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The common *TMEM173 HAQ, AQ* alleles rescue CD4 T cellpenia, restore T-regs, and prevent *SAVI (N153S)* inflammatory disease in mice

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Abstract

The significance of STING (encoded by the *TMEM173* gene) in tissue inflammation and cancer immunotherapy has been increasingly recognized. Intriguingly, common human *TMEM173* alleles R71H-G230A-R293Q (*HAQ)* and G230A-R293Q (*AQ*) are carried by ~60% of East Asians and ~40% of Africans, respectively. Here, we examine the modulatory effects of *HAQ*, *AQ* alleles on STING-associated vasculopathy with onset in infancy (SAVI), an autosomal dominant, fatal inflammatory disease caused by gain-of-function human *STING* mutations. CD4 T cellpenia is evident in SAVI patients and mouse models. Using STING knock-in mice expressing common human *TMEM173* alleles *HAQ*, *AQ*, and *Q293*, we found that *HAQ*, *AQ*, and *Q293* splenocytes resist STING-mediated cell death *ex vivo*, establishing a critical role of STING residue 293 in cell death. The *HAQ/SAVI(N153S)* and *AQ/SAVI(N153S)* mice did not have CD4 T cellpenia. The *HAQ/SAVI(N153S)*, *AQ/SAVI(N153S)* mice have more (~10-fold, ~20-fold, respectively) T-regs than *WT/SAVI(N153S)* mice. Remarkably, while they have comparable TBK1, IRF3, and NFκB activation as the *WT/SAVI*, the *AQ/SAVI* mice have no tissue inflammation, regular body weight, and normal lifespan. We propose that STING activation promotes tissue inflammation by depleting T-regs cells *in vivo*. Billions of modern humans have the dominant *HAQ*, *AQ* alleles. STING research and STING-targeting immunotherapy should consider *TMEM173* heterogeneity in humans.

Teaser

Common human *HAQ*, *AQ TMEM173* alleles dominate the gain-of-function *SAVI(N154S) TMEM173* mutant in mice.

eLife assessment

This study describes **useful** mouse models of knock-ins of human STING1 variants and an assessment of these variants' action in mouse immune cells. While the implications of the variants in the inflammatory response are of significant interest, limitations are still found in the authors' interpretation and conclusions made, and the evidence for the conclusion remains **incomplete**.

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Introduction

STING drives cytosolic DNA-induced type I IFNs production [1]. Recent research revealed that STING promotes inflammation in a variety of inflammatory diseases, including nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, kidney injury, neurodegenerative diseases, cardiovascular diseases, obesity, diabetes, and aging [2–9]. The type I IFNs-independent function of STING has also emerged [9–12]. For example, initially described as a type I interferonopathy [13], recent studies in STING-associated vasculopathy with onset in infancy (SAVI) mouse models showed that SAVI is largely independent of type I IFNs [14–17]. In a *STING N153S* mouse model of SAVI, crossing *N153S* mice to IRF3/IRF7, and IFNAR1 knockout mice, N153S mice still developed spontaneous lung diseases [14]. JAK inhibitors were used to block type I IFNs signaling for SAVI patients with mixed success [17–20]. For example, in a review of JAK inhibition in 18 SAVI patients, incomplete response to treatment happened in 7/18 (38%) of patients [21]. Furthermore, 2 patients died of respiratory failure despite this treatment [21]. Both radioresistant parenchymal and/or stromal cells and hematopoietic cells influence SAVI pathology in mice [22,23]. The observation is important because it predicts that allogeneic stem cell transplantation may not work in human SAVI patients. Indeed, lung transplantations did not show improvement in SAVI patients [18,24]. Patients died at 3- and 9 months post-lung transplant [18,24]. How STING drives inflammation *in vivo,* independent of type I IFNs, remains unknown. Consequently, there is no curative care for SAVI.

