when the pulmonary cytokine levels in all animals had decreased. Thus, pre-infection with live-virus by a non-productive route reduced inflammation-mediated disease, irrespective of the route and the accompanying viral load (Fig.2).

Heterologous antibody responses are induced by live-virus vaccination and are greater by the IM route.

In evaluating the effect of immunisation route on antibody production, sera were collected from mice that were previously inoculated with PR8 via the IP or IM route and assayed for reactivity against intact virus by HI assay and viral proteins by ELISA. Both routes of inoculation were effective at inducing high levels of specific antibody that could inhibit the hemagglutination of the homologous PR8 virus (Fig.5A). Titers >40 are used as a correlate of protective virus-neutralising antibody. As expected, the titers of these same sera against the heterologous subtype virus X31 were undetectable (<10). However, when measured in an ELISA against inactivated viral components, low but significant levels of cross-reactive antibodies were generated against the HA of X31 in both IM and IP infected mice (Fig.5B), and these were significantly higher in animals inoculated via the IM route. The presence of elevated levels of X31-specific antibodies induced via the IM route corresponds to the greater reduction in lung viral titres upon challenge with heterologous virus.

DISCUSSION

Recent outbreaks caused by the emergence of swine-origin A/H1N1 and highly pathogenic avian influenza A/H5N1 and A/H7N9 subtypes highlighted the need to develop vaccine strategies that induce cross-protective immunity to previously un-encountered viral strains. The intranasal administration of live-attenuated influenza vaccines adapted to replicate at lower physiological temperatures suited to the upper respiratory tract can induce a broader range of systemic and mucosal antibody and limited T cell-mediated responses [28-31], show some protection against drifted seasonal [32] and highly pathogenic strains [28,33]. However, due to the potential risk of attenuated vaccine strains to replicate in the lower respiratory tract, those in greatest need of vaccination, infants and the elderly, are excluded from using these vaccines. Harris et al. [19] showed that live virus delivered by the non-productive IM route was much better at inducing virus-specific $CD8^+$ T cell responses as well as protecting against heterologous challenge than inactivated virus given by the same route, suggesting that the use of live-virus immunisation may be a practical means of vaccinating paediatric populations to provide a breadth of protection.

The successful outcome of vaccination can be largely influenced by the vaccine formulation, the type of adjuvant and the inoculation route. While one inoculation route may be efficacious for a particular vaccine, such route may not be as effective for vaccine-induced protection in a different disease context [34,35]. This notion holds true for the use of live-virus vaccines, where different inoculation routes not only affect the overall protective effect of the vaccine but also the type, range and localization of ensuing responses [36-38].

We characterized immune responses induced by live non-attenuated influenza virus administered via two inoculation routes that result in non-replicative infection. We sought to understand the degree of heterologous protection obtained by vaccinating and the likely cross-protective mechanisms involved. We showed that inoculation of live-virus via the IM route was more effective than via IP route at inducing cross-protective responses, demonstrated by the faster clearance of the heterologous challenge virus from the lung. This was despite the fact that the two routes of inoculation induced distinct priming patterns. Antigen presentation following IM was localized to LNs proximal to and draining the injection site, while inoculation via the IP route resulted in antigen presentation occurring at sites including those distal to the inoculation site such as the mediastinal, mesenteric, inguinal and lumber LNs, indicative of the systemic dissemination of antigen. Unexpectedly, this difference did not affect the induction of the resultant primary CTL responses as each route induced similar levels of cytokine producing D^bNP_{366}- and D^bPA_{224}-specific CTLs. Moreover, the quality and magnitude of CTLs recalled to the lungs following heterologous viral challenge did not appear to be affected by the inoculation route.

