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Citation	Tian, Tian, Krista Dubin, Qiushuang Jin, Ali Qureshi, Sandra L. King, Luzheng Liu, Xiaodong Jiang, George F. Murphy, Thomas S. Kupper, and Robert C. Fuhlbrigge. 2012. Disruption of TNFα/TNFR1 function in resident skin cells impairs host immune response against cutaneous vaccinia virus infection. The Journal of Investigative Dermatology 132(5): 1425-1434.
Published Version	doi:10.1038/jid.2011.489
Accessed	February 19, 2015 11:54:06 AM EST
Citable Link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:10579378
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Disruption of TNFα/TNFR1 function in resident skin cells impairs host immune response against cutaneous vaccinia virus infection

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Abstract

One strategy adopted by vaccinia virus (VV) to evade the host immune system is to encode homologs of TNF receptors (TNFR) that block TNFα function. The response to VV skin infection under conditions of TNFα deficiency, however, has not been reported. We found that TNFR1−/− mice developed larger primary lesions, numerous satellite lesions and higher skin virus levels after VV scarification. Following their recovery, these TNFR1−/− mice were fully protected against challenge with a lethal intranasal dose of VV, suggesting these mice developed an effective memory immune response. A functional systemic immune response of TNFR1−/− mice was further demonstrated by enhanced production of VV-specific IFNγ and VV-specific CD8+ T cells in spleens and draining lymph nodes. Interestingly, bone marrow (BM) reconstitution studies using WT BM in TNFR1−/− host mice, but not TNFR1−/− BM in WT host mice, reproduced the original results seen in TNFR1−/− mice, indicating that TNFR1 deficiency in resident skin cells, rather than hematopoietic cells, accounts for the impaired cutaneous immune response. Our data suggest that lack of TNFR1 leads to a skin-specific immune deficiency and that resident skin cells play a crucial role in mediating an optimal immune defense to VV cutaneous infection via TNFα/TNFR1 signaling.

Introduction

Vaccinia virus (VV) is a large double stranded DNA virus belonging to the poxvirus family. Like other poxviruses, VV has evolved elegant and sophisticated mechanisms to evade detection and destruction by the host immune system. Among the evasion strategies adopted by VV is to encode homologs of cytokines, chemokines and their receptors that play crucial

Conflict of interest: The authors have declared that no conflicts of interest exist.

roles in control of the immune response (Seet et al., 2003). Since TNFα is a potent inflammatory cytokine that can orchestrate communication between innate and adaptive immune responses, it is not surprising that VV has developed mechanisms to interfere with the TNFα/TNFR pathway. The best characterized anti-TNFα strategy employed by VV is to encode homologs of TNFR, termed vTNFR, that can bind and sequester TNFα (Reading et al., 2002). In contrast, recombinant VV expressing the gene for murine TNFα demonstrated attenuated virulence *in vivo* (Sambhi et al., 1991).

Within the normal host, TNFα is produced principally by macrophages and activated T cells. TNFα-mediated effects are critically important in the response to a variety of infections caused by bacteria, protozoa, fungi and viruses (Schluter and Deckert, 2000). For VV in particular, it has been reported that mice deficient in TNFα or TNF receptors are more susceptible to intraperitoneal (i.p.) and intravascular (i.v.) infections. (Nie et al., 2009; Ruby et al., 1997). The mechanism and outcome of cutaneous VV infection is not necessarily expected to be the same as these systemic infections and the role of TNFα following cutaneous infection is less well defined. Although routine vaccination against smallpox was discontinued in the United States in 1972, the potential for use of the smallpox virus as a bioterror agent and the endemic presence of monkeypox virus in Africa has resulted in continued use of smallpox vaccines in medical, military and at-risk populations (Rimoin et al., 2010). Effective smallpox vaccination in humans is commonly achieved by inoculating skin with live VV via scarification. Inoculation via other routes, or use of killed virus vaccines, is known to result in inferior protective responses in humans (McClain et al., 1997). We have also demonstrated that VV scarification elicits stronger T cell and humoral responses, compared to other routes of immunization in mice (Liu et al., 2010). However, smallpox vaccination by scarification can lead to uncontrolled virus replication, known as eczema vaccinatum (EV), in patients with atopic dermatitis (AD) and immune deficiency disorders, resulting in devastating morbidity and mortality (Engler et al., 2002).

This study explores the impact of TNFα/TNFR1 deficiency on the outcome of cutaneous VV infection. After VV scarification, TNFR1−/− mice demonstrated an impaired cutaneous immune response with a phenotype that resembled EV. Investigation of innate and adaptive immune responses in TNFR1−/− mice showed no difference in effector or memory immune responses. The results of bone marrow reconstitution studies suggest that the impaired cutaneous response was caused by TNFR1 deficiency of resident skin cells.

Results

1. TNFR1 deficient mice developed markedly larger skin lesions with higher virus counts compared to WT mice following VV scarification

Mice were scarified with WR-VV at the base of the tail and observed for the development of pox lesions. Seven days after VV scarification, TNFR1−/− mice exhibited lesions that were at least 2-fold larger than lesions on WT control mice. At day 14, the differences in skin lesions were more dramatic, as multiple satellite lesions appeared on the TNFR1−/− mice and the lesions on WT mice started to heal. By day 21, TNFR1−/− mice showed signs of skin healing, but their lesions were still markedly larger than WT controls (Figures 1a, 1b and 1c). To confirm the effect of TNFα on skin lesions, WT mice were treated with function-blocking anti-TNFα mAb or control Ab. Anti-TNFα mAb treatment led to significantly larger lesions in WT mice following VV scarification, consistent with the results seen in TNFR1−/− mice (Figure 1d). In both studies, all mice ultimately survived and skin lesions resolved by day 28 – 35. These data indicate that disruption of the TNFα/TNFR1 pathway leads to exacerbation of skin lesions following VV scarification.

