Expression of a constitutively active human STING mutant in hematopoietic cells produces an Ifnar1-dependent vasculopathy in mice

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STING-associated vasculopathy with onset in infancy (SAVI) is an autoinflammatory disorder characterized by blood vessel occlusions, acral necrosis, myositis, rashes, and pulmonary inflammation that are the result of activating mutations in the STimulator of Interferon Genes (STING). We generated a transgenic line that recapitulates many of the phenotypic aspects of SAVI by targeting the expression of the human STING-N154S-mutant protein to the murine hematopoietic compartment. hSTING-N154S mice demonstrated failure to gain weight, lymphopenia, progressive paw swelling accompanied by inflammatory infiltrates, severe myositis, and ear and tail necrosis. However, no significant lung inflammation was observed. X-ray microscopy imaging revealed vasculopathy characterized by arterial occlusions and venous thromboses. Type I interferons and proinflammatory mediators were elevated in hSTING-N154S sera. Importantly, the phenotype was prevented in hSTING-N154S mice lacking the type I interferon receptor gene (Ifnar1). This model, based on a mutant human STING protein, may shed light on the pathophysiologival mechanisms operative in SAVI.

DOI 10.26508/lsa.201800215 | Received 11 October 2018 | Revised 11 June 2019 | Accepted 12 June 2019 | Published online 20 June 2019

Introduction

As an important component of a sensing mechanism for cytosolic dsDNA derived from viruses, bacteria, or the host, the STING protein has the ability to trigger potent type I interferon responses (Ishikawa et al, 2009; Abe et al, 2013; Gao et al, 2013; Xiao & Fitzgerald, 2013). However, de novo activating mutations in the STING molecule have also been identified as being responsible for a monogenic autoinflammatory syndrome (Jeremiah et al, 2014; Chia et al, 2016; Fremond et al, 2016; Picard et al, 2016; Konig et al, 2017) known as SAVI (Liu et al, 2014). This autosomal dominant genetic disease has been attributed to a number of distinct gain-of-function STING mutations (also known as TMEM173) leading to the constitutive activation of the STING protein (Liu et al, 2014). The SAVI phenotype is characterized by blood vessel inflammation and damage, development of inflammatory skin lesions, losses of ear and nasal cartilages, as well as ulceration and necrosis of digits that often require amputation (Jeremiah et al, 2014; Liu et al, 2014; Chia et al, 2016). Additional features can include a lupus-like syndrome (Konig et al, 2017), arthralgias, myositis (Liu et al, 2014; Fremond et al, 2016), and potentially fatal lung disease (Liu et al, 2014; Picard et al, 2016).

Laboratory features of SAVI can include increased levels of inflammatory markers such as C-reactive protein and the erythrocyte sedimentation rate (Liu et al, 2014; Munoz et al, 2015), anemia, lymphocytopenia, thrombocytosis, hyper-globulinemia, evidence of immune complex deposition, and the presence of antinuclear antibodies (ANAs), anti-cardiolipin antibodies, and rheumatoid factor (Jeremiah et al, 2014; Liu et al, 2014; Munoz et al, 2015; Chia et al, 2016; Fremond et al, 2016; Picard et al, 2016; Konig et al, 2017). Constitutive activation of STING, with the downstream activation of tank-binding kinase-1 and nuclear factor-kB, leads to raised levels of type I interferons and various cytokines and chemokines (Ishikawa et al, 2009; Abe et al, 2013; Gao et al, 2013; Xiao & Fitzgerald, 2013). SAVI is relatively refractory to glucocorticoids; however, partial responses to Janus kinase (JAK) inhibitors have been observed (Munoz et al, 2015; Fremond et al, 2016; Konig et al, 2017).

Murine models for SAVI and other autoinflammatory syndromes will facilitate studies of disease pathogenesis and the development of therapeutic strategies. Herein, we have generated a model for SAVI via the transgenic expression of a SAVI-associated hSTING mutation (N154S) in murine hematopoietic cells. Similar to SAVI (Liu et al, 2014; Fremond et al, 2016; Konig et al, 2017), hSTING-N154S transgenic mice exhibited the following: acral necrosis, dermal...
infiltrates, myositis, vasculopathy, lymphopenia, and elevated proinflammatory mediators and type I interferons. Unlike humans with activating mutations of STING (including the N154S hSTING mutation), hSTING-N154S mice failed to develop significant lung pathology. Importantly, and in keeping with constitutive STING activation being classified as an interferonopathy, the observed phenotype failed to develop in hSTING-N154S mice lacking the type I interferon α receptor subunit 1 (Ifnar1).