It is unclear why no differences in cell-mediated responses were observed despite different

priming patterns. Studies examining the homing ability of CTLs into tissues from which the antigen originated would suggest the opposite [39,40]. Previously, rapid systemic LCMV infection via the IP route resulted in the induction of responses at secondary lymphoid organs identifying the mediastinal LN as the primary site of CTL activation [41]. As a result, memory T cells are preferentially recruited back to the mediastinal LN, where they remain poised to provide host defence against further encounter with this pathogen. However, even though cross-reactive CTL numbers induced by immunisation with Flumist via the intranasal route are not sufficient to mediate heterologous protection per se, these cells rapidly develop memory characteristics and thus can be boosted efficiently by viral antigen [42]. It appears that influenza viruses given via either IP or IM routes establish influenza virus-specific $CD8^+$ memory pools capable of mediating robust recall responses after the pulmonary viral challenge.

We also ruled out the possibility that the enhanced protection against heterologous challenge observed using the IM route was attributed to the hyper-induction or dysregulation of proinflammatory cytokine production and vaccination by either route was responsible for reducing particular cytokines often associated with severe infection [43]. We did, however, discover that the enhanced viral clearance was accompanied by the presence of heightened levels of cross-reactive antibodies against heterologous HA, indicating that while both routes of inoculation were similarly effective at inducing CTLs and homologous antibody responses, delivery via the IM route is advantageous for inducing heterologous antibody-mediated immunity.

As expected [44,45], the cross-reactive antibodies did not display HI activity against X31 virus and are therefore unlikely to be directed against the receptor-binding domain in the HA head region which have direct virus-neutralising function. One possibility is that the antibodies are recognising the highly conserved stalk domain of HA [46,47] comprising the fusion active subunit of HA2 [48,49] or other conserved parts of the HA (and other surface molecules, NA and M2) that can be accessed more efficiently on infected cell surfaces displaying an array of viral glycoproteins prior to budding. Although viral antigen present at the cell surface on abortively-infected DC and macrophages would be common to productive and non-productive routes of infection, it is possible that the absence of large amounts of nascent free virus at the non-productive sites may allow some antibody response to be focused towards the abundant display of cell-associated antigens.

How these different priming patterns of IP and IM routes affect the induction of heterologous antibody responses remains unclear. Considering distinct DC subsets in the mediastinal LN have different abilities to induce anti-viral CTLs following intranasal influenza infection [50], it is possible that this could also be the case for the establishment of memory $CD4^+$ T cell responses that can support the generation of cross-protective antibody production by B cells [4,51]. We have in fact found that the frequencies of each DC subset and their ability to activate

antigen-specific T cells following IP or IM immunization (Supplementary Fig.2A) are significantly different. Notable differences were observed in the numbers of T cells activated by CD11b+ and $CD103^+$ DCs in the mediastinal node and $CD8^+$ and $CD11b^+$ DCs in the inguinal node following IP immunization compared to IM immunization, and between all subsets in the mesenteric node following IM immunization compared to IP immunization (Supplementary Fig 2B-C).

Direct DC-B cell interactions may play a role. DC-mediated presentation of both T cell-dependent and -independent antigens to B cells *in vitro* and *in vivo* can promote B cell proliferation, differentiation and isotype-class switching [52-54]. Splenic $CD8\alpha^-$DCs can initiate extrafollicular B cell responses to antigen, highlighting the possibility that individual lymphoid-derived DC subsets may play distinct roles in directly influencing B cell antibody production [53]. Alternatively, the differences in IP and IM priming on cross-protective antibody production may simply be that the number of B cell precursors capable of recognising conserved epitopes are low and the greater concentration of antigen following IM delivery may facilitate the chances of their activation.

Overall, the characterization and comparison of the priming patterns and resultant immune responses induced by two inoculation routes has provided us with an insight into how non-replicative routes of infection can be used to induce both the cross-protective CTLs and strong strain-specific antibody responses. We also emphasize the importance of immunization route in the generation of cross-protective antibody production that may contribute to greater heterologous virus clearance. Given the prevailing concerns associated with the use of live-attenuated influenza virus vaccines given intranasally, this indicates that inoculation via an alternate route could minimize the risk of replication and still be sufficiently immunogenic. Such strategy could be useful during an outbreak of any unpredicted influenza virus when stocks of inactivated vaccines are unavailable and the use of live non-systemically spreading viruses, of homologous or even heterologous strains, as vaccines could be a rapid emergency measure to lessen the global burden of disease.