To investigate whether the enhanced skin lesions were associated with virus replication and dissemination, we measured the virus load of inoculated tail skin and skin adjacent to the pox lesion. We found that TNFR1−/− and WT mice possessed comparable levels of virus in inoculated skin one week after VV scarification. Two weeks after scarification, TNFR1−/− mice had slightly higher virus loads in the inoculated skin than WT controls. (Figure 1e). In contrast, analysis of skin samples adjacent to scarification sites revealed that the skin of TNFR1−/− mice contained at least 100 fold more virus than that of WT mice one week after VV scarification and 10 times more virus two weeks after scarification (Figure 1f). Thus, TNFR1−/− mice have a defect in controlling local VV replication and dissemination, which might contribute to the formation of larger skin lesions. To investigate whether the impaired cutaneous response of TNFR1−/− mice to VV scarification reflected a global impairment or a skin-specific immune dysfunction, we measured viral load in peripheral organs and found that TNFR1−/− mice and WT mice had comparable viral burdens in ILN, spleen, lung and liver for up to two weeks post scarification and both cleared VV from peripheral organs by three weeks (Figure S1). The impaired host response of TNFR1−/− mice to VV scarification, therefore, appears to be restricted to skin.

2. Humoral immune response was reduced in TNFR1−/− mice following VV scarification, although survival from intranasal challenge was not affected

To investigate the mechanisms underlying the impaired cutaneous response of TNFR1−/− mice after VV scarification, we compared the production of VV-specific Ab between TNFR1−/− and WT mice following VV scarification. Total VV-specific IgG levels in TNFR1−/− mice were similar to those in WT mice up to three weeks after VV scarification but significantly less than those in WT mice at four weeks and later. Interestingly, TNFR1−/− mice did not generate significant VV-specific IgG1 at any time point, yet their production of IgG2a was comparable to WT controls (Figure 2a). These results indicate that the humoral immune response in TNFR1−/− mice was impaired following VV scarification. However, the observation that TNFR1−/− and WT mice produced comparable levels of VV-specific Ab during two weeks following scarification indicates that the difference in lesion size and character seen in TNFR1−/− mice was not caused by inferior VV-specific Ab production. To confirm generation of systemic immunity, we challenged TNFR1−/− and WT mice six weeks after VV scarification with a lethal intranasal dose of WR-VV. All of the immunized mice survived the challenge while unimmunized mice lost body weight at the same rate and died within one week (Figure 2b). Thus, the reduced humoral response did not impair systemic immune protection to subsequent lethal challenge in TNFR1−/− mice.

3. Type 1 T cell immune response was enhanced in TNFR1−/− mice following VV scarification

To determine whether the altered cutaneous response of TNFR1−/− mice was due to a defect in T cell immune responses, we used *in vitro* restimulation assays to detect VV-specific cytokine production and VV $B8R_{20-27}$ peptide (TSYFESV)/MHC pentamer staining to enumerate VV-specific CD8$^+$ T cells in spleens and ILNs. Similar levels of VV-induced IFNγ were produced by spleen cells from TNFR1−/− and WT mice 7 days following WR-VV scarification. Surprisingly, by days 14 and 30, the level of IFNγ secreted by spleen cells from TNFR1−/− mice was significantly higher than that for WT mice. ILN cells from VV scarified TNFR1−/− mice produced significantly more IFNγ than WT ILN cells at all observed time points (Figure 3a). No significant production of IL-4 or IL-10 was detected from the spleen or ILN cells of VV inoculated mice of either strain (data not shown).

Consistent with the results of *in vitro* restimulation assays, spleens of TNFR1−/− mice contained similar numbers of VV-specific CD8$^+$ T cells as compared to WT mice at day 7

post scarification. At day 14 and 30, TNFR1−/− mice had significantly more VV-specific CD8^{+} T cells evident in the spleen. TNFR1−/− mice displayed a greater number of VV-specific CD8^{+} T cells in the ILN at all observed time points (Figure 3b, 3c). These data suggest TNFR −/− mice are not deficient in production of CD8^{+} effector cells.

To address the possibility that the increased Th1 response in TNFR1−/− mice could be due to greater antigen levels, resulting from the higher virus counts in the skin, we scarified mice with MVA, a strain of VV that does not replicate in mammalian cells. As seen with VV, spleen cells of MVA-scarified TNFR1−/− mice produced similar levels of IFNγ at day 7 and higher levels at days 14 and 30 when compared to WT mice. ILN cells of TNFR1−/− mice also produced more IFNγ than WT mice at day 7 (Figure 3d). These results indicate that the enhanced Th1 cytokine production observed in TNFR1−/− mice is independent of antigen load. Furthermore, lesion size was similar between TNFR1−/− and WT mice following MVA scarification (data not shown), suggesting that the larger lesions and satellite lesions observed in TNFR1−/− mice after VV scarification reflect the cytopathic effect of replicating vaccinia virus. Collectively, these data indicate that the altered cutaneous response of TNFR1−/− mice following VV scarification was not caused by a defect of T cell activation, production of VV-specific CD8^{+} effector cells or Th1 helper response. In fact, TNFR1−/− mice had an enhanced type 1 T cell response as compared to WT mice.

4. T cell homing to the inoculation site was not altered by TNFR1 deficiency

The impaired cutaneous response of TNFR1−/− mice might indicate a defect in T cell homing to skin following VV scarification. However, comparable CD3^{+} T cell infiltration was found in the lesions of TNFR1−/− mice and WT mice six days post VV scarification (Figure 4a). To assess the kinetics of T cell skin homing, we performed FACS analysis on cells recovered from tail skin at different time points following VV scarification and found similar ratios of CD4^{+} and CD8^{+} T cells recruited to the inoculated skin of TNFR1−/− and WT mice (Figure 4b). Given that the total number of skin cells recovered was slightly higher in TNFR1−/− mice than WT mice (data not shown), these results suggest that TNFR1−/− mice could recruit CD4^{+} and CD8^{+} T cells to skin at least as efficiently as WT mice.

To further explore the influence of TNFR1 deficiency on VV-specific CD8^{+} T cell skin homing, we also performed B8R-pentamer staining. At day 3 post VV scarification, very few CD8^{+} T cells were present in the inoculated skin of either TNFR1−/− or WT mice (Figure 4c). At day 7, both TNFR1−/− and WT had substantial numbers of CD8^{+} T cells present in the inoculation site, with a similar fraction of CD8^{+} T cells staining with B8R pentamer (TNFR1−/−: 18.7%; WT: 19.5%) (%B8R within CD8^{+} = %B8R^{+}CD8^{+}/ (%B8R^{+} CD8^{+} + %B8R^{−} CD8^{+}) * 100). On days 14 and 30, TNFR1−/− mice continued to show slightly higher %B8R-pentamer positive CD8^{+} T cells than WT controls.

