Airway T cells protect against RSV infection in the absence of antibody.

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Conflict of Interest Statement

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Running title: Trm protect against RSV
Abstract

Tissue resident memory T cells (Trm) act as sentinels and early responders to infection. RSV specific Trm have been detected in the lungs after human RSV infection, but whether they play a protective role is unknown. To dissect the protective function of Trm, BALB/c mice were infected with RSV; infected mice developed antigen specific CD8⁺ Trm (CD103⁺/CD69⁺) in the lungs and airways. Intranasally transferring cells from the airways of previously infected animals to naïve animals reduced weight loss on infection in the recipient mice. Transfer of airway CD8 cells led to reduced disease and viral load and increased IFNγ in the airways of recipient mouse, whilst CD4 transfer reduced TNFα in the airways. Because DNA vaccines induce a systemic T cell response, we compared vaccination with infection for the effect of memory CD8 cells generated in different compartments. Intramuscular DNA immunization induced RSV specific CD8 T cells, but they were immunopathogenic not protective. Notably, there was a marked difference in the induction of Trm; infection but not immunization induced antigen specific Trm in a range of tissues. These findings demonstrate a protective role for airway CD8 against RSV and support the need for vaccines to induce antigen specific airway cells.
Introduction

Respiratory Syncytial Virus (RSV) is the leading cause of morbidity from lower respiratory tract viral infection in infants and children less than 5 years old. There is as yet no RSV vaccine and whilst a number of candidates are in late stages of clinical trials, the ideal immune correlates of protection are not fully characterized. The main target of vaccine induced adaptive immunity has traditionally been antibody. Antibody is a key correlate of protection against RSV, for example maternal antibody levels closely correlate with the risk of RSV infection in the infant. But protection can occur in the absence of detectable serum antibodies and infection can occur when serum neutralizing antibodies are high. This suggests a role for protective T cell immunity, supported by the observation that children with defective T cell responses were unable to clear the virus for several months and suffered from more severe disease.

Their protective capacity should make the induction of CD8 T cells an attractive goal for an RSV vaccine; and yet, there is a substantial body of evidence suggesting that excess T cell responses can be detrimental. One possibility is that the wrong types of T cells, either functionally or spatially, are being induced. Memory T cells can be phenotypically characterized by their cell surface marker expression, with different subsets behaving differently on re-exposure to the pathogen. Recently a novel population of tissue resident memory T (Trm) cells has been defined. These cells move from the circulation into tissues, leading to the upregulation of CD69, the downregulation of S1P1 and normally the upregulation of the integrin CD103, which leads to their retention in the tissue. Functionally, these cells are primed to respond more rapidly to pathogen, with 20-times higher affinity for antigen than effector memory T cells, allowing them to detect cells expressing low levels of antigen, such as those in the early stages of infection. Once a pathogen is detected, Trm cells contribute to the early immune response by secreting cytokines such as interferon gamma (IFNγ); for example influenza-specific lung resident T cells respond rapidly upon reactivation, producing multiple cytokines. It has recently been shown that the presence of RSV specific CD8+ T cells in the lungs, but not the blood, of human adults correlates with less severe disease upon RSV challenge infection. Less research has been performed on CD4+ Trm in the lungs, but they have been shown to have a protective role in influenza and Nippostrongylus brasiliensis.

The aim of this study was to investigate the functional role of Trm in protection against RSV infection. We demonstrate for the first time that transferring airway CD4 or CD8 cells is sufficient to protect against disease after RSV infection. We then compared the effect of memory CD8 cells generated after lung infection or systemic vaccination and saw that systemic DNA vaccines induce pathogenic not protective CD8 T cells. Comparing vaccination and infection, we see significant differences in the localisation and type of antigen specific CD8 cells, which may contribute to their different effects. From this we conclude that airway resident T cells are sufficient to protect against RSV and their induction should be a goal of vaccination.
Results

Prior RSV infection protects against subsequent infection and induces both RSV specific antibody and CD8 T cells