Results

Gross morphological abnormalities of hSTING-N154S mice

By 8–10 wk of age, three of the five hSTING-N154S founder lines exhibited growth impairment, a failure to gain weight, and a reduced lifespan as a result of complications associated with the disease (Fig 1A–C). However, overall survival could not absolutely be determined in our hSTING-154S mice as the time to endpoint (e.g., sacrifice because of the severity of disease) was somewhat variable. We also observed that the disease in these three lines affected males and females equally. To reduce variability, we selected the 1,501 line (the most severe phenotype), and herein, all experimental observations are centered on this line only. In addition, all three lines developed progressive paw swelling (Fig 1D–F), accompanied by acral necrosis that was manifested by losses of ear cartilage as well as tail inflammation and shortening (Fig 1G and H). The progressive paw swelling that occurred in the three lines demonstrated that this was not the result of a gene insertion site defect (Fig S1A).

Paw inflammation in hSTING-N154S mice

In contrast to WT paws (Fig 2A), hSTING-N154S paws exhibited edema and dense inflammatory cell infiltration of the dermis (Fig 2B), with areas of necrosis, including bone marrow necrosis (Fig 2B and C). A prominent inflammatory myositis, accompanied by muscle fiber loss, was invariably present (Fig 2C–E). There were only rare foci of pulmonary infiltrates (Fig S1B and C) and mild hind foot joint synovitis along with synovial lining cell hyperplasia and hyper trophy (Fig S1D), whereas proximal muscles only showed rare foci of infiltrates in interstitial areas (Fig S1E). We did not find evidence of inflammatory infiltrates or tissue necrosis in our surveys of other mouse tissues.

Paw vasculopathy in hSTING-N154S mice

X-ray microscopy (XRM) imaging of Microfil®-perfused hSTING-N154S mouse forepaws revealed dilation of large draining veins, often containing defects consistent with sizable venous thrombi, as well as multiple sites of small arterial and venous vessel stenoses and occlusions (Fig 3A–C). Consistent with the XRM imaging, there was histopathological evidence of paw vessel inflammation and damage (Fig 3D), as well as arteriolar luminal occlusions by organizing bland thrombi (Fig 3E and F). We did not find convincing evidence of internal elastic lamina disruption that would be typical of a transmural vasculitis; hence, the findings were compatible with the diagnosis of a vasculopathy. We did not find evidence of vessel occlusions or tissue necrosis in our surveys of other mouse tissues.

Human STING expression in whole splenic tissue and selected cell populations

To examine mutant hSTING expression in the various splenic populations, including CD3+ (T cells), CD11b+ (macrophages), and CD19+ (B cells), as well as CD31+ endothelial cells that were isolated from the lung, we used a human-specific STING fluor-conjugated antibody (Fig S2A and B). When STING expression was assessed in the various cell populations derived from the spleen, for example, CD3+ (T cells), CD11b+ (macrophages), and CD19+ (B cells), we discerned that only the transgene-positive cells expressed the human STING (Fig 4A–C). Percentages were relatively low possibly owing to (i) technical reasons associated with the efficiency of the intracellular staining process in different cells types; (ii) expression levels per cell being below the detection threshold of this method; and (iii) the possibility of variagated transgene expression. As there was a possibility that the Vav1 promoter could have resulted in the expression of mutant hSTING in the endothelium, we isolated CD31+ CD4+ T endothelial cells from the lung; human STING protein was not detected in isolated endothelial cells (Figs 4C and S2A). We also examined the splenic protein expression of STING in WT, hSTING-154S, and hSTING-N154S mice that had been crossed onto an mSting-KO background. We observed no significant increases in splenic STING expression in any of the mice that expressed the hSTING-N154S transgene as compared with WT mice (Fig 4D). This was due to the relatively low levels of transgene-derived mutant STING expression, best illustrated when hSTING-N154S mice were crossed onto an mSting-KO background. As expected, mSting protein expression was absent in the spleens of mSting-KO mice (Fig 4D).

Lymphopenia in lymphoid tissues of hSTING-N154S mice

Because lymphopenia is a feature of SAVI, we investigated whether this would be reflected in the lymphoid tissues of hSTING-N154S mice. CD4+ and CD8+ abundance and ratios were, thus, determined for spleen, thymus, and lymph nodes of hSTING-N154S mice, their WT littermates, and mSting-KO mice. We found a marked reduction in the number of CD4+ and CD8+ cells in the spleen and lymph nodes of hSTING-N154S mice, but no differences were observed in the thymus. No significant differences in the percentages of these populations were seen when WT and mSting-KO mice were compared (Fig 5A–C).

Serum type I interferons in hSTING-N154S mice

We also examined whether the hSTING-N154S phenotype was accompanied by the production of type I interferons. In RNA derived from the spleen, we found that IFNβ transcripts were modestly increased in hSTING-N154S mice as compared with WT littermates (Fig 5D). In addition, using a 13-plex Luminex assay, we found that the IFN-β levels were elevated in the sera of hSTING-N154S mice (Fig 5E), and using an ELISA, we were able to detect multiple murine
Figure 1. hSTING-N154S mice show impaired weight gain, paw swelling, and acral necrosis. 

(A, B) Both female and male hSTING-N154S mice demonstrated a failure to gain weight starting at 8–10 wk of age. As discussed in the Results section, the “n” of mice used to calculate each time point was variable as all mice did not survive to endpoint (e.g., sacrifice due to the severity of disease); WT littermates were euthanized at these same time points as controls. 