Alternative explanations for the impaired cutaneous immune response in TNFR1 −/− mice could reflect enhanced recruitment of regulatory T cells (Tregs) to the site of inoculation (Rouse et al., 2006) or altered functions of the VV-specific CD8 effector cells present in the site. We found the fraction of CD4^{+}CD25^{+}FoxP3^{+} Tregs in inoculated skin were comparable in WT and TNFR1−/− mice (23.4% and 22.6%, respectively) (Figure S2a). Furthermore, CD8^{+}B8R^{+} T cells from TNFR1−/− mice produced similar levels of IFNγ and granzyme B in response to in vitro stimulation as compared to WT mice. (Figure S2a and S2b). These results suggest that VV-specific CD8^{+} T cells recruited to the inoculated skin are not defective or suppressed and general T cell homing to the skin is operational in TNFR1−/− mice following VV scarification.

5. Impaired cutaneous response to VV scarification is associated with TNFR1 deficiency of resident skin cells and not of BM-derived hematopoietic cells

The development of pox lesions in VV scarified mice involves both BM derived cells that are recruited to the skin as well as skin stromal cells, including keratinocytes and fibroblasts. To distinguish which of these two cell populations utilized TNFα/TNFR1 signaling to control virus dissemination and skin lesion formation, we generated three groups of chimeric mice: (1) TNFR1−/− BM →WT host, (2) WT BM→ TNFR1−/− host and (3) WT BM→ WT host. Chimeric mice were inoculated with VV approximately 8 weeks after irradiation and bone marrow reconstitution. By day 12 after VV scarification, WT BM→TNFR1−/− mice developed multiple satellite lesions and had significantly larger lesions than both WT BM → WT and TNFR1−/− BM →WT mice (Figure 5a), a phenotype that is strikingly similar to that of non-irradiated/ non-reconstituted TNFR1−/− mice.

Assessment of viral load in lesional and adjacent skin confirms the gross impression. VV DNA copy number was similar within lesional skin of all three chimeric mouse populations. Assessment of adjacent skin, however, shows high viral load in WT BM→TNFR1−/− mice while TNFR1−/− BM →WT and WT BM → WT chimeric mice contained only trace amounts of virus (Figure 5b). From this data, we conclude that the lack of TNFR1 in radioresistant skin cells, as opposed to circulating BM-derived cells, is the key factor associated with more vigorous local virus replication and dissemination and leads to the development of larger skin lesions and satellite lesions in VV scarified TNFR1−/− mice.

Since the impaired cutaneous response to VV scarification of TNFR1 deficient mice appeared to reflect a malfunction of skin stromal cells, we explored potential mechanisms that might lead to this susceptibility. Toward this end, we performed immunohistochemical staining of mouse skin inoculated with VV-GFP. Interestingly, the degree of keratinocyte staining was similar in WT and TNFR1−/− mice. In contrast, VV-GFP was detected in numerous dermal mesenchymal cells in the superficial and deep layers of skin in TNFR1−/− mice while these areas were devoid of staining in WT mice (Figure 5c).

Given the evidence that the critical cells responding to TNF in this model are radioresistant, we entertained the possibility that the increase in VV replication and dissemination observed in TNFR1−/− mice could be due to an inability to upregulate innate antiviral mechanisms in response to VV scarification. The antimicrobial protein cathelicidin (or CRAMP in mice) has been shown to posess critical antiviral activity against VV in both human and mouse skin. Murine CRAMP knockout mice exhibited reduced ability to control VV replication and dissemination in skin (Howell et al., 2006; Howell et al., 2004). We compared CRAMP gene expression in skin from WT and TNFR1−/− mice and found that 12 hours following VV scarification, CRAMP gene expression was approximately 4-fold higher in the inoculated skin of WT mice than TNFR1−/− mice (Figure 5d). CRAMP gene expression was low in distant skin of both groups at all observed time points. These results suggest that the inability of skin stromal cells to upregulate CRAMP in response to TNF stimulation may be the fundamental defect in TNFR1 deficient mice.

Discussion

In this study, we have shown that WT mice were able to control VV replication in epidermis after scarification and that VV-specific T cells were generated and able to eliminate VV by week 3. In the absence of TNFR1, VV was able to infect a broader area of skin including epidermal, dermal and subcutaneous layers. T cells still developed and were able to eliminate virus, but larger lesions and tissue damage were observed. We went on to show that this outcome in TNFR1−/− mice is related to innate immune mechanisms inherent in

skin, specifically in skin stromal cells and not hematopoietic cells, and is likely related to the capacity of CRAMP to restrict VV dissemination.

TNFα exerts its effects through binding to a pair of specific receptors, TNFR1 and TNFR2 (Faustman and Davis, 2010). We elected to perform our initial experiments in TNFR1−/− mice because VV is commonly used to vaccinate humans via skin scarification and keratinocytes express almost exclusively TNFR1. It has been reported that keratinocytes can serve as a first line of defense following VV skin infection in that they are less-permissive to VV infection and replication *in vitro* as compared to dermal fibroblasts and microvascular endothelial cells (Liu et al., 2005). The epidermis of normal skin contains low levels of TNFα, while cutaneous infection can greatly increase its synthesis and release from keratinocytes (Kock et al., 1990; Nestle et al., 2009). One explanation of our results is that disruption of the TNFα/TNFR1 pathway makes skin stromal cells more susceptible to VV infection and leads to virus dissemination to dermal and subcutaneous tissues. Reduced expression of the CRAMP gene in the skin of TNFR1−/− mice following VV scarification supports this hypothesis.

Specifically, VV-specific Ab production and T cell recruitment and function did not prove to cause the impaired cutaneous immune response to VV seen in TNFR1−/− mice. We did observe, however, that TNFR1−/− mice did not produce IgG1 subclass antibodies to VV, resulting in an overall lower total VV-specific Ab titer. Similar defects in primary antibody responses have been reported for TNFR1−/− mice exposed to Herpes Simplex virus (Lundberg et al., 2007) and schistosome vaccine (Street et al., 1999). These authors proposed that TNFR1−/− mice have defects in the follicular dendritic cell network and germinal center formation in lymph nodes, which could affect Ab responses. However, the overall humoral response was not significantly different between TNFR1−/− and WT mice in the two week following scarification when the lesions differences became manifest, and was sufficient to protect mice from subsequent lethal intranasal VV challenge. The results of T cell studies were more intriguing because TNFR1−/− mice showed an enhanced T cell response to VV scarification as compared to WT mice. TNFα has been described as a negative regulator of type 1 immune responses in other infectious models. TNFα −/− mice exhibited an uncontrolled type I immune syndrome to pulmonary mycobacterium infection (Zganiacz et al., 2004) and TNFR1/R2 double knockout mice displayed expanded viral specific T cells in the spleen following choriomeningitis virus (LCMV) infection (Singh and Suresh, 2007). The mechanism by which TNFα regulates T cell activity is not fully understood, and the reason why TNFR1−/− mice have enhanced type I responses following VV scarification remains to be elucidated.