Intranasal infection with wild type RSV protects against subsequent exposures and antibody plays a role in this protection, but the role of T cells is unclear. The aim of the study was to investigate the role of lung resident T cells in protection against RSV infection. The response to RSV was compared after one, two or three exposures to RSV, each 21 days apart. As expected, mice infected for the first time with RSV lost significantly more weight (Fig 1A), had more viral load on day 4 after infection (Fig 1B) and significantly lower anti-RSV IgG in serum after infection (Fig 1C). To distinguish between circulating and local cells, mice were pretreated with intravenous antibody prior to collecting organs. CD8 T cells were recruited into both the lungs (Fig 1D) and airways (Fig 1E), peaking at day 8 of mice infected with RSV for the first time. There were significantly more total CD8 cells in the lungs or BAL after a single infection than two or three infections. However, the proportion of CD8 cells that were resident memory cells (Trm, defined as CD69⁺ and CD103⁺) was significantly greater in the lungs (Fig 1F) and airways (Fig 1G) of mice previously exposed to RSV than primary infection at days 4 and 8 after infection. This combination of markers has been used to identify Trm after viral infection in both human and murine studies, though it may also pick up some effector cells at the acute timepoints. Interestingly, the proportion of CD69⁺/CD103⁻ cells was the same 21 days after the first, second or third exposure to RSV – rising to approximately 10% of CD8 cells after one exposure or contracting to the same proportion after two or three exposures. Of the CD69⁺/CD103⁻ CD8 Trm cells, a high proportion were specific for the immunodominant peptide of the RSV M protein (Fig 1H and I). The proportion that was pentamer positive were significantly greater after re-exposure to RSV. There were no differences seen in the response after the second or third exposure to RSV. Therefore RSV specific Trm are induced in the lungs and airways after infection.

Airway cell transfer reduces disease after RSV infection.

The aim of the study was to define the role of airway cells in protection against RSV disease. Mice were infected with RSV, 4 weeks later T cells were depleted with antibody prior to re-infection with RSV. Depleting CD8⁺ or CD4⁺ led to a significant increase in early weight loss compared to the RSV-RSV group, indicating they have a role in the reduction of disease (Fig 2A). However, these mice had anti-RSV antibody which may mask any protective effect of T cells. We used transfer studies to test cells in isolation. Donor mice were infected two times with RSV or sham infected with PBS; 3 weeks after the second infection mice were culled and cells were collected from the airways or spleens. 10⁶ cells were instilled into the airways of naive animals, of these 22% were lymphocytes and of the CD8 T cells transferred, 25% were RSV specific Trm accounting for 10⁴ cells transferred (Supp Fig 1). One day after cell transfer the recipient animals were infected with RSV. Mice receiving cells from the airways of RSV exposed animals lost significantly less weight on days 6 and 7 compared to mice receiving splenocytes from RSV exposed animals or cells from either the airways or spleens of PBS treated animals (Fig 2B). There were significantly more CD8 (Fig 2C) and CD4 (Fig 2D) Trm in mice receiving cells from the airways of RSV infected animals, seven days after infection of the recipient mice. To determine the fate of transferred airway cells from naive or RSV infected, they were labelled with cell trace violet prior to intranasal transfer into naive animals. Recipient mice were infected with RSV one day after cell transfer and then culled two days after RSV infection. Labelled
cells were detectable at a low frequency in the airways of all mice, with significantly more cells in the airways of RSV infected animals receiving cells from RSV donors (Supp Fig 1D).

To determine the relative contribution of CD4 and CD8 cells in protection against disease following RSV infection, airway cells from previously RSV infected mice were sorted prior to intranasal transfer to naïve mice (Supp Fig 2). Both CD4 and CD8 cell transfer reduced weight loss compared to control mice (Fig 2E). There were, however, phenotypic differences between mice receiving CD4 or CD8 cells. Mice that received CD8 cells had significantly lower viral load on day 4 of infection with RSV (p<0.01, Fig 2F) and significantly greater IFNγ in the airways on day 1 of infection (p<0.01, Fig 2G). Strikingly transfer of CD4+ cells led to a significant reduction in airway Tumor Necrosis Factor alpha (TNFα) (p<0.05, Fig 2H), but no effect on IFNγ or viral load. Transferring sorted cells from the airways of PBS exposed animals had no protective effect (Fig 2I). Curiously, transfer of unsorted cells from the airways of RSV infected mice also reduced weight lost, but without an impact on viral load, IFNγ or TNF. From this we conclude that airway CD8 are protective against reinfection and airway CD4 can reduce disease.

