# Interleukin-1β But Not Tumor Necrosis Factor is Involved in West Nile Virus-Induced Langerhans Cell Migration from the Skin in C57BL/6 Mice

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**Langerhans cells are bone marrow-derived epidermal dendritic cells. They migrate out of the epidermis into the lymphatics and travel to the draining lymph nodes where they are responsible for the activation of T cells in the primary immune response. Tumor necrosis factor and interleukin-1β, have previously been shown to be responsible for Langerhans cell migration in response to contact sensitizers in BALB/C mice; however, which cytokines are responsible for mediating Langerhans cell migration in response to a replicating cutaneously acquired virus such as the West Nile Virus, are not known. We have devised a method for identifying Langerhans cells in the draining lymph nodes using E-cadherin labeling and flow cytometry. We infected tumor necrosis factor-deficient gene knockout mice (tumor necrosis factor$^{-/-}$) intradermally with West Nile Virus and found that levels of Langerhans cell emigration and accumulation in the draining lymph nodes were similar to wild-type C57BL/6 mice. This was borne out by the finding that high levels of systemic neutralizing anti-tumor necrosis factor antibody failed to inhibit the migration of Langerhans cells from the epidermis and their accumulation in the draining lymph nodes in wild-type C57BL/6 mice. In West Nile Virus-infected, tumor necrosis factor$^{-/-}$ mice treated with systemic neutralizing anti-interleukin-1β antibodies, however, migration of Langerhans cells from the epidermis and their accumulation in the draining lymph nodes were significantly inhibited compared with control antibody-treated, infected animals. The results indicate that Langerhans cell migration, accumulation in the draining lymph nodes and the initiation of lymph node shut-down in response to a cutaneous West Nile Virus infection is dependent on interleukin-1β and can occur in the absence of tumor necrosis factor.** ***Key words: cytokines/dendritic cells/E-cadherin/epidermis/flavivirus. J Invest Dermatol 117:702–709, 2001***

Langerhans cells are bone marrow-derived dendritic cells (DC) that reside in the epidermis of the skin. They are professional antigen-presenting cells, responsible for activating naïve T cells in a primary immune response to an antigen (Steinman, 1991).

Work with contact sensitizers shows that Langerhans cells take up antigen and migrate from the skin to the local draining lymph nodes where they present that antigen to T lymphocytes (Stingl *et al*, 1978, 1980). It is less clear, however, what happens when a replicating antigen such as an arthropod-borne flavivirus, is deposited in the epidermis and dermis by the bite of an infected mosquito or tick. It has recently been demonstrated that human Langerhans cells are directly infected with a closely related flavivirus, Dengue (Wu *et al*, 2000). This group suggests that Langerhans cells are the primary targets of flavivirus infection in the skin. Such infection is common in many areas of the world, with neurotropic flaviviruses, such as West Nile virus (WNV), St Louis encephalitis, Murray Valley encephalitis, tick-borne encephalitis, and Japanese encephalitis viruses, causing significant morbidity and mortality in both humans and animals. Some, such as WNV, endemic in Third World Africa and India, cause serious periodic epidemics outside these areas. An outbreak of WNV encephalitis occurred for the first time in New York in 1999 with 50 documented cases and at least five fatalities (Anderson *et al*, 1999; Briese *et al*, 1999; Lanciotti *et al*, 1999). The likelihood of outbreaks of infection with these viruses in nonendemic areas has increased markedly with tourism and as global warming extends the environment permissive for the mosquito vector (Anderson *et al*, 1999; Hubalek and Halouzka, 1999).

Work from our laboratory has shown *in vitro* infection with WNV is accompanied by cytokine-independent upregulation of the major histocompatibility complex class I and II (MHC I, II) antigens, the cellular adhesion molecules, CD54 (ICAM-1) and CD106 (VCAM-1), and the selectin, CD62-E (E-selectin), variously, depending on the cell type (King and Kesson, 1988; Shen *et al*, 1997). Furthermore, this virus-induced response is accompanied by an increased susceptibility to cytotoxic T cell lysis (Douglas *et al*, 1994), consistent with increasing evidence for an

Manuscript received July 10, 2000; revised March 20, 2001; accepted for publication May 6, 2001.

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Abbreviations: DC, dendritic cells; i.d., intradermal; WNV, West Nile Virus.

immunopathologic basis for flavivirus disease (Bres, 1988; Andrews *et al*, 1999).

More recently, therefore, our efforts have been directed at defining the earliest events in the initiation of the immune response to cutaneous WNV infection in an *in vivo* BALB/C mouse model. We have found that epidermal Langerhans cells significantly increase expression of MHC II, ICAM-1, and the costimulatory molecule, CD80 (B7-1) (Johnston *et al*, 1996), and migrate from the epidermis to the local draining lymph nodes (Johnston *et al*, 2000). Others meanwhile have highlighted the importance of DC in initiating an immune response specifically against viruses (Ludewig *et al*, 1998).

Which factors induce epidermal Langerhans cell migration to the draining lymph nodes in response to WNV are unknown. Cytokines, however, involved in Langerhans cell migration in response to contact sensitizers include tumor necrosis factor (TNF) and interleukin (IL)-1β, thought to work separately, but in tandem. This has been shown by intradermal injection of recombinant TNF or IL-1β into the skin of BALB/C mice. Furthermore, systemically administered neutralizing TNF and/or IL-1β antibodies inhibit Langerhans cell migration from the epidermis to draining lymph nodes in this model (Cumberbatch *et al*, 1997a, b).

Thus, in this study we investigated the possible role of TNF and IL-1β in Langerhans cell migration from the epidermis to draining lymph nodes following a cutaneous WNV infection in C57BL/6 $TNF^{+/+}$ and $TNF^{-/-}$ mice. We show that in $TNF^{-/-}$ mice, Langerhans cell migration in response to WNV is quantitatively comparable with $TNF^{+/+}$ controls, but that IL-1β is required. Furthermore, we show that TNF is not required for WNV-induced Langerhans cell migration in $TNF^{+/+}$ mice.