(C) Generalized growth impairment was seen in hSTING-N154S mice (left) relative to WT littermates (right). 

(D–F) hSTING-N154S mice also developed progressive paw swelling that was first evident by ~6 wk of age (red arrows). Paw thickness was determined by dorsoventral measurement (yellow arrow) using digital calipers. 

(G, H) hSTING-N154S mice developed tail inflammation and swelling with ensuing necrosis that lead to tail shortening (E). 

These mice also exhibited losses of ear cartilage. For the paw thickness data, a one-way ANOVA with Tukey’s multiple comparisons post hoc test was used. *** $P < 0.001$ versus WT, n ≥ 13 per group.
IFN-α variants (1, 2, 4, 5, 6) and significant increases of IFN-α in hSTING-N154S sera (Fig S5).

Serum hyper-cytokinemnia in hSTING-N154S mice

Compared with littermate control sera, serum samples from hSTING-N154S mice contained elevated levels of several chemokines (CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL9, and CXCL110) and cytokines (TNF-α, IL-6, G-CSF, and IL-5) (Fig 6). Aside from IL-6 and IL-5, levels of cytokines and chemokines that were found in N154S mice (Fig S4). This revealed that 15 of 19 transgenic animals were ANA+, with titers varying between 1:1,600 and 1:1,280. A proportion (4) of 11) of littermate controls also were ANA+, although the decrease in CD19+ cells (consisting of neutrophils) were significant differences being observed between littermate controls and N154S mice. Furthermore, lymphocytes and Ly6G+ cells were not detected in the BAL fluid of mice from either group (data not shown).

ANAs in hSTING-N154S mice

Since ANAs have been observed in human SAVI, we undertook an analysis of hSTING-N154S and littermate control sera (Figs S5 and S6). This revealed that 15 of 19 transgenic animals were ANA+, with titers varying between 1:1,600 and 1:1,280. A proportion (4) of 11) of littermate controls also were ANA+, although the decrease in CD19+ cells (consisting of neutrophils) were significant differences being observed between littermate controls and N154S mice. Furthermore, lymphocytes and Ly6G+ cells were not detected in the BAL fluid of mice from either group (data not shown).

Phenotype hSTING-N154S mice depends on IFNAR1

To determine whether the observed phenotype required intact type I IFN receptor signaling, we interbred Ifnar1−/− (C57BL/6) and N154S (C57BL/6) mice to place the hSTING-N154S transgene onto an ifnar1−/− background. As before (Fig 1A-C), the hSTING-N154S offspring were smaller than either the age-matched (13-22 wk-old) littermate controls or the hSTING-N154S/Ifnar1−/− KO mice (Fig 8A). Importantly, the hSTING-N154S/Ifnar1−/− KO mice were indistinguishable from the WT littermate controls and failed to develop evidence of acral necrosis or the marked paw swelling characteristic of hSTING-N154S mice (Fig 8A-C). In keeping with this result, histological examination of hSTING-N154S/Ifnar1−/− KO paws revealed no evidence of dermal inflammation, necrosis, or myositis (Fig S7A). Body weights of hSTING-N154S/Ifnar1−/− KO mice (37.7 ± 3.83 g) were similar to those of WT mice (36.3 ± 2.00 g) and differed significantly from those of age-matched hSTING-N154S mice (25.5 ± 0.56 g, P < 0.05) (Fig 8D). Caliper measurements of hind paws also showed no differences between the hSTING-N154S/Ifnar1−/− KO and WT littermates. In contrast, the hind paws of the hSTING-N154S mice were ~30% thicker than those of both WT (P < 0.05) and hSTING-N154S/Ifnar1−/− KO mice (P < 0.01) (Fig 8E). Splenic enlargement observed in hSTING-N154S mice was not observed in the hSTING-N154S/Ifnar1−/− KO mice (1.2 ± 0.05 mg/g versus hSTING-N154S 3.6 ± 0.19 mg/g, P < 0.01) (Fig 8F). Last, we found that the hSTING-N154S/Ifnar1−/− KO mice did not have elevated serum levels of cytokines and chemokines that were found in N154S mice (Fig 9).