**Vaccines inducing systemic T cells do not protect against RSV infection**

Having observed that T cell transfer reduced disease after RSV infection, we wished to test whether vaccination could induce T cell mediated protection. DNA vaccines have been shown to induce strong cellular responses in the systemic compartment and so we used them to test whether vaccine induced anti-RSV T cells could protect against RSV disease in mice. Mice were immunized intramuscularly with 5 μg plasmid encoding RSV M2 in a prime-boost-boost regime. 3 weeks after the second immunization, mice were infected with RSV. RSV M2 immunized mice lost more weight, more rapidly than untreated mice (Fig 3A). DNA immunization reduced the viral load at d4 after infection, but not to the same magnitude as previous infection with RSV (Fig 3B). Immunization with RSV M2 induced a significant population of RSV M2 specific CD8 cells, this was greater than in animals previously infected with RSV (Fig 3C). But DNA vaccination induced significantly fewer RSV specific CD8 cells expressing Trm markers (Fig 3D). The DNA vaccine induced RSV specific cells were highly inflammatory, producing significantly more TNFα (Fig 3E) or IFNγ (Fig 3F) than cells from naïve or previously exposed mice. It has been previously been demonstrated that excess CD8 cells in the airways can lead to enhanced disease in RSV. We wished to determine whether DNA vaccine induced T cells were causing disease. When CD8 cells were depleted using antibody during RSV infection in spite of an increase of viral load (Fig 3G) there was a significant reduction in weight loss (Fig 3H). From this we conclude that systemically induced T cells can cause disease rather than protect.

**Immunization and infection induce different antigen specific T cells populations in different tissues**

During infection, the proportion of airway CD8 T cells specific for the immunodominant M2 epitope was similar between RSV infected and DNA vaccinated animals, but there were significant differences in the proportion of those that expressed the Trm markers CD69 and CD103. To determine whether these differences occurred at the time of initial exposure to RSV antigens and whether there were differences in the cellular distribution we compared PBS immunized, RSV infected and M2 DNA immunized animals. Mice receiving DNA were immunized three times at two week intervals in the left anterior tibialis muscle in the hind limb. There were some differences in timing between exposure and recovery of cells: PBS or RSV groups were culled seven weeks after the
initial exposure, DNA vaccinated mice were culled 3 weeks after the final exposure, all animals were culled and samples analyzed at the same time.

The phenotype of CD8 cells in the site of immunization (the left hind limb muscle and covering skin) was compared with the distal, right hind limb muscle and skin, airways, lung, blood and spleen. No RSV specific CD8 cells were detected after PBS delivery (Fig 4A). RSV infection led to populations of RSV specific cells in all tissue compartments (right muscle wasn’t measured due to technical complications). DNA vaccination with a plasmid encoding the M2 gene, induced a population of M2 specific CD8 cells in the immunized muscle (left) but not the distal, right muscle. DNA immunization induced a larger population of RSV specific cells in the blood and lungs than the airways or spleen. RSV specific cells in the lungs after vaccination may represent blood contamination because the lungs were not flushed prior to analysis. RSV infection induced a population of RSV specific CD8 T cells in all tissues assessed. Strikingly, infection induced significantly more RSV specific CD8 Trm cells in the lungs and skin sections than DNA vaccination (Fig 4B). Therefore infection and immunization induce qualitatively different cell populations recognizing the same epitope, with only infection leading to the induction of a protective CD8 T cell subset in the lungs.
Discussion

In the current study we demonstrate for the first time that airway T cells are sufficient to protect against infection, even in the absence of antibody. Transferring CD8 T cells from mice previously infected with RSV to naïve animals reduced weight loss and viral load on exposure to RSV. This is an important finding because of the implication it has for RSV vaccine strategies. It ties in closely with a recent study that demonstrated that intranasal vaccination with a recombinant virus expressing RSV proteins was protective against infection and induced RSV specific tissue resident memory cells. However, caution needs to be taken with T cell targeting vaccines for RSV because the induction of CD8 T cells in the systemic compartment led to enhanced disease on exposure to RSV infection.

The transfer studies demonstrate that cells resident in the airways are protective against RSV infection. Functionally, there was an increase in IFNγ in the airways after CD8 transfer, which may lead to the recruitment of other antiviral cells to the airway, though the CD8 cells themselves may be having a direct antiviral effect. It was of note that transferring CD4 T cells from the airways reduced disease, without affecting viral load. There was a reduction in TNFα levels following CD4 T cell transfer; we have previously shown that blocking TNFα reduces disease and it may be that some of the CD4 cells transferred are regulatory T cells as we have shown that Treg depletion increases TNFα levels and disease. Total cell transfer also reduced weight loss, so either T cells are working in concert or there is a role for other cell types in the airways, for example macrophages. Macrophages make up the majority of airway cells even after RSV infection and the transferred macrophages may also release cytokines locally and promote antiviral responses. Splitting the cells into different subsets prior to transfer would enable the identification of which memory or effector T cells are protective.

