**Pan-viral protection against arboviruses by activating skin macrophages at the inoculation site**

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One-sentence summary

Activation of innate immune responses to arbovirus at the mosquito bite prevents efficient systemic dissemination of virus.


## Abstract

Arthropod-borne viruses (arboviruses) are important human pathogens for which there are no specific antiviral medicines. The abundance of genetically distinct arbovirus species, coupled with the unpredictable nature of their outbreaks, has made developing virus-specific treatments challenging. Instead, we have defined and targeted a key aspect of the host innate immune response to virus at the arthropod bite that is common to all arbovirus infections, potentially circumventing the need for virus-specific therapies. Using mouse models and human skin explants, we identify innate immune responses by dermal macrophages in the skin as a key determinant of disease severity. Post-exposure treatment of the inoculation site by a topical TLR7 agonist suppressed both the local and subsequent systemic course of infection with a variety of arboviruses from the Alphavirus, Flavivirus, and Orthobunyavirus genuses. Clinical outcome was improved in mice following infection with a model Alphavirus. In the absence of treatment, anti-viral interferon expression to virus in the skin was restricted to dermal dendritic cells. In contrast, stimulating the more populous skin-resident macrophages with a TLR7 agonist elicited protective responses in key cellular targets of virus that otherwise proficiently replicated virus. By defining and targeting a key aspect of the innate immune response to virus at the mosquito bite site, we have identified a putative new strategy for limiting disease following infection with a variety of genetically-distinct arboviruses.

## Introduction

Emerging and re-emerging arboviruses pose an increasing threat to human health. There has been a substantial increase in both the incidence and geographical range of medically-important arboviruses spread by mosquitoes, which infect hundreds of millions of people each year and include the Zika (ZIKV), dengue (DENV) and chikungunya (CHIKV) viruses. Arboviruses are a large, genetically-diverse group of viruses that cause a wide spectrum of diseases in humans (1–5). Despite their genetic diversity, it is nonetheless difficult to clinically differentiate between these infections in the early stages of disease, as they are either asymptomatic or present as a non-specific febrile viral illness. In many geographic areas this is compounded by the widespread co-circulation of distinct species of arboviruses in the same geographic area (6). Together, these factors complicate the use of putative virus-specific anti-virals, which for acute infections are most often only efficacious when given during early stages (7). This, when combined with our inability to accurately predict the timing and location of future epidemics (8), makes stockpiling future virus-specific drugs and vaccines difficult. Currently, there are few vaccines and no antivirals available for arbovirus infections. We suggest that due to the diversity of arbovirus genetics, their common clinical features and their unpredictable epidemiology, the development of a pan-viral medicine that is efficacious for multiple arbovirus infections would be highly advantageous.

Infected mosquitoes deposit virus into the skin dermis as they probe for a bloodmeal, triggering activation of distinct inflammatory pathways in response to mosquito biting and to virus sensing (9–11). We and others have demonstrated that host responses in the skin to mosquito biting, or mosquito saliva, has a defining influence on the systemic course of infection for a wide variety of genetically-distinct arboviruses including flaviviruses,

alphaviruses and bunyaviruses *(10–14)*. As such, this is a key stage of infection during which virus replicates rapidly before disseminating to the blood and remote tissues. Indeed, the majority of virus in the blood at 24 hours post infection of the skin is likely derived from replication at this site *(9)*. However, some of the original virus inoculum also disseminates directly to the blood and the draining lymph node (dLN) where replication is also established prior to systemic dissemination *(9, 13)*. Thus, it is not clear what role skin-specific innate immune responses, activated by virus sensing at the mosquito bite, has on modulating the subsequent systemic course. In this study, we wanted to define the relevance of skin virus-sensing pathways and determine whether this can be targeted to modulate outcome of infection.

Innate immune sensing of virus activates immune pathways that are distinct to those activated by mosquito biting, resulting in expression of type I interferons (IFN) and the anti-viral genes they upregulate, IFN-stimulated genes (ISG). Despite the evolution of multiple strategies by arboviruses to counteract IFN they are nonetheless highly sensitive to them, suggesting that therapies designed to target these pathways could be generalisable to a range of viruses *(15–18)*. Systemic administration of type I IFN has been used to treat hepatitis C virus, although febrile-like side effects are common *(19)* and as such it is not suitable for e.g. long-term prophylactic use. However, previous attempts to inhibit arbovirus infection by systemic administration of innate immune agonists or type I IFN have only been efficacious when given prior to infection *(20–24)*. In contrast, we suggest targeting processes at the inoculation site, which represents a discreet identifiable locale that can be targeted post-mosquito bite, prior to the systemic dissemination of virus. However, targeting these pathways is challenging as

the coordination of early innate immune responses to virus at mosquito bites are not well defined.

Studying innate immune responses at the tissue- and system-wide level to arboviruses that are medically-important in humans, has been frequently complicated by their inability to replicate in immunocompetent mice (10). Therefore, we chose to primarily study host innate immune responses to a prototypic model arbovirus Semliki Forest virus (SFV; genus Alphavirus). Unlike most human arboviral pathogens, SFV is capable of efficiently replicating, disseminating systemically within, and causing clinically-observable disease in immunocompetent mice *(25)*. SFV is a close relative of CHIKV that has a large number of genetically-modified clones and has been used extensively to study host response to infection *(26)*. SFV replicates quickly following infection of mouse skin, with viremia peaking by 24 hours post-infection (hpi). Dissemination of SFV to brain tissue can result in encephalitis, neurological signs and death *(9, 25)*. Clinical outcome of SFV infection in mice most likely reflects the efficiency by which virus seeds brain tissue.

In this study, we define key aspects of host response to virus at mosquito bites and demonstrate that this can be therapeutically modulated to suppress viral replication and the development of clinical disease. We suggest that for virus to successfully disseminate from the skin to the blood at a sufficient level to induce a high-titre viremia, sufficiently robust replication must occur at the inoculation site prior to a host IFN induced anti-viral state. Using an immune-competent mouse model of arbovirus infection and *ex vivo* infection of human skin explants, we investigate therapeutic manipulation of skin IFN pathways by post-exposure topical application of a widely-used generic innate immune agonist for infection with multiple genetically-distinct arboviruses.

## Results

### *Skin innate immune responses to virus infection at the inoculation site are a key determinant of the systemic course*

We wanted to determine whether targeting early innate immune responses in the skin had any observable effect on the later systemic course of infection. However, it was first necessary to define the kinetics and magnitude of endogenous host ISG responses to infection with SFV6 (a virulent strain of SFV), in the absence of therapies. Following infection of the foot skin, ISG expression in response to virus was slow and of low-magnitude, with ISG expression not significantly elevated until 24 hpi or later, peaking at 48 hpi (Fig 1A-D). In comparison, the dLN rapidly and robustly upregulated ISG, with amounts peaking by 16 hpi. Thus, upregulation of anti-viral ISG in the skin could be detected only after virus had disseminated systemically (9). It is not clear what relevance skin-specific innate immune responses to arbovirus have on the subsequent systemic course of infection, nor whether virus replication in the skin is absolutely necessary to establish viremia. To determine whether therapeutic induction of anti-viral innate immune pathways at the inoculation site could have any effect on the systemic course of infection, we injected or topically applied to a defined cutaneous site a range of innate immune agonists one hour prior to infection with SFV6 (Fig 1E-K). The TLR7 agonist imiquimod (IMQ) was administered either as a subcutaneous (s.c.) injection or applied topically as a cream (Aldara, topical IMQ). To assess success of virus infection viral RNA was measured by qPCR and infectious virus assayed by plaque assay. Virus RNA measured by qPCR represented sum total of genome and replicated viral transcripts. To define the contribution made by the original inoculum towards the level of viral RNA assayed by qPCR at 24hpi, mice were

inoculated with the UV-inactivated virus (which can infect cells but does not replicate). Copy number of viral RNA were close to the detection limit of the assay at 24 hpi, suggesting non-replicating virus was rapidly cleared *in vivo* (Fig S1A).