Discussion

We have generated a mouse model of human SAVI by expressing a constitutively active human STING mutant in hematopoietic cells. Despite mutant STING expression being restricted to the hematopoietic compartment, our hSTING-N154S mice exhibited many of the characteristics that have been observed in SAVI. These similarities include growth failure, dermal inflammation, acral necrosis with tissue loss due to a vasculopathy with vessel thrombosis, myositis, increased proinflammatory cytokine/chemokine accumulation, and lymphopenia (Jeremiah et al, 2014; Liu et al, 2014; Munoz et al, 2015; Omoymini et al, 2015; Chia et al, 2016; Fremond et al, 2016; Picard et al, 2016; König et al, 2017). For example, Liu et al (2014) reported that serum concentrations of several proinflammatory cytokines, such as CXCL10 and TNFα, were significantly
XRM imaging of hSTING-N154S paws, together with histological analysis, revealed evidence of a severe vasculopathy. Thus, after perfusion of the mice with a radio-opaque monomer that polymerizes in the cold, XRM was used to obtain high-resolution images of the vasculature. This revealed that hSTING-N154S paws contained widespread stenoses and obstructions of both arterioles and venules, together with the presence of prominent thrombi in large veins. Histological demonstration of arteriolar lumen occlusions by bland organizing clots was also obtained. An as of yet unresolved question revolves around the reason(s) for the acral distribution of pathology in the hSTING-N154S mice. The predicted reduced temperature of extremities (tail, ears, and paws) might be an etiological factor. In this regard, it is interesting that the acral lesions in SAVI are aggravated by cold weather, raising the possibility that cryoprotein(s) might be an etiological factor in the pathology (Munoz et al, 2015; Picard et al, 2016; König et al, 2017). This possibility, or perhaps the small vessel vasculopathy in combination with cold-induced vasoconstriction, might account for the chilblains, Raynaud phenomenon, and livedoid rashes seen in SAVI (Stoffels & Kastner, 2016), although we were not able to visualize any cryoprecipitates after prolonged cooling of hSTING-N154S sera (data not shown). Reducing the temperature of the mouse holding room is a possibility because this could aggravate or accelerate disease progression in the hSTING-N154S mice. However, further studies are required to determine whether cryoproteins, and/or factors associated with anti-phospholipid syndrome, are present in these mice.

Because various STING mutations have been reported to result in interstitial pulmonary inflammation and fibrosis (Jeremiah et al, 2014; Liu et al, 2014; Picard et al, 2016), we examined the lungs of hSTING-N154S mice. Unlike humans with activating STING mutations, hSTING-N154S mice failed to develop significant lung inflammation or fibrosis. Only very rare foci of hematopoietic cell infiltrates were present, even in mice >6 mo of age. This was consistent with our finding that the lungs and BAL fluid of hSTING-N154S mice did not show significant increases in hematopoietic cell numbers, except for neutrophils in the former. Because hSTING-N154S expression is confined to hematopoietic cells, the absence of significant pulmonary disease in the mice suggested that expression of constitutively active STING protein in lung parenchymal cells may be required for development of interstitial lung disease and fibrosis.

Regarding lung involvement, Warner et al (2017) described the phenotype of mice having an N153S knock-in mutation of mSTING. These mice developed severe lung inflammation, nonacral skin ulceration, as well as hyper-cytokinemia and lymphopenia (Warner et al, 2017). Similar to the hSTING-N154S mice, 4–6-mo-old mSTING-N153S mice had elevated serum proinflammatory mediators (Warner et al, 2017), albeit at lower levels than that of hSTING-N154S mice. The elevated, as they were in the hSTING-N154S mice we generated. Interestingly, and in contrast to myositis models requiring immunization with myosin protein plus adjuvant (Allenbach et al, 2009; Kang et al, 2015), hSTING-N154S mice spontaneously developed severe myositis of interossei muscle.

Transgenic model of STING-associated vasculopathy Martin et al.
mSting-N153S mice were not reported to develop acral inflammation and necrosis, vasculopathy, or myositis. Also, deletion of Irf3 did not block the phenotype of these mice, likely owing to redundancy in the pathways involved in mediating the type I interferon responses. Modest increases in interferon-stimulated genes were seen when fibroblasts from the mSting-N153S mice or from humans with STING-N154S–associated SAVI were evaluated (Warner et al., 2017). Similarly, we found increases in both IFN-α and IFN-β in hSTING-N154S sera, as well as increased levels of CXCL10, a marker that often accompanies interferon production (Luster & Ravetch, 1987; Vanguri & Farber, 1990).

Bouis et al. (2018) recently reported the generation of mice having a V154M knock-in of mSting. These mice demonstrated an increased mortality rate, weight loss, and evidence of both lung and renal hematopoietic cell infiltrates. They also developed pronounced lymphopenia, resulting in a SCID-like phenotype with hypogammaglobulinemia and NK cell depletion. The reported phenotype did not describe acral inflammation and necrosis, vasculopathy, or myositis. Interestingly, the SCID-like phenotype was not reversed by interbreeding mSting-V154M mice with Ifnar1-knockout mice, although the inhibitory effect of mutant mSting activation on T cells was partially reversed. These results are consistent with other reports (Cerboni et al., 2017), and our unpublished in vitro observations, indicating that the negative effects of STING activation on T cells is relatively independent of an autocrine type I interferon effect. Furthermore, agonist-mediated mSting protein activation was shown to be toxic to mouse B lymphocytes (Tang et al., 2016).

Lastly, Motwani et al. (2019) developed two mSting-mutant knock-ins that developed similar phenotypic features as the previous two mSting mutant knock-ins, although these too did not develop acral necrosis (Motwani et al., 2019). They also found that the phenotype was present in the absence of the type I IFN receptor. Similar to these various mSting knock-ins, we also observed significant decreases in lymphocytes in the peripheral blood, lungs, spleen, and lymph nodes of hSTING-N154S mice. These findings are consistent with reports of peripheral blood lymphopenia in individual carrying SAVI mutations.