Characterized as an innate immune sensor, STING expression is, paradoxically, high in CD4 T cells [13,25]. Furthermore, STING activation kills mouse and human CD4 T cells *ex vivo* [26–28]. SAVI patients and mouse models had CD4 T cellpenia [13,28]. STING was first discovered as MPYS for its cell growth inhibition and cell death function in mouse B lymphoma cells [29]. STING-mediated cell death is cell type dependent. For example, while STING activation kills human endothelial cells, primary and cancerous T cells, it does not kill mouse MEFs, BMDCs, or BMDMs [30–32]. Second, STING-mediated cell death is type I IFNs-independent [28,30,32]. Multiple cell death pathways, *i.e.,* apoptosis, necroptosis, pyroptosis, ferroptosis, and PANoptosis, are proposed [32–34]. Last, the *in vivo* biological significance of STING-mediated CD4 T cell death is not clear [28,32]. In humans, SAVI patients with constitutively activated STING have low CD4 T cell numbers [13], and type I IFNs are dispensable for STING-mediated human CD4 T cell death [28]. Different from SAVI mice, SAVI patients (*N154S* or *V155M*) had normal counts of CD8 T and B cells [13].

The human *TMEM173* gene is highly heterogeneous [35,36]. ~50% of people in the U.S. carry at least one copy of non-*WT TMEM173* allele [35]. Among them, the R71H-G230A-R293Q (*HAQ*) is the 2nd most common *TMEM173* allele carried by ~23% of people in the U.S. [35]. However, in East Asians, *WT/HAQ* (34.3%), not *WT/WT* (22.0%), is the most common *TMEM173* genotype [36]. Critically, the *HAQ* allele was positively selected in modern humans outside Africa [37].

Anatomically modern humans outside Africa are descendants of a single Out-of-Africa Migration 50,000~70,000 years ago. ~1.4% of Africans have the *HAQ* allele, while ~63.9% of East Asians are *HAQ* carriers [37]. Haplotype analysis revealed that *HAQ* was derived from G230A-R293Q (*AQ*) allele [37]. Importantly, the *AQ* allele was negatively selected outside Africa [37]. ~40.1% of Africans are *AQ* carriers, while ~0.4% of East Asians have the *AQ* allele [37]. *TMEM173* alleles often have a dominant negative effect likely because the protein STING exists as a homodimer [29,38]. SAVI is an autosomal dominant inflammatory disease [13]. *WT/HAQ* individuals had reduced Pneumovax23®-induced antibody responses compared to *WT/WT* individuals (NCT02471014) [39]. Notably, *AQ* responds to CDNs and produces type I IFNs *in vivo* and *in vitro*[37,40,41], but the *AQ* allele was negatively selected in non-Africans [37]. In contrast, the *HAQ* allele, defective in CDNs-type I IFNs responses [35,36,39,42,43], was positively selected in non-Africans [37]., indicating that the CDNs-type I IFNs independent function of STING was essential for the survival of early modern humans outside of Africa.

In this study, we discovered, surprisingly, that the *HAQ*, *AQ* splenocytes are resistant to STING-mediated cell death. We generated *HAQ/SAVI(N153S)* and *AQ/SAVI(N153S)* mice and found that the *HAQ*, *AQ* alleles prevent CD4 T cellpenia, increasing/restoring T-regs and alleviating/stopping tissue inflammation in SAVI mice, thus providing evidence for the *in vivo* significance of type I IFNs-independent, STING-mediated cell death and potential *AQ*-based curative therapy for SAVI patients.

Results

STING activation kills mouse spleen CD4, CD8 T, and CD19 B cells *ex vivo*

We first used the synthetic non-CDNs STING agonist diABZI [44] to induce lymphocyte death because diABZI induces cell death without the need for lipid transfection or detergent for cell permeabilization [31,45] and diABZI is in clinical trials (NCT05514717). Splenocytes from C57BL/6N mice were treated with diABZI in culture, and cell death was determined by Annexin V and Propidium Iodide stain. Splenocyte cell death could be detected as early as 5 hours post diABZI treatment (Figure S1A). Dosage responses showed that ~25ng/ml diABZI could kill 70% of splenocytes (Figure S1B). Similarly, STING agonists DMXAA and synthetic CDNs RpRpss-Cyclic di-AMP killed mouse spleen CD4, CD8 T cells, and CD19 B cells (**Figure 1A**). Thus, STING activation readily induces mouse lymphocyte death *ex vivo*.