At 24 hpi, only topical IMQ and poly(I:C)-treated mice exhibited lower viral RNA at the skin inoculation site, whereas all innate immune agonists decreased virus in the dLN (Fig 1E). Poly(I:C) was particularly potent at decreasing viral RNA in most tissues analyzed, although viremia (Fig 1G) and survival to infection (Fig 1K) was not significantly affected. In comparison, only topical IMQ significantly decreased viral replication at both local tissues (skin and dLN, $p<0.05$), tissues remote from the inoculation site (non-dLN ($p<0.01$), spleen($p<0.01$) and brain ($p<0.05$)), reduced infectious virus in blood ($p<0.001$), and limited the development of clinical disease (Fig 1H, $p<0.05$). As SFV6 is highly virulent in mice, it does not model all arboviral disease seen in humans that are often non-fatal. Therefore, to better model the human situation we also tested the ability of IMQ to suppress infection with SFV4, a less virulent strain than SFV6 (Fig S1B). Again, application of topical IMQ at the site of SFV4 inoculation significantly ($p<0.01$) suppressed infection.

*Post-exposure targeting of the inoculation site by topical application of an innate immune agonist protects mice from infection with virus*

Treatment modalities that target early stages of infection are likely to involve post-exposure treatment, e.g. once the erythema of a bite is apparent. Because pre-bite application of topical IMQ was most efficacious in increasing host resistance to infection, we next determined its efficacy when applied post-infection. In addition, as arbovirus infection of skin always occurs in the context of an arthropod bite, we used a mouse model that additionally

incorporates biting *Aedes aegypti* mosquitoes. Host response to mosquito bites include edema and an influx of leukocytes that enhances host susceptibility to infection with virus *(9, 10, 27)*. Because topical IMQ is a cream that can become removed by cage bedding, it was first applied at 1 hpi and then re-applied once at 6 hpi to maintain dosing. In this model, application of topical IMQ was highly efficacious in lowering quantities of SFV6 RNA in all tissues analysed and infectious virus in the blood by 24 hpi (Fig 2A-B). For mice in which infection was allowed to progress, topical IMQ application resulted in a significant delay to the onset of neurological signs (Fig 2C, ($p<0.0001$). Consistent with this, analyses of brain tissue at post infection day 7 revealed extensive expression of SFV6-encoded mCherry throughout the brain in untreated mice compared to treated mice (Fig 2D).

Similarly, topical IMQ at 1hpi post infection with the less virulent SFV4 strain significantly reduced virus by 24 hpi in tissues and the blood ($p<0.01$) and substantially decreased mortality ($p<0.001$), with the majority of mice surviving infection (Fig 2E-G). Importantly, protection by topical IMQ was time-limited, as treatment 5 hpi significantly lowered viral RNA ($p<0.05$ in skin and spleen), but treatment delayed until 10 hpi did not significantly modulate the level of viral replication or dissemination by 24 hpi (Fig 2H).

Although these experiments were designed to test the ability of immune modulators to target the inoculation site, it is possible that mice inadvertently consumed some topical IMQ orally e.g. during grooming. In the above studies, mice were given topical IMQ at 1 hpi, during which the mice were anesthetised. Mice did not fully recover from anaesthesia until after 5 hpi, during which the majority of topical IMQ would have been absorbed and was not available for oral consumption. Nonetheless, to define the contribution that inadvertent oral consumption of topical IMQ had on susceptibility to virus infection, we washed away

unabsorbed topical IMQ from the inoculation site using a mild detergent aqueous wash immediately prior to recovery from anaesthesia. In addition, a separate group of mice received topical IMQ as an oral gavage (10% Aldara in water). Mice washed at 5 hpi still exhibited significantly ($p<0.05$) reduced amounts of virus at the skin inoculation site, spleen and the blood by 24hpi. In comparison, those mice given IMQ as an oral gavage exhibited no significant reduction of virus (Figure 2I). Together this suggests that topical IMQ was primarily modulating the course of virus infection through responses activated at the site of application.

The above studies involved virus infection of mouse skin by needle at sites of mosquito biting, because mosquito saliva has profound modulatory effects on host susceptibility to virus *(10, 11)*. Because infection of the mosquito salivary gland by virus can alter salivary protein content *(28)*, it is possible that virus inoculated by infected mosquitoes themselves has additional effects on modulating virus infection in the mammalian host. To determine if topical IMQ is also efficacious in altering host susceptibility to virus in the presence of saliva from infected mosquitoes, we obtained saliva from SFV-infected mosquitoes and co-inoculated it with a SFV6 into mouse skin (Figure S1C). To ensure that we inoculated similar quantities of virus as in our other experiments, infected saliva was firstly irradiated with UV to inactivate salivary gland-derived virus, and then a defined dose of SFV6 added prior to inculcation into mice. By 24hpi, those mice that received topical IMQ treatment exhibited significantly ($p<0.01$) less virus in the serum and spleen. Thus, irrespective of changes in virus-induced salivary gland gene expression, topical IMQ was effective at inhibiting virus infection in mice. Together these data identify the inoculation site as a key site for viral replication during the first 24 hpi and suggests that, in untreated mice, skin IFN responses are not sufficiently robust to prevent systemic dissemination. Therapeutic targeting of this site with topically applied innate

immune agonists was therefore highly effective at suppressing both the local and subsequent systemic course of infection, significantly improving survival.

*Topical IMQ induces upregulation of cutaneous ISG via canonical type I IFN receptor signalling*

We next wanted to determine the mode of action, as a detailed mechanistic understanding of this protection could inform the development of more targeted strategies. Topical IMQ contains a mixture of the TLR7 agonist IMQ (5%) and isostearic acid (25%), both of which can be inflammatory in a type I IFN-independent manner *(29, 30)*, suggesting that IFN may be dispensable for some of the beneficial effects described here. However, we found that by 24 hours post application at a mosquito bite, topical IMQ caused widespread induction of ISG expression, including the prototypic ISGs *cxcl10, ifit1, isg15* and *rsad2* (Fig. 3A), some of which have been identified as key responders to alphavirus infection *(17, 31)*. *Cxcl10* could only be detected significantly upregulated by 24 hours (Fig. S2A, $p<0.01$). Interestingly however, despite a clear upregulation of ISG, we could not detect any increase in type I (*ifna4, ifnb1, ifne, ifnk, ifnz*) or II (*ifng1*) IFN transcripts in whole skin biopsies, as measured by Taqman assays or custom SYBR green qPCR assays (Fig. 3A, S2A,B), suggesting topical IMQ may activate ISG expression independent of IFN receptor signalling in the skin *(32)*. Conceivably, our inability to detect type I IFN transcripts in wild type mice skin may be due to large numbers of IFN-negative cells that diluted the IFN signal, or because IFN-producing cells migrated out of the skin, rendering IFN undetectable.

Anti-viral ISG can be induced by a variety of distinct receptors, including signalling through IFN-αβ-R, IFNGR, IFNLR1 and other poorly-defined non-canonical pathways *(16, 32)*. However,

we found that activation of skin ISG was dependent on IFN-αβ-R signalling, as mice deficient in this receptor could not upregulate these prototypic ISGs in response to topical IMQ (Fig. 3B). Importantly, loss of IFN signalling was associated with a loss of protection from infection, as topical IMQ had no impact on virus replication in IFN-αβ-R null mice (Fig. 3C,D). In addition, to specifically show that the IMQ component was essential for protection against virus, we treated mouse skin with a cream mimic that lacked IMQ but contained 25% w/w isostearic acid *(29)*, which did not protect mice from infection (Fig. 3E). Nonetheless, it's likely that these excipients are required for a maximal IFN response, as injection of IMQ alone had little or no effect on ISG expression (Fig. S2C-F), nor did it have any effect on virus replication, as shown above (Fig. 1E-G, and Fig. S1B).