Our BM reconstitution experiments revealed that the primary cells responding to TNFα/TNFR1 signaling following VV scarification to limit viral propagation are resident skin cells, not hematopoietic cells. In preliminary studies we have also demonstrated that TNFα −/− mice develop large skin lesions after VV scarification similar to those seen in TNFR1−/− mice (Figure S3a). We set up BM reconstitution experiments with TNFα −/− mice to further study this phenotype. Consistent with the results of WT BM → TNFR1−/− mice, WT BM → TNFRα−/− mice developed significantly larger skin lesions with higher virus load at adjacent skin than the control WT BM → WT mice following VV scarification. However, unlike TNFR1 BM → WT mice, TNFα−/−BM → WT mice also developed larger skin lesions with higher virus counts compared to control chimeric mice (Figure S3b and S3c). Therefore, while the target of TNFα action is restricted to skin stromal components, the source of TNFα produced in response to VV infection would appear to include both hematopoietic cells and radioresistant skin elements. More work is needed to understand the difference between TNFR1−/− mice and TNFα−/− mice upon VV scarification.

An important technical note for reconstitution experiments involves Langerhans cells (LC), long-lived bone marrow-derived epidermal dendritic cells. As LC are relatively radioresistant, we may expect that a fraction of the epidermal LC present in the chimeric mice should be of host origin (Poulin et al., 2007). However, the fact that LC express predominantly TNFR2, and thus would not show altered function in TNFR1 −/− mice, would indicate that altered LC function is not the source of impaired cutaneous response in TNFR1−/− mice or WT BM→ TNFR1−/− chimeric mice (Wang et al., 1996).

To our knowledge, this is previously unreported that TNFR1 is essential for efficient and effective host defense to cutaneous VV infection. Our results indicate that restriction of VV propagation by resident skin cells is the specific point in the immune cascade that is defective in TNFR1−/− mice. We feel the data presented indicate TNF signaling plays an important role in regulating local innate immune responses in skin and should be the target of investigation in other models of skin infection. Understanding the immune mechanisms of VV resistance in skin and the role of TNFα will support the development of safer and more effective vaccination strategies. Furthermore, these findings may have important implications regarding the use of TNFα antagonists in the treatment of chronic inflammatory disorders, which may present a currently unappreciated risk for the development of EV-like responses.

Materials and Methods

Mice

All mice used have a C57BL/6 genetic background. TNFR1 deficent (TNFR1−/−) mice, TNFα deficient (TNFα −/−) mice, Thy1.2$^+$ CD45.2$^+$ B6 mice and Thy1.2$^+$ CD45.1$^+$ congenic B6 mice were originally obtained from Jackson Laboratory (Bar Harbor, Maine) and bred in a biosafety level 1 (BL-1) facility at Harvard Medical School (HMS). All mice were handled in accordance with guidelines set out by the Center for Animal Resources and Comparative Medicine at HMS.

Viruses and Viral infection

Recombinant VV expressing EGFP (GFP-VV) and WR-VV stocks were expanded and titered by standard procedures (Liu et al., 2005). Modified Vaccinia Ankara (MVA: ACAM3000MVA) was expanded and titered using DF-1 cells (ATCC, Manassas, VA). The mice were immunized with 5×10^6 pfu of VV or MVA by scarification at the base of the tail as described (Tian et al., 2009). In some experiments, C57BL/6 WT mice were injected i.p. with 250µg anti-mouse TNFα Ab (clone XT 3.11,) or isotype control antibody (clone HRPN) (Bio X cell, West Lebanon, NH) 1h before and every 3 d after WR-VV scarification. This dose was selected as the standard for *in vivo* blockade of TNFα in other mouse models (Lundberg et al., 2007). Following VV scarification, lesions were measured with a millimeter ruler every 2–4 days. For challenge experiments, mice were anesthetized with isoflurane and inoculated intranasally with 2×10^6 pfu of WR-VV as previously described (Tian et al., 2009).

Quantitative PCR for determination of viral load

Viral load of various organs was determined by real-time PCR, as previously described (Tian et al., 2009).

Measurement of vaccinia virus-specific antibody

Vaccinia virus-specific antibody levels were determined at the indicated time points by ELISA, as previously described (Tian et al., 2009).

In vitro restimulation assay

VV-specific cytokines were determined by *in vitro* restimulation assay, as previously described (Tian et al., 2009).

Flow cytometry

Single cell suspension from VV inoculated tail skin was prepared as previously described (Liu et al., 2010). To detect VV-specific CD8+ T cells, single cell suspensions from ILN, spleen and skin of immunized mice were stained with PE-conjugated B8R-pentamer (Proimmune, Oxford, U.K) as previously described (Freyschmidt et al., 2010). To detect Tregs, the cells were stained with antibodies to mouse FoxP3 (APC) (eBioscience, San Diego, CA) as well as CD4(FITC), CD25 (PE) and CD3 (PerCP) (BD Biosciences, San Diego, CA) according to the manufacture's protocol. For intracellular IFNγ and Granzyme B staining, skin cells were fist stimulated with PMA and ionomycin (eBioscience) in the presence of GolgiStop (BD Biosciences) for 5h, then stained with antibodies to mouse IFNγ (APC) or Granzyme B (APC) in addition to CD8 (FITC), B8R-pentamer (PE) and and B220 (PerCP) from BD Biosciences. The stained cells were acquired on a FACSCanto (BD Biosciences) and analyzed with FlowJo (Version 6.4.7, Tree star, Ashland, OR).

Immunohistochemistry

Inoculated skin was harvested from three to four mice per group at each time point. Skin was preserved in formalin, embedded in paraffin, sectioned, and stained with H&E, anti-CD3 antibody (DAKO, Carpinteria, CA) and anti-GFP (Clontech, Palo Alto, CA) as previously described (Tian et al., 2009).