TBK1 activation is required for STING-mediated mouse spleen cell death *ex vivo*

STING activation can lead to apoptosis, pyroptosis, necroptosis, or ferroptosis [28–34,45,46]. We then treated mouse splenocytes with apoptosis, pyroptosis, necroptosis inhibitors, STING inhibitors H-151, C-176, and palmitoylation inhibitor 2-bromopalmitate (2-BP), followed by diABZI stimulation. Inhibitors for NLRP3 (MCC950), RIPK1 (Necrostatin-1), RIPK3 (GSK872), Caspase-1 (VX-795), Caspase-3 (Z-DEVD-FMK), Caspase 1,3,8,9 (Q-VD-Oph), ferroptosis (liproxstatin-1) did not affect diABZI-induced splenocyte cell death *ex vivo* (Figure S1B, S1C). The STING inhibitors H-151, C-176, and 2-BP also could not prevent diABZI-induced cell death (Figure S1C) though they inhibited diABZI-induced IFNβ production (Figure S1D). Instead, the TBK1 inhibitor BX-795 abolished diABZI-induced splenocyte death (Figure S1C).

BX-795 is a multi-kinase inhibitor, including 3-phosphoinositide-dependent protein kinase 1 (PDK1) and TBK1 (IC_{50s} = 6 and 11 nM, respectively). However, the treatment of PDK1 inhibitor GSK2334470 (IC50 = 10nM) did not prevent diABZI-induced splenocyte death (**Figure 1B**). In