In comparison to skin responses, topical IMQ upregulated both IFN and ISG in the dLN; IFNγ was significantly elevated by 8h post application ($p<0.05$, Fig S2G), while type I IFNs and ISGs were detectable by 24 hours (Fig. 3F, Fig. S2G). In agreement with previous studies in humans *(33)*, there was a limited systemic response to topical IMQ, although our qPCR assay was sufficiently sensitive to detect some type I IFN expression in distal lymphoid tissue sites, but not e.g. remote skin or joints (Fig. 3G).

*Skin resident cells detect topically-applied IMQ and are sufficient for mediating protection to virus*

We next wanted to identify the cellular basis by which virus is detected and an anti-viral state induced and modulated by topical IMQ at mosquito bites. Although a variety of cultured skin cells are known to express RNA-virus pattern recognition receptors *(34)*, the cellular coordination of innate immune responses to arbovirus at the mosquito bite are not described. We concentrated on studying cells of the dermis, as the majority of virus transmitted by

mosquito is deposited here, and because epidermal cells do not express the IMQ receptor TLR7 *in vivo (35)*. To identify whether bite-recruited leukocytes or skin-resident cells are sufficient for detecting IMQ and eliciting protection from virus, we developed a skin model in which freshly biopsied mouse skin was taken from either resting skin or mosquito-bitten skin (4 h post bite, during leukocyte influx (9)) and infected with Gaussia-luciferase expressing SFV6 (SFV6-Gluc), which enables longitudinal detection of virus at high sensitivity, *in vitro*. Mosquito biting results in the rapid recruitment of leukocytes including neutrophils and monocytes (9), and as shown here small numbers of pDCs (Fig 4A). Infection of skin explants resulted in replication of virus, as measured by qPCR of viral RNA, functional expression of virus-encoded luciferase and release of new infectious virus into the surrounding tissue culture medium (Fig 4B-E, S2H). Although cultured skin explants do not replicate all aspects of *in vivo* responses, they do enable the study of cell-cell interactions that occur between distinct skin-resident cell types in the absence of leukocytes that would otherwise be recruited during mosquito-biting and virus infection *in vivo*.

We applied topical IMQ to the epidermis of explant cultured skin at 1hpi (at the same dosing as above for the *in vivo* studies). Topical IMQ application resulted in robust induction of ISGs in skin explants, albeit at lower amounts than that seen *in vivo* by 24 hours (Fig S2I); perhaps a consequence of tissue culture media removing some of the applied cream. Importantly, application of topical IMQ resulted in a significant ($p<0.01$), decrease in viral RNA, virus-encoded luciferase and a complete block in the release of new virus into the supernatant, for both explants derived from resting- and mosquito bitten-skin (Fig 4B-E). The magnitude of fold decrease in viral RNA was similar in *ex vivo* explants derived from resting skin, as compared to that in mosquito-bitten skin *in vivo* (Fig. 4F). This suggests that although ISG

induction was less robust in explants (Fig S2I), it was nonetheless sufficient to reduce virus titer. Thus, topical IMQ reduced viral replication irrespective of leukocyte recruitment triggered by either infection or by mosquito bite, suggesting tissue-resident cells were sufficient for mediating topical IMQ -induced protection from virus in the skin.

We next wanted to define which skin-resident cells were necessary for activating ISG expression in response to topical IMQ. Skin-resident leukocytes include populations of γδ-T cells and ROR-γT innate lymphocytes that are necessary for some inflammatory responses to repeated topical IMQ application *(36)*. However, we found that NOD SCID Gamma (NSG) mice, which lack all functional lymphocytes and innate lymphoid cells, were similarly protected from infection at 24hpi by topical IMQ (Fig. 4G). This suggests early skin responses to topical IMQ are activated by either stromal cells or non-lymphoid leukocytes, of which myelomonocytic cells are the most populous in the dermis. Skin dermis-resident myelomonocytic cells are derived from either bone marrow (BM) precursors (requiring expression of chemokine receptor CCR2), or are derived from non-BM embryonic sites of haematopoiesis (not requiring CCR2 expression) *(37)*. To determine if BM-derived skin resident myelomonocytic cells are required to mediate topical IMQ -protection, we infected skin explants from *ccr2*-null and WT mice and treated them with IMQ. Topical IMQ was similarly efficacious irrespective of *ccr2* status, suggesting that the vast majority of skin-resident dermal DC and monocytic-derived cells were not required (Fig. 4H). Indeed, IFN and ISG induction in response to topical IMQ application was similar in *ccr2*-null skin as compared to WT skin (Fig. 4I,J), suggesting that IMQ-responding cells were skin-resident cells not derived from myeloid BM-precursors.

*Skin macrophages and dendritic cells communicate with stromal cells to mediate topical IMQ-induced protection from virus*

Together, the above data suggests that the skin-resident cell type responding to topical IMQ was either a population of non-BM derived macrophages, mast cells, stromal cells or a combination of these. It is not possible to deplete all these cell types; therefore, we additionally undertook a comprehensive approach to define responses of all these cell populations. This involved analysing the gene expression profile of skin inoculation-site cells isolated by FACS, to reveal the cell-specific basis for innate immune response to virus and separately to topical IMQ (Fig. 5).

Although skin-resident cells are sufficient to mediate IMQ-responses, this does not necessarily exclude a role for mosquito-bite elicited responses at later stages. For this reason, and because arboviruses are all naturally transmitted into skin bitten by arthropods, we studied cellular responses to virus and topical IMQ in mosquito-bitten skin. We adapted a previously-defined FACS gating strategy *(37)*, to isolate populations of macrophages, dendritic cells, stromal cells, and all other CD45+ leukocytes as a bulk population (Fig. S3A,B). These isolated populations were then interrogated for their expression of *ifna4, ifnb1, isg15* and *rsad2* because they have been previously implicated as key for host responses to alphaviruses *(31, 38, 39)* and also the prototypic ISG *cxcl10*.

The cellular coordination of innate immune responses to virus at the tissue-wide level in mosquito-bitten skin are poorly defined. Therefore, we firstly analysed responses of skin cells to virus infection alone in the absence of topical IMQ (Fig 5A). At 24hpi, sorted stromal cells and macrophages were the only cells that expressed high amounts of virus structural gene transcript E1, indicative of active replication, while DCs expressed only very low amounts of

viral RNA (Fig. S3C). This, along with our previous studies (9), suggest that stromal cells and macrophages are key targets for viral replication of SFV at mosquito bites *in vivo*. Importantly, the only cell type expressing type I IFN in response to virus were DCs, which was limited to *ifnb1* only, as no *ifna4* was detected. Thus, DCs were the primary initiators of anti-viral ISG responses at virus-infected mosquito bites. However, dermal DC were present at low numbers in the skin at 24hpi, suggesting their total contribution to the tissue-wide IFN response was minimal (Fig. S3B). This agrees with our whole tissue analysis that demonstrated little IFN expression before 24 hpi described above. Activated DCs are highly migratory and rapidly leave the skin via the lymphatics, which may part explain why skin-wide IFN expression was so low. In summary, although stromal cells and macrophages exhibited high expression of viral RNA they did not make detectable type I IFN. Nonetheless they expressed the ISG *rsad2, cxcl10 and isg15* by 24hpi (Fig. 5A), presumably in response to DC-expressed IFN cues. All other leukocytes included in the bulk sort did not express IFN or upregulate ISG in response to virus.