## MATERIALS AND METHODS

**Animals** Female C57BL/6 mice aged between 6 and 10 wk were obtained from the Animal Resource Center (Perth, WA, Australia). Female TNF-knockout mice ($TNF^{-/-}$) bred on a C57BL/6 background (Korner *et al*, 1997) aged between 6 and 10 wk were obtained from Dr. Jonathon Sedgwick at the Centenary Institute of Cancer Medicine and Cell Biology (Sydney, NSW, Australia). Animals infected with WNV were housed in Hepa cages (Allentown Caging, NJ) and given access to standard mouse food and distilled water *ad libitum*. All experiments were conducted with approval from the University of Sydney Animal Ethics Committee.

**Virus infection** WNV (Sarafend strain) was grown (King and Kesson, 1988; Shen *et al*, 1997) and used for cutaneous infection (Johnston *et al*, 2000) as previously described. Briefly, 4 ml of stock virus was seeded on to washed monolayers of Vero cells cultured in 175 $cm^2$ flasks (Nunc, Roskilde, Denmark). Cultures were overlaid with 10 ml of Dulbecco's minimal Eagle's medium (CSL, Melbourne, Victoria, Australia) supplemented with 10% fetal bovine serum (CSL), and incubated for 60 min at 37°C 5% $CO_2$. After a further 30 h incubation under these conditions, the supernatant was removed and centrifuged to remove cellular debris. The supernatants were then stored at −70°C until required. Titers of virus stocks were determined by serial dilutions on Vero cell monolayers as previously described (Taylor and Marshall, 1975). Anesthetized mice (2,2,2-tribromo-ethanol; Aldrich Chemical, Milwaukee, WI), were injected intradermally (i.d.) into the dorsal side of their ears with 50 μl of WNV (approximately $5 \times 10^5$ PFU), using a 30 gauge needle (Becton Dickinson, Franklin Lakes, NJ). Control animals were mock-infected with 50 μl of vehicle (Dulbecco's minimal Eagle's medium with 10% fetal bovine serum from mock-infected vero cell cultures). Mice were killed 12, 18, or 24 h later by cervical dislocation and tissue was processed immediately.

**Treatment of mice with neutralizing anti-TNF or anti-IL-1β antibodies** Mice received three 100 μl intraperitoneal (i.p.) injections of neutralizing antibody 2 h prior to, at the time of, and 6 h after, WNV injection. Wild-type C57BL/6 mice ($TNF^{+/+}$) received $2 \times 10^4$ neutralizing units of polyclonal rabbit anti-mouse TNF in 100 μl (Genzyme Diagnostics, Cambridge, MA) per injection. $TNF^{-/-}$ mice received 20 μg of polyclonal goat anti-mouse IL-1β in 100 μl (R&D Systems, Minneapolis, MN) per injection. Control mice received either normal rabbit serum (Zymed Laboratories, San Francisco, CA) or total goat IgG (R&D Systems). Antibodies were diluted at an equal IgG concentration in phosphate-buffered saline (PBS). Mice were killed 18 h after WNV or vehicle injection.

***In vivo* antibody neutralization of lipopolysaccharide (LPS) - induced TNF or IL-1β release** To confirm that the anti-TNF and anti-IL-1β antibodies successfully neutralized TNF and IL-1β, respectively, *in vivo*, groups of three $TNF^{+/+}$ or three $TNF^{-/-}$ mice that had received anti-TNF or anti-IL-1β neutralizing or control antibody, as described above, were injected i.p. with LPS (100 μg in 100 μL PBS) derived from *Escherichia coli* (Sigma, St Louis, MO). These mice were injected 2 h and 8–10 h after the final anti-TNF and anti-IL-1β antibody treatment, respectively. Mice were then carefully monitored for signs of shock with the endpoint of both experiments being 18 h after the second antibody injection. At this point, the mice were bled. The level of serum TNF or IL-1β was determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Nakane *et al*, 1992). For the TNF ELISA, hamster anti-mouse TNF monoclonal antibody (TN3; Genzyme Diagnostics) and rabbit anti-mouse TNF polyclonal antibody (IP-400; Genzyme Diagnostics) were used as the capture and detecting antibody, respectively. Antibody binding was detected using biotinylated donkey anti-rabbit IgG (Sigma). The serum level of bioactive TNF was also estimated by a cytotoxicity assay using the TNF sensitive WEHI-164 cell line, as previously described (Espevik and Nissen-Meyer, 1986; Rubel *et al*, 1998), using recombinant murine TNF (Endogen, Woburn, MA) as a standard. For the IL-1β ELISA, an IL-1β DuoSet was used (murine-IL-1β DuoSet; R&D Systems).

**Identification of Langerhans cells in draining lymph nodes** Subauricular ear skin draining lymph nodes were removed and mechanically disrupted in 1 ml of Dulbecco's minimal Eagle's medium with 10% fetal bovine serum using two 21 gauge hypodermic syringe needles. Total cell counts were performed using a hemocytometer, with dead cells being identified by trypan blue (0.4%) dye exclusion. This was to monitor lymph node shutdown, one of the first indicators of initiation of an immune response. Lymph node shutdown is characterized by an increase in cellular input to the node, combined with an initial decrease in cellular output, resulting in an increase in the total number of leukocytes within the node (Smith and Morris, 1970; Cahill *et al*, 1974). Langerhans cells in the draining lymph nodes were identified by two-color flow cytometry analysis. Approximately $10^6$ cells were placed in glass tubes and centrifuged at $460 \times g$ for 3 min at 4°C. The pellet was then resuspended in 50 μl of anti-E-cadherin rat IgG1 antibody (Decma-1; Sigma) (Tang *et al*, 1993) and the anti-mouse I-A rat IgG2b antibody TIB120 (American Type Culture Collection; ATCC; Rockville, MD) at a concentration of 1–2 μg of antibody per $10^6$ cells. The cells were incubated with the antibodies for 45 min at 4°C, washed by underlaying with 200 μl of fetal bovine serum and centrifuging at $460 \times g$. The cells were then incubated for 45 min at 4°C with goat anti-rat-IgG1-fluorescein isothiocyanate (RG11/39.4; PharMingen, San Diego) and goat anti-rat-IgG2b-biotin (RG7/11.1; PharMingen). The cells were washed and incubated with streptavidin–phycoerythrin (Jackson Immunoresearch Laboratories, West Grove, PA) for 45 min at 4°C. Isotype-matched control antibodies rat IgG1 (R3-34; PharMingen) and rat IgG2b (R35-38; PharMingen) were used in parallel with the above primary antibodies. All samples were acquired and analyzed using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Files with $5 \times 10^4$ events were collected and isotype controls were subtracted from positively stained cells to determine the percentage of positive cells. E-cadherin$^+$ cells were gated using forward scatter *vs* fluorescence dot plots.