In view of these discrepancies, an important question remains: why is the phenotype of our transgenic model different from the three mSting knock-in transgenics that have not been reported to develop acral necrosis? One obvious possibility concerns the use of an ectopic gene promoter to drive hSTING-N154S expression. The Vav1 gene promoter is unlikely to be subject to the same regulation as the endogenous mSting gene promoter, which possibly could have led to higher-than-normal WT levels of mutant hSTING protein expression. However, upon analyses of total STING expression in splenic lysates, we found no significant increases in Vav1-hSTING-N154S–directed protein expression. Furthermore, when hSTING-N154S mice were placed on an mSting-KO background, relatively low levels of STING expression were seen using an antibody that detects both human and murine STING. Although it is possible that the constitutively active mutant hSTING protein undergoes rapid degradation, another possibility, as suggested by the disease-attenuating effect of antibiotic treatment reported for the mSting-V154M mouse (Bouis et al., 2018), is that differences in microbiota between the various transgenics might account, at least in part, for their phenotypic variability.

Why do our transgenics invariably develop prominent paw inflammation and acral necrosis? It has been reported that the Vav1 promoter may be expressed in endothelial cells (Joseph et al., 2013) and thus may have been responsible for the observed vascular pathology. Although it is possible that mutant hSTING protein expression levels were below the ability of the intracellular detection method we used, hSTING expression in the endothelial cells that had been isolated from the lung was undetectable. Furthermore, if acral necrosis was indeed dependent on ectopic Vav1 promoter-directed mutant hSTING expression in the endothelium, why would the mSting-mutant knock-ins lack endothelial expression, given that mSting is thought to be ubiquitously expressed?

Unlike the mSting mutant knock-in models (Warner et al., 2017; Bouis et al., 2018; Motwani et al., 2019), we did not observe any significant lung inflammation. One possibility is that mutant STING expression in the lung parenchymal cells is required. Because
Vav1-hSTING-N154S expression is primarily confined to hematopoietic cells, the lack of lung disease in our model suggests that the expression of constitutively active STING in lung parenchymal cells may be necessary for the lung inflammation to develop.

In addition to decreased T lymphocyte levels, there were reductions in peripheral blood and pulmonary B lymphocytes in hSTING-N154S mice, although in the lungs, this did not reach statistical significance. In contrast, there were increased pulmonary and peripheral blood Ly6G+ cells (neutrophils) in hSTING-N154S mice compared with controls. Recently, Kim et al (2018) demonstrated that B lymphocytes, via direct interaction with neutrophils in the lungs, facilitate the clearance of aging cells. Moreover, depletion of B lymphocytes resulted in the accumulation of aged PMNs within the lungs, which promoted fibrotic interstitial lung disease (Kim et al, 2018). B-cell lymphopenia may thus be contributing to the increased level of neutrophils seen in the hSTING-N154S samples. However, increased levels of mediators such as G-CSF, via their ability to increase bone marrow generation and mobilization of granulocytes (Bendall & Bradstock, 2014), may have also promoted neutrophil numbers.

Figure 5. T-cell lymphopenia and type I interferon levels in hSTING-N154S mice. (A–C) Whereas CD4+ T cell numbers were moderately reduced in the thymi of hSTING-N154S mice (A), there were marked reductions in the number of CD4+ and CD8+ cells in the spleens (B) and lymph nodes (C) when compared with WT mice. There were no differences in these T-cell populations when WT and mSTing-KO mice were compared. One-way ANOVA with Tukey’s multiple comparisons post hoc tests were used to analyze group differences. WT and hSTING-N154S, n = 10; mSTing-KO, n = 4. Horizontal lines represent the mean ± SEM with significant differences denoted as *P < 0.05 or **P < 0.001 versus WT. (D) Using quantitative RT-PCR analysis of splenic tissues, we observed that IFN-β transcripts were modestly increased in the hSTING-N154S mice (n = 7) relative to those in WT littermates (n = 7). (E) 13-plex Luminex assay of serum showed that mIFN-β levels were elevated in the sera of 8 of 13 hSTING-N154S mice (n = 13) compared with 4 of 10 WT littermates (n = 10). (F) Compared with WT littermates, there was also a significant increase in mIFN-α levels as detected via ELISA in the sera of hSTING-N154S mice (n = 12) (LumiKine Xpress mIFN-α ELISA kit). Horizontal lines represent the mean ± SEM serum concentrations (pg/ml) of murine IFN-β or IFN-α. Data are pooled from five independent experiments (n = 1–5 for each group). Unpaired t test was carried out between hSTING-N154S and WT groups where *P < 0.05.
Consistent with reports of positive ANA tests in SAVI, we found that 15 of 19 transgenic animals were ANA+ (with titers ranging between 1:160 and 1:1,280), whereas 4 of 11 of the transgene-negative littermate controls were ANA+ (titers of 1:320 or less). C57BL/6 wild-type mice are known to be ANA+ to varying degrees (Bygrave et al., 2004). Interestingly, one of the transgenic sera also contained reactivity towards Jo-1, PL-7, and SRP, markers associated in humans with autoimmune myositis and/or interstitial lung disease (Richards et al., 2009; Benveniste et al., 2016). A full breakdown of the autoimmune profiles of the hSTING mutant mice can be found in Fig S5.