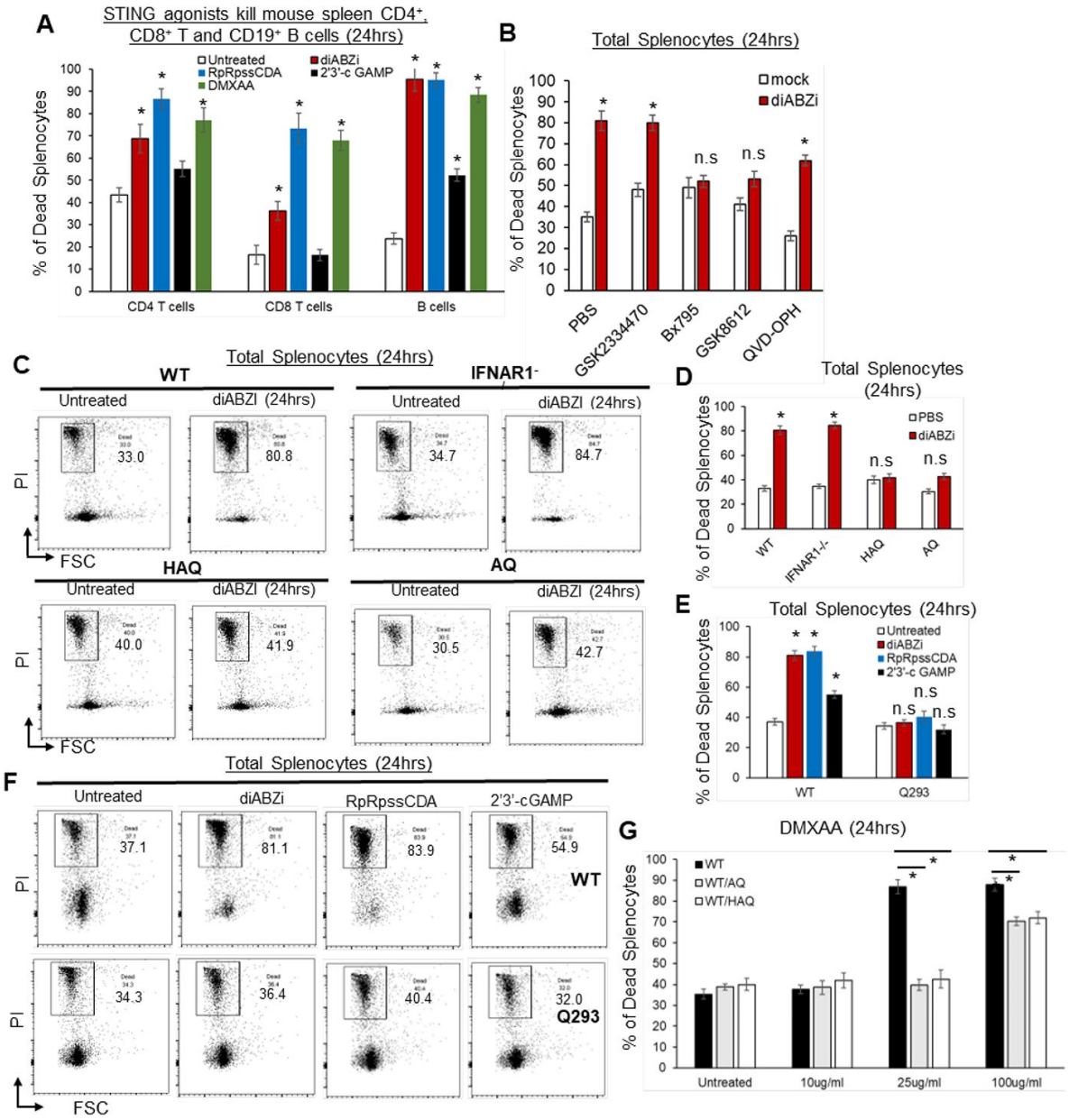

Fig. 1.

Splenocytes from *HAQ, AQ*, and *Q293* mice are resistant to STING-mediated cell death *ex vivo*.

(A). C57BL/6N splenocytes were treated directly (no transfection) with diABZI (100ng/ml), RpRpss-Cyclic di-AMP (5 µg/ml) or 2'3'-cGAMP (10µg/ml), DMXAA (25µg/ml) for 24hrs in culture. CD4, CD8 T cells and CD19 B cells death were determined by PI staining. (**B)**. Splenocytes from C57BL/6N mice were pre-treated with indicated small molecules, GSK2334470 (1.25µM), GSK8612 (2.5µM), Bx-795 (0.5µM), QVD-OPH (25µM) for 2hrs. diABZI (100ng/ml) was added in culture for another 24hrs. Dead cells were determined by PI staining. (**C-D).** Flowcytometry of *HAQ*, *AQ*, IFNAR1$^{-/-}$ or C57BL/6N splenocytes treated with diABZI (100ng/ml) for 24hrs. Cell death was determined by PI staining. (**E-F)**. *Q293* or the *WT* littermates splenocytes were treated with diABZI (100ng/ml), RpRpss-Cyclic di-AMP (5 µg/ml) or 2'3'-cGAMP 10µg/ml) for 24h. Cell death was determined by PI staining. (**G).** *WT/HAQ*, *WT/AQ*, or *WT/WT* littermates splenocytes were treated with DMXAA (10, 25 or 100µg/ml) for 24h. Cell death was determined by PI staining. Data are representative of three independent experiments. Graphs represent the mean with error bars indication s.e.m. *p* values are determined by one-way ANOVA Tukey's multiple comparison test (**A, E, G**) or unpaired student T-test (**B, D**). * p<0.05. n.s: not significant.

contrast, GSK8612, a highly potent and selective inhibitor for TBK1, prevented diABZI-induced splenocyte death (**Figure 1B**). Thus, TBK1 activation is likely critical for STING-mediated splenocyte cell death *ex vivo*.

HAQ, AQ, Q293 STING knock-in mouse splenocytes are resistant to STING-mediated cell death *ex vivo*

HAQ and *AQ* are common human *TMEM173* alleles [35–37]. Previously, we reported that *HAQ* knock-in mice are defective in CDNs-induced immune responses, while CDNs responses in *AQ* knock-in mice are similar to WT mice [37]. We treated splenocytes from *HAQ* and *AQ* mice with diABZI *ex vivo* and found, surprisingly, that both *HAQ* and *AQ* splenocytes were resistant to diABZI-induced cell death (**Figure 1C**, 1D). In comparison, IFNAR1$^{-/-}$ splenocytes were killed by diABZI, confirming that STING-mediated lymphocytes death are type I IFNs-independent (**Figure 1C**, 1D) [28,30,32].

HAQ and *AQ* share the common A230 and Q293 residues changes. We thus generated a *Q293 TMEM173* knock-in mouse. Notably, the Q293 splenocytes were resistant to STING agonists 2'3'-cGAMP, RpRpss-Cyclic di-AMP, and diABZI-induced cell death (**Figure 1E**, 1F). Thus, the residue 293 of STING is critical for its cell death function.