Owing to variability in fluorescence between different flow cytometry experiments, results for the number of E-cadherin$^+$ Langerhans cells in draining lymph nodes were normalized. To do this, Langerhans cell migration in the control-treated WNV-infected groups (positive controls) were regarded as "100%" migration, and all other levels of migration were normalized against and then compared with this result. Mathematically, normalization is as follows:

$$(a/b) \times 100$$

where a is the experimental value and b is the mean of the positive control.

**Identification of Langerhans cells in epidermal sheets** Twelve, 18, or 24 h following injection of virus or vehicle, mice were killed and ears surgically removed. Epidermal sheets were prepared as described previously (Halliday *et al*, 1992). In brief, the dorsal surface of the ear that was injected with virus was tape-stripped three to five times with cellophane tape (BDF Australia, Australia) to remove the stratum

corneum. The ear was then cleaved along the cartilage and incubated in PBS (pH 7.3) supplemented with 20 mM ethylenediamine tetraacetic acid (Sigma) for 3 h at 37°C. The epidermis was mechanically separated from the dermis and fixed in acetone for 20 min at −20°C. After washing three times with PBS, the epidermis was incubated in hybridoma supernatant containing anti-I-A antibody (TIB120) overnight. The epidermis was then washed as above and incubated with biotinylated goat anti-rat antibody (Caltag Laboratories, San Francisco, CA) diluted in PBS containing 1% bovine serum albumin (PBS-bovine serum albumin; Sigma) for 2 h. After this the epidermis was washed as above and incubated with streptavidin–alkaline phosphatase (Amersham, Buckingham, U.K.) in PBS-bovine serum albumin for 1 h. The epidermal sheets were then washed again as above and incubated in a New Fuchsin-based substrate for 20 min. All incubations were performed at room temperature. Following washing with distilled water, the epidermal sheets were placed on glass microscope slides, air dried, and mounted in Histomount (National Diagnostics, Riverstone, NSW, Australia). Stained epidermal sheets were blinded and counted using a true color, fully automated image analysis system (Chromatic Color Image Analysis System, LR. Javis, Wild-Leitz, Sydney, NSW, Australia). For each epidermal sheet, randomly selected fields were counted until the total area evaluated approximated 1 $mm^2$. Isotype controls were included to ensure specificity of staining.

**Data analysis** Statistical differences between groups was assessed by ANOVA, which will take into account variability between groups. For analysis of ELISA data, an unpaired Student's t test was used. In both methods, values of $p < 0.05$ were considered significant.

## RESULTS

**WNV induces a reduction in epidermal Langerhans cells and an increase in lymph node Langerhans cells in both $TNF^{+/+}$ and $TNF^{-/-}$ mice** To investigate the role of TNF in Langerhans cell migration from C57BL/6 skin in response to WNV, groups of wild-type ($TNF^{+/+}$) and TNF knockout ($TNF^{-/-}$) mice were each infected with WNV or mock infected, and the frequencies of Langerhans cells, both in the epidermis and the draining lymph nodes, were determined at various time points. The epidermis of mock-infected $TNF^{-/-}$ mice contained significantly higher numbers of Langerhans cells (700 per $mm^2$), when compared with similarly treated $TNF^{+/+}$ mice (550 per $mm^2$). In $TNF^{+/+}$ mice, WNV caused a significant reduction in the density of epidermal Langerhans cells at 18 h, but not 12 or 24 h after infection (**Fig 1**). Interestingly, a reduction in Langerhans cells

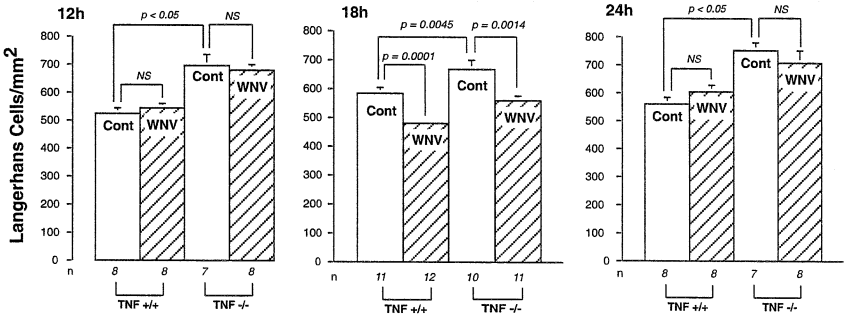


**Figure 1. WNV causes a reduction in epidermal Langerhans cells in both $TNF^{+/+}$ and $TNF^{-/-}$ mice.** C57BL/6 wild-type ($TNF^{+/+}$) and TNF knockout ($TNF^{-/-}$) mice were injected with WNV or vehicle control. Twelve, 18, or 24 h postinfection, epidermal sheets were prepared and stained for Langerhans cells with anti-MHC II antibody. Langerhans cells were enumerated by image analysis. For 12 h and 24 h time points, results were pooled from two separate experiments. For the 18 h time point, results are a pool of three separate experiments. All results are presented as mean ± SEM, and were statistically analyzed by ANOVA. NS, not significant. The number of injected ears per group (n) is indicated below the x-axis.

