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Enhancement of CD8$^+$ T-cell memory by removal of a vaccinia virus NF-κB inhibitor

Short title: **Enhancing CD8$^+$ T-cell immunity**

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Abbreviations: vaccinia virus, VACV;

Summary

Factors influencing T-cell responses are important for vaccine development but incompletely understood. Here vaccinia virus (VACV) protein N1 is shown to impair the development of both effector and memory $CD8^+$ T-cells and this correlates with its inhibition of nuclear factor kappa B (NF-κB) activation. Infection with VACVs that either have the *N1L* gene deleted (vΔN1) or containing an I6E mutation (vN1.I6E) that abrogates its inhibition of NF-κB resulted in increased central and memory $CD8^+$ T-cell populations, increased $CD8^+$ T-cell cytotoxicity and lower virus titres after challenge. Furthermore, $CD8^+$ memory T-cell function was increased following infection with vN1.I6E, with more IFNγ production and greater protection against VACV infection following passive transfer to naïve mice, compared to $CD8^+$ T-cells from mice infected with wild type virus (vN1.WT). This demonstrates the importance of NF-κB activation within infected cells for long-term $CD8^+$ T-cell memory and vaccine efficacy. Further, it provides a rationale for deleting N1 from VACV vectors to enhance $CD8^+$ T-cell immunogenicity, while simultaneously reducing virulence to improve vaccine safety.

Introduction

Immunological memory provides protection against reinfection by pathogens encountered previously and in mammals is conferred by specific leukocyte populations that endure long after clearance of infection [1, 2]. Naïve T-cell clones expand rapidly after T-cell receptor ligation and, whilst most die subsequently after clearance of the specific antigen, some survive to become long-lasting memory cells that protect against future infection [3, 4]. Induction of strong T-cell memory is desirable for vaccine development but factors that influence this are not fully understood. Two populations of

memory T-cells, called central and effector memory $CD8^+$ and $CD4^+$ T-cells (T_{CM} and T_{EM} respectively), are defined by expression of specific surface markers. In mice these are CD62L and CD44 (T_{CM} being $CD44^{hi}CD62L^{hi}$ and T_{EM} being $CD44^{hi}CD62L^{lo}$) and are induced well by acute viral infections [5, 6]. These subsets are functionally distinct because T_{CM} cells mediate long term protection, whilst T_{EM} cells provide immediate protection [6] and they have distinct locations with T_{CM} being resident mainly in lymph nodes, whilst T_{EM} are predominantly in peripheral tissue. $CD8^+$ T_{CM} and T_{EM} cells confer protection against several pathogens, although the T_{CM} subset may be more broadly protective [7-10].

Vaccinia virus (VACV) was the live vaccine used to eradicate smallpox [11] and induces long-lasting protective immunity [12-15], for review see [16]. Consequently, VACV is useful for studying the induction of immunological memory. Moreover, the engineering of VACV to express foreign antigens has made VACV a popular vector for vaccine development [17, 18]. VACV expresses about 200 proteins [19] and many of these inhibit innate immunity [20]. Studying such immunosuppressive proteins provides insight into how the innate immune system functions and may be suppressed [21-23], and their manipulation can improve VACV immunogenicity. For instance, deletion of genes encoding the chemokine binding protein A41 [24], the IL-1β binding protein B15 [25], or the IRF3 inhibitor C6 enhanced immune responses [26, 27]. Such viruses are useful tools for studying how the innate immune response shapes adaptive immunity [28].

This paper concerns VACV protein N1 and shows that its deletion or mutation can simultaneously reduce virus virulence and induce stronger $CD8^+$ T-cell responses that confer enhanced protection

against virus challenge. N1 is present in many, but not all, VACV strains and orthopoxviruses, for details see reference [29], and is, for instance, present in VACV strain modified virus Ankara (MVA) but is shortened from 117 to 113 amino acid residues due to a frameshift mutation that removes the last 27 residues and replaces these with 23 unrelated residues [30]. N1 is an intracellular homodimer expressed early during infection [29] that inhibits activation of NF-κB [31-33], suppresses apoptosis [33, 34] and contributes to virus virulence [29, 33, 35]. The crystal structure of N1 revealed a Bcl-2 fold [34, 36] and structure-based mutagenesis showed that inhibition of NF-κB activation and apoptosis are separable [33]. N1 mutants unable to block NF-κB activation (I6E) or apoptosis (R58Y and Q61Y) were described and analysis of recombinant VACVs expressing these mutant N1 proteins showed that inhibition of NF-κB activation, rather than apoptosis, was the predominant mechanism by which protein N1 contributed to VACV virulence [33]. An additional mutant, R71Y, affected neither inhibition of apoptosis nor NF-κB activation and a virus bearing this mutation has wild type virulence [33].

Here, these VACVs are utilised to study how NF-κB activation during infection influences development of cellular immunity, immunological memory and resistance to reinfection. NF-κB is crucial in regulating inflammation and cell proliferation, but there is little direct evidence of its role in development of immunological memory due to gross developmental defects in mice where NF-κB signalling is suppressed. Recombinant VACVs with altered ability to suppress NF-κB enable circumvention of this problem. Data presented show that intradermal (i.d.) infection with VACV lacking N1 (vΔN1) or bearing the I6E mutation (vN1.I6E) caused increased activation of $CD8^+$ T-cells compared to WT virus (vN1.WT), illustrating the importance of NF-κB signalling for induction of

T-cell responses. Furthermore, mice infected with vN1.I6E or vΔN1 developed increased numbers of $CD8^+$ T_{CM} and T_{EM} cells that mediated enhanced protected against VACV challenge. This study illustrates how the innate immune response to viral infection driven by NF-κB has a profound impact on the development of T-cell memory and provides a rationale for deleting the *N1L* gene, and possibly other inhibitors of NF-κB, from VACV-based vaccines.

Materials and Methods

Ethics statement

This work was conducted under licence PPL 70/7116 from United Kingdom Home Office according to the Animals (Scientific Procedures) Act 1986.

Mice and cell lines

Female C57BL/6 (B6) mice (Harlan) were housed under pathogen-free conditions. EL4 (H-2b) and P815 (H-2d) cells (both ATCC) were cultured in RPMI 1640 (Gibco) containing 10% foetal bovine serum (FBS) (Harlan Seralab) and penicillin/streptomycin (50 µg/ml; Gibco).

Viruses

VACV strain WR recombinants vN1.WT and vΔN1 [29] and VACVs expressing N1 mutant protein I6E, R58Y, Q61Y and R71Y were described [33]. Virus infectivity was titrated by plaque assay on BSC-1 cells.

Murine infection models

Female C57BL/6 mice (6-8 weeks) were infected i.d. with 10^4 plaque-forming units (PFU) in both ear pinnae [37, 38]. Virus doses used to infect animals were always re-titrated to confirm the infectious dose administered. *In vivo* data shown are from one representative experiment, and all experiments were performed at least twice. To determine virus titres, infected ears were ground with a tissue homogenizer, subjected to three cycles of freezing and thawing and sonication, and the resulting homogenate was titrated on BSC-1 cells [37, 38]. To evaluate the degree of protection induced by i.d. infection, immunised mice were challenged by i.n. infection with the indicated dose of VACV strain WR as described [39].