WT/HAQ, *WT/AQ* mouse splenocytes are partially resistant to STING-mediated cell death *ex vivo*

WT/HAQ (34.3%) is the most common human *TMEM173* genotype in East Asians, while *WT/AQ* (28.2%) is the 2nd most common *TMEM173* genotype in Africans [36]. We generated *WT/HAQ*, *WT/AQ* mice and treated their splenocytes with mouse STING agonist DMXAA. *WT/HAQ* and *WT/AQ* splenocytes were protected from 25μg/ml DMXAA-induced cell death (**Figure 1G**). 100μg/ml DMXAA could kill *WT/HAQ* and *WT/AQ* splenocytes, albeit less than *WT/WT* cells (**Figure 1G**). Thus, the *HAQ* and *AQ* alleles are dominant and likely impact STING activation even in heterozygosity.

STING activation kills primary human CD4 T cells but not CD8 T or CD19 B cells

STING agonists-based clinical trials in humans have been disappointing (NCT02675439, NCT03010176, NCT05514717) [47,48]. We showed that the human *TMEM173* gene might undergo natural selection during the out-of-Africa migration [37] sensitive to evolutionary pressure. Thus, we investigated STING-mediated death in primary human lymphocytes.

Human explant lung cells from the *WT(R232)/WT(R232)* donors were treated with STING agonists 2'3-c GAMP, RpRpss-Cyclic di-AMP, diABZI for 24 hours in culture. Lymphocyte cell death was determined by Propidium Iodide staining. Different from mouse lymphocytes, diABZI and RpRpss-Cyclic di-AMP killed human CD4 T but not CD8 T or CD19 B cells (**Figure 2A**, 2B). Human CD8 T and CD19 B cells are resistant to 500ng/ml diABZI-induced cell death (Figure S2A).