Next, to determine which cells were activated by topical IMQ, mosquito-bitten mouse skin was treated with topical IMQ alone (Fig. 5B, Fig. S3E). Crucially, in contrast to virus infection, topical IMQ induced *ifnb1* expression in both the more populous skin-resident macrophages and dermal DC, while stromal cells and all other leukocytes lacked type I IFN transcripts. In response, stromal cells substantially upregulated *rsad2* and *isg15*. The ability of this assay to detect type I IFN transcripts is in contrast to that found in whole skin biopsies where IFN expression was not detected (Figure 3). This most likely reflects the enhanced sensitivity that is provided by analysing sorted/purified populations of cells.

As viruses have evolved mechanisms to antagonise IFN signaling we next wanted to determine if virus infection modulated this response to topical IMQ. Therefore, we infected mosquito bites with virus, either with or without topical IMQ application at 1 hpi and assayed type I IFN and ISG expression at 24 hpi in each cell type (Fig 5C). Despite the lower titres of virus in topical IMQ-treated skin by 24 hpi (and therefore less activation of IFN signalling via virus sensing), we found equal or higher fold increases in the expression of both type I IFNs and ISG in the macrophage population. ISG upregulation was also more robust in macrophages, DC and stromal cells, with the exception of *rsad2* in DC, suggesting that IMQ-induced upregulation during virus infection was ISG and cell type-specific. Activation of higher IFN expression in macrophages was important for two reasons; firstly, macrophages were significantly more numerous in the skin than dermal DC at 24 hpi (Fig. S3B, $p<0.001$) and secondly, they constituted an important source of virus replication in the first 24 hours of infection (Fig S3C and (9)). IMQ-elicited IFN appeared to be functional, as type I IFN negative stromal cells exhibited a significant decrease in viral RNA with topical IMQ treatment by 24hpi, as did virus in infected macrophages (Fig. S3C $p<0.05$).

Together, this shows that topical IMQ upregulated type I IFN in skin-resident macrophages that, in addition to responses of the less frequent dermal DCs, acted to induce ISG expression and thereby reduce viral replication in the skin. Furthermore, because dermal DC-deficient *ccr2*-null skin *(37)* was not compromised in its ability to express ISG or reduce virus titres in response to topical IMQ, as described above, we suggest that skin-resident macrophages alone are sufficient to mediate protection. Thus, based on these associations, it is likely that responses by skin-resident macrophages to topical IMQ are sufficient to confer protection against virus infection.

## Stromal cells integrate cues from IMQ-treated leukocytes to resist infection with virus

We next wanted to determine whether signals from IMQ-activated macrophages and DCs are alone sufficient to confer protection on stromal cells from virus infection and whether this required cell-to-cell contact. The majority of dermal stromal cells are either fibroblasts or keratinocytes, both of which can be experimentally infected with a range of arboviruses *(10, 40, 41)*, including SFV4 (Fig. 6A-D). Some cultured fibroblasts have been described to express a variety of receptors that sense virus, including the IMQ receptor TLR7 *(42)*. However, we found that FACS-isolated stromal cells *ex vivo* lacked detectable TLR7 (Fig. S3D). In addition, our cultured primary dermal fibroblasts (Fig. 6B) and keratinocytes (Fig. 6E) did not show any response to IMQ following SFV infection; IMQ did not protect cells from infection; ISG *cxcl10* expression was not induced (even at high doses, Fig. 6C); and media from fibroblasts pre-treated with IMQ for 24 hours did not confer protection to other stromal cells from subsequent virus infection (Fig. 6D). Thus, both our *in vivo* and *in vitro* analyses suggest stromal cells were not able to respond to IMQ alone.

To determine if soluble factors from IMQ-stimulated DCs or macrophages could influence the susceptibility of primary cultures of skin fibroblasts to infection, primary cultures of leukocytes were stimulated with IMQ for 24 hours and their supernatant given to fibroblasts at 1 hpi with luciferase-expressing SFV6-Gluc (Fig. 6D). While supernatant from resting leukocytes had no effect on fibroblast susceptibility to infection, tissue culture supernatant from DC or macrophages treated with IMQ protected fibroblasts from infection, as measured by virus-encoded luciferase.

As infected myeloid cells and DC are susceptible to arbovirus-encoded anti-IFN mechanisms *(43, 44)*, we next determined if infected DC were still able to provide IMQ-induced protection and trigger an anti-viral state in stromal cells. To do this we used a transwell system in which DCs were separated from stromal cells by a cell-impermeable membrane. All cells were infected with SFV4(Xho)-EGFP and treated at 1 hpi with either 0.2 μg/ml IMQ or saline control (Fig. 6E-H). DC cultures themselves became infected with SFV, exhibiting high level expression of viral RNA (Fig 6G). Nonetheless, DC were able to resist infection upon addition (1hpi) of IMQ and consequently expressed higher amounts of the ISG *cxcl10*. Importantly, while the addition of IMQ alone, or DC alone, had no effect on the ability of co-cultured stromal cells to resist infection, the combined presence of both IMQ and DC did protect keratinocytes (Fig. 6E-F and Fig S4) and fibroblasts (Fig. 6H). This was evident by a significant decrease in viral RNA expression (Fig 6E,H, $p<0.05$) and by microscopic inspection of the stromal cell monolayer that otherwise became decimated by 24hpi, with the remaining intact cells also positive for virus-encoded eGFP (Fig. 6F, Fig. S4). Protection from virus in stromal cells occurred in conjunction with the induction of stromal ISG expression (Fig. 6I). IMQ-treated keratinocytes only increased IFN and ISG expression with the addition of DCs to the transwell insert. Thus, IMQ-induced stromal cell protection from virus occurs in conjunction with the induction of ISG expression that was licenced by extracellular cues derived from IMQ-responsive leukocytes.

## Topical IMQ protects mice and human skin from infection with a variety of genetically-distinct medically important viral pathogens

We finally wanted to determine whether activating IFN pathways by topical IMQ at the skin inoculation site could have potential applicability to a variety of genetically distinct arboviruses of medical importance. We therefore analysed the ability of topical IMQ to modulate the outcome of infection to a relevant representative of each of genetically-distinct groups of arboviruses in mice and human skin explants. The prototypic Bunyamwera virus (BUNV, genus Orthobunyavirus, family Peribunyaviridae, order Bunyavirales), which like SFV is also transmitted by *Aedes* mosquitoes, replicates efficiently at mosquito bites in the skin and causes viremia in mice *(9)*. BUNV has well-described potent IFN antagonism (4) and therefore may be resistant to topical IMQ treatment. However, when mosquito-bitten BUNV-infected inoculation site was treated with topical IMQ at 1hpi, there was a significant decrease in viral RNA in target tissues ($p<0.01$ to $p<0.05$) and blood ($p<0.001$ to $p<0.05$) from 24 to 72 hpi (Fig. 7A-C). Similarly, we determined if topical IMQ could prevent dissemination of arthritogenic CHIKV (a medically important pathogen of the Togaviridae family that has caused widespread outbreaks of disease) to mouse joints that were remote from the inoculation site (Fig. 7D-G). By day 5 post infection, mice treated with topical IMQ 1 hpi exhibited significantly lower amount of CHIKV RNA (qPCR) and infectious virus in the ankle joints and wrists of the forelimbs (Fig. 7D-G and S5, $p<0.0001$ to $p<0.05$), irrespective of whether mice were co-inoculated with mosquito saliva.

Finally, to determine if topical IMQ could also reduce viral replication in human skin, we cultured explants of freshly biopsied human skin, infected them with virus and treated with topical IMQ to the epidermis at 1 hpi (Fig. 7H-I). Application of topical IMQ resulted in a significant decrease in viral replication for both CHIKV and ZIKV (genus Flavivirus of the family Flaviviridae), as measured by viral RNA and also infectious virus at 24 and 48 hpi ($p<0.001$ to

$p<0.05$). Taken together these studies demonstrate that an innate immune agonist that primarily targets inoculation-site skin-resident macrophages, has a significant post-exposure prophylactic effect on the replication and dissemination of a variety of genetically distinct medically important viruses.