One of the striking findings was that after infection, RSV specific cells T were detectable in all tissues sampled. After infection cells from the lungs or the skin had a greater proportion of CD69+/CD103+ cells. The skin is a known site for tissue resident cells and it may be that effector cells induced in the lungs track to other tissues where they take on the characteristics of resident memory cells. It was of note that although RSV infection induces antigen specific cells in the spleen, transferring splenocytes was not protective: a similar phenomenon was seen when protection was transferred with airway cells, but not splenocytes from influenza infected mice. It will be important to determine the factors that lead to the induction of Trm in the lungs and to determine whether these can be replicated artificially for vaccine strategies. In the skin, the cytokines TGF-β and IL-15 are required for Trm development, but their role in lung Trm formation is not known.

One approach that has promise for the induction of resident memory cells is mucosal vaccination. It was striking that intramuscular DNA vaccination, whilst it did induce antigen specific cells in the immunized tissue and the blood, did not induce tissue resident memory cells in any site tested, even the site of immunization. Route has a significant effect on the qualitative response to DNA vaccination. Intranasal immunization with influenza DNA in a 100 µl volume also led to the induction of airway resident memory cells. Likewise in a recent study, intranasal delivery of a recombinant viral vector expressing RSV M led to the induction of tissue resident RSV specific cells, but only when delivered in a large enough volume to reach the lungs (100 µl); intraperitoneal immunization with the same vaccine did not induce Trm. These studies suggest that the vaccine needs to get into the lungs to induce the correct type of memory cells and that responses in the upper airway are not the same as the lower airway. Vaccination at other mucosal sites can also lead
to the induction of Trm, intravaginal immunization with human papillomavirus vectors (HPV) expressing RSV antigen led to the induction of RSV specific Trm in the vagina, but when the same antigen was delivered intramuscularly, Trm were not induced. One potential strategy is to induce the formation of memory CD8+ T cells in circulation that can then be ‘pulled’ to a local site, however we had limited success using this strategy to recruit antigen specific B cells to the mucosa.

Ultimately an RSV vaccine needs to be effective in early life, when the bulk of severe disease happens. The infant immune response, in particular the cellular response is different to the adult immune response. We have previously shown that neonatal RSV infection primes for an immunopathogenic CD8 response, similar to the phenotype seen after DNA vaccination. How the immune environment in the neonatal lung differs to the adult lung with regards to the priming of T cell responses is an important research topic for the development of optimum vaccines. Whilst RSV vaccines that are closest to the clinic are being developed for pregnant women with a view of passing on passive humoral immunity to the newborn, based on the data presented here, we would also advocate strategies that induce local T cells.
Methods

Mouse immunization and infection

6–8 week old female BALB/c mice were obtained from Harlan UK Ltd (Oxford, UK) and kept in specific-pathogen-free conditions in accordance with the United Kingdom’s Home Office guidelines. All work was approved by the Animal Welfare and Ethical Review Board (AWERB) at Imperial College London. RSV A2 virus were grown using the human laryngeal carcinoma cell line, HEp-2. Viral titer was calculated by an immuno-plaque assay using biotinylated goat anti-RSV polyclonal antibody (AbD Serotec, Oxford, UK) to detect plaques. For infections, mice were anesthetized using isoflurane and infected intranasally (i.n.) with $10^5$ plaque forming units (pfu) RSV A2 in 100 µl. For DNA vaccination, mice were injected 5 µg plasmid DNA expressing the M2-1 gene in 20 µl intramuscularly into the left anterior tibialis muscle. Mice were DNA immunized three times, two weeks apart. In some studies, to distinguish between circulating and tissue resident cells, mice were pre-treated intravenously with 3µg anti-CD45.2 (A700 fluorophore) in 200µl 3 minutes prior to sacrifice.