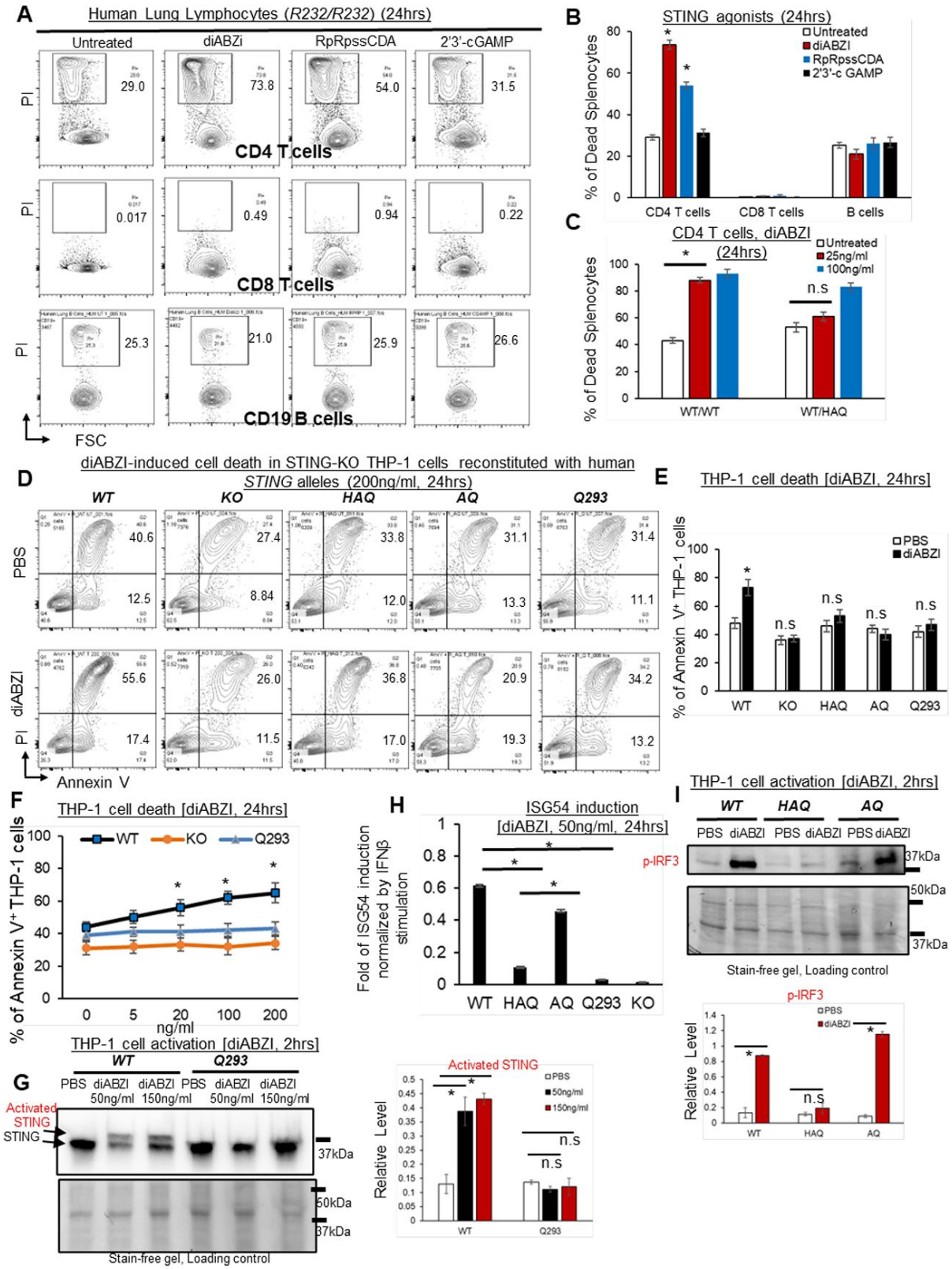
A
Human Lung Lymphocytes (R232/R232) (24hrs)
Untreated
diABZi
RpRpssCDA
2'3'-cGAMP
PI
29.0
73.8
54.0
31.5
CD4 T cells
0.017
0.49
0.94
0.22
CD8 T cells
25.3
21.0
25.9
26.6
CD19 B cells
FSC
B
STING agonists (24hrs)
Untreated
diABZI
RpRpssCDA
2'3'-c GAMP
% of Dead Splenocytes
CD4 T cells
CD8 T cells
B cells
C
CD4 T cells, diABZI (24hrs)
Untreated
25ng/ml
100ng/ml
n.s
WT/WT
WT/HAQ
D
diABZI-induced cell death in STING-KO THP-1 cells reconstituted with human STING alleles (200ng/ml, 24hrs)
WT
KO
HAQ
AQ
Q293
PBS
diABZI
40.6
12.5
27.4
8.84
33.8
12.0
31.1
13.3
31.4
11.1
55.6
17.4
26.0
11.5
36.8
17.0
20.9
19.3
34.2
13.2
Annexin V
E
THP-1 cell death [diABZI, 24hrs]
% of Annexin V+ THP-1 cells
PBS
diABZI
n.s
WT
KO
HAQ
AQ
Q293
F
THP-1 cell death [diABZI, 24hrs]
WT
KO
Q293
% of Annexin V+ THP-1 cells
ng/ml
H
ISG54 induction [diABZI, 50ng/ml, 24hrs]
Fold of ISG54 induction normalized by IFNβ stimulation
p-IRF3
WT
HAQ
AQ
Q293
KO
I
THP-1 cell activation [diABZI, 2hrs]
WT
HAQ
AQ
PBS
diABZI
37kDa
50kDa
Stain-free gel, Loading control
p-IRF3
Relative Level
G
THP-1 cell activation [diABZI, 2hrs]
WT
Q293
PBS
diABZI 50ng/ml
diABZI 150ng/ml
Activated STING
STING
37kDa
50kDa
Stain-free gel, Loading control
Activated STING
Relative Level
n.s

Fig. 2.