## Discussion

We demonstrate that innate immune responses to virus in the skin is a limiting factor for suppressing subsequent systemic infection. Critically, we show that therapeutic modulation of these pathways in the first hours of infection can suppress disease and improve clinical outcome in mice. This work therefore identifies the inoculation site as an important site of arboviral replication and that skin innate immune virus-sensing pathways must be sufficiently robust to act as determinants of the clinical course. In addition to the basic biological insights that these studies provide, we suggest that therapies that either target IFN induction, or alleviate the virus-encoded mechanisms that inhibit IFN, may therefore be efficacious. Targeting a common pathway at the inoculation site, as shown here to suppress infection by several genetically distinct arboviruses, may circumvent the need to develop multiple species-specific antivirals, as their development and future use remains challenging due to the unpredictable nature of arbovirus outbreaks and difficulty associated with reaching an accurate and timely clinical diagnosis.

In this study, we identified the key skin cell types in mice that coordinate anti-viral immune responses to virus and those that respond to topical IMQ. We found that skin type I IFN expression to virus was restricted to the relatively rare DC population. In contrast, macrophages and stromal cells could not elicit IFN expression to virus alone, despite a high

level of viral RNA in these cells. Thus, skin-resident DC are key activators of the type I IFN response at the inoculation site to virus. Such specificity may reflect either cell-type specific susceptibility to virus-encoded mechanisms to antagonise IFN activation or the particular cell-tropism of our model arbovirus, SFV.

We suggest that the observed efficacy of our exemplar immune-modulator, topical IMQ (Aldara), was explained by activation of dermal macrophages. These cells were not only otherwise deficient in their IFN expression in response to virus alone, but also replicate virus (9). We found that IFN signalling was key for IMQ-induced protection from virus, while skin-resident macrophages were the only cell type that increased IFN-β in response to IMQ, and also that their addition to cultures of skin fibroblast were sufficient to confer IMQ-induced protection. We suggest that therapies that specifically target dermal macrophages may provide yet enhanced efficacy, for example by increasing retention of the drug within the dermis or via delivery within liposomes . Furthermore, as the IMQ receptor TLR7 exhibits a relatively restricted expression in specific cells, those innate immune agonists that target more widely expressed virus-sensing receptors, or their pathways, may further increase efficacy.

Topical IMQ was no longer efficacious where treatment was delayed for 10 hours in our mouse model. We suggest that this timing most likely reflects specific aspects of our model system that supports rapid dissemination of SFV, itself a mouse-adapted strain. It is not clear how these kinetics apply to human infection by mosquito, although it is widely-accepted that virus may take considerably longer (several days) to disseminate from human skin to the blood *(45)*. This in turn may mean that the window available for post-exposure intervention in humans is conveniently longer than that observed in our model, although virus-, host- and environment-specific factors are likely to define its length. However, because *Aedes* sp. mosquitoes are

day-biting and their bites are typically visible within minutes, it is possible that there is a sufficient time-window for post-exposure prophylactic treatment between infection/biting and awareness of a bite/application of cream in humans. Targeting skin responses later during infection, e.g. once clinical signs have become apparent, is unlikely to have any effect on the severity of infection, as virus wold have already disseminated systemically. Furthermore, it is important to note that it may not be possible for individuals to notice all mosquito bites sufficiently quickly.

Although the data presented here are primarily derived from studies using mice, we suggest that therapeutic intervention at the inoculation site represents a feasible strategy for targeting mosquito-borne virus infection that merits further investigation. Putatively, treatment for either mosquito-, or perhaps also tick- borne virus, could be employed topically in the first few hours after an arthropod bite is noticed. We suggest that with refinement, a modified form of our exemplar immune-modulator (topical IMQ, Aldara) may have potential as a treatment strategy for this globally important disease category. Thus, further work that refines the most efficacious method for stimulating IFN responses in macrophages, opens up the possibility of broadly applicable, cost-effective, therapeutic interventions for arbovirus infection.

We maintained application of topical IMQ for only five hours to a single site, suggesting systemic side effects are less likely than with prolonged exposure. However, effective post-exposure prophylaxis might entail treatment of multiple, discrete, suspected arbovirus-infected mosquito bites, even with e.g. vector repellent strategies employed. Thus, it will be important to refine any future formulations such as to render the application of treatment practical. However, it should be noted that Aldara has been widely used for many years for a

variety of dermatological conditions, including in primary care, and is generally well-tolerated by patients, even when repeatedly applied at the same site for several weeks *(46)*. Additionally, our approach would likely entail topical immunomodulation over a smaller surface area than those typically treated with Aldara for its licensed indications. Nonethless, this approach may involve repeated activation of IFN responses, which even if done at discrete localised sites, may come with associated concomitant side effects. Finally, it is noteworthy that outbreaks of arbovirus infection are explosive in nature and can be highly seasonal, enabling possible use of any prophylactic strategies to be more intensively promoted when risk of infection is known to be high. Furthermore, it is possible that those patients deemed at higher risk of complications following arbovirus infection, such as immunosuppressed individuals, or e.g. those that are more susceptible to severe dengue, may particularly benefit from this strategy.

## Materials and Methods

*Study design*

This study was initiated to determine whether anti-viral innate immune response to arbovirus at the mosquito bite can be therapeutically manipulated to suppress the subsequent systemic course and improve outcome of infection in mice. We investigated mammalian host responses to representative viruses from three genetically divergent groups of arboviruses. Two of the viruses (ZIKV and CHIKV) are medically important emerging arboviruses, whereas the others (SFV and BUNV) are model viruses that replicate efficiently in immune competent mice. As previously described (9), *Ae. aegypti* bite sites were infected with mosquito cell culture-derived virus in a sub-μl volume by hyperfine needle. Infected mosquitoes were not used to inoculate virus to mice as the inoculum supplied by biting mosquitoes was too variable and unpredictable to allow effective comparisons; infected mosquito saliva was used as a co-

inoculum with virus in some studies here. All experiments involving mice had been subject to rigorous review by our local welfare and ethical review committee and additionally approved by the UK Home Office (licence PA7CF4E75).

On the basis that immune responses in mice may not always resemble those in humans, we also investigated the ability of interventions to suppress infection with the human pathogens ZIKV and CHIKV in human skin explants. Human studies were performed following ethical approval and in accordance with all applicable regulations (research ethics committee number 10/H1306/88 and 16/HY/0086). Sample sizes and end points were selected on the basis of our published experience with arbovirus infection. Wherever possible, preliminary mouse experiments were performed to determine requirements for sample size, considering the available resources and ethical use of animals. Female animals (age-matched) were assigned randomly to experimental groups. For plaque assays, luciferase assays, and qPCR, samples were coded prior to analysis, to limit bias. For qPCR, each result represents the median of 3 or 4 technical replicates of one biological replicate. For plaque assay, viral stocks and biological samples was serially diluted and each dilution assayed in duplicate. Biological replicates from mice were excluded from analysis if injection of virus inadvertently punctured a blood vessel (although this was rare and occurred with a frequency of <1%). No outliers were removed from these studies. Primary data are reported in data file S1.

### Cell culture

Baby hamster kidney (BHK)-21 cells and C6/36 mosquito cells were grown as previously described (9). Mouse leukocytes were derived from bone marrow-precursors, using 10ng/ml M-CSF for 6 days (macrophages); 20ng/ml GM-CSF for 6 days or 200 ng/ml Flt3L for 10 days (dendritic cells). C57Bl/6-derived primary keratinocytes (Cell biologics) were grown in

complete epithelial cell media as per manufacturer's instructions. Skin fibroblasts were derived from adult C57Bl/6 dermis, digested with collagenase D (1 mg/ml), dispase II (0.5 mg/ml), and DNAse (0.1 mg/ml) in HBSS to release cells and adherent fibroblasts grown for 12 days in complete DMEM.