Until recently, therapeutic treatment of vasculopathies has been relatively unsuccessful. However, the use of JAK inhibitors in vitro, such as tofacitinib, ruxolitinib, and baricitinib, has shown some promise as they reduced the transcription of IFNB1 and several other interferon response genes in fibroblasts obtained from human patients (Liu et al., 2014). In the clinical setting, JAK inhibitors were shown to be of some therapeutic benefit owing to their ability to down-regulate the type I interferon receptor-initiated signal transduction pathway (Munoz et al., 2015; Fremond et al., 2016; Konig et al., 2017). IFNAR1, a member of the helical cytokine class II family of receptors, is a critical component of the IFN signaling pathway (Novick et al., 1994; Peng et al., 2015). This receptor activates intracellular signal transduction in response to all type I interferons, including, but not limited to, IFN-β and the various IFN-α subtypes (Garcia-Sastre & Biron, 2006). In keeping with the phenotype of the hSTING-N154S mice being dependent on type I interferons, we found that absence of Ifnar1 prevented the development of the dramatic phenotype seen in the hSTING-N154S mice. Thus, paw

Figure 6. Increased cytokine levels in hSTING-N154S mice.
Serum cytokine levels in hSTING-N154S mice (n = 13) and WT littermate controls (n = 12) were measured by 31-plex murine cytokine array. Each symbol represents an individual mouse and horizontal lines represent the mean ± SEM of serum concentrations for each cytokine (pg/ml). Data are pooled from five independent experiments (n = 1–5 for each group). Unpaired t test was carried out between hSTING-N154S and WT groups where *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. See Fig S3 for the remainder of the Luminex results from these mice.
inflammation, acral necrosis, myositis, and proinflammatory cytokine/chemokine production was absent in hSTING-N154S mice lacking the Ifnar1.

Extrapolating to humans, our results suggest that IFNAR1 inhibition is likely to be of therapeutic benefit in SAVI. Indeed, the development and testing of anti-IFNAR1 antibodies such as anifrolumab (Peng et al, 2015; Furie et al, 2017) for use in humans is currently an active area of investigation and clinical trials. In this vein, work on the development of STING inhibitors also offers considerable promise (Haag et al, 2018). The SAVI model we have generated, based on the activity of a mutant human STING protein, represents not only a model system for dissecting mechanisms involved in the pathogenesis of SAVI but will also serve as a useful preclinical tool for the in vivo evaluation of therapeutics aimed at curtailing abnormal STING activity.

Materials and Methods

Mice

These studies were conducted in accordance with the guidelines of the Health Sciences Animal Care Committee of the University of Calgary. C57BL/6, Golden ticket (TMEM173gt:gt) on a C57BL/6 background, and B6.129S2-Ifnar1tm1Agt/Mm (backcrossed to C57BL/6 for at least five generations) were from Jackson Laboratories. WT refers to mice that were littermate controls for the transgenic animals being analyzed. Mice were fed standard laboratory chow, allowed water ad libitum, and maintained in independently ventilated micro-isolator units at 22 ± 1°C, 65–70% humidity, and a 12-h light/dark cycle.

Generation of hSTING-N154S transgenic lines

Transgene generation involved inserting the hSTING-N154S mutant cDNA downstream of the murine Vav1 gene promoter to obtain hematopoietic cell-specific expression. The 2.3/4.4(HS21/45) Vav-hCD4 (Clone#2) 11.2-Kb vector generously provided by Dr. Jerry Adams (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) (Ogilvy et al, 1999) was used (Fig S7B). The Vav-hCD4 construct was digested with SfiI and NotI to remove the hCD4 cDNA, and this was replaced with a synthetic (Celtek) cDNA encoding the consensus hSTING sequence plus the N154S point mutation reported in Liu et al (2014). The SfiI site within the mutant STING cDNA was eliminated via codon substitution to facilitate cloning of the cDNA.
into the Vav1 backbone using a Sfi/NotI digestion (Fig S7C). Bacteria carrying the plasmid were grown in Luria broth (244620; BD Difco) with 100 μg/ml ampicillin (A9518; Sigma-Aldrich) and purified with the PureLink HiPure Plasmid Midiprep Kit (K210015; Invitrogen). The DNA fragment containing the transgene (Fig S7B) were removed from the vector by HindIII digestion and purified using Promega’s Wizard SV Gel and PCR Clean-Up System A9281. Transgenic lines were produced via pronuclear microinjection of the Vav1 gene promoter-hSTING-N154S construct into C57BL/6 × DBA F1 embryos at the University of Calgary’s Clara Christie Centre for Mouse Genomics.