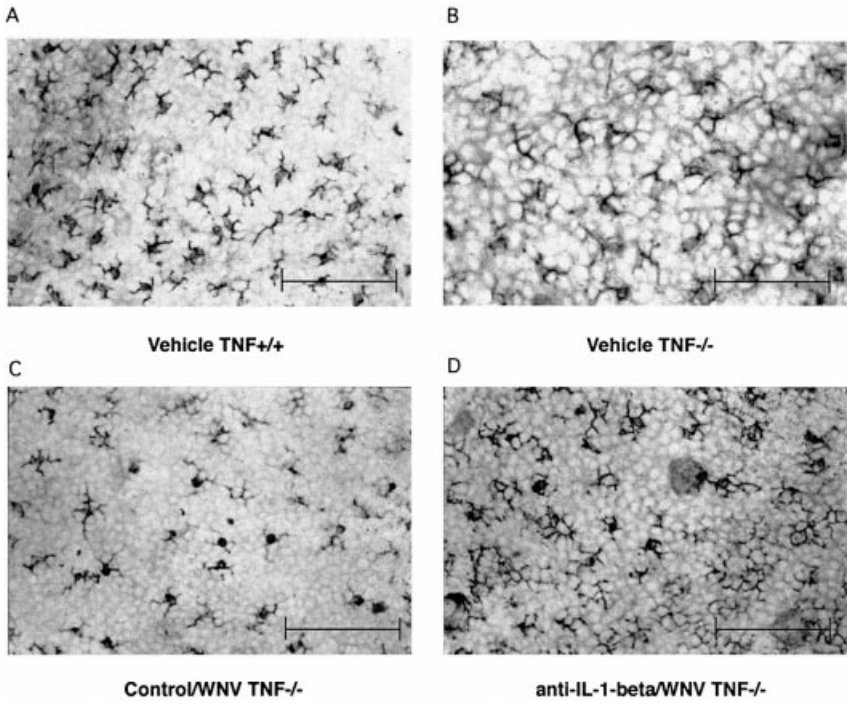


**Figure 2. WNV causes morphologic changes in epidermal Langerhans cells.** Epidermal sheets from 18 h WNV-infected or mock-infected $TNF^{+/+}$ and $TNF^{-/-}$ mice were stained for MHC class II. $TNF^{+/+}$ (*A*) or $TNF^{-/-}$ mice (*B*) treated with vehicle display long extended dendrites. Control IgG treated, $TNF^{-/-}$ mice infected with WNV (*C*) display a more activated appearance with smaller dendrites and rounded cell bodies when compared with vehicle controls. Anti-IL-1β treated WNV-infected $TNF^{-/-}$ mice (*D*) had reduced numbers of Langerhans cells with an activated appearance compared with control IgG treated WNV-infected mice (*C*). *Scale bar*: 50 μm.

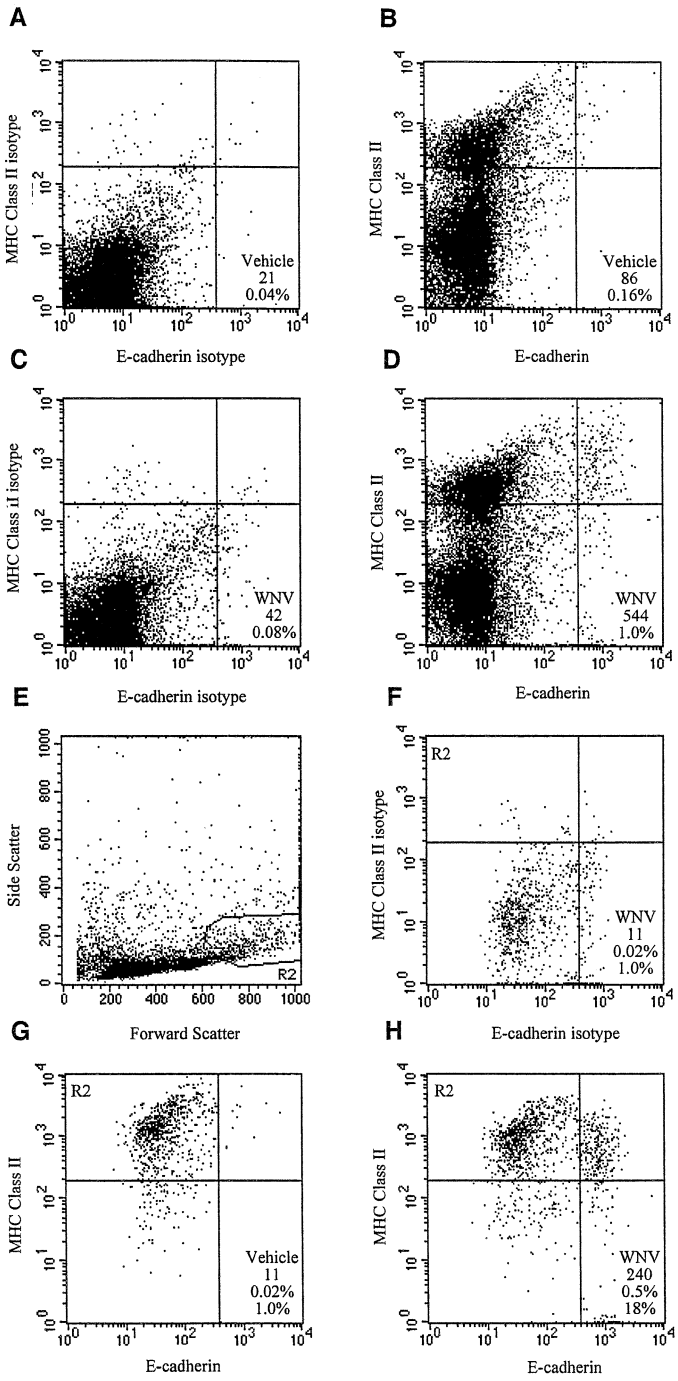


**Figure 3. Langerhans cells in the draining lymph nodes of WNV-infected mice express high levels of both E-cadherin and MHC class II molecules.** Lymph node cell suspensions were prepared from $TNF^{+/+}$ mice infected with vehicle or WNV for 18 h. Cells were stained with anti-E-cadherin and anti-MHC II antibodies (or isotype control antibodies) and analyzed by flow cytometry. Parts (*A*)–(*D*) show ungated populations of lymph node cells. Numbers in each figure show statistics for the right upper quadrant. These represent the absolute number of cells in the sample of 50,000 events and the absolute percentage of cells. Part (*E*) shows the gate (R2) used to select events with high forward *vs* high side scatter typical of DC. Parts (*F*)–(*H*) show events within R2 only. Numbers in each figure show the absolute number of events in R2, absolute percentage of cells and percentage of cells within R2, from top to bottom, respectively. Representative data are shown here, and these were similar in three replicate experiments.

density in response to WNV infection was observed in $TNF^{-/-}$ mice with similar kinetics. At 18 h, but not at the 12 and 24 h time-points, some of the Langerhans cells in WNV-infected $TNF^{+/+}$ and $TNF^{-/-}$ mice displayed a more activated appearance with smaller dendrites and rounded cell bodies compared with mock-infected mice (**Fig 2**).