***HAQ*, *AQ*, and *Q293* human cells are resistant to STING agonists-induced death.**

(A-B). Total human Lung cells from *WT/WT* individuals were treated with diABZi (100ng/ml) for 24hrs. Cell death in CD4, CD8 T cells and CD19 B cells were determined by PI staining. (**C**). Total lung cells from a *WT/HAQ* (2 individuals) and a *WT/WT* (3 individuals) were treated with diABZi (25, 100ng/ml) for 24h. Cell death in CD4 T cells was determined by PI staining. (**D-E**). STING-KO THP-1 cells (Invivogen,, cat no. thpd-kostg) were stably reconstituted with human *WT (R232), HAQ*, *AQ*, *Q293*. Cells were treated with diABZI (200ng/ml) in culture for 24 hrs. Dead cells were determined by Annexin V staining. (**F).** STING-KO THP-1 cells stably reconstituted with human *WT (R232), Q293* were treated with indicated dose of diABZI for 24hs in culture. Dead cells were determined by Annexin V staining. (**G**). STING-KO THP-1 cells stably reconstituted with human *WT (R232), Q293* were treated with indicated dose of diABZI for 2hs in culture. STING activation was detected by anti-STING antibody (Proteintech, #19851-1-AP). (**H**). STING-KO THP-1 cells stably reconstituted with human *WT (R232), HAQ*, *AQ*, *Q293* were treated with 50ng/ml diABZI in culture for 24hrs. ISG-54 reporter luciferase activity was determined in cell supernatant and normalized to 10ng/ml IFNβ-stimulated ISG-54 luciferase activity. (**I).** STING-KO THP-1 cells stably reconstituted with human *WT (R232), HAQ*, *AQ* were treated with 50ng/ml diABZI in culture for 2hrs. STING and IRF3 activation were determined by anti-STING antibody and anti-p IRF3 antibody (CST, Ser396, clone 4D4G). Densitometry was determined by ImageLab 5. Data are representative of three independent experiments. Graphs represent the mean with error bars indication s.e.m. *p* values determined by one-way ANOVA Tukey's multiple comparison test (**B, C, F, H, G**) or unpaired student T-test (**E, I**). * $p<0.05$, n.s: not significant.

WT/HAQ human CD4 T cells are resistant to low doses of diABZI-induced cell death

WT/HAQ mouse splenocytes are resistant to low-dose diABZI-induced cell death (**Figure 1G**). To extend our observation into primary human T cells, we obtained lung explants from *WT/WT* and *WT/HAQ* individuals (Figure S2B) and treated them with diABZI in culture. 25ng/ml diABZI killed *WT/WT*, but not *WT/HAQ*, human lung CD4 T cells (**Figure 2C**).

diABZI induces cell death in STING-KO human THP-1 cells reconstituted with WT human STING (R232) but not HAQ, AQ or Q293 human STING allele

To further determine cell death influenced by human *TMEM173* alleles *HAQ*, *AQ*, and *Q293*, we used the *STING-KO* THP-1 cell line because STING agonist induces type I IFNs and cell death in *STING-KO* THP-1 cells expressing *WT* human STING [34]. (Figure S2C, S2D). We, thus, generated stable THP-1 *STING-KO* lines expressing *HAQ, AQ, WT,* or *Q293 TMEM173* allele. Cell death was determined by Annexin-V staining. diABZI killed THP-1 *STING-KO* lines expressing *WT* but not *HAQ*, *AQ*, or *Q293 TMEM173* allele (**Figure 2D**, 2E). No cell death was induced in the *Q293* THP-1 cells stimulated by 20 to 200 ng/ml of diABZI (**Figure 2F**). diABZI also did not induce STING activation in *Q293* THP-1 cells (**Figure 2G**). Notably, 50ng/ml diABZI induced p-IRF3 activation and type I IFNs in *AQ* THP-1 cells but not *HAQ* THP-1 cells (**Figure 2H**, 2I), indicating that the STING-cell death and STING-IRF3-Type I IFNs pathways can be uncoupled.

HAQ and *AQ* alleles rescue the lymphopenia and suppress myeloid cell expansion in *SAVI(N153S)* mice.

The *in vivo* significance of the STING/MPYS-cell death is unclear. Furthermore, multiple cell death pathways, i.e., apoptosis, necroptosis, pyroptosis, ferroptosis, and PANoptosis, are proposed [32–34]. The uncertainty likely results from studies using different cell types (primary cells vs cancer

cell lines); species (human vs mouse); STING agonists (cGAMP, which requires cell permeabilization by detergents or lipid transfection, vs diABZi, DMXAA that can directly cross the membrane) [26,27,30,31,49]. Critically, which mechanism is relevant *in vivo*, causing T cellpenia is not known. To clarify the *in vivo* significance and mechanisms of STING-mediated cell death, we turned to SAVI mice.