Founders were identified using the following primers: SOEcolchF 5'-GGC GGT GGT GAA GGA ACG AG-3' and SOEcolchR 5'-CCT TGA TGC CAG CAC GGT CA-3', 5% DMSO with a cycling program of 95°C 3 min (95°C 15 s, 69°C 15 s, and 72°C 30 s) × 35 s, 72°C 5 min, using the KAPA (D-MARK KK7352) Hot Start genotyping system. Five hSTING-N154S founders were identified and three of these that showed paw swelling had been backcrossed onto the C57BL/6 background for between five and eight generations during these experiments. All transgenic mice used were hemizygous for the transgene.

Histology

Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, with 4-μm sections before staining. See figure legends for specific stains used.

Real-time PCR

Spleens from hSTING-N154S, WT, and mSting-KO mice (Golden-ticket, gt:gt) were mechanically disrupted and lysed in QIAzol lysis reagent (QIAGEN) before chloroform and isopropanol RNA extraction. For cDNA synthesis, 1 μg of RNA was treated with DNase (Promega) followed by RT-PCR with 10-mM dNTPs, random primers, and Superscript II reverse transcriptase (Invitrogen). Real-time PCR of cDNAs was carried out using the LightCycler FastStart DNA MasterPLUS SYBR Green Kit (Roche). Data were normalized to β-actin mRNA and experimental transcripts expressed as the relative fold-change in mRNA compared with controls. Primer sequences were as previously used (Downey et al, 2014).
Figure 9. Absence of systemic hyper-cytokinemia in hSTING-N154S/Ifnar1-KO mice. Serum cytokine levels (pg/ml) in age-matched WT, hSTING-N154S (N154S), and hSTING-N154S/Ifnar1-KO mice as measured by Luminex 31-plex murine cytokine array. Each symbol represents an individual mouse, and horizontal lines represent the mean ± SEM, n = 3 for WT and hSTING-N154S; n = 8 for hSTING-N154S/Ifnar1-KO. Statistical

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Serum cytokine assays

Blood was collected by cardiac puncture from deeply anesthetized 3–6-mo-old mice and transferred to 1.6-ml Eppendorf tubes. Coagulated blood was centrifuged at 8,765 × g for 10 min at 4°C, and serum supernatant was collected and stored at −20°C until assayed. The samples were assayed using 31- and 13-plex Milliplex murine cytokine/chemokine arrays (Millipore) on a Luminex 200 system (Luminex Corp.) by Eve Technologies (Eve Technologies), and ELISA for mIFN-α (LumiKine Xpress; InvivoGen) and mIFNβ (LumiKine; InvivoGen). Results are presented in picograms per milliliter.

XRM

MicrofiR perfusion was performed as we previously described (Downey et al, 2012). High-resolution imaging of paws from mice perfused with the radio-opaque MicrofiR was carried out with a Zeiss Xradia 520 Versa. XRM is distinct from the traditional microcomputed tomography as it combines both geometric magnification and optical objectives of microscopy to achieve higher spatial resolution at a relatively longer working distance (Sakdinawat & Attwood, 2010; Zhu et al, 2015). For XRM, front paws were sealed in centrifuge tubes containing neutral buffered saline. Both low-energy (40 kVp voltage, 3 W power) and high-energy (150 kVp voltage, 10 W power with a custom filter) XRM scans were performed on the same sample sequentially using the 0.4# objective, which is sensitive to high-energy photons. To achieve a high signal-to-noise ratio, 2,501 projections were collected per rotation scanner (FSC/SSC) and samples measured with a minimum of 10⁴ counts.

Intracellular staining of hSTING in hematopoietic cells

Single-cell suspensions were prepared from spleens and lungs of hSTING-N154S, WT control, and mSTing-KO mice (n = 6, n = 3, and n = 3, respectively). The lungs were flushed with 10 ml of saline through the right ventricle, then harvested and minced with surgical scissors, placed in 5 ml of PBS on ice, and then processed with the gentleMACS tissue dissociator. To further digest the tissue, the lung samples were incubated with dispase (2.5 U/ml) for 30 min at 37°C in a CO₂ incubator. The lung homogenate was then passed through a 100-μm filter into a 50-ml Falcon tube containing 10 ml PBS, centrifuged (650 g, 4°C, 5 min), and were treated with 1× RBC Lysis Buffer (BioLegend) for 3 min. For spleen, these tissues were placed in 1× PBS, ground between the rough sides of frosted glass slides, and then transferred to a 50-ml Falcon tube, centrifuged (234 g, 4°C, 5 min), and treated with hemolysis buffer (ACK cell lysis buffer) for 5 min at room temperature. The samples were then washed thrice with 1× PBS and incubated with anti-CD16/32 (FcBlock-BioXCell) for 30 min on ice. After washing the samples thrice with FACS wash (PBS, 2% FBS, and 0.002 M EDTA), the samples were transferred to a 96-well plate where antibody cocktails for cell surface markers were added to the corresponding wells and stained on ice for 30 min. All antibodies were used at 1:200 dilutions. The cells from the spleen were stained with FITC-CD3, PerCPCy5.5-CD11b, and PE-CD19 and from the lung, with PE-CD3, and PE-cy7-CD45. After washing the samples thrice with FACS wash, the cells were fixed and permeabilized using the Foxp3 fixation/permeabilization working solution from eBioscience Foxp3/ transcription factor staining buffer set (Invitrogen 00-5522-00). After