Bone Marrow Chimeras

Adult congenic B6 (CD45.1) mice were lethally irradiated (900–1000 Rad) and injected intravenously with $5–10 \times 10^6$ bone marrow (BM) cells from age and gender matched donor TNFR1−/− (CD45.2) or TNFα−/− (CD45.2) mice. In the case of WT BM→TNFR1−/− or WT BM→ TNFα−/− chimeric mice, TNFR1−/− or TNFα−/− mice were used as recipients and reconstituted with BM cells from donor congenic B6 (CD45.1) mice. Recipient B6 mice reconstituted with BM from donor B6 mice that express different hematopoietic markers (CD45.1 or CD45.2 accordingly) were used as controls. The level of blood chimerism was determined as previously described ((Poulin et al., 2007). 96–98% chimerism for all mice can be achieved 8 wks after BM reconstitution.

Real-time RT-PCR to detect CRAMP gene expression

Total RNA was extracted from skin with RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). CRAMP gene expression was measured as previously described (Howell et al., 2006). CT values for the CRAMP gene were normalized to the reference gene cyclophilin A as described previously (Liu et al., 2006). CRAMP gene expression in the distant skin of WT mice was used as a baseline.

Statistics

The significance of observed differences between indicated groups was assessed by an unpaired Student's *t* test. Tests were 2-tailed with a confidence interval of 95%. *P* values < 0.05 were considered significant. Statistical analyses were performed with Prism software (v4.0, Graphpad Software, Inc., La Jolla, CA)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Nonstandard abbreviations used

AD	atopic dermatitis
EV	eczema vaccinatum
ILN	inguinal lymph node
LC	Langerhan cell
MVA	Modified Vaccinia Ankara
TNFR	TNF receptor
VV	vaccinia virus
WR	Western Reserve strain

Acknowledgments

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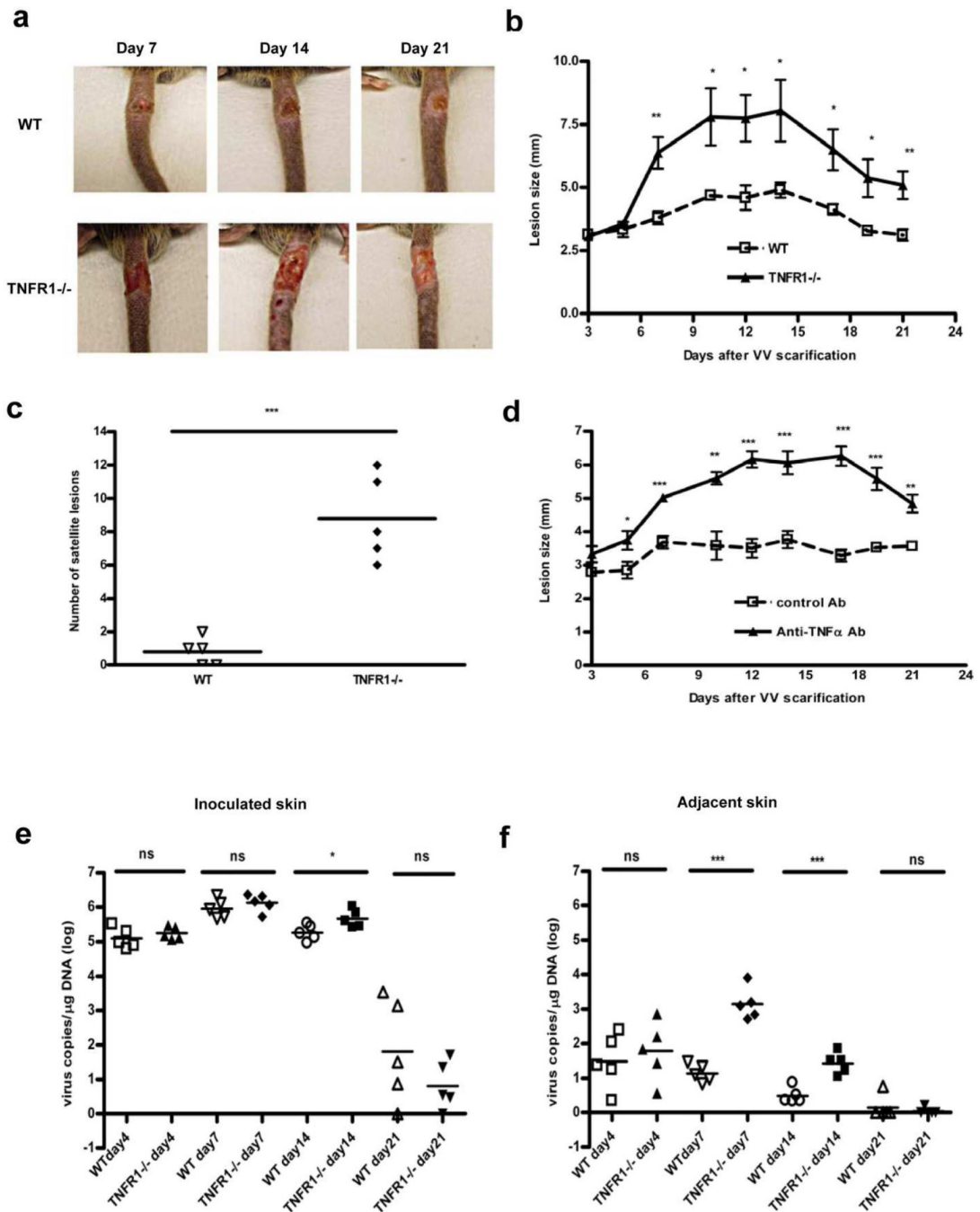
Figure 1. TNFR1−/− mice developed markedly larger skin lesions with higher virus counts following VV scarification