Flow cytometry was used to detect E-cadherin$^+$ Langerhans cells in the draining lymph nodes as described previously (Johnston *et al*, 2000). Pooled lymph node cell suspensions from $TNF^{+/+}$ mice injected with either vehicle (**Fig 3*A***, ***B***, ***G***) or WNV (**Fig 3*C***, ***D***, ***F***, ***H***), were double-labeled with anti-MHC class II and anti-E-cadherin antibodies (**Fig 3*B***, ***D***, ***G***, ***H***) (or appropriate isotype-matched control antibodies) (**Fig 3*A***, ***C***, ***F***). In these samples, all E-cadherin$^+$ cells expressed high levels of MHC class II antigens. On back-gating, these double-positive cells had forward *vs* side-scatter profiles typical of DC (data not shown). Moreover, when this high forward scatter *vs* high side scatter population was forward-gated (**Fig 3*E***), these cells were MHC class II$^{high}$ and E-cadherin$^+$ (**Fig 3*F***, ***G***, ***H***). Very small numbers of cells were detectable in mice that received vehicle (**Fig 3*G***), but a clear population of MHC class II$^{high}$ and E-cadherin$^+$ cells were detected in WNV-infected mice (**Fig 3*H***). Similar results were observed in $TNF^{-/-}$ mice (data not shown). This indicates that E-cadherin can be used to detect epidermally derived DC in draining lymph nodes.

Significantly higher numbers of E-cadherin$^+$ Langerhans cells in the draining lymph nodes of WNV-infected, compared with mock-infected animals, were observed at all time points (12, 18, or 24 h) in both wild-type and knockout mice (**Fig 4**). There was no statistically significant difference between $TNF^{+/+}$ and $TNF^{-/-}$ mice at any of these time points.

WNV-infected groups of $TNF^{+/+}$ and $TNF^{-/-}$ mice had significantly higher total numbers of lymph node cells than mock-infected mice at all time points (**Fig 5**), indicating that lymph node shut-down occurred in conjunction with the appearance of E-cadherin$^+$ Langerhans cells in the draining lymph nodes.

**Neutralizing anti-TNF antibody does not inhibit Langerhans cell migration in $TNF^{+/+}$ mice** Wild-type C57BL/6 ($TNF^{+/+}$) mice were injected with anti-TNF neutralizing antibodies or control serum prior to infection with WNV or vehicle control as described. Neutralization by anti-TNF antibody was confirmed in $TNF^{+/+}$ mice by measuring serum levels of TNF in response to LPS (**Table I**). Anti-TNF antibody significantly reduced the levels of LPS-induced TNF in mouse serum. These ELISA results were further confirmed by bioassay (data not shown). Epidermal Langerhans cells, lymph node Langerhans cells, and total lymph node cell numbers were determined 18 h after infection. **Figure 6** shows that anti-TNF neutralization did not inhibit WNV-induced Langerhans cell migration in $TNF^{+/+}$ mice. In mice that received neutralizing anti-TNF or control antibody, the density of epidermal Langerhans cells decreased in each to a similar extent. Furthermore, in both groups, there was a significant increase in the number of E-cadherin$^+$ Langerhans cells found in the draining lymph nodes after WNV infection, compared with mock infection. Mice infected with WNV and treated with neutralizing anti-TNF or control antibody had a significant increase in total lymph node cell numbers compared with their respective controls, suggesting that lymph node shutdown was independent of TNF (**Fig 6**).

**Epidermal Langerhans cell migration and accumulation in the draining lymph nodes of $TNF^{-/-}$ mice is dependent on IL-1β** IL-1β, in concert with TNF, has been shown to be involved in epidermal Langerhans cell migration to the lymph nodes in response to contact sensitizers (Cumberbatch *et al*, 1997a, b). Therefore, to investigate the role of IL-1β alone in the migration of Langerhans cells after WNV infection, $TNF^{-/-}$ mice were pretreated with neutralizing anti-IL-1β or control IgG prior to WNV or mock infection. Hence, the actions of both TNF and IL-1β were blocked in these experiments. Neutralization by anti-IL-1β antibody was confirmed in $TNF^{-/-}$ mice by measuring serum levels of IL-1β in response to LPS by ELISA (**Table I**). This treatment reduced IL-1β levels during LPS stimulation back to the

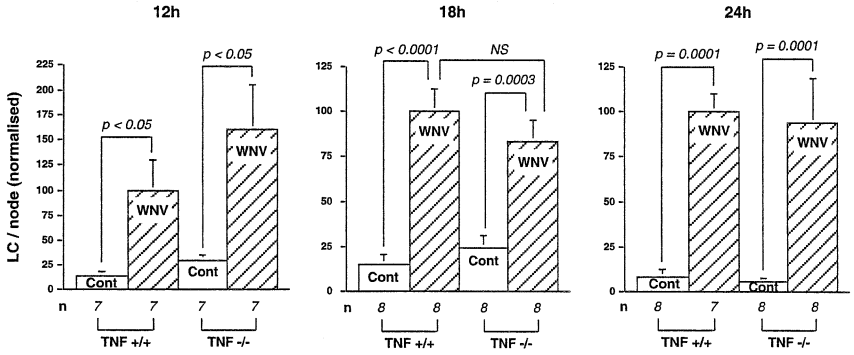


**Figure 4. WNV increases the number of Langerhans cells in draining lymph nodes of $TNF^{+/+}$ and $TNF^{-/-}$ mice.** $TNF^{+/+}$ and $TNF^{-/-}$ mice were injected with WNV or vehicle control. The draining lymph nodes were removed and analyzed for E-cadherin$^{+}$ Langerhans cells by flow cytometry 12, 18, or 24 h postinfection. Results from three separate experiments were normalized to the WNV-treated $TNF^{+/+}$ groups, presented as mean ± SEM, and were statistically analyzed by ANOVA. The number of nodes per group (n) is indicated below the x-axis. There was no significant difference between control groups for Langerhans cells/node.