Isolation of cell populations

Mice were euthanised and the liver, spleen, lung and lymph nodes were removed. Hepatic lymphocytes were prepared as described [40]. Splenocytes and lymph node suspension cells were obtained by forcing the organ through a stainless steel mesh. Splenocytes were treated with 0.2% NaCl solution to remove erythrocytes. Lung pieces were incubated in RPMI 1640 with 5% FBS, 100 U/ml penicillin/streptomycin, 10 mM HEPES, 50 μM 2-ME, 20 mM L-glutamine containing 20 U/ml collagenase (Type Ia) and 1 μg/ml DNase (Type I) for 30 min prior to passing through a mesh. For preparation of cells for passive transfer to recipient mice, the mouse $CD4^+$ or $CD8^+$ T-cell isolation kit was used as indicated by the manufacturer (Miltenyi Biotec) to deplete non-$CD4^+$ or non-$CD8^+$ cells on an autoMACS instrument.

Antibodies, cell staining and flow cytometry

Anti-mouse CD3 (clone 145-2C11), CD4 (GK1.5), CD8 (5H10-1), B220 (RA3-6B2), NK1.1 (PK136), CD11b (M1/70), Ly-6G/Ly-6C (RB6-8C5), CD44 (IM7), CD62L (MEL-14), granzyme B (GB11), CD16/32 (2.4G2) and IFN-γ (XMG1.2) mAb were purchased from BD Biosciences or Biolegend. The mAbs were purified or conjugated with FITC, PerCP/cy5.5, APC, PE-Cy7, BV650 C or BV421. Isotype controls were used as negative controls. For intracellular staining, cells were incubated with Golgistop (BD Pharmingen) for 5 h before analysis. After surface staining, samples were fixed, permeabilised using Cytofix/Cytoperm intracellular staining kit (BD Pharmingen), and incubated with the indicated mAb. Then cells were stained intracellularly for 30 min, washed and fixed in 1% paraformaldehyde (Sigma-Aldrich). Flow cytometry was performed with a BD LSR Fortessa (BD Biosciences), and data were analysed with FlowJo software (Tree Star Inc.). LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies) was used to exclude nonviable cells from analysis.

DimerX assay to detect VACV specific $CD8^+$ T-cells

Recombinant soluble dimeric mouse H-$2K^b$:Ig fusion proteins were purchased from BD Biosciences and the DimerX assay was performed according to the manufacturer's instructions. Briefly, 2 µg of H-2Kb:Ig fusion proteins were incubated overnight at 37°C in PBS with a 40 molar excess of $B8_{20}$ peptide (TSYKFESV). Peptide-loaded dimers were then incubated for 1 h at room temperature with PE-coupled anti-mouse IgG1 (clone A85-1, BD Biosciences). Cells were labelled with DimerX and anti-CD8 (clone 53-6.7, BD Biosciences) for 1 h on ice and washed twice before acquisition using a BD LSR Fortessa (BD Biosciences). Analysis was done using FlowJo software (Tree Star Inc.). Events were gated for live lymphocytes on FCS × SSC followed by $CD8^+$ T-cells × $DimerX^+$ cells.

Backgrounds as determined using irrelevant peptides were in the order of 0.5 to 0.8% and were subtracted from the values presented for test samples.

^{51}Cr release cytotoxic assay

Cytotoxic T lymphocyte (CTL) activity was assayed by ^{51}Cr-release assay [24]. VACV-infected EL4 cells were used as targets for VACV-specific CTL lysis. In some experiments, $CD8^+$ cells were depleted from liver and spleen cell suspensions by incubation with anti-CD8 mAb (clone 3.115) together with human complement. An isotype control mAb was used in parallel. Flow cytometry confirmed >95% depletion of the desired cells. The remaining cells were used for cytotoxicity assays without adjustment for alteration in number during depletion. The cytotoxicity of purified NK cells was tested on VACV-infected P815 cells by ^{51}Cr release assay. The percentage of specific ^{51}Cr release was calculated as specific lysis = [(experimental release−spontaneous release)/(total detergent release−spontaneous release)]×100. The spontaneous release values were always <15% of total lysis.

Cell depletion by antibody *in vivo*

Rat anti-CD8 (YTS169) or rat anti-CD4 (YTS 191.1) mAbs were concentrated from tissue culture supernatant by ammonium sulphate precipitation and quantified by ELISA. Depleting antibodies (0.3 mg) were injected into the peritoneal cavity of naïve recipient mice 10, 8 and 6 days prior to transfer of 10^6 $CD8^+$ or $CD4^+$ T-cells from immunised or naïve mice. The depletion of specific T-cell populations was analysed by FACS and showed >95% of specific cells were depleted.

Serum antibody titration

To measure the neutralising titre of anti-VACV antibodies, vaccinated mice were exsanguinated at 28 day p.i., and sera were prepared and heated at 56 ºC for 30 min to inactivate complement. Two-fold dilutions of sera in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 2% FBSwere prepared and were incubated with VACV intracellular mature virus (purified by sucrose density gradient centrifugation) for 1 h at 37 ºC before plaque assay on BS-C-1 cells. ND_{50} values represent the reciprocal of the serum dilution giving 50% reduction in plaque number compared to virus incubated without serum.

Statistical analyses

Data were analysed using GraphPad Prism 5 software, represented as mean with the standard error of the mean (SEM), and assessed for significance using the Mann–Whitney U or student's t-test statistics. *p*-values less than 0.05 were considered statistically significant. *< 0.05, **< 0.01.

Results

Deletion of VACV N1 increases effector $CD8^+$ T-cell numbers during acute infection

Intradermal infection with vN1.WT, vΔN1 or viruses with single amino acid mutations in the N1 protein did not affect virus replication *in vivo* early (2 days) post infection p.i. (Supplementary Figure 1), as noted earlier for vN1.WT, vΔN1 or revertant viruses [29]. To investigate if blocking NF-κB or apoptosis affected virus immunogenicity, mice were immunised i.d. with these viruses to mimic dermal vaccination and splenic T-cells were analysed one month thereafter. Total splenic cells increased substantially early after infection (being maximal 7-10 days p.i.), but there were no

differences between vN1.WT and the N1 mutant viruses in the magnitude or kinetics of this response (Figure 1A, B). The absolute numbers of T-cells ($CD3^+CD4^+$, $CD3^+CD8^+$), B cells ($CD3^-B220^+$) and NK cells ($CD3^-NK1.1^+$) in the spleen at 7 days p.i. increased after infection (Figure 1C), but no differences were observed between the viruses. The proportion of splenic T-cells (expressed as a percentage of total lymphocytes) increased after infection, whereas this value decreased for B cells (Figure 1D). The proportions of splenic macrophages ($CD11b^+Ly6G^-$) and neutrophils ($CD11b^+Ly6G^+$) (not shown) were similar to mock infection for all viruses. Analysis of cells in the posterior cervical lymph nodes proximal to the infection site showed similar results for the total cell numbers (Supplementary Figure 2A), $CD8^+$ T-cells (Supplementary Figure 2B) and other cell subsets including $CD4^+$ T-cells, B cells, NK cells, macrophages and neutrophils (not shown).