5×10^6 pfu of WR-VV was applied to the base of the tail by scarification for all studies. At indicated time points,(a) Photographs were taken and (b) skin lesions were measured. (c) Numbers of satellite lesions were recorded on day 14. (d) WT mice were treated with anti-TNFα or control Ab before and following VV scarification and lesion size was measured. VV DNA copy number was determined by quantitative PCR in (e) inoculated and (f) adjacent skin following VV scarification. Error bars represent mean ± SEM. n=5 per group. * P < 0.05, ** P < 0.01, *** P < 0.001, ns= not significant.
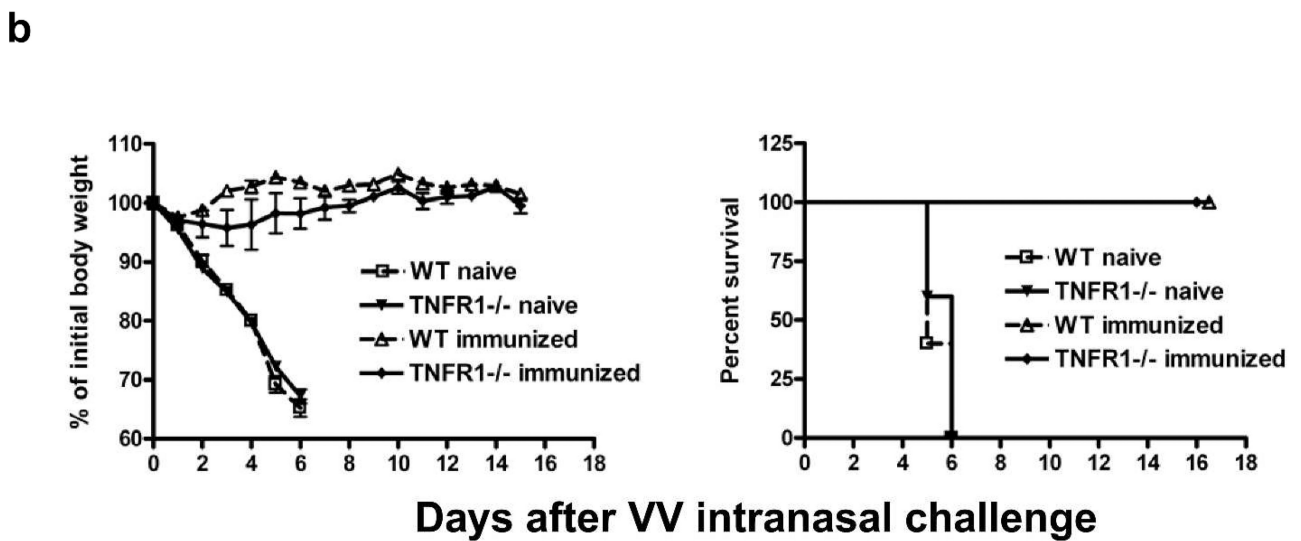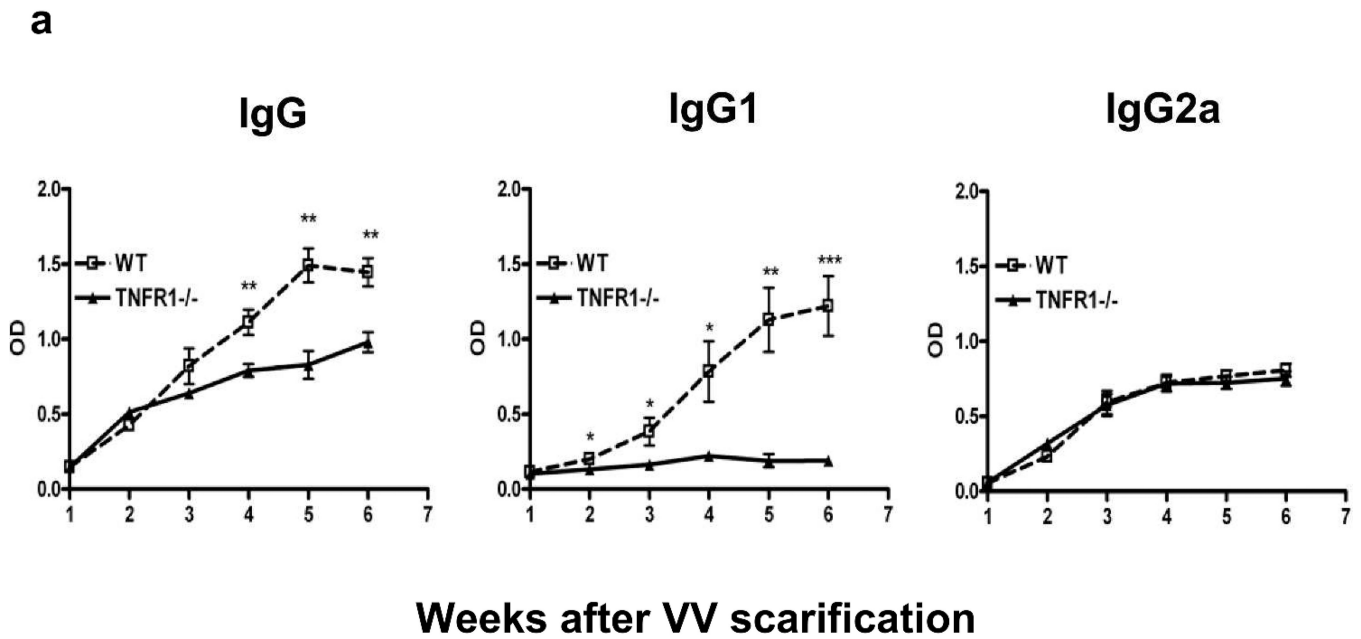
a