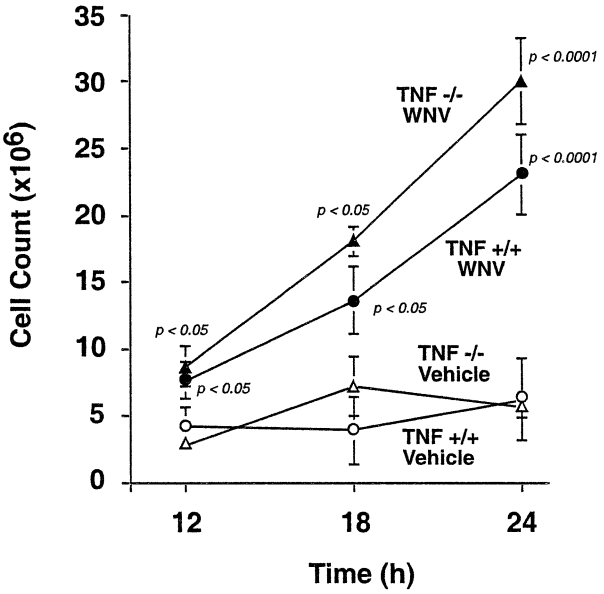


**Figure 5. Total lymph node cell numbers increase with time after WNV infection in both $TNF^{+/+}$ and $TNF^{-/-}$ mice.** $TNF^{+/+}$ and $TNF^{-/-}$ mice were injected with WNV or vehicle control. Twelve, 18, or 24 h postinfection, the total number of cells in the draining lymph nodes were quantitated using a hemocytometer, with dead cells being excluded with trypan blue (0.4%). Results were pooled from two separate experiments, presented as mean ± SEM, and were statistically analyzed by ANOVA. p-values are compared with the relevant controls at the same time point.

approximate limits of detection using this ELISA. Epidermal Langerhans cells, lymph node Langerhans cells and total lymph node cell numbers were determined 18 h after infection. The results in **Fig 7** show that Langerhans cell migration from the epidermis in response to viral infection is reduced by treatment with anti-IL-1β antibody. Control IgG-treated, but not IL-1β-neutralized mice, showed a significant reduction in numbers of epidermal Langerhans cells in response to cutaneous WNV infection. Thus there were significantly larger numbers of Langerhans cells in the epidermis of WNV-infected, IL-1β-treated mice, compared with WNV-infected control IgG-treated animals. Interestingly, when comparing the two vehicle-injected groups in these experiments, there was a significant difference ($p = 0.0413$) between anti-IL-1β-treated and control IgG-treated Langerhans cells densities. Analysis of lymph node Langerhans cells

**Table I. Neutralizing anti-TNF and anti-IL-1β antibodies successfully neutralized LPS induced serum TNF or IL-1β as measured by ELISA**

| | Treatment | |
|---|---|---|
| | Control-treated | Antibody-treated |
| TNF (pg per ml) | 815 ± 209[a] | 101 ± 7 ($p < 0.05$)[b] |
| IL-1β (pg per ml) | 402 ± 43 | 240 ± 10 ($p < 0.05$) |

[a]Mean cytokine levels (pg per ml) ± SEM for three mice/group.
[b]Statistical analysis was done by the Student's t test with $p < 0.05$ considered significantly different.

showed that whereas WNV increased the numbers of Langerhans cells in both control (3-fold increase) and anti-IL-1β-treated mice (2-fold increase), compared with each other, there were significantly fewer Langerhans cells in the lymph nodes of anti-IL-1β-treated mice ($p = 0.0034$). Total lymph node cell numbers were also significantly lower in the WNV-infected anti-IL-1β antibody group compared with WNV-infected control IgG $TNF^{-/-}$ mice ($p = 0.0179$).

## DISCUSSION

The cytokines, TNF and IL-1β, have been implicated in Langerhans cell migration to draining lymph nodes in response to contact sensitizers (Cumberbatch *et al*, 1997a). This study examined the role of these two cytokines in Langerhans cell migration in response to a replicating, cutaneously acquired arbovirus, WNV, in C57BL/6 $TNF^{+/+}$ and $TNF^{-/-}$ mice. Eighteen hours after cutaneous WNV infection *in vivo*, we found a significant reduction in epidermal Langerhans cell numbers, a corresponding significant increase in Langerhans cell numbers in the draining lymph node, as well as lymph node shutdown, compared with mock-infected controls. Interestingly, there was no difference in these parameters between $TNF^{+/+}$ and $TNF^{-/-}$ groups in their response to WNV infection. Systemic administration of neutralizing anti-TNF antibodies in WNV-infected $TNF^{+/+}$ mice failed to inhibit Langerhans cell migration following WNV infection in these mice, further supporting the findings in $TNF^{-/-}$ mice that TNF was not involved in Langerhans cell migration. On the other hand, IL-1β neutralization significantly inhibited emigration of Langerhans cells from the epidermis, appearance of Langerhans cells in the lymph node, and lymph node shutdown in WNV-infected $TNF^{-/-}$ mice. Whereas previous reports show that TNF plays a crucial part in Langerhans cell migration in response to contact sensitizers