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FIGURE LEGENDS

Figure 1. Priming of T cells following IM immunisation is localised to the draining LNs, while IP immunisation results in the systemic distribution of antigen to distal sites.

C57BL/6 mice (5 per group) were infected with live PR8-OVA virus (1.5×10^7 p.f.u.) via the IM or IP route. DCs were isolated from various lymphoid tissues 24 hr later and 5×10^3 cells co-cultured for 60 hr with 5×10^4 CFSE-labelled $CD8^+$ T cells from OT-1 transgenic mice (A). Proliferating OT-1 T cells were then determined by measuring the CFSE levels of the $CD8^+$ cells by flow cytometry. Numbers within each representative histogram indicate the percentage (± SD) of proliferating ($CFSE^{low}$) cells from triplicate samples (B). In addition, 1×10^6 CFSE-labelled $CD8^+$ T cells from OT-1 transgenic mice were transferred intravenously into naïve C57BL/6 mice one day before inoculation with live PR8-OVA virus via the IM or IP route (C). Lymph nodes and spleens of animals were harvested 3 d later and $CD8^+$ T cells analysed for CFSE levels by flow cytometry. Results presented as bar graphs depict the mean (± SD) of the number of proliferating ($CFSE^{low}$) OT-1 cells (D) and the total number of OT-I T cells present in each organ (E). (* indicates $p<0.05$).

Figure 2. Accelerated viral clearance after IM immunisation with live influenza virus.

C57BL/6 mice (n=10 per group) were immunised with 1.5×10^7 p.f.u. PR8 (H1N1) virus via the intra-peritoneal (IP; squares) or intramuscular (IM; triangles) route, or inoculated with PBS (circles) prior to intranasal challenge with 1×10^4 p.f.u. X31 (H3N2) virus 6 weeks later. On day 6 or 8 following challenge, lung viral titres were measured in a plaque formation assay. Viral titres from individual animals are presented with the mean value (± SD) of the group represented by the horizontal bar (* indicates $p<0.05$ and < indicates below the threshold of detection).

Figure 3. Virus-specific $CD8^+$ T cell responses are similar following IM and IP immunization.

The magnitude of virus-specific $CD8^+$ T cell responses in the spleen of C57BL/6 mice (5 per group) were determined 10 and 30 days following primary PR8 virus (1.5×10^7 p.f.u.) immunisation via the IM or IP route (1°) and in the spleen and BAL washes 8 days after secondary intranasal challenge (2°) with X31 (1×10^4 p.f.u.) 6 weeks later. Flow cytometric dot plots show the percentage (± SD) of D^bNP_{366} or D^bPA_{224}-specific $CD8^+$ T cell populations after staining for epitope-specificity with MHC class 1 tetramers (A). The data are also represented as bar graphs depicting the mean (± SD) of the total number of epitope-specific T cells in each organ (B). IFN-γ secretion from D^bNP_{366} or D^bPA_{224}-specific $CD8^+$ T cells in the presence of antigen was enumerated in an intracellular

cytokine assay (C). The number of IFN-γ-producing $CD8^+$ T cells that secrete TNF-α and IL-2 was also determined (D).

Figure 4. Similar cytokine levels were found in the lungs of IP- and IM-immunised mice following X31 virus challenge.

C57BL/6 mice (5 per group) were infected with PR8 virus (1.5×10^7 p.f.u.) via the IM or IP route prior to intranasal challenge with X31 virus (1×10^4 p.f.u.) 6 weeks later. Lungs were harvested from mice 6 or 8 days after challenge and homogenised. Concentrations of cytokines in the supernatant of clarified homogenates were determined in a cytokine bead array assay. Box and whisker graphs represent the average amount (± SE) of each cytokine in each group.

Figure 5. Both immunisation routes induce similar homologous antibody responses but immunisation via the IM route induces greater cross-reactive antibody responses.