During acute VACV infection activated $CD8^+$ T-cells have high granzyme B (GzmB) and low CD62L expression ($GzmB^{hi}CD62L^{lo}$) and this population can be detected without the requirement for *ex vivo* peptide antigen stimulation [41]. These cells were analysed at different times p.i and in naïve mice, ~95% of splenic $CD8^+$ T-cells were in the resting, $GzmB^{lo}CD62L^{hi}$, population and less than 1% were $GzmB^{hi}CD62L^{lo}$. However, 7 days p.i. with vN1.WT, vN1.R58Y or vN1.R71Y approximately 45% of splenic $CD8^+$ T-cells were $GzmB^{hi}CD62L^{lo}$. Notably, after infection with vΔN1 or vN1.I6E this population increased to >60% and the differences between these viruses and vN1.WT, vN1.58Y and vN1.R71Y were statistically significant (Figure 2A, B). Similar results were obtained 14 days p.i. when vΔN1 and vN1.I6E caused an increased proportion of activated $CD8^+$ T-cells. By days 21 and 28 p.i. the activated $CD8^+$ T-cell population had decreased to resting levels in all groups (Figure 2). In conclusion, deletion of N1 or I6E mutation increased the number of activated $CD8^+$ splenocytes

(Figure 2B). To quantify VACV-specific $CD8^+$ T-cells, DimerX reagent loaded with the immunodominant VACV $B8_{20-27}$ peptide was used. Consistent with a previous study [41], VACV-specific $GzmB^{hi}CD62L^{lo}$ $CD8^+$ T-cells were identified in the spleen at day 7 p.i., and their proportion and absolute number increased upon deletion of N1 or I6E mutation (Figure 3A, B). In the draining lymph nodes this difference was more pronounced ($p<0.01$) (Figure 4). Therefore, either removal of N1, or its mutation to ablate inhibition of NF-κB, induced greater numbers of $CD8^+$ effector T-cells following infection.

VACVΔN1 induces enhanced development of immunological memory

To test if changes induced by N1 mutation influenced protection against reinfection, mice were immunised i.d. with WT or mutant viruses and challenged i.n. 4 weeks later (when activated $CD8^+$ T-cells had returned to resting levels) with a dose of VACV WR (5×10^6 p.f.u) representing >200 LD_{50} for naïve mice [29, 42]. Mice vaccinated with vΔN1 or vN1.I6E showed better protection against challenge, characterised by reduced weight loss and more rapid recovery, compared to those immunised with vN1.WT, vN1.R58Y or vN1.R71Y (Figure 5A). Also, the virus titre in lungs 4 days after virus challenge was lower following immunisation with vΔN1 or vN1.I6E compared to other groups (Figure 5B). No virus was detected in spleen after challenge for any of the virus groups (data not shown). Collectively, although deletion or I6E mutation of N1 reduced virulence, these changes enhanced immunological memory following vaccination.

To understand the basis of enhanced protection, VACV-specific antibodies were measured at 28 days p.i by plaque reduction neutralisation assay [43]. This showed that all groups of immunised mice had

high serum antibody titres, but titres induced by immunisation with vΔN1 or vN1.I6E were lower ($p<0.05$) than mice infected with vN1.WT or vN1.R58Y and vN1.R71Y (Figure 5C). Therefore, antibody responses did not explain the enhanced protection induced by vΔN1 and vN1.I6E, suggesting cellular immunity might be responsible.

Deletion of VACV N1 results in enhanced $CD8^+$ T-cell effector functions

To investigate T-cell effector functions, the killing activity of $CD8^+$ T-cells was assessed 28 days p.i with vN1.WT or the mutant N1 viruses by *ex vivo* cytotoxicity assay (Figure 6A). Splenic lymphocytes from mice immunised with vΔN1 or vN1.I6E showed significantly higher cytotoxicity against VACV-infected autologous target cells compared with cells derived from mice immunised with vN1.WT, vN1.R58Y or vN1.R71Y (Figure 6A). Notably, differences between groups and the cytotoxicity of lymphocytes were abolished by $CD8^+$ T-cell depletion with specific mAb (Figure 6B). Analysis of hepatic lymphocytes gave similar results (not shown). Consistent with their enhanced killing activity, splenic $CD8^+$ T-cells expressed significantly greater CD107a at 28 days p.i. with vΔN1 or vN1.I6E ($p<0.01$) than with vN1.WT, vN1.R58Y or vN1.R71Y (Figure 6C). NK cell responses were not responsible for the enhanced protection because, although splenic NK cells from immunised mice lysed VACV-infected targets better than mock-infected cells, there were no differences between mice immunised with vN1.WT or vΔN1 (Figure 6D).

vΔN1 and vN1.I6E induce increased populations of $CD8^+$ T_{CM} and T_{EM} cells

The enhanced cytotoxicity of $CD8^+$ T-cells 28 d p.i. with vΔN1 and vN1.I6E indicated phenotypic differences between these cells and those from mice infected with the other viruses despite activation

markers having returned to baseline by day 21 p.i. (Figure 2). Therefore, $CD8^+$ T_{CM} and T_{EM} cells in the spleen and draining lymph node were analysed 28 d after i.d. infection. After vN1.I6E infection 10.4±1.5% of splenic $CD8^+$ T-cells were $CD44^{hi}CD62L^{lo}$ (T_{EM}) and 20.6±1.9% were $CD44^{hi}CD62L^{hi}$ (T_{CM}) and similar results were obtained with vΔN1. However, only 7.1±1.3% $CD8^+$ T_{EM} and 14.7±2.2% of $CD8^+$ T_{CM} cells were induced by vN1.WT, vN1.R58Y or vN1.R71Y and the differences between these groups and the vN1.I6E/vΔN1 groups were statistically significant ($p<0.05$) (Figure 7A). In the draining lymph nodes this enhancement was even more pronounced ($p<0.01$) (Figure 7B). In contrast, $CD4^+$ T_{CM} and T_{EM} populations were indistinguishable between viruses (Figure 7A, B). Analysis by DimerX staining demonstrated a higher proportion and absolute number of VACV-specific $CD8^+$ T ($DimerX^+CD8^+$) cells at 28 days p.i. with vΔN1 and vN1.I6E compared to vN1.WT, vN1.R58Y or vN1.R71Y ($p<0.05$) (Figure 8A, B). In this population there were no naïve T-cells ($CD44^{lo}CD62L^{hi}$) and only T_{EM} and T_{CM} remained (Figure 8A lower panels). Infection with vΔN1 or vN1.I6E induced greater numbers of T_{EM} and T_{CM} cells than vN1.WT, vN1.R58Y or vN1.R71Y ($p<0.05$) (Figure 8C). In conclusion, N1 reduces development of $CD8^+$ T-cell central and effector memory and this correlates with inhibition of NF-κB.

$CD8^+$ T-cell effector functions are enhanced after challenge of vΔN1- or vN1.I6E-vaccinated mice

To address if enhanced $CD8^+$ T-cell memory influences their effector function, IFN-γ production by these cells was investigated 4 days after reinfection. Cells from lungs and spleen were stimulated with peptides from VACV protein B8 (the IFN-γ receptor [44]) that are recognised by MHC class I restricted $CD8^+$ T-cells [45] and IFN-γ production was quantified by intracellular cytokine staining.

IFN-γ production by cells from both organs was higher following vΔN1 and vN1.I6E infection compared to other viruses (Figure 9). Similar data were obtained with $CD8^+$ T-cells from the draining lymph nodes (data not shown). Therefore, $CD8^+$ T-cell memory induced by vΔN1 or vN1.I6E infection correlates with enhanced effector function of these cells in response to secondary infection.