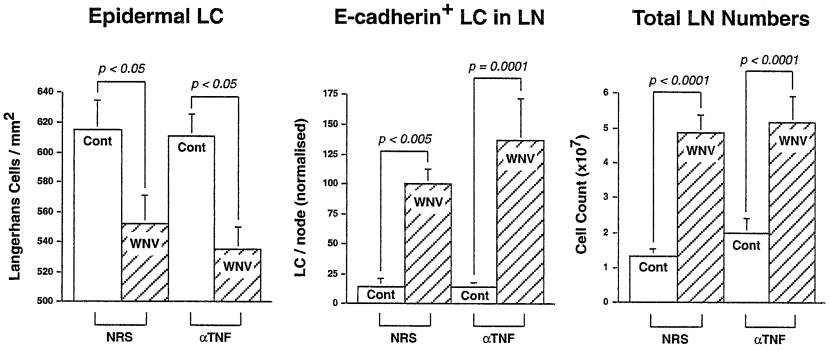


**Figure 6. Neutralizing anti-TNF antibody does not inhibit Langerhans cell migration from the epidermis or accumulation in the draining lymph nodes of TNF$^{+/+}$ mice at 18 h.** Groups of TNF$^{+/+}$ mice were treated with either control serum (NRS) or anti-TNF neutralizing serum (αTNF) and injected with WNV or vehicle control (see *Materials and Methods*). Eighteen hours after WNV infection, mice were killed and analyzed for epidermal Langerhans cell density, E-cadherin$^{+}$ Langerhans cells in draining lymph nodes, and total lymph node cell number. For all groups, n = 8. Results were pooled from two separate experiments, are presented as mean ± SEM, and were statistically analyzed by ANOVA. There was no significant difference between control groups for Langerhans cells/node.

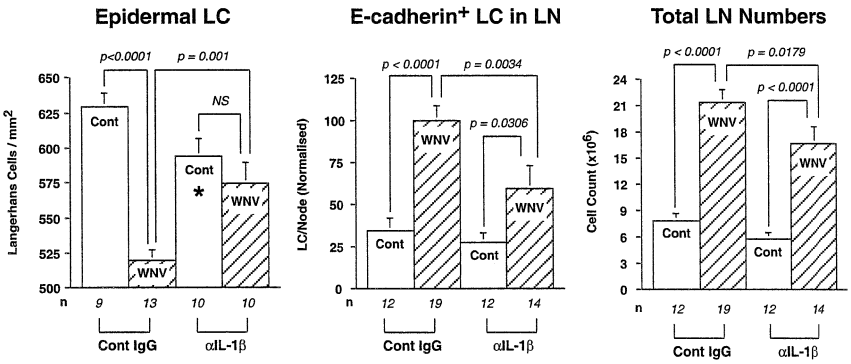


**Figure 7. Neutralizing anti-IL-1β antibody inhibits Langerhans cell migration from the epidermis and accumulation in the draining lymph nodes of TNF$^{-/-}$ mice at 18 h.** Groups of TNF$^{-/-}$ mice were treated with either control serum (Cont IgG) or anti-IL-1β neutralizing serum (αIL-1β) and injected with WNV or vehicle control. Eighteen hours after WNV infection, mice were killed and analyzed for epidermal Langerhans cell density, E-cadherin$^{+}$ Langerhans cells in draining lymph nodes, and total lymph node cell number. The number of nodes or sheets per group (n) is indicated below the x-axis. Results were pooled from three separate experiments for epidermal Langerhans cells, normalized and pooled from five separate experiments for E-cadherin$^{+}$ Langerhans cells in lymph nodes. Five separate experiments were pooled for total lymph node numbers. Results are presented as mean ± SEM, and were statistically analyzed by ANOVA. There was no significant difference between control groups for Langerhans cells/node. (*indicates $p < 0.05$ comparing the two control groups for Langerhans cell densities).

(Kimber and Cumberbatch, 1992; Cumberbatch *et al*, 1994; Wang *et al*, 1996), some groups have described similar findings to those described here (Stoitzner *et al*, 1999). These results were obtained using an *in vitro* migration system and showed that normal migration of DC occurred in skin explants taken from TNF/lymphotoxin-deficient C57BL/6 mice. In relation to this study, these results corroborate our findings that in response to a cutaneous-acquired viral infection, TNF is not crucial. In most *in vivo* studies, contact sensitizers are used to induce Langerhans cell migration, whereas in this study we used a live, replicating virus, which may utilize different signals to induce Langerhans cell migration. Moreover, previous studies have mostly been in BALB/C mice, whereas we have used C57BL/6 mice in this study. The work presented here has produced results that appear to contradict those found by Cumberbatch *et al* (1994, 1997a, b). Rather, we believe that our unique model of virally induced Langerhans cell migration has only reinforced the crucial part that TNF and IL-1β plays in mediating Langerhans cell migration in response to a variety of cutaneous-acquired infections. Thus, although our model is unique, it has not withstanding a more universal application. The use of a second strain has highlighted the range of overlapping function in cytokines related to Langerhans cell migration and illustrates the necessity to canvas more than one mouse strain for definitive results. By exploring the variety of possibilities, namely using genetically different inbred strains of mice, we hopefully can also further our understanding of the range of possible responses in humans.

Reports over the last 2 decades indicate clear differences between BALB/C and C57BL/6 mice. Differences in the TNF locus are proposed to account for sensitivity to ultraviolet-induced immunosuppression between BALB/C (ultraviolet-resistant) and C57BL/6 (ultraviolet-susceptible) mice (Streilein and Bergstresser, 1988). *In vitro*, IL-1α and TNF are not required by C57BL/6 DC to drive IL-12-dependent Th1 development when antigen is presented, whereas BALB/C DC are unable to activate Th1 cells without these cytokines (Shibuya *et al*, 1998). It is therefore possible that Langerhans cells in these strains migrate preferentially in response to different cytokines. Our results clearly indicate that TNF is not required for Langerhans cell migration in response to WNV in C57BL/6 mice.

In BALB/C mice, IL-1β also mediates Langerhans cell migration, in tandem with TNF (Cumberbatch *et al*, 1997a, b).