RSV viral load

Viral load in vivo was assessed by extracting RNA from frozen lung tissue disrupted in a TissueLyzer (Qiagen, Manchester, UK) using Trizol extraction and then converting it into cDNA. Quantitative RT-PCR was carried out using bulk viral RNA, for the RSV L gene and mRNA using 900 nM forward primer (5’-GAACCTCAGTGTAGGTAGAATGTTTGCA-3’), 300 nM reverse primer (5’-TTCAGCTATCATTTTCTGGCAAT-3’) and 100 nM probe (5’-FAM-CTTGAACCTGTCTGAACAT-TAMRA-3’) on a Stratagene Mx3005p (Agilent technologies, Santa Clara, CA, USA). L-specific RNA copy number was determined using a RSV L gene standard.

Semi-quantitative antigen-specific ELISA

Antibodies specific to RSV were measured in sera using a standardized ELISA. MaxiSorp 96-well plates (Nunc) were coated with 1 µg/ml RSV lysate or a combination of anti-murine lambda and kappa light chain specific antibodies (AbDSerotec, Oxford, UK) and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS. Bound IgG was detected using HRP-conjugated goat anti-mouse IgG (AbD Serotec). A dilution series of recombinant murine IgG was used as a standard to quantify specific antibodies. TMB with H$_2$SO$_4$ as stop solution was used to detect the response and optical densities read at 450 nm.

Cell isolation

Lungs, airway cells and spleen. Mice were culled using 100µl intraperitoneal pentobarbitone (20 mg dose, Pentoject, Animalcare Ltd. UK). Spleens, lung tissue and BAL collected as previously described. Lungs and spleens were homogenized by passage through 100 μm cell strainers, then centrifuged at 200 x g for 5 minutes. Supernatants were removed and the cell pellet treated with red blood cell lysis buffer (ACK; 0.15 M ammonium chloride, 1 M potassium hydrogen carbonate, and 0.01 mM EDTA, pH 7.2) before centrifugation at 200 x g for 5 minutes. The remaining cells were resuspended in RPMI 1640 medium with 10% fetal calf serum, and viable cell numbers determined by trypan blue exclusion.
Blood. For antibody specified time points post-immunization, blood samples were taken by tail vein bleed and sera isolated after clotting by centrifugation. For cell isolation, blood was collected in heparinized capillary tubes (Hirschmann Laborgeräte), followed by ACK lysis.

Skin and muscle recovery. Sacrificed mice had both their legs shaved with electric clippers. A 2cm² area of skin over each of the anterior tibialis muscles was excised and placed in a well containing DMEM + 10% FCS (D10) in a 12 well plate kept on ice. The anterior tibialis muscles were also excised. Skin and muscle samples were chopped up into 3mm³ sections. 1 ml of the digestion cocktail containing 12.5 μg/ml Liberase TL in SF media, DNAse at 200 μg/ml, hyaluronidase at 50 μg/ml was added to each sample as described previously 37. Samples were placed in a shaking block at 37°C for 1 hour and then the digested sample was filtered through a 70 μm cell strainer and centrifuged at 528g for 5 minutes. The supernatant was discarded and cell pellet resuspended in 5 ml ACK lysis buffer for 5 minutes. 1.5X volume D10 was added and cells were again centrifuged at 528g for 5 minutes.

Airway cell transfer

Cells were collected from BAL or spleen, washed and resuspended in sterile PBS. Mice were anaesthetized and 10⁶ cells in 100 μl were delivered intranasally with a Gilson pipette. Isolated cells from the airways were negatively sorted for CD4⁺ and CD8⁺ populations using the MACS CD4⁺ and CD8⁺ T cell isolation kits as per manufacturers’ instructions from 10⁷ airway cells using LS columns. The purity of isolated CD4⁺ or CD8⁺ cells were analyzed by flow cytometry. To track cells; prior to transfer 5 μM Celltrace™ Violet dye (Invitrogen) was added to 10⁶ cells, they were incubated with gentle agitation, washed and resuspended in sterile PBS.

Flow cytometry

Cells were stained with Fixable Violet Dead Cell Stain (Life Technologies, Paisley, UK), washed, suspended in Fc block (Anti-CD16/32, BD) in PBS-1% BSA and then stained with surface antibodies: RSV M2 82-90 Pentamer R-PE (Proimmune, Oxford, UK), CD3-FITC (BD, Oxford UK), CD4- PE/Cy7 (BioLegend, CA, USA), CD8-APC-H7 (BD), CD69-APC (BioLegend), CD103- PerCP Cy5.5 (BioLegend). Analysis was performed on an LSRFortessa flow cytometer (BD). FMO controls were used for surface stains.