$CD8^+$ T-cells from vN1.I6E-vaccinated mice confer enhanced protection

Finally, the ability of T-cell subsets to confer protection against challenge with VACV was examined by passive transfer. Splenic $CD8^+$ and $CD4^+$ T-cells were purified from naïve or vaccinated mice 28 d p.i. and transferred to naïve mice that were challenged with VACV. Mice infused with an equivalent number of additional naïve $CD8^+$ or $CD4^+$ T-cells were equally susceptible to subsequent virus challenge (data not shown), showing that more naïve cells *per se* did not influence outcome. However, mice that received $CD8^+$ or $CD4^+$ T-cells from vN1.WT immunised mice responded differently to challenge. First, these animals lost weight sooner than mice receiving naïve cells (Figure 10). This effect has been observed repeatedly following i.n. challenge of VACV immunised mice and was attributable to lung immune pathology [24-26]. Passive transfer of anti-VACV Ab prior to challenge did not enhance disease symptoms [46], suggesting the effect was via cellular immunity. Figure 10 shows this phenomenon is mediated by T-cells and that $CD8^+$ cells play a greater role than $CD4^+$ cells. Second, mice receiving either $CD8^+$ or $CD4^+$ T-cells from immunised mice were protected better than those that received equivalent cells from naïve mice, and this was characterised by a lower weight loss, lower virus titres and more rapid recovery (Figure 10A). Notably, the transfer of $CD8^+$ T-cells from mice immunised with vN1.I6E enhanced this protection, and reduced virus titres further, whilst the transfer of $CD4^+$ T-cells from these mice conferred no additional benefit over those from vN1.WT

immunised mice (Figure 10A).

This beneficial effect of $CD8^+$ T-cells from vN1.I6E immunised mice was also seen if the recipient mice were depleted of $CD8^+$ T-cells by addition of mAb prior to passive transfer (Figure 10B). Flow cytometry showed that this treatment had depleted >95% of $CD8^+$ T-cells at the time of challenge, and the mAb had declined to levels unable to affect the function of infused $CD8^+$ T-cells upon transfer (A Cooke, personal communication). As above, $CD8^+$ T-cells from vN1.I6E immunised mice conferred greater protection than cells from vN1.WT immunised mice, but the N1.I6E mutation did not influence protection from $CD4^+$ cells (Figure 10A, B). Therefore, $CD8^+$ and $CD4^+$ memory T-cell populations confer protection against VACV challenge and the I6E mutation enhances protection from the $CD8^+$ population. Hence, the inhibition of NF-κB by N1 correlates with a profound and specific effect on the generation of $CD8^+$ T-cell memory.

Discussion

Despite encoding scores of immunomodulatory proteins, deletion of individual VACV genes can impact on virulence and immunogenicity [20]. Previous studies showed VACV protein N1, or its ectromelia virus counterpart, contributed to virulence in several infection models [29, 35, 47-49], affected T-cell and NK cell responses during primary infection [47, 48] and a N1 deletion mutant induced better protection from challenge [49]. However, it was unknown if these effects of N1 were attributable to inhibition of apoptosis or NF-κB or both activities, whether this phenotype was evident following immunisation by dermal vaccination and challenge via a heterologous route, and what mechanism mediated the enhanced protection. Subsequently, using VACV N1 mutants that

discriminate between inhibition of apoptosis and NF-κB, it was shown that virulence correlated with inhibition of NF-κB [33]. Here, these mutants were used to investigate the consequence of inhibiting NF-κB on the development of adaptive immunity using i.d. infection, mimicking dermal vaccination. N1 is shown to hinder the development of $CD8^+$ T-cell effector and memory functions and this correlates with its ability to block NF-κB activation.

During primary infection with VACV, deletion of N1 or an I6E mutation resulted in enhanced $CD8^+$ T-cell (but not $CD4^+$ or NK) cytotoxicity, increased numbers of $CD8^+$ (but not $CD4^+$) T_{CM} and T_{EM} cells, and better protection against challenge with VACV. In contrast, mutation of the BH3 binding groove of N1, which obviated the ability of N1 to bind Bad and Bid and to disrupt apoptotic signalling [33], did not affect T-cell responses. Therefore, N1 influences immunological memory, specifically $CD8^+$ T-cells, and this correlates with inhibition of NF-κB signalling. Passive transfer demonstrated that $CD8^+$ and $CD4^+$ T-cells from immunised mice provided protection against VACV challenge, but $CD8^+$ T-cells transferred from vN1.I6E-immunised mice conferred enhanced protection compared to $CD8^+$ T-cells from vN1.WT-immunised mice. Therefore, enhancement of the $CD8^+$ T-cell response correlates with loss of NF-κB inhibition.

During acute virus infection the inflammatory environment influences the proliferation and development of effector and memory T-cell populations. Inflammation is the third signal required for optimal T-cell activation, where antigen stimulation and co-stimulation comprise signals one and two, respectively [50, 51]. IL-2, IL-12 and IFNs influence the differentiation of naïve T-cell precursors into their effector and memory populations and other cytokines may also contribute [50, 52].

Co-stimulation is provided mainly by 4-1BB and CD27, although CD40, CD28 and OX40 can also contribute to signal 2 [5, 50]. Following clearance of antigen, IL-15 and IL-7 drive the proliferation and maintenance of the $CD8^+$ memory populations and anti-apoptotic factors, such as $Bcl_{XL,}$ are essential to avoid activation-induced death of these cells [5, 53]. Here we show that modulation of NF-κB signalling by VACV protein N1 during primary infection affects the effector and memory $CD8^+$ T-cell response, and this is consistent with NF-κB regulating the expression of many cytokines, co-stimulatory molecules and maintenance factors needed for $CD8^+$ T-cell responses [54]. Transgenic mouse models for studying the impact of NF-κB on these processes are hampered by the profound effects on the development of haematopoietic cells and/or systemic hyperinflammation deriving from deletion or inhibition of NF-κB [55-57]. Hence, using mutant viruses to modify specific signalling pathways during infection provides an alternative way to assess the effects of NF-κB, or other inflammatory responses, on immunological memory development in a wild-type host. In addition, data produced in this study showed an unexpected specificity for enhancing $CD8^+$ T-cell function without modulating $CD4^+$ T-cell function to the same degree by direct modification of NF-κB during acute infection. In the future, mutant VACVs will allow aspects of the mechanisms of $CD8^+$ and $CD4^+$ T-cell responses during acute virus infection to be dissected.

The impact of innate immunity on the development of immunological memory is of great interest for vaccine development. Adjuvants help memory development [28, 58] by activating pattern recognition receptors and thereby transcription factors, including NF-κB, which induce a favourable inflammatory cytokine environment at the site of antigen exposure during vaccination. The activity of N1 during VACV infection, namely blocking NF-κB and inhibiting $CD8^+$ T-cell memory, is opposite to the

activity of a vaccine adjuvant. Therefore, deleting N1 from VACV is a logical strategy to improve immunogenicity, especially if cytotoxic T-cell activity is required. Indeed, removal of VACV immunomodulators can enhance memory responses during vaccination [26, 27, 59]. There are at least ten different VACV intracellular inhibitors of NF-κB activation [31-33, 60-67] and several other proteins that block IRF3 activation [23, 65, 67-69] and therefore it is likely that removal of some of these immunomodulators alone or in combination may improve immunological memory. Thus, these mutant viruses are valuable tools to identify factors promoting immunological memory as well as functioning as improved vaccines.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. N1 mutation does not affect splenic lymphocyte numbers following infection. Groups of 5 mice were infected i.d. with the indicated VACVs and splenic lymphocyte populations were counted at 7, 14, 21 and 28 d p.i. (A, B) Total cell numbers. (C, D) T and B cell populations presented as total cell number (C) and as percentages of total lymphocytes (D). Results are expressed as mean ± SEM. Statistical comparison of cells from mock-infected mice with virus-infected mice: * $p<0.05$, ** $p<0.01$.