Application of the contact sensitizer, trinitrochlorobenzene, to BALB/C skin increases the mRNA expression of IL-1β in epidermal Langerhans cells (Enk *et al*, 1993) and IL-1β decreases E-cadherin expression preceding Langerhans cell migration out of the epidermis (Jakob and Udey, 1998). Mice deficient in IL-1β have impaired contact hypersensitivity to trinitrochlorobenzene (Shornick *et al*, 1996). Interestingly, IL-1β, but not TNF has been shown to enhance the migration of human Langerhans cells in a skin explant culture system (Rambukkana *et al*, 1996). Our results indicate that IL-1β mediates Langerhans cell migration to the draining lymph nodes and lymph node shutdown in response to cutaneous WNV infection in C57BL/6 mice in the absence of TNF. These outcomes were significantly inhibited but not completely blocked by high concentrations of systemically administered neutralizing antibody to IL-1β (3-fold *vs* 2-fold increase in Langerhans cell migration for control-treated and antibody-treated $TNF^{-/-}$ animals, respectively). These findings are consistent with those found by Stoitzner *et al* (1999) in that they were also able to inhibit but not completely block Langerhans cell migration *in vitro* with anti-IL-1β antibodies. Failure to abrogate these completely may have a technical basis or may indicate that there are yet other cytokines that mediate or augment these activities in this strain.

The identity of Langerhans cells in the epidermis was made by MHC class II staining and morphologic criteria at light microscopy; those reaching the draining lymph node were identified flow cytometrically, using two-color MHC II and E-cadherin labeling. E-cadherin anchors Langerhans cells to keratinocytes in the epidermis (Udey, 1997). It is downregulated when Langerhans cells are activated by antigen, enabling them to migrate out of the epidermis (Tang *et al*, 1993). Used with MHC II, it is a reliable marker for lymph node Langerhans cells, as only those $MHC\ II^{+}$ Langerhans cells that have migrated from the epidermis display E-cadherin and, although downregulated, levels of E-cadherin are readily detectable by flow cytometry (Borkowski *et al*, 1994; Blauvelt *et al*, 1995; Johnston *et al*, 2000).

In both $TNF^{+/+}$ and $TNF^{-/-}$ groups, the density of epidermal Langerhans cells decreased only transiently, and returned to baseline levels by 24 h following WNV infection. Langerhans cells are continually replenished from bone marrow precursors and it is likely that repopulation returned epidermal Langerhans cell numbers to normal by 24 h. Similar bone marrow precursor renewal of the airway DC population also occurs (Holt *et al*, 1994); however, a significant difference was observed in baseline epidermal Langerhans cell densities between $TNF^{+/+}$ and $TNF^{-/-}$ mice. Previous studies have implicated TNF in the differentiation and recruitment of human DC precursors (Caux *et al*, 1993). Recent evidence suggests that the CC chemokine, macrophage inflammatory protein 3α (MIP-3α) is important in the localization of Langerhans cell precursors to the epidermis (Charbonnier *et al*, 1999), whereas MIP-3β is important in the trafficking of Langerhans cells to the draining lymph nodes (Kellermann *et al*, 1999). As TNF downregulates the surface expression of CCR6, the receptor for MIP-3α (Carramolino *et al*, 1999), $TNF^{-/-}$ mice may recruit more Langerhans cell precursors in to the epidermis, resulting in higher baseline Langerhans cell densities. We are currently investigating these possibilities.

It can be seen in **Fig 7** that treatment of vehicle-injected (negative control) $TNF^{-/-}$ mice with anti-IL-1β resulted in lower Langerhans cells densities than mice treated with control IgG. This result may be explained by data from a recent study by Homey *et al* (2000) that shows that keratinocytes upregulate MIP-3α in response to IL-1β. As MIP-3α recruits Langerhans cells to the skin, our data suggests that anti-IL-1β antibody inhibited the continuing recruitment of Langerhans cell precursors into the epidermis in this experiment, resulting in the discrepancy in epidermal Langerhans cell numbers between the vehicle-injected, anti-IL-1β-treated, and control IgG-treated mice.

It follows that if anti-IL-1β antibody were not inhibiting emigration of Langerhans cells from the epidermis in WNV-infected mice, there would be a greater reduction in epidermal Langerhans cell numbers in the WNV-infected, anti-IL-1β antibody-treated mice, compared with the WNV-infected, control IgG-treated mice. Our results, however, showed that this antibody significantly inhibited the WNV-induced emigration of Langerhans cells from the epidermis, compared with both the WNV-infected, control IgG-treated and the vehicle-injected, anti-IL-1β antibody-treated groups.

The mechanisms of cytokine-induced Langerhans cell migration and lymph node shutdown are not fully understood. Lymph node shutdown (Smith and Morris, 1970; Cahill *et al*, 1974; Hay and Hobbs, 1977) increases the peripheral T cell pool in the node, thus increasing the likelihood of an antigen-presenting Langerhans cell coming into contact with antigen-specific T cells. In this study, lymph node shutdown was contemporaneous with the appearance of Langerhans cells from WNV-infected skin in the draining node in WNV-infected but not vehicle-injected control mice. Neutralizing IL-1β antibodies in $TNF^{-/-}$ mice inhibited both Langerhans cell migration and lymph node shutdown. Thus whether Langerhans cell mobilization triggered lymph node shutdown, or whether IL-1β was independently responsible, is not known.

Recent evidence suggests that chemokines may play an important part in DC migration and trafficking. Sozzani *et al* (1998) showed that DC activated with IL-1, TNF, LPS, or CD40L had a strongly augmented chemotactic response to EBI1 ligand chemokine. Others have shown enhanced *in vitro* migration of human DC to MIP-3β and the CXC chemokine, stromal cell derived factor-1α, following preculture of DC with TNF, LPS, or IL-1β (Lin *et al*, 1998). In a contact sensitizer model, Yamazaki *et al* (1998) demonstrated that the chemokines RANTES (Regulated on activation, normal T-expressed and secreted) and monocyte chemoattractant protein-1, as well as TNF, were able to stimulate Langerhans cell migration. Thus, it is likely that IL-1β influences Langerhans cell migration via both direct and indirect mechanisms. Further experiments are required, however, to establish whether chemokines are involved in the WNV-induced Langerhans cell migration studied here.

*The authors would like to thank Dr. Ron Sluyter and Dr. Jonathon Sedgwick for their critical review of the manuscript, also to Anna Hanson and Shannon Burke for their help with the ELISA and bioassay. This work was supported by the National Health and Medical Research Council (NHMRC) (no. 970757).*

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