Weeks after VV scarification

b

Days after VV intranasal challenge

Figure 2. The altered vaccinia virus-specific antibody response observed in TNFR1−/− mice following scarification did not affect survival of immunized mice challenged with intranasal VV
(a) 5×10^6 pfu of WR-VV was applied to TNFR1−/− and WT mice by scarification. The level of VV-specific antibody in the sera of individual mice was measured by ELISA. (b) Six weeks after scarification, TNFR1−/− and WT mice were challenged with 2×10^6 pfu of WR-VV by intranasal inoculation. Naive TNFR1−/− and WT were used as controls. The body weight and survival of the mice were recorded daily and plotted. Error bars represent mean ± SEM. n=5 per group. Representative results of three independent experiments are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
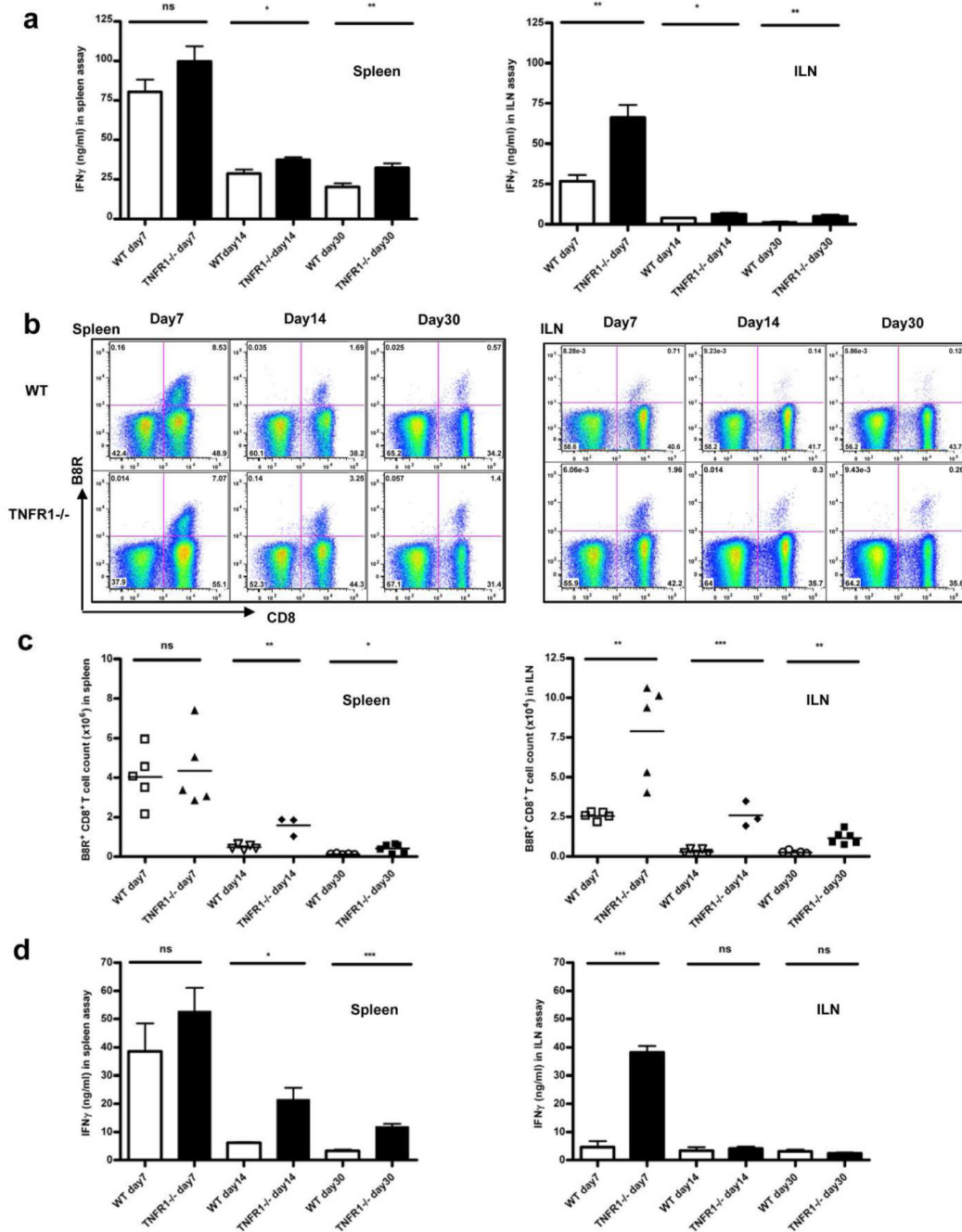
Figure 3. Type 1 T cell immune response was enhanced in TNFR1−/− mice following VV scarification

5×10^6 pfu of WR-VV was applied to TNFR1−/− and WT mice by scarification. At indicated time points, spleens and ILNs were harvested for (a) *in vitro* restimulation to detect VV-specific IFNγ and (b) B8R-pentamer staining to identify VV-specific CD8+ T cells. (c) The absolute number of CD8+B8R+ cells was plotted . (d) 5×10^6 pfu of MVA was applied to TNFR1−/− and WT mice by scarification. At indicated time points, *in vitro* restimulation assay was performed to detect VV-specific IFNγ from spleens and ILNs. Error bars represent mean ± SEM. n=3-5 per group. * P < 0.05, ** P < 0.01, *** P < 0.001, ns = not significant.
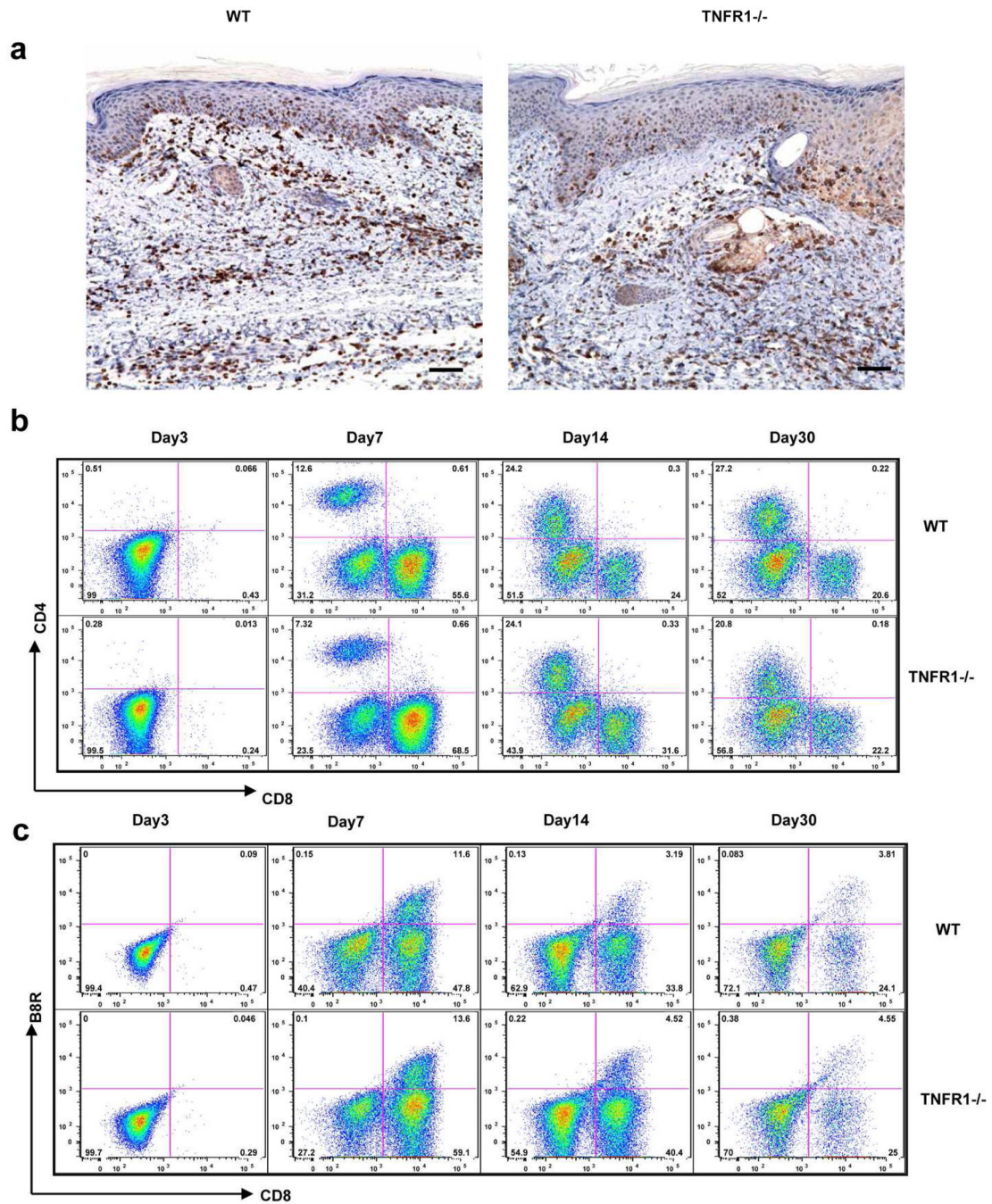
Figure 4. T cell homing to the VV inoculation site did not depend on TNFR1