Figure 2. Infection with vΔN1 or vN1.I6E induces enhanced numbers of effector CD8$^+$ T-cells. Mice were infected i.d. with the indicated VACVs and populations of splenic GzmB^{hi}CD62L^{lo} CD8$^+$ T-cells were counted at 7, 14, 21 and 28 days p.i. (A) Flow cytometry scatter plots from representative samples from individual mice. The arrows emphasise the greater percentage of GzmB^{hi}CD62L^{lo} CD8$^+$ T-cells following infection with vN1.I6E or vΔN1 compared to other viruses. (B) Graphs showing the proportion of total CD8$^+$ T-cells that were GzmB^{hi}CD62L^{lo} (left) and the absolute numbers of GzmB^{hi}CD62L^{lo} cells (right panel) (mean ± SEM)**.** *, $p<0.05$, n=5.

Figure 3. Infection with vΔN1 or vN1.I6E induces enhanced numbers of VACV-specific effector CD8$^+$ T-cells. Mice were infected i.d. with the indicated VACVs and populations of splenic DimerX$^+$CD8$^+$, and GzmB^{hi}CD62L^{lo} of DimerX$^+$CD8$^+$ T-cells were counted at 7 days p.i. (A) Flow cytometry scatter plots from representative samples from individual mice. The arrows emphasise the greater percentage of DimerX$^+$CD8$^+$ and GzmB^{hi}CD62L^{lo} of Dimer$^+$CD8$^+$ T-cells following infection with vN1.I6E or vΔN1 compared to other viruses. (B) Graphs showing the absolute numbers of DimerX$^+$CD8$^+$ T-cells (left) and of GzmB^{hi}CD62L^{lo} cells (right panel) (mean ± SEM). *, p<0.05, n=5.

Figure 4. Infection with vΔN1 or vN1.I6E induces enhanced numbers of effector CD8$^+$ T-cells in the VACV-specific CD8$^+$ T-cells in the draining lymph nodes. Mice were infected i.d. with the indicated VACVs and populations of DimerX$^+$CD8$^+$, and GzmB^{hi}CD62L^{lo} of DimerX$^+$CD8$^+$ T-cells in the draining lymph nodes were counted at 7 days p.i. (A) Flow cytometry scatter plots from representative samples from individual mice. The arrows emphasise the greater percentage of DimerX$^+$CD8$^+$ and GzmB^{hi}CD62L^{lo} of DimerX$^+$CD8$^+$ T-cells following infection with vN1.I6E or vΔN1 compared to other viruses. (B) Graphs showing the absolute numbers of DimerX$^+$CD8$^+$ T-cells (left) and GzmB^{hi}CD62L^{lo} of DimerX$^+$CD8$^+$ T-cells (right panel) (mean ± SEM). **, p<0.01, n=5.

Figure. 5. VACVs lacking N1 or expressing I6E induce better protection to virus challenge. (A) Groups of 5 mice were infected i.d. with the indicated viruses. At 28 d p.i. mice were challenged i.n. with VACV (5×10^6 p.f.u of VACV WR) and weight change was monitored. Each mouse was compared to its original weight on day zero and data are expressed as the percentage ± SEM. (B) Groups of 5 mice were infected and challenged as (A), sacrificed on d 1 or 4 post challenge (p.c.) and

virus titres in the lungs were measured by plaque assay. Data are mean titre ± SEM, **,p<0.01. (C) Sera from mice infected as in (A) were collected 28 d p.i and assayed for neutralisation of VACV strain WR. The median value for each population is represented by a horizontal black bar. Significant differences between groups are shown, Mann–Whitney test. *, p<0.05, n=15.

Figure 6. Deletion or I6E mutation of N1 enhances CD8$^+$ T-cell cytotoxicity. (A) Mice were infected i.d. with the indicated viruses splenic lymphocytes were harvested at 28 d p.i. and tested for their ability to lyse VACV-infected EL4 cells by chromium release assay. Data are presented as percentage cell lysis at various effector to target (E:T) cell ratios. *, p<0.05, **, p<0.01, n=5. (B) Lymphocytes were prepared as in (A) and pre-incubated with a CD8 blocking mAb before cytotoxic activity was assayed as in (A). (C) Lymphocytes were prepared as in (A). Histograms showing the absolute number of CD8$^+$ cells expressing CD107a following stimulation with VACV B8 peptide. (D) Cytotoxicity assay as in (A) but with purified splenic NK cells and using VACV-infected P815 cells as targets. Results are expressed as mean ± SEM.

Figure 7. Deletion or I6E mutation of N1 enhances CD8$^+$ T_{EM} and T_{CM} populations. Mice were infected i.d. with the indicated viruses and 28 d later populations of CD8$^+$ or CD4$^+$ CD44^{hi}CD62L^{lo} (T_{EM}) and CD44^{hi}CD62L^{hi} (T_{CM}) cells were counted from (A) the spleen or (B) the draining lymph nodes. These data are presented as scatter plots with arrows indicating the gates corresponding to CD44^{hi}CD62L^{lo} populations (black arrow) or CD44^{hi}CD62L^{hi} (blue arrow, top panels), or as histograms showing absolute numbers of the specific populations as a proportion of total CD8$^+$ or CD4$^+$ cells. *, p<0.05, n=5. Results are expressed as mean ± SEM.

Figure 8. Deletion or I6E mutation of N1 enhances VACV-specific $CD8^+$ T_{EM} and T_{CM} cell populations. Mice were infected i.d. with the indicated VACVs and populations of splenic lymphocytes that were $DimerX^+CD8^+$, and T_{EM} or T_{CM} of $DimerX^+CD8^+$ T-cells, were counted at 28 days p.i.. (A) Flow cytometry scatter plots from representative samples from individual mice. The arrows emphasise the greater percentage of $DimerX^+CD8^+$ following infection with vN1.I6E or vΔN1 compared to other viruses. (B) Graphs showing the absolute numbers of $DimerX^+CD8^+$ T-cells. (C) Graphs showing the absolute numbers of T_{EM} and T_{CM} of $DimerX^+CD8^+$ T-cells (mean ± SEM). *, $p<0.05$; **, $p<0.01$, n=5.

Figure 9. Deletion or I6E mutation of VACV N1 enhances the effector function of memory $CD8^+$ T-cells. Mice were infected i.d. with the indicated viruses and challenged i.n. 28 d p.i. with 5×10^6 PFU of VACV WR. Lung or splenic lymphocytes were isolated 4 d later and the $CD8^+$ T-cell populations were assayed by intracellular cytokine staining (ICS) for IFNγ production. Data are presented as scatter plots (top panels) and as histograms indicating the percentage of $IFN\gamma^+$ $CD8^+$ T-cells (middle panels), or the absolute cell number of $IFN\gamma^+$ $CD8^+$ T-cells (bottom panels). *, $p<0.05$, n=5. Results are expressed as mean ± SEM.

Figure 10. $CD8^+$ T-cells from vN1.I6E-infected mice confer enhanced protection. (A) Mice were infected i.d. with vN1.WT or vN1.I6E or mock infected and either $CD8^+$ or $CD4^+$ cells were isolated 28 d p.i and 10^6 cells were transferred into naïve recipient mice. The recipient mice were challenged 6 h later with 3×10^3 PFU. of VACV WR and weight change (middle panels) was monitored. *, $p<0.05$, n=5. Lower panels show virus titres in the lungs 5 d post challenge. Data are mean titre ± SEM, * $p<0.05$, ** $p<0.01$. NS = non-significant. (B) As (A), except the naïve recipient mice were depleted

for $CD8^+$ or $CD4^+$ T-cells by administration of mAb at 10, 8 and 6 d prior to transfer of cells. $CD4^+$ T-cells were transferred to mice depleted of $CD4^+$ T-cells, or $CD8^+$ T-cells were transferred to mice depleted of $CD8^+$ T-cells. *, $p<0.05$, n=5. Results are expressed as mean ± SEM.