**significance between data sets was assessed by one-way ANOVA followed by Tukey's multiple comparisons post hoc test between all groups. Significant differences between hSTING-N154S mice and WT or hSTING-N154S/Ifnar1−/− mice were denoted by *P < 0.05, **P < 0.01, ***P < 0.001; WT and hSTING-N154S/Ifnar1−/− differences denoted by ϕ P < 0.05. LIX, EOTAXIN, MIP-2, M-CSF, GM-CSF, IFNγ, VEGF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-7, IL-9, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, and LIF were also measured; however, no differences were observed between the groups (data not shown).**

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1-h incubation, the cells were washed in 1× permeabilization buffer and incubated with Alexa Fluor 647–hSTING (1:40 dilution; BD phamnogen) for 30 min, they were washed and analyzed using a flow cytometer (BD FACS Canto). Flow cytometry data were analyzed using FlowJo software (version 10.2).

Western blot analysis

To examine STING protein expression, splenic homogenates (10% wt/vol) from the various mouse phenotypes were prepared in extraction buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton-X 100, and 10 mM Tris–HCl, pH 7.4) with the addition of a protease inhibitor cocktail (Complete, Roche Diagnostic GmbH). Protein concentrations were determined by Bradford assay. Proteins were separated on 12% SDS-polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked in 3% BSA/TBST and then incubated with an anti-STING rabbit polyclonal antibody (1:1,500 dilution; Cell Signaling Technology Inc.) that recognizes both human and mouse STING for 24 h at 4°C. As positive controls, two human colorectal cancer cell (CRC) lines known to express hSTING protein were used: HT29 and HCT116. An HRP-conjugated donkey anti-rabbit secondary antibody was used. STING protein bands were then visualized with SuperSignal West Pico chemiluminescence substrate and quantified using a calibrated imaging densitometer equipped with Quantity One software (Bio-Rad). The membranes were then stripped and re-probed with an anti-β-actin antibody as a loading control (Sigma-Aldrich). For spleen analyses, 40 μg of protein/lane and for CRC cell protein, 10 μg/lane were loaded.

ANA testing used HEP-2 cell substrates (HEp-2000; ImmunoConcepts Inc.) to screen for mouse autoantibodies by indirect immunofluorescence (IIF) at a screening dilution of 1/160. All available samples were also tested for ANA specificities included in the ENA screening panel (chromatin, ribosomal P, Sm, U1RNP [ribonucleoprotein], SS-A/Ro60, Ro52/TRIM21, SS-B/La, Scl-70 [topoisomerase I], Jo-1 [histidyl tRNA synthetase]) by addressable laser bead immunoassay (FDIS; TheraDiag), other myositis-related antibodies (O), TIF1γ, PL-12, SAE, EJ, MDAS, PL-7, SRP, NXP2, MI-2) by line immunoassay (Euroimmun GmbH), anti-centromere by IIF pattern on HEP-2 cells, and dsDNA by the C. luciliae IIF test (ImmunoConcepts). Antibodies to DFS70/LEDGF were detected by chemiluminescence immunoassay (QUANTA Flash DFS70; INOVA Diagnostics).

Statistics

Statistical analysis was performed using GraphPad PRISM software (v5.0b). Variance between sample sets was estimated by a one-way ANOVA followed by Tukey’s multiple comparisons post hoc test. Analysis of data from the qRT-PCR experiments was performed using a paired t test. Unpaired t tests were used to compare mean serum cytokine levels and leukocyte counts between hSTING–N154S mice and littermate controls. P-values of <0.05 were considered significant.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.201800215.

Acknowledgements

We are very grateful to Meifeng Zhang for performing the ANA determinations, the assistance of Wei Liu with the XRM, to Dragan Ponjevic for histological sectioning, to Elaine De Heuvel for her assistance with the histopathology preparation, to Cameron Fielding for generating the transgenic lines in the Transgenic Core Facility in the Clara Christie Centre for Mouse Genomics, and to the Flow Cytometry Core Facility at the University of Calgary. K Henare was the recipient of a Postdoctoral Fellowship from the Health Research Council of New Zealand (Project Grant Number 15/446), administered by the University of Auckland. BG Yipp was supported by a Tier II Canada Research Chair in Pulmonary Immunology, Inflammation and Host Defense. The Zeiss Xradia 520 versa XRM instrument was obtained via a grant from the Canadian Foundation for Innovation. This study was supported by an Operating Grant from the Canadian Institutes for Health Research (to FR Jirik).

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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