Cytokine detection

Cytokine responses in the airway and lung cells post transfer and RSV infection were analyzed using a TH1/TH2 Group 1 Bio-Plex Pro™ Mouse Cytokine Assay kit according to manufacturers’ instructions.

Statistical Analysis

Calculations as described in figure legends were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).
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Author Contributions

EK, LC and JT designed the studies. EK, LL, JM and HC performed the experimental studies. EK and JT wrote the manuscript.

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Figure legends

**Figure 1. Prior RSV infection protects against subsequent infection and induces both RSV specific antibody and CD8 T cells.** Mice were infected with 10^5 pfu RSV intranasally in 100μl once (blue), twice (red) or three (green) times with RSV, with a 21 day gap between exposures. Weight was measured daily after infection (A). Viral load was measured on day 4 after infection (B). Anti-RSV antibody responses were measured on day 7 after infection (C). CD8^+ T cells (D,E), that were Trm: CD69^+ /CD103^+ (F,G) and antigen specific (H, I) in the lungs (D, F, H) or airways (E, G, I) respectively were measured by flow cytometry on days 4, 8 and 21 after infection. Points/ bars represent mean +/- SEM of n=5 animals. * p<0.05 comparing RSVx1 with RSVx2, # p<0.05 comparing RSVx1 with RSVx3.

**Figure 2. Airway cell transfer reduces disease after RSV infection.**

BALB/c mice were infected with 10^5 pfu RSV; prior to re-infection groups received either anti-CD8 or anti-CD4 antibodies on days -1, +2 and +5 post infection and % weight measured (A). Naïve mice received 10^6 cells intranasally from airways and spleens of RSV exposed or PBS exposed mice prior to infection with 10^5 pfu RSV (B). CD8^+ (C) and CD4^+ (D) Trm T cells in the airways of recipient mice 7 days after RSV infection. Cells were sorted from airways of RSV exposed prior to infection RSV. Weight loss (E), lung viral load at day 4 after RSV infection (F), IFNγ (G) and TNFα (H) levels in the airways 1 day in recipient mice after infection. Cells from PBS exposed mice were sorted and transferred into mice before infection, weight loss (I). Points and bars represent n = 5 animals +/- SEM and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 as measured via one-way ANOVA with Tukey’s multiple comparison post-test. Weight loss significance was calculated by two-way ANOVA: for (A) significance is shown between RSVx2 and anti-CD4 (*) or anti-CD8 (#); (B) significance is shown between RSV BAL and control (*); for (E) significance is shown between control and CD4 (#) or CD8 (*).

**Figure 3. Vaccines inducing systemic T cells do not protect against RSV infection.**

BALB/c mice were intramuscularly vaccinated with electroporation using a 3 dose regimen with 5 μg RSV M DNA vaccine. Mice were infected with 10^5 pfu RSV and compared to naïve (RSVx1) or previously exposed (RSVx2) animals, weight loss after RSV challenge (A). Lung RSV viral load at day 4 post-infection (B). Airway CD8^+ T cells specific for RSV M82-90 pentamer (C), that were Trm (D). Lung CD8 cells producing TNF (E) or IFNγ (F). Mice were vaccinated three times with RSV M DNA prior to challenge CD8 cells were depleted. Lung RSV viral load at day 5 post-infection (G). Weight loss during RSV infection after M DNA vaccination and CD8+ T cell depletion (H). For (A) significance is shown between RSVx2 (*) and RSVx1. Points and bars represent n = 5 animals +/- SEM and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 as measured via one-way ANOVA with Tukey’s multiple comparison post-test. Weight loss significance was calculated by two-way ANOVA.

**Figure 4. Immunisation and infection induce different antigen specific T cells populations in different tissues.** BALB/c mice were sham inoculated with PBS i.n. (white bars), or infected with 10^5 pfu RSV Strain A2 (black bars) or vaccinated with 5μg RSV M DNA (grey bars) in the left leg muscle in a prime-boost regimen with two week intervals. 7 weeks after start of the study, all mice were sacrificed. The left and right flank skin and muscle, blood, spleen, airway and lung cells were analysed. CD8^+ T cells specific for M82-90 pentamer* (A) and M specific CD8^+ T cells displaying Trm markers (B). Points represent n = 5 animals +/- SEM and *p<0.05 by multiple weight t-test.
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