Sera were obtained from C57BL/6 mice that were infected 4 weeks previously with PR8 virus (1.5×10^7 p.f.u.) via the IM or IP route. Sera were tested for antibodies with HI activity against PR8 and X31 viruses (A) and binding activity by ELISA against viral proteins derived from PR8 virus or the HA of X31 virus (B). Results are presented as the average antibody titre (± SD) from all sera samples within each group. (* indicates $p<0.05$).

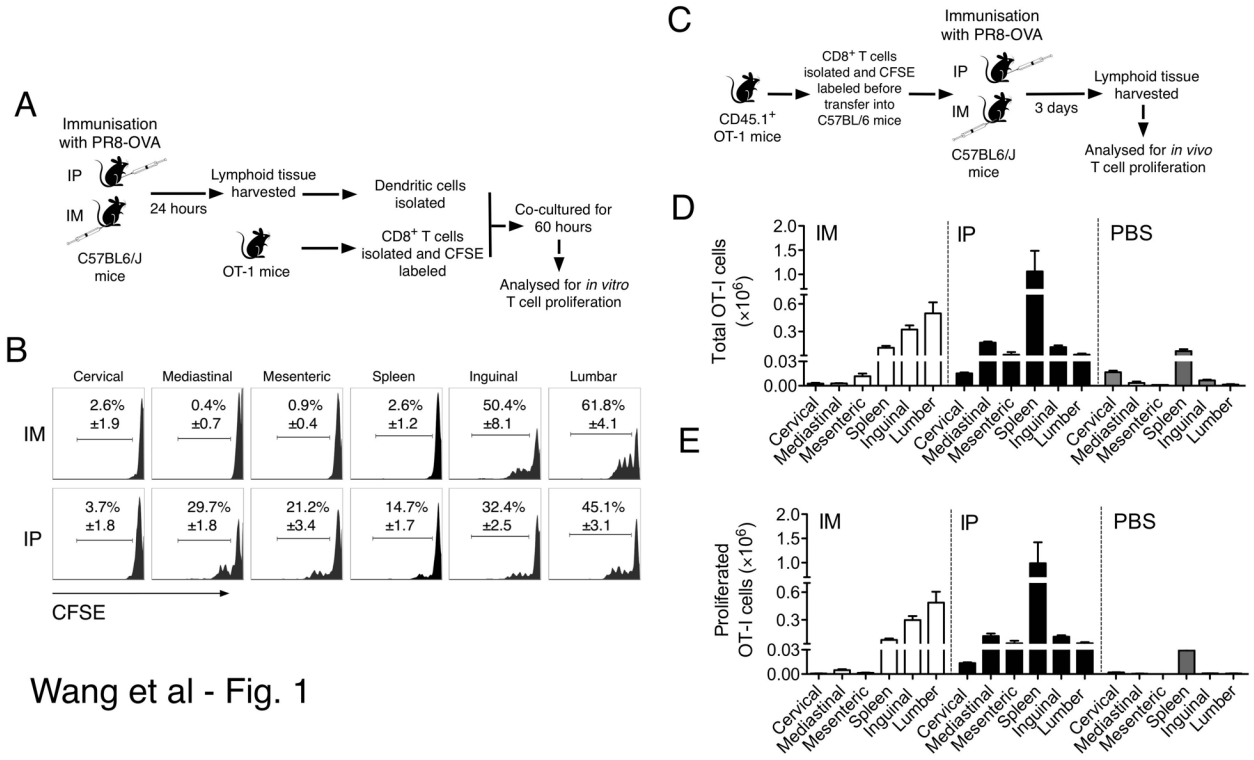
SUPPLEMENTARY DATA LEGENDS

Supplementary Figure 1. Numbers of $CD8^+$ T cells in lymphoid organs following IM and IP priming. 1×10^6 CFSE-labelled $CD8^+$ T cells from OT-1 transgenic mice were transferred intravenously into naïve C57BL/6 mice one day before immunisation with PR8-OVA virus via the IM or IP route. Lymph nodes and spleens of animals were harvested 3d later and the total number of $CD8^+$ T cells in each organ was enumerated by staining with a fluorochrome conjugated anti-CD8 antibody and analysed by flow cytometry. Results are presented as bar graphs depicting the average number (±SD).