## Viruses

The pCMV-SFV4 and pCMV-SFV6 backbone for production of SFV has been previously described *(25, 47)*. The full-length infectious cDNA clone of SFV4, was engineered by Peter Liljeström (Karolinska Institutet) and was used to construct a stabilized infectious SFV plasmid, pCMV-SFV4 *(47, 48)*. SFV6 is a copy of non-mouse adapted SFV L10 (a polyclonal virulent isolate), derived by making 6 nonsynonymous nucleotide changes to SFV4. The derived virus, SFV6, generates a high-titer viremia in mice, is efficiently neuroinvasive and highly virulent *(25)*. The EGFP marker gene was inserted into the C- terminal region of SFV nsP3 via a naturally-occurring XhoI site. mCherry and Gaussia luciferase (Gluc) were separately inserted as a cassette under the control of a duplicated sub-genomic protomer 3' with resulting viruses referred to here as SFV6-mCherry and SFV6-Gluc *(49)*. Plasmids were electroporated into BHK-21 cells to generate infectious virus. SFV clones that have been genetically altered in this way are stable and there is no evidence of virus attenuation or genetic instability *(50)*. A such, the use of these reporter viruses allowed the replication, dissemination and localisation of SFV to be studied in high detail. SFV4 is the prototypic, less virulent strain of the virus, whereas SFV6 is a copy of a virulent strain *(25)*. Both strains cause disease in mice when inoculated in the skin at a mosquito bite *(9)*.

Wild type BUNV was derived as previously described *(51)*. A low passage wild type ZIKV isolate *(52)* was derived from a patient displaying classical disease symptoms in Recife (ZIKV PE243)

and has been sequenced, kindly supplied by Prof Alain Kohl (MRC-University of Glasgow Centre for Virus research). In CHIKV mouse experiments, CHIKV Indian Ocean strain 899 (FJ959103.1) was used (Prof. C. Drosten, University of Bonn, Germany) *(53)*. For human explant studies, CHIKV was made from the infectious clone derived from the LR2006_OPY1 isolate (DQ443544). These strains were chosen as they have both been passaged only a few times since isolation and likely represent wild type circulating virus. We estimate that the CHIKV and ZIKV used here has not been passaged more than 5 times in total since isolation. In all cases, viruses were grown once in BHK-21 cells, then passaged once in C6/36 Aedes mosquito cells and titrated prior to use, as mosquito cell derived virus has distinct glycosylation and because insect cells impose distinct evolutionary constraints on viral progeny *(54)*. SFV4 and SFV6 were used at passage 2.

### Mice

In all cases, results have been generated by infecting mice with the same passage of virus. Unless otherwise specified, all mice were 6-8-week-old wild type mice (C57bl/6J). C57bl/6J and Nod-SCID gamma (NSG) mice were derived from a locally bred-colony maintained in a pathogen-free facility, in filter-topped cages and maintained in accordance with local and governmental regulations. To prevent genetic drift, mice were have been re-derived using externally-supplied mice (Charles River). Ccr2-deficient mice were originally obtained from the Jackson Laboratory (stock number 004999). Ifnar1-deficient and wild type (WT) counterparts on a 129S7/SvEvBrdBkl-Hprtb-m2 background (B&K Universal) were maintained in Tecniplast 1284 L Blue line IVC cages at Biological Services, University of Glasgow. All mice had a 12 h light/dark cycle and provided ad libitum with sterile food and water.

### Aedes aegypti mosquito biting and virus infection of mice

We used our previously established model of arbovirus infection at mosquito bites *(9)*. This model was specifically developed to consistently model natural infection by arbovirus, including: mimicking the same dose delivered by mosquitoes (between 100 and 100,000 PFU for alphaviruses), using mosquito-cell derived virus, injecting small 1 μl inoculum volume, and by including the presence of a mosquito bite at the site of inoculation. To ensure mosquitoes bit a defined area of skin (upper side of the left foot), anesthetized mice were placed for up to ten minutes onto a mosquito cage containing *Ae. aegypti* mosquitoes (locally bred colony derived from the Liverpool strain) as previously described (9). Biting was restricted to defined area of the left foot by covering all other mouse skin with an impenetrable barrier. Bitten skin (3-5 mosquito bites/foot) was injected with virus in a 1 μl volume into the skin, using either 250 PFU SFV6, 1x10^4 PFU SFV4, or 2.5x10^4 PFU BUNV. These doses represent those that are known to initiate high titre viremia by 24 hpi and, for SFV, clinical signs within days post infection. Total RNA input (copy number) from inoculum is roughly 10-fold higher than PFU *(55)*. For survival curves, mice were monitored closely and culled when they reached more than three clinically defined end-points of disease. Clinical signs included; body weight loss >10%; subdued behaviour when provoked; hunching; convulsions; limb paralysis; prostration >1hour; and vocalisation. For CHIKV infection of mice, 3-4 week-old mice were infected by s.c. injection of $10^6$ PFU of CHIKV in the hind-left foot pad with or without *Aedes aegypti* mosquito saliva.

### Infection of mosquitoes and mosquito saliva extraction

Washed male rabbit blood (ENVIGO) was mixed with 2mM ATP (ThermoScientific) and 7.8*10^7 PFU/ml SFV4. Blood was loaded into a hemotek feeder (HEMOTEk) and placed on cages containing *Ae. aegypti* mosquitoes for 45 minutes to allow mosquitoes to feed. Fully

engorged female mosquitoes were then transferred into boxes and maintained at 28°C, 80% humidity for 7 days. Mosquito saliva was extracted in oil from salivating mosquitoes and corresponding individual mosquito heads were harvested and submerged in TRIzol (Life Technologies). Saliva from groups of 5 mosquitoes were pooled together and stored at -80°C. RNA from mosquito heads was extracted using PureLink RNA micro columns as per manufacturer's instructions (Life Technologies). Mosquito heads were homogenised in TRIzol using a precellys 24 tissue homogeniser (Bertin Instruments) with glass beads (VWR), followed by purification using PureLink micro columns (Life Technologies). RNA was converted into cDNA using the High Capacity RNA-to-cDNA kit (Life Technologies). Expression of SFV E1 gene was undertaken using custom designed SYBR-green based qPCR assay using PerfeCTa (Quanta). Samples were analysed using ΔΔCT against negative control mosquito heads (CT>35). Saliva groups from SFV E1 positive mosquito heads were then pooled and UV inactivated for 10 minutes to remove virus and then mixed with a defined dose of 10,000 PFU SFV4 for inoculation into mouse skin.

### Administration of innate immune agonists

Anesthetized mice were injected with either 6μg poly(I:C), poly(dA:dT) or IMQ (Invivogen) into skin as a 4μl aqueous volume using Hamilton syringes, at the same site as virus infection (using upper skin of left foot *(9)*). Poly (I:C) and poly (dA:dT) were purchased pre-complexed with transfection reagent Lyovec. 2mg topical IMQ (Aldara, 5% w/w IMQ; 25% w/w isostearic acid) was applied to the site of virus inoculation (upper side of left foot). In most cases, because topical IMQ is a cream that can become removed by cage bedding, it was re-applied once at 6 hpi to maintain dosing. Although it is difficult to define the exact dose of IMQ provided using topical application of cream, previous studies have shown that dosing results in limited

systemic absorption, with IMQ concentration peaking at 0.4 ng/ml blood in humans *(33)*, with most being retained in the absorbent stratum corneum (the outer dead keratinized layer) or otherwise removed by external processes.