SAVI is an autosomal dominant, inflammatory disease caused by one copy of a gain-of-function *TMEM173* mutant (*WT/SAVI*) [13]. CD4 T cellpenia was found in SAVI patients and SAVI mouse models [13,14]. STING activation in SAVI mice is independent of ligands and happens *in vivo*. We thus generated *HAQ/SAVI(N153S)* and *AQ/SAVI(N153S)* mice aiming to establish the *in vivo* significance and mechanism of STING-cell death.

First, *HAQ/SAVI(N153S)* and *AQ/SAVI(N153S)* mice had reduced splenomegaly compared to *WT/SAVI(N153S)* mice though their spleens were still larger than the littermates *WT/HAQ* and *WT/AQ* (**Figure 3A**, 3B). Next, *HAQ/SAVI(N153S)* and *AQ/SAVI(N153S)* mice had similar spleen B cells and CD4 T cell numbers as the *WT/HAQ*, *WT/AQ* littermates (**Figure 2B**, 2C). Their $CD8^+$ T cells were lower than their *WT* littermates but much higher than the *WT/SAVI(N153S)* mice (**Figure 3D**). Third, spleen myeloid cell numbers, i.e., neutrophils, $Ly6C^{hi}$ monocytes and F4/80 macrophages, were all reduced by half compared to *WT/SAVI(N153S)* mice (**Figure 3E** ~3H). Last, splenocytes from *HAQ/SAVI*, *AQ/SAVI* mice were partially resistant to diABZI, DMXAA-induced cell death *ex vivo* (Figure S3). Notably, the *HAQ/SAVI(N153S)* and *AQ/SAVI(N153S)* mice also had restored bone marrow monocytes (Figure S4). Thus, *HAQ* and *AQ* alleles prevent lymphopenia and suppress myeloid cell expansion in *SAVI(N153S)* mice.

The *HAQ* allele alleviates and the *AQ* allele prevents *SAVI(N153S)* disease in mice

SAVI(N153S) disease is characterized by early onset, failure to thrive (low body weight), persistent lung inflammation, decreased lung function, and young death in humans and mouse models [13,17,49,50]. The *HAQ/SAVI(N153S)* mice weighed more and had an improved lifespan than the *WT/SAVI(N153S)* mice (**Figure 4A**, 4B). The lifespan, airway resistance, and tissue inflammation (lung, liver) were also improved in *HAQ/SAVI(N153S)* mice compared to the *WT/SAVI(N153S)* mice (**Figure 4C**, 4D, 4J, 4K). However, the pulmonary artery pressure was still elevated in *HAQ/SAVI(N153S)* mice (**Figure 4E**). Remarkably, the *AQ/SAVI(N153S)* mice had similar body weight and lifespan as the *WT/AQ* mice (**Figure 4F**, 4G). The airway resistance, pulmonary artery pressure, and tissue inflammation in *AQ/SAVI(N153S)* were similar to the *WT/AQ* littermates (**Figure 4H**, 4I, 4J, 4K). Thus, the *HAQ* allele alleviates and the *AQ* allele prevents inflammatory SAVI disease in mice. Interestingly, lungs from *HAQ/SAVI, AQ/SAVI* had similarly reduced infiltration of $Ly6G^+CD11B^+$ neutrophils and $Ly6G^-Ly6C^+CD11B^+$ inflammatory monocytes compared to *WT/SAVI* mice (Figure S5).

diABZI induces similar STING, TBK1, IRF3, NFκB activation in the *AQ/SAVI(N153S)* and *WT/SAVI(N153S)* bone-marrow-derived-macrophage (BMDM)

SAVI was characterized as type I interferonopathy [17]. However, several studies showed that type I IFN signaling and IRF3 activation were dispensable for SAVI disease [14,15,23,50]. *AQ* allele prevents SAVI disease (**Figure 4**). However, diABZI-treated *AQ/SAVI(N153S)* and *WT/SAVI(N153S)* BMDM had similar TBK1-IRF3 activation and IFNβ production (**Figure 5A**, 5B). diABZI treatment caused IκBα degradation, and similar TNF production in *WT/SAVI* and *AQ/SAVI* BMDM (**Figure 5C**, 5D). Furthermore, diABZI activation led to STING protein degradation in *WT/SAVI* and *AQ/SAVI* BMDM (**Figure 5E**). Last, using cleavable crosslinker dithiobis (succinimidyl propionate