Supplementary Figure 2. Frequency and ability of dendritic cell subsets from lymph nodes of immunised mice to activate T cells. C57BL/6 mice (n=15 per group) were infected with PR8 virus (1.5×10^7 p.f.u.) via the IM or IP route. DC subsets ($CD8^+$, $CD11b^+$ and $CD103^+$ DCs) were isolated from pooled lymph nodes 24 hours later (A). The frequencies of $CD8^+$ and $CD103^+$ DCs are also represented in the FACS plots (B) The absolute number of DC of different subsets in the inguinal, mediastinal or mesenteric lymph nodes pooled from 15 mice in each group is also shown (C). DC subsets were then co-cultured with CFSE-labelled OT-1 T cells at a 1:2 ratio for 60 hours and T cell proliferation determined by analysis of CFSE levels in OT-1 T cells (D).

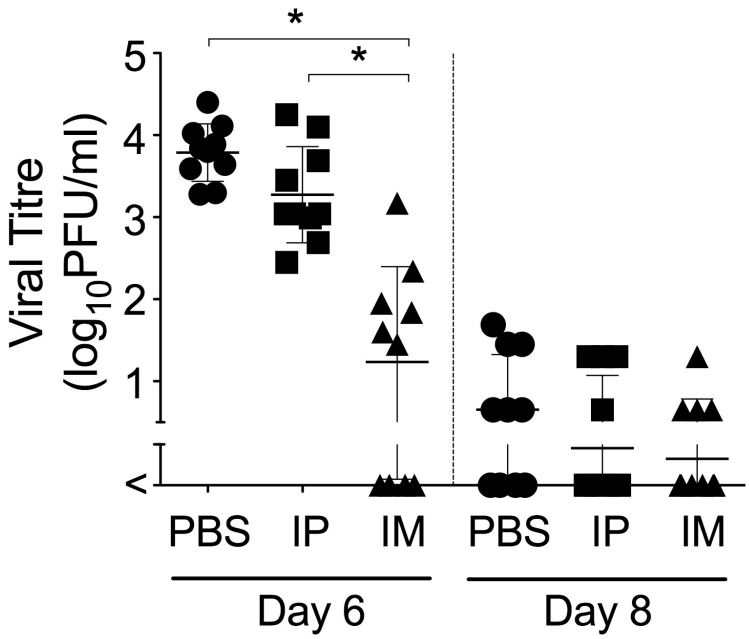
FIGURES

Figure 1



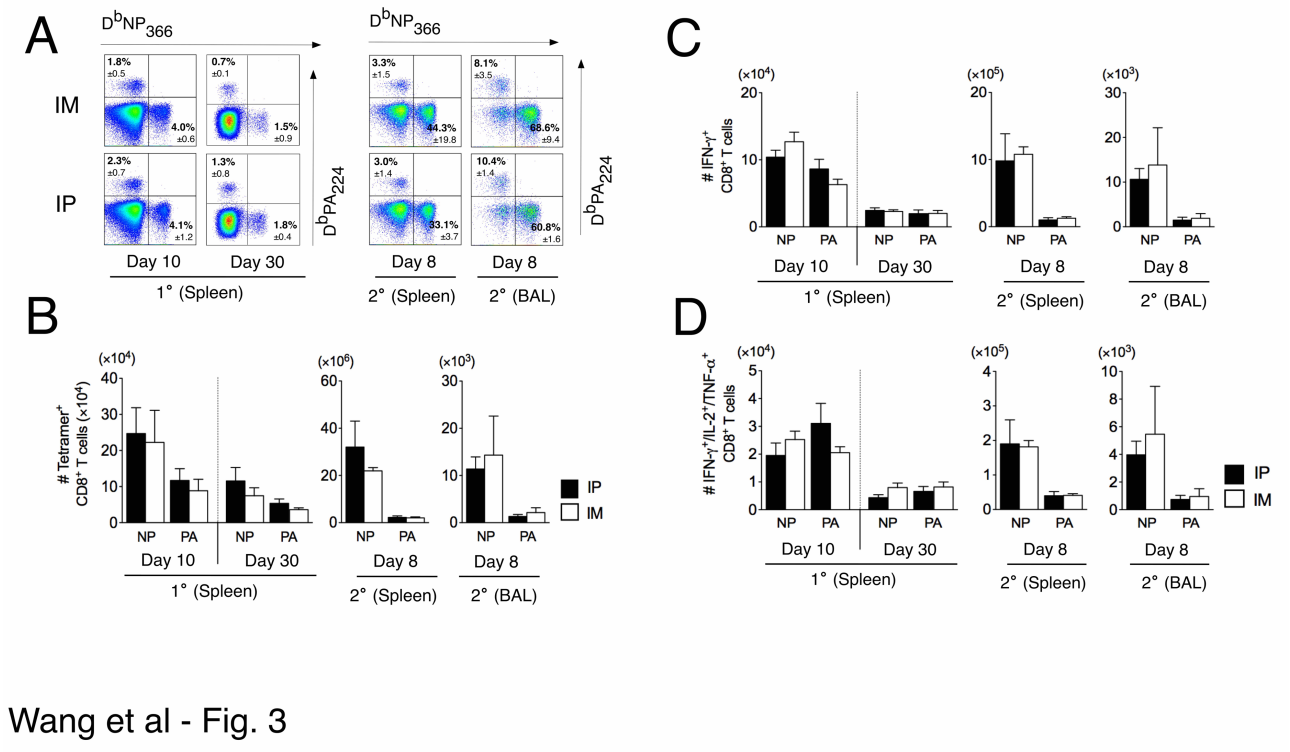
Wang et al - Fig. 1

Figure 2



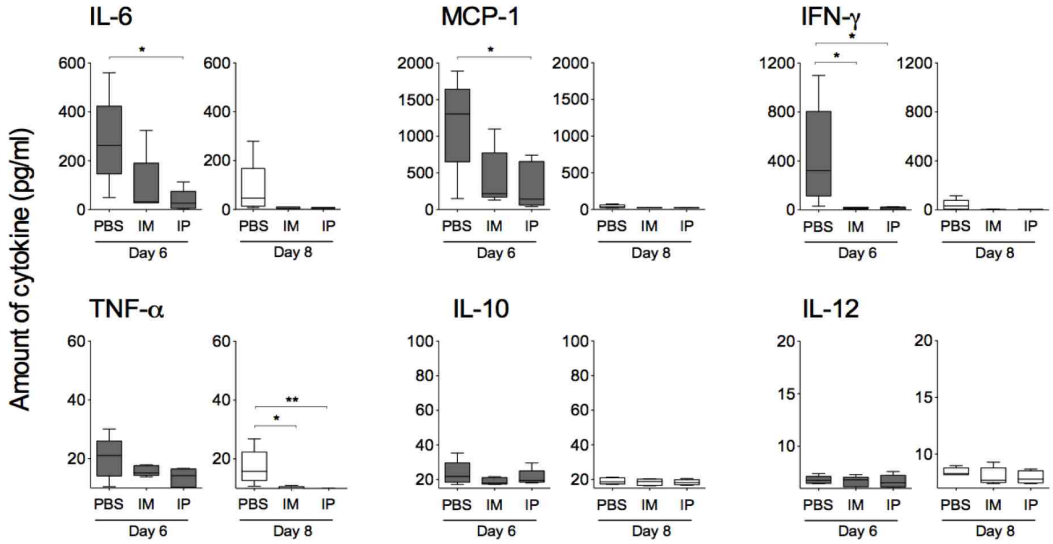
Wang et al - Fig. 2

Figure 3



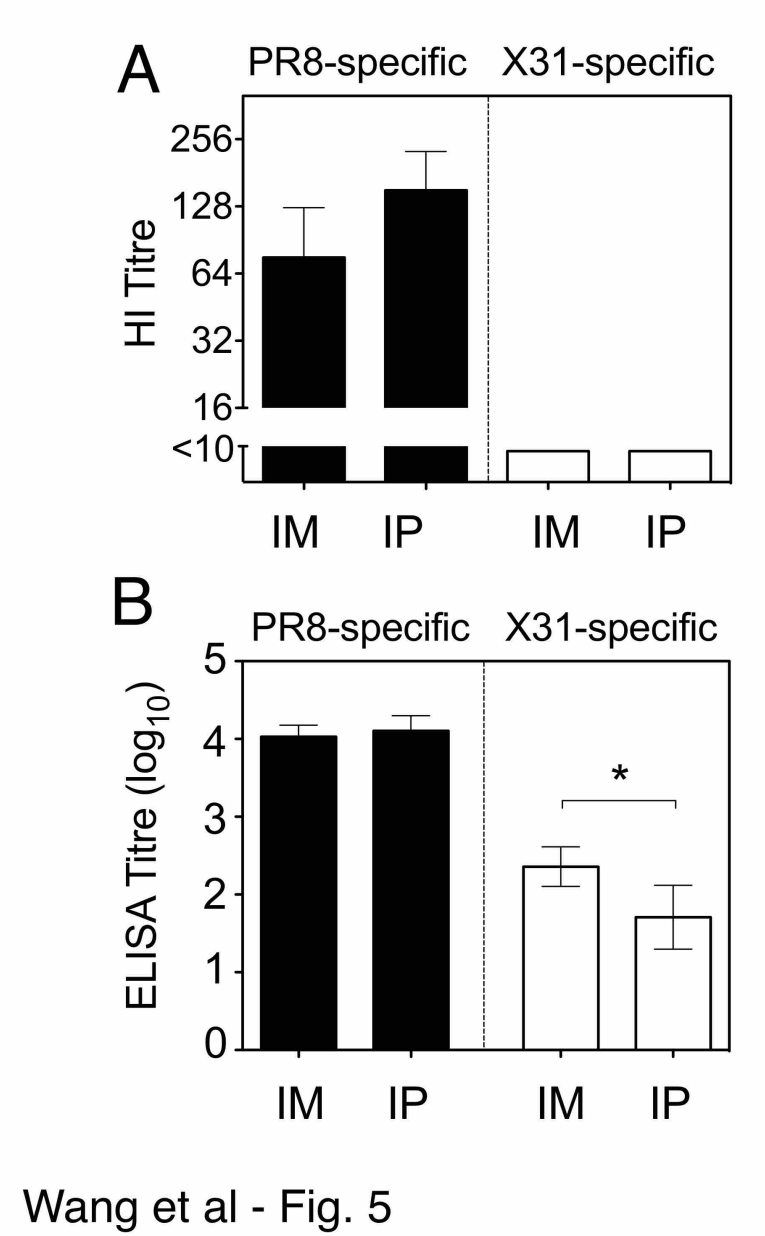