5×10^6 pfu of WR-VV was applied to TNFR1−/− and WT mice by scarification. (a) At day 6, CD3^{+} cells (brown) were identified in the inoculated skin using immunohistochemistry. Representative photographs of three mice per group are shown. Bar = 20µm (b) CD4^{+} and CD8^{+} T cells and (c) VV-specific CD8^{+} T cells (CD8^{+} B8R-pentamer^{+}) recruited to the inoculation site were measured using flow cytometry at different time points. Plots shown are gated on leukocytes using side and forward scatter. Representative results of two independent experiments are shown. n= 3–5 per group.
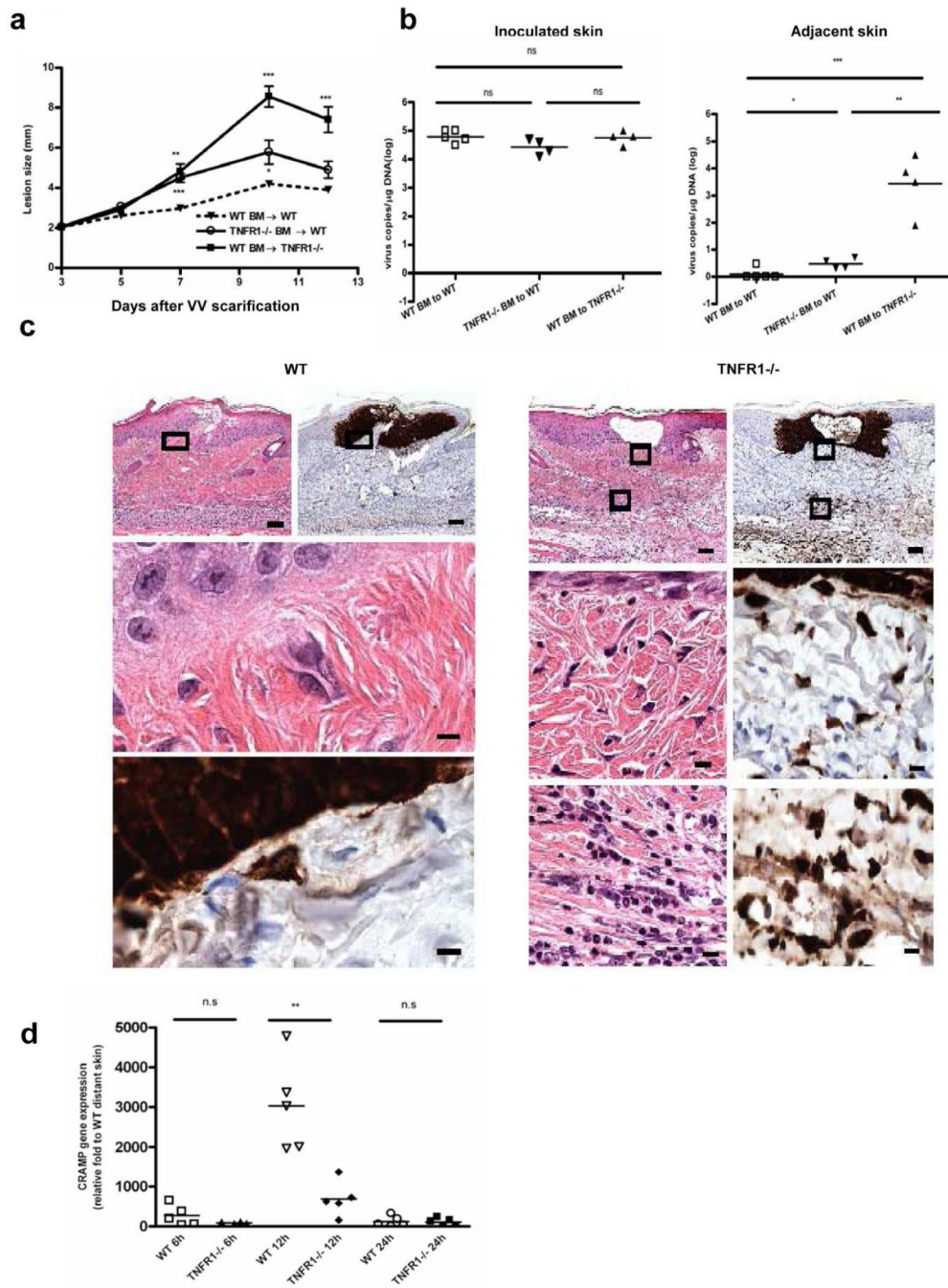
Figure 5. Impaired cutaneous response to VV scarification of TNFR1−/− mice is related to resident skin cells
WR-VV was applied to BM reconstituted chimeric mice by scarification. (a) Lesion size and (b) Virus load at day 12 were determined. (c) GFP-VV was applied to WT and TNFR1−/− mice by scarification, H&E and anti-GFP Ab staining were used to identify VV infected cells (brown) at day 5. Bars: WT top = 100 µm, middle and bottom = 25µm; TNFR1−/− top = 100 µm, middle and bottom pairs = 10µm. (d) skin samples from WR-VV inoculated mice were harvested for measurement of CRAMP gene expression. Error bars represent mean ± SEM. n=4–5 per group. * P < 0.05, ** P < 0.01, *** P < 0.001, ns= not significant.