Figure 1

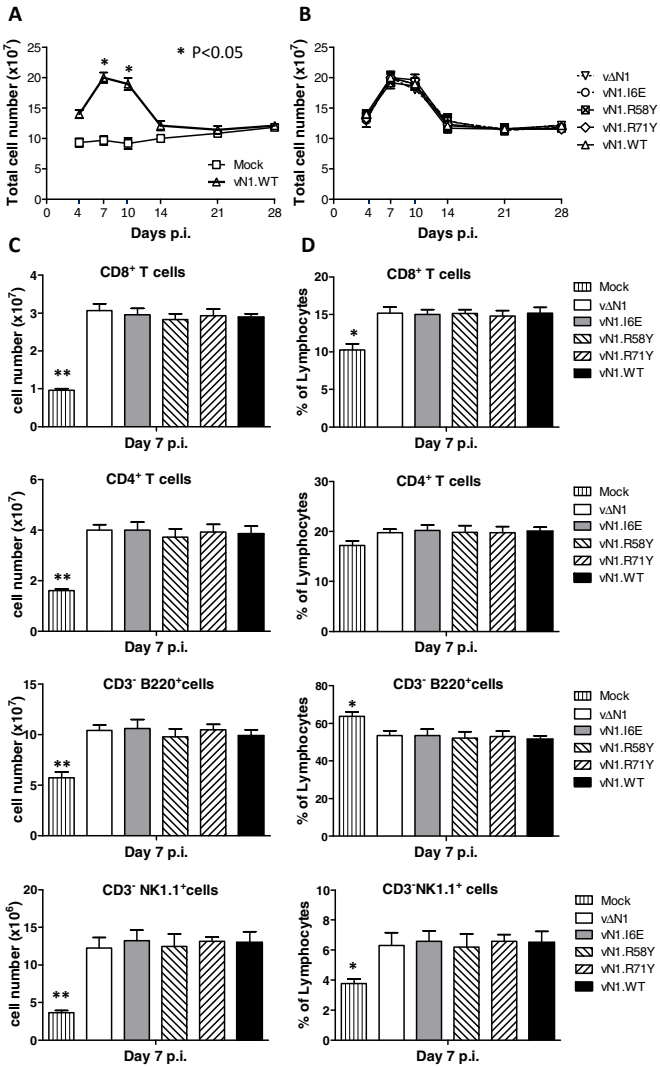
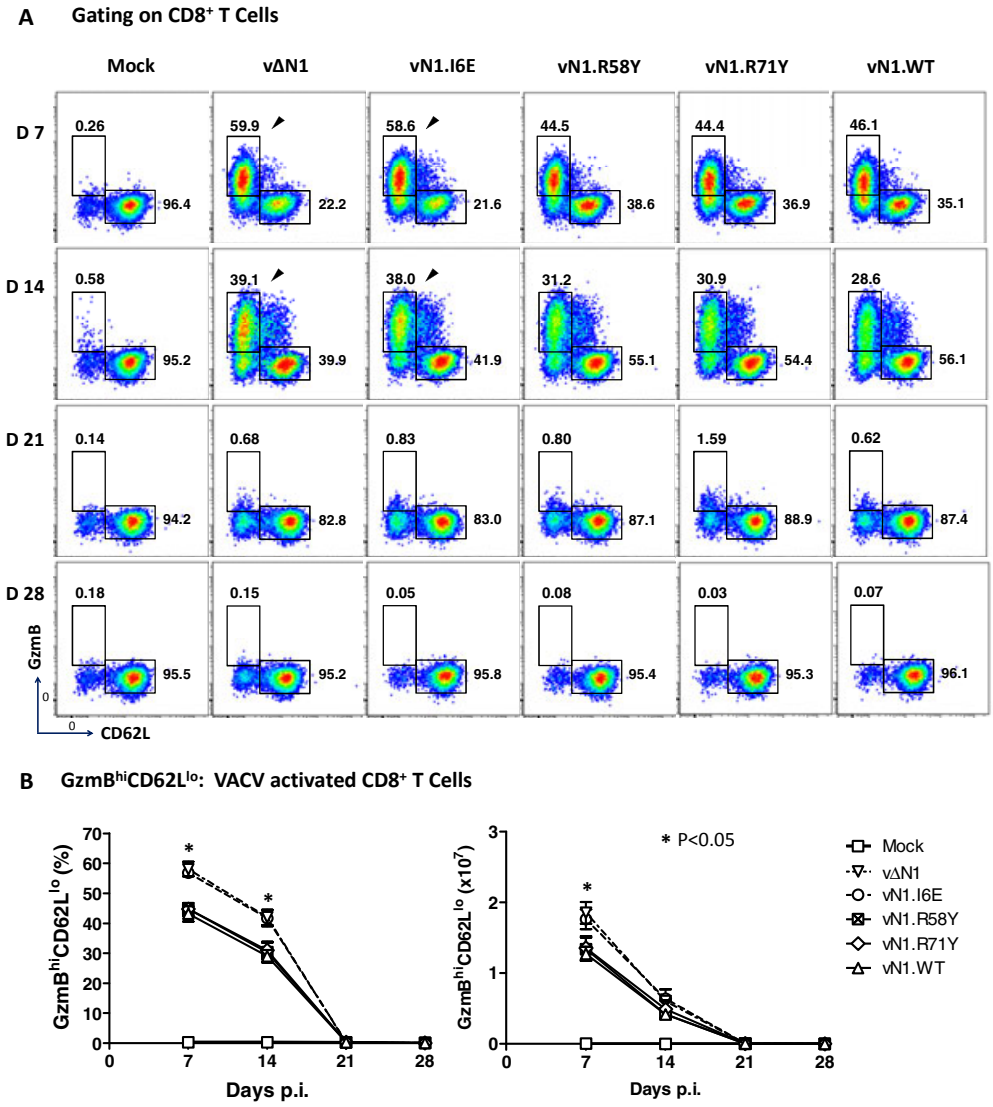


Figure 2



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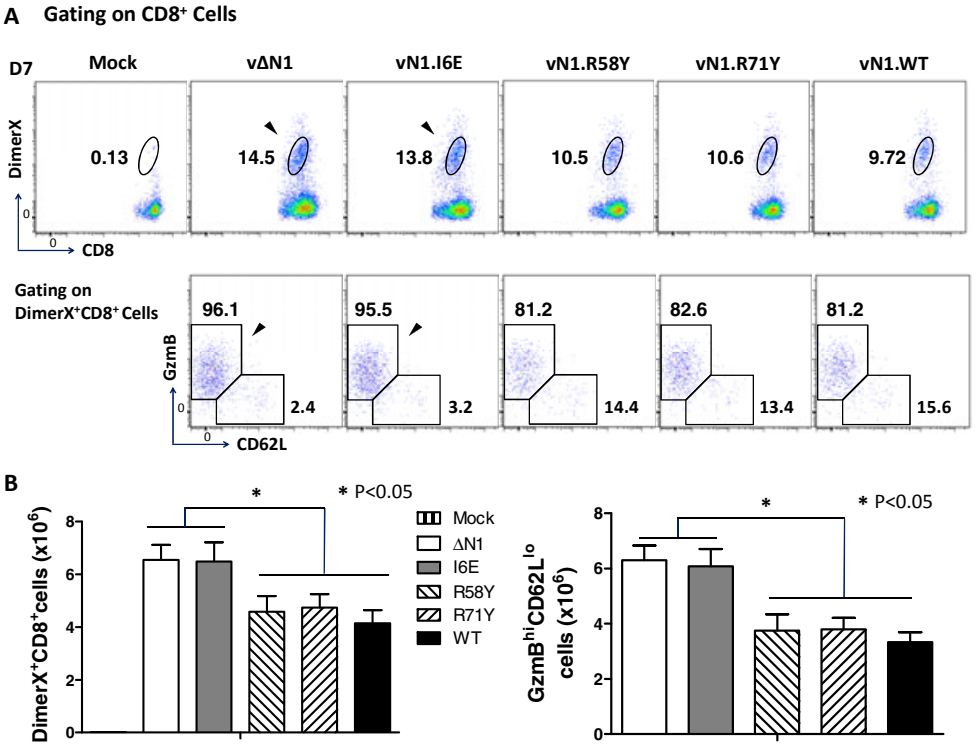