### Skin explant studies

For human explants, informed consent was obtained from 16 volunteer donors, and skin sourced from areas that were relatively protected from environmental insult and had no obvious lesions (upper inner arm). Human studies were performed following ethical approval and in accordance to all applicable regulations. For mouse studies, skin was derived from either resting skin, or from mosquito-bitten skin (biopsied at 4 hour post bite) . For both human and mouse studies, skin was dissected to remove sub-dermal tissues, to leave dermis exposed, and immediately infected with virus by placing lower dermis into virus-containing solution (0.75% BSA in PBS, PBSA) for one hour. Explants were then washed in saline and the epidermal surface of the skin explant was briefly dried. Similar to our mouse-based in vivo studies, 2mg topical IMQ (Aldara) was applied topically to the epidermis (at 1 hpi) until absorbed. Explants were then cultured in 24 well plates containing complete DMEM media at 37°C/5% $CO_2$ for up to 48 hours.

### Gene expression analysis

Viral RNA and hosts gene transcripts were quantified by qRT-PCR and infectious virus by end-point titration, as described previously *(53)*. Tissue generated up to 100μg of total RNA, of which 1μg of RNA was used to create cDNA, of which 1% was used per qPCR assay (10 ng RNA equivalent). qPCR primers for SFV and CHIKV amplified a section of E1 and primers for BUNV targeted segment M (9), while primers for ZIKV amplified a section of the *env* gene. For

SFV, CHIKV and ZIKV, qPCR assays measured the sum value of both genome and sub-genomic RNA.

### Flow cytometry

For FACS, skin tissue samples were enzymatically digested with collagenase D (1 mg/ml), dispase II (0.5 mg/ml), and DNAse (0.1 mg/ml) in HBSS for 50 minutes at 37°C. Enzymes were quenched with serum, cells washed, treated with FcR block (Miltenyi Biotec) and stained with antibodies and a viability dye (9). Cells were analysed on a CytoFLEX (Beckman Coulter Life Sciences). For cell sorting, skin was digested and stained on ice in presence of a transcriptional inhibitor (actinomycin D) and sorted to 90-100% purity using an Influx cell sorter (BD). See fig. S3 for the gating strategy.

### Immunohistochemistry and histology

Tissues were fixed in 4% methanol-free paraformaldehyde (Thermo Scientific) then dehydrated in an increasing concentration of sucrose. Tissue was embedded in Optimal Cutting Temperature (OCT) compound (Agar Scientific) and sectioned. Tissue sections were stained with DAPI mounting media and imaged on a Zeiss Axioskop.

### Statistical analysis

Data were analyzed using Prism Version 7 software. Copy numbers of viral RNA and infectious titres from virus-infected mice were not normally distributed (with data points often spread over orders of magnitude) and were accordingly analyzed using the non-parametric based tests Mann-Whitney or Kruskal-Wallis test with Dunn's multiple comparison test where appropriate, unless otherwise stated in figure legends. All such column plots show the median value +/- interquartile range. Where data were normally distributed (as determined by using either the Shapiro-Wilk test or by simple visual inspection), data were analyzed using ANOVA

with Holm-Sidak's multiple comparison test and plotted with mean values. Survival curves were analyzed using the logrank (Mantel Cox) test. All plots have statistical significance indicated *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.

## Supplementary Materials

Fig. S1. Pre-exposure of skin to topical IMQ increased host resistance to infection with SFV4.

Fig. S2. Type I IFN and ISG expression in skin and draining LN following IMQ application.

Fig. S3. Gene expression analysis of skin inoculation site-derived FACS isolated cells

Fig. S4. IMQ-mediated protection against virus infection in keratinocytes is dependent on help from leukocytes.

Fig. S5. Topical IMQ prevents systemic dissemination of CHIKV to joint tissue remote from inoculation site.

Data file S1. Primary data

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**Fig. 1. Immunomodulation of the skin inoculation site enhances host resistance to virus infection.**

(A-D) Mice were infected subcutaneously (s.c.) with 250 plaque forming units (PFU) SFV6 in the upper skin of the left foot. Copy number of host (A) *ifnb1*, (B) *cxcl10* (C) *ifng* (D) *cxcl9* and 18S RNA in skin and draining popliteal lymph node (dLN) was determined by qPCR at various timepoints.

(E-K) 1h prior to infection with SFV6 viruses, mice were pretreated with a single 2mg topical administration of IMQ, or injected s.c. with 6µg of either; aqueous IMQ, poly(dA:dT) or poly(I:C). (E-G) At 24hpi, copy number of SFV RNA and host 18S was determined by qPCR at 24 hpi (E,F) and virus titers in the serum quantified by plaque assay (G). Non-draining LN was the popliteal LN contralateral to infection (n=6). (H-K) Survival of mice was assessed when defined clinical endpoints were reached (n=10). PID=post infection day.

Groups that were significantly different to 0 h (A-D) or to SFV infection alone (E-K) are marked; *p<0.05, **p<0.01, ***p<0.001, ns=not significant (Kruskal-Wallis and logrank (Mantel Cox) test).

**Fig. 2. Targeted post-exposure immunomodulation suppresses the local and systemic course of infection and improves clinical outcome in mice.**

(A-D) Mosquito-bitten mouse skin was infected with 250 PFU SFV6 and treated with topical IMQ at 1 hpi (with one re-application at 6hpi) or left untreated. (A) SFV RNA and host 18S were quantified by qPCR at 24hpi (n=7). (B) Plaque assay of serum at 24hpi (n=7). (C) Survival of

mice (n=10). (D) Mid-sagittal sections of the brain from mice (n=5) infected with SFV6-mCherry (red) stained with DAPI (blue); upper composite image was assembled from multiple photographs, while lower images show typical individual images at higher magnification.

(E-G) Mosquito-bitten mouse skin was infected with 10 000 PFU SFV4 and treated with topical IMQ at 1 hpi (with one re-application at 6hpi) or left untreated. (E) SFV RNA and host 18S were quantified by qPCR at 24hpi. (F) Serum virus quantified by plaque assay (n=6). (G) Survival of mice (n=11).

(H) Mosquito-bitten mouse skin was infected with 10 000 PFU SFV4 and treated with topical IMQ at at either 5 or 10 hpi. SFV RNA and host 18S were quantified by qPCR at 24hpi. (n=5-6)

(I) Mosquito-bitten mouse skin was infected with 250 PFU SFV6 and then either left; untreated; treated with topical IMQ at 1 hpi (with one re-application at 6hpi); or similarly treated with topical IMQ and residual cream removed by an aqueous-detergent wash 30 minutes after second dosing; or administered as an oral gavage of topical IMQ (10% of total dose in water) n=6.

*p<0.05, **p<0.01, ***p<0.001, ****(p<0.0001) ns=not significant (Mann-Whitney, Kruskal-Wallis or logrank (Mantel Cox) test).

**Fig. 3. Protection by topical imiquimod is dependent on type I IFN signaling.**

(A) Mosquito-bitten skin was treated with a single topical application of IMQ. Fold change gene expression of ISG in skin was determined by qPCR at 24h post treatment compared to untreated controls (n=4).

(B) *ifnar1* -/- mice and syngeneic wild type controls were treated with a single 2mg application of topical IMQ. Copy number of host ISG and 18S RNA were determined by qPCR at 24h post treatment (n=10).

(C,D) Mosquito-bitten *ifnar1* -/- skin (n=5) was infected with 250 PFU SFV6 and treated with topical IMQ at 1 hpi (with one re-application at 6hpi) or left untreated. (C) SFV RNA and host 18S quantified by qPCR at 24hpi. (D) Serum virus quantified by plaque assay.