Wang et al - Fig. 3

Figure 4



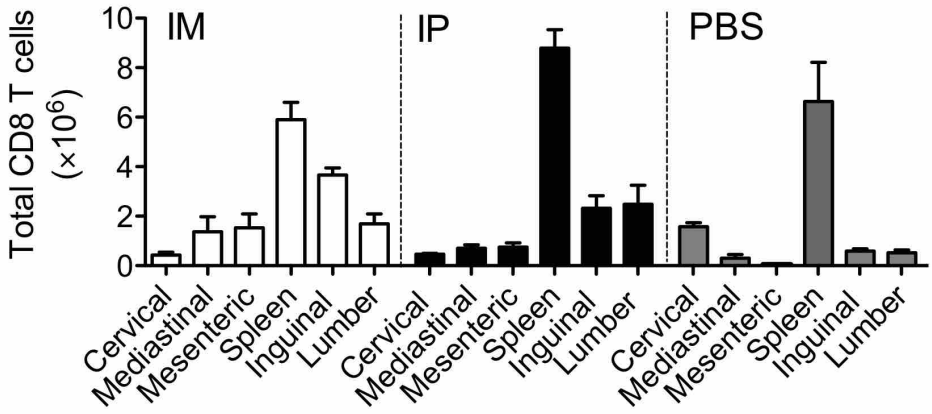
Wang et al - Fig. 4

Figure 5



Wang et al - Fig. 5

SUPPLEMENTARY DATA



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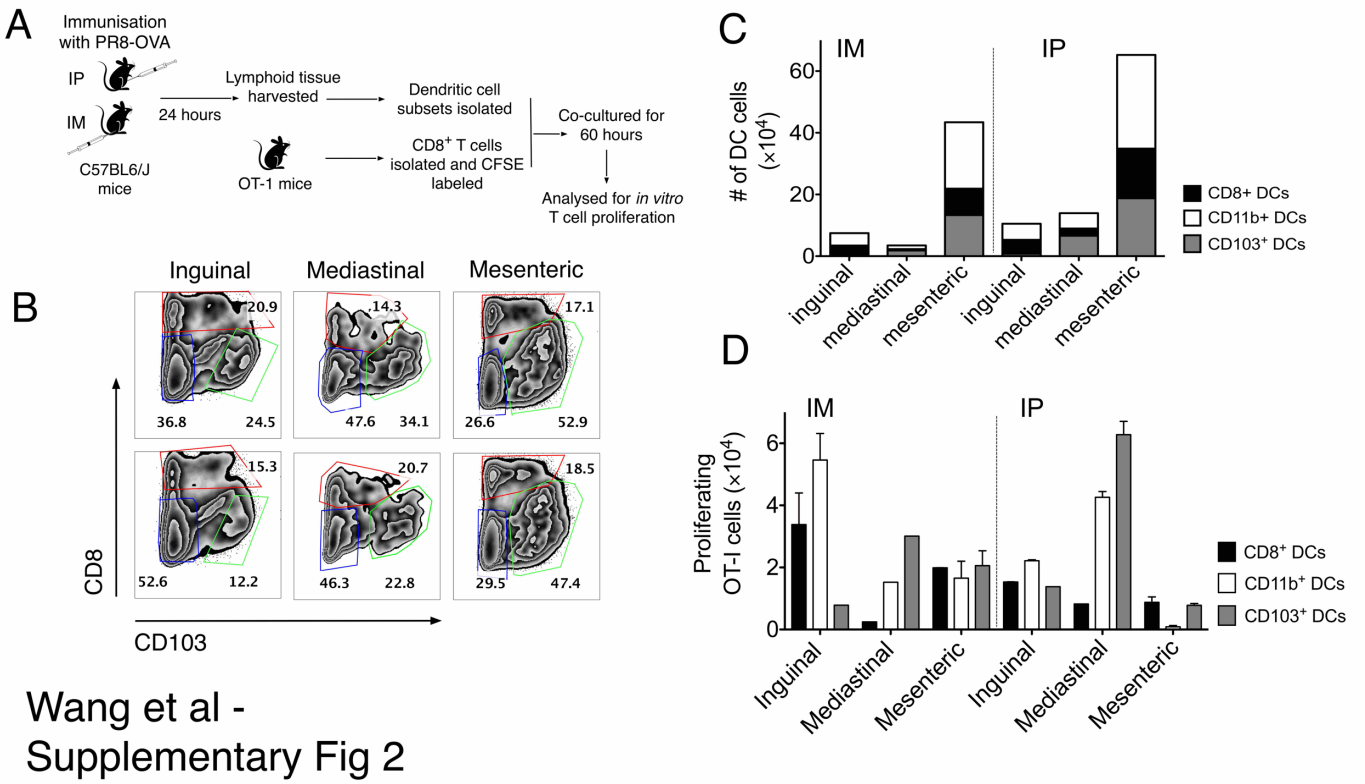
Supplementary Fig 1



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Supplementary Fig 2