Figure 4

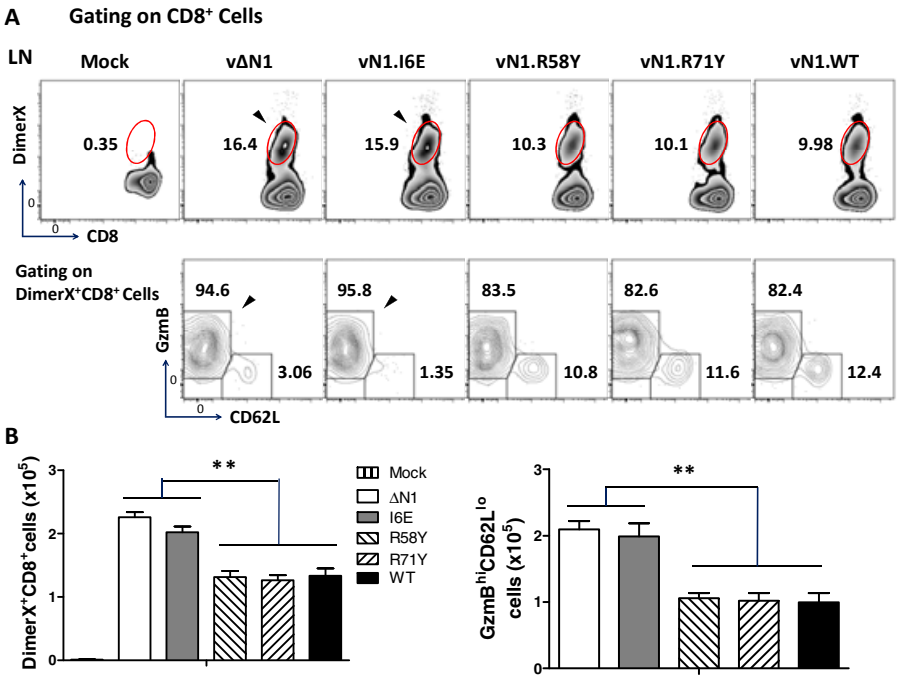

Figure 5

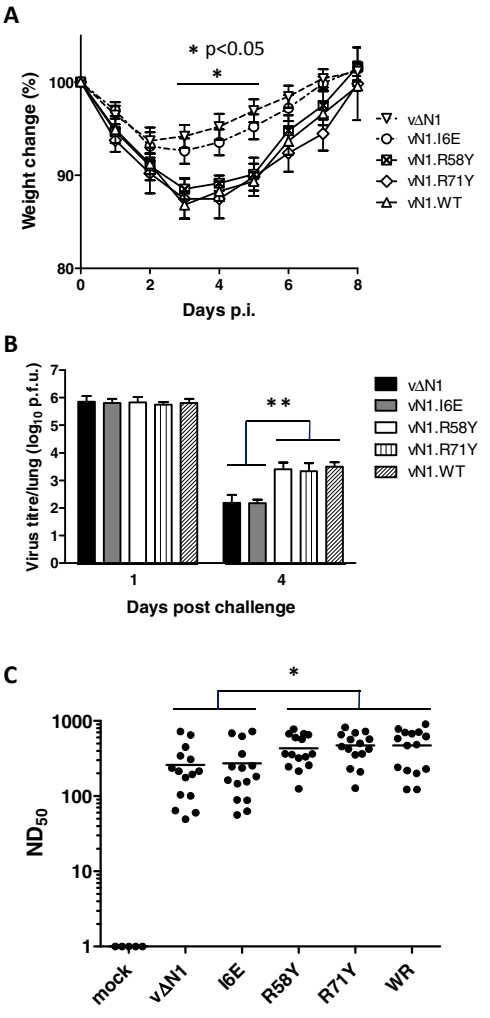

Figure 6

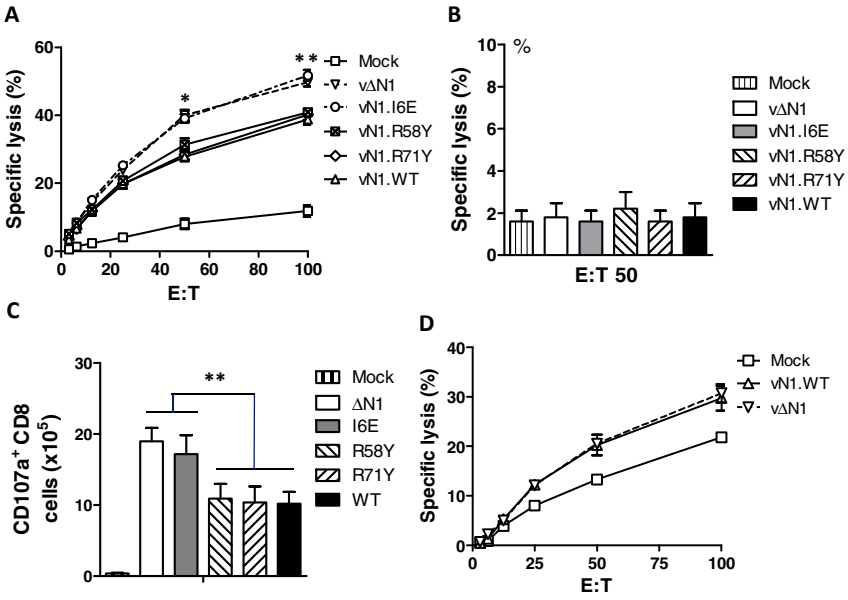

Figure 7

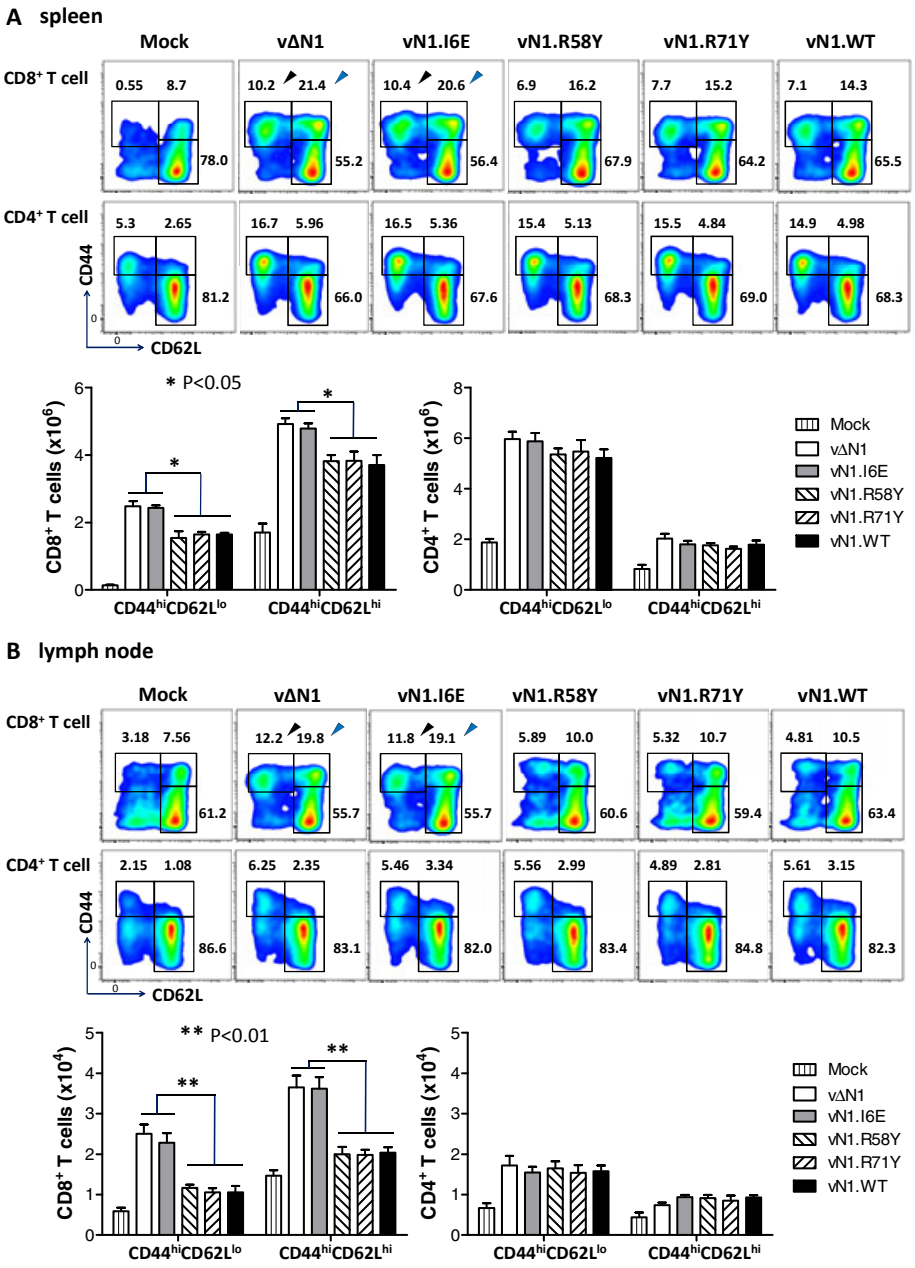

Figure 8

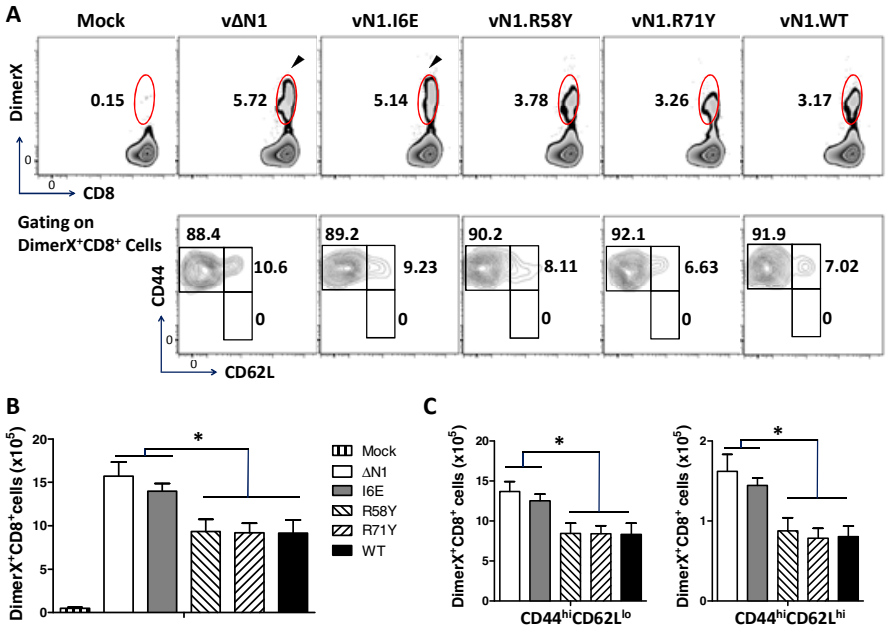

Figure 9

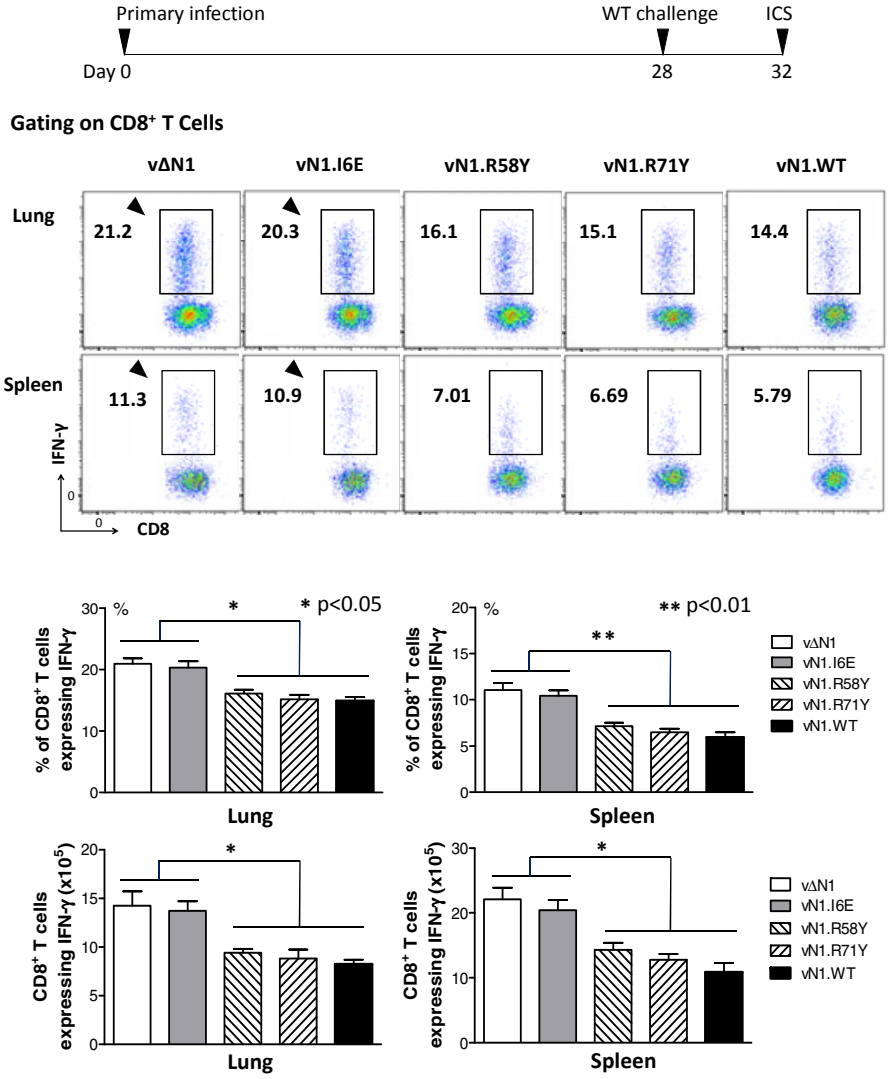

Figure 10