(E) Mosquito-bitten WT mouse skin was infected with 250 PFU SFV6 and then treated with either topical IMQ at 1 hpi, or control cream (25% w/v isostearic acid), with one re-application of each at 6hpi. SFV RNA and host 18S was determined by qPCR at 24 hpi (n=7-8).

(F) Mice were bitten with mosquitoes and the site treated with a single topical application of IMQ. Fold change gene expression of ISG in dLN was determined by qPCR at 24h post treatment compared to untreated controls (n=4).

(G) Mosquito-bitten WT mice were treated with topical IMQ and *ifnb1* determined by qPCR at 24 h (n=5-12). Each graph represents a separate experiments.

*p<0.05, **p<0.01, ****p<0.0001, ns=not significant (Mann-Whitney and Kruskal-Wallis test).

**Fig. 4. Host responses to IMQ by skin-resident cells are sufficient to mediate protection**

(A) Mosquito-bitten mouse skin was infected with 250 PFU SFV6. At 24 hpi, skin from the inoculation site was digested to release cells and numbers of pDCs (CD45+ CD11c+ SiglecH+) quantified by FACS per $10^5$ live cells.

(B-E) Mosquito-bitten skin, at 4 hours post bite, or resting mouse skin, was biopsied and resulting 6mm skin explants infected *ex vivo* with 1x $10^5$ PFU SFV6-Gluc. At 1 hpi, the explants were removed from the media, briefly dried, and the epidermis treated with a single application of topical IMQ. Treated explants were then placed resting in tissue culture media

and at 24hpi: (B) copy numbers of SFV RNA were determined by qPCR, (C) virus titers in the media quantified by plaque assay (n=6), and (D) virus-encoded Gluc assayed (n=6). (E) SFV RNA were also quantified by qPCR in skin explants at 48 hpi.

(F) Mice were either infected *in vivo* at mosquito bites with or without a single application of topical IMQ (at 1 hpi), or 6mm explants derived from resting skin (with no associated bite-recruited leukocytes were infected with 250 PFU SFV6 *ex vivo* by needle and then treated with a single application topical IMQ (at 1 hpi). Copy number of SFV RNA and host 18S was determined by qPCR at 24 hpi and compared to respective untreated infected controls (n=6-7).

(G) Mosquito-bitten skin of NSG mice were infected *in vivo* with 250 PFU SFV6 and then treated with topical IMQ at 1 hpi (with one re-application at 6hpi). Mice were left for 24 hours and SFV RNA were quantified by qPCR in skin and spleen at 24 hpi, and infectious virus in the serum by plaque assay (n=6).

(H-J) 6mm skin explants from WT and *ccr2*-null mice were infected with $1x10^5$ PFU SFV6 and treated with a single 2 mg application of topical IMQ *ex vivo*. At 24 hpi, copy number of (H) SFV RNA, (I) *ifnb1*, and (J) host ISG (*cxcl10*, *isg15*, *rsad2*) were determined by qPCR (n=6).

*p<0.05, **p<0.01, ns=not significant (Mann-Whitney and Kruskal-Wallis test).

## Fig. 5. Topical IMQ targets dermal macrophages and dendritic cells to activate tissue-wide ISG expression

(A-C) Mosquito-bitten mouse skin was digested to release cells and then FACS-sorted at 4°C into "stroma", "macrophage", dendritic cell "DCs" and "other leukocyte" compartments as a bulk sort. All steps were undertaken in presence of transcriptional inhibitors. Copy number

of gene transcripts were determined by qPCR for *ifna4*, *ifnb1*, *isg15*, *cxcl10*, *rsad2* and 18S RNA (n=4). Cells were sourced from mosquito-bitten skin that was either; (A) infected with 250 PFU SFV6 alone; (B) treated with topical IMQ alone (at 1h and reapplied at 6h); (C) infected with 250 PFU SFV6 and treated with topical IMQ at 1hpi (with one re-application at 6hpi). *p<0.05, ns=not significant (Mann-Whitney).

Fig. 6. Protection of skin stromal cells from virus by IMQ requires signals from myeloid cells

(A) Primary mouse keratinocytes were infected with SFV4(Xho)-EGFP at an MOI of 0.1. At 6 hpi replication complexes (green) were present throughout cytoplasm (DAPI, blue).

(B,C) Primary mouse embryonic fibroblasts were infected with SFV6 in vitro at an MOI of 0.1. At 1hpi, cells were treated with either 0.5, 2 or 10 μg/ml IMQ and at 24 hpi; (B) copy number of SFV RNA determined by qPCR (n=6) and infectious virus in the media were quantified by plaque assay; and (C) copy number of host *cxcl10* gene transcripts was determined by qPCR (n=6).

(D) Primary mouse dermal fibroblasts and BM-derived Flt3L DCs, GM-CSF DCs, and M-CSF macrophages were treated with 0.5 μg/ml IMQ for 24 hours. The resulting conditioned media was aspirated and placed on SFV6-Gluc-infected fibroblasts (1 hpi with at an MOI of 0.01). Virus-encoded Gluc was assayed at 24 hpi (n=6).

(E-G) Primary keratinocytes were infected *in vitro* with SFV6 (E,G) or SFV4(Xho)-eGFP (F) at an MOI of 0.1, then at 1 hpi treated with 0.5 μg/ml IMQ in the presence or absence of Flt3-L DC's separated by a 0.5 μm transwell membrane. (E) Copy number of SFV RNA and host 18S RNA in the keratinocytes were determined by qPCR at 24 hpi (n=5-6). (F) SFV-encoded eGFP shown as green, with cell nuclei counter-stained with DAPI (blue). (G) Copy number of SFV RNA and *cxcl10* transcripts were determined in DC by qPCR at 24hpi (n=5-6).

(H,I) Primary fibroblasts (H) or keratinocytes (I) were infected *in vitro* with SFV6 at an MOI of 0.1 in the presence or absence of Flt3L-derived DCs separated from stromal cells by a transwell membrane. At 1 hpi cells were either left untreated or given 0.5 μg/ml IMQ. (H) Copy number of SFV RNA were determined by qPCR at 24hpi (n=5-6). (I) Gene expression of SFV-

infected keratinocyte ISG were determined by qPCR (n=4) and significance determined by 2-way ANOVA (*<p0.05, ****p<0.0001).

*p<0.05, **p<0.01, ****p<0.0001, ns=not significant (Mann-Whitney and Kruskal-Wallis test).

**Fig. 7. Topical IMQ protects both mouse and human skin from varied, genetically distinct arboviral threats**.

(A-C) Mosquito-bitten skin of mice (n=7-8) were infected with 25 000 PFU BUNV and treated with topical IMQ at 1 hpi (with one re-application at 6hpi). Copy number of BUNV RNA was determined by qPCR in skin (A) and dLN (B); and infectious virus in blood determined by plaque assay up to 72hpi (C).

(D-G) Mice were co-inoculated with $10^6$ PFU of CHIKV (s.c.) and mosquito saliva (derived from 5 *Ae.aegypti* mosquitoes) and treated with topical IMQ at 1hpi (with one re-application at 6hpi). Dissemination of virus to distal joints were assessed at 5 days post infection in the right ankle joint (D,E) and both wrist joints (F,G). CHIKV RNA were quantified by qPCR and infectious virus titers quantified by TCID50 (n=10).

(H,I) Bisected human skin biopsies from 16 different donors were cultured as explants and infected with virus ($1\times10^5$ PFU CHIKV (H) or $0.5x10^5$ ZIKV (I)). One half of each biopsy was treated with a single application (2 mg) of topical IMQ *ex vivo* 1hpi; while the other half was left untreated. CHIKV and ZIKV RNA was quantified by qPCR at 24 and 48 hpi and virus titers in the media quantified by plaque assay.

*p<0.05, **p<0.01, ****p<0.0001, ns=not significant (Mann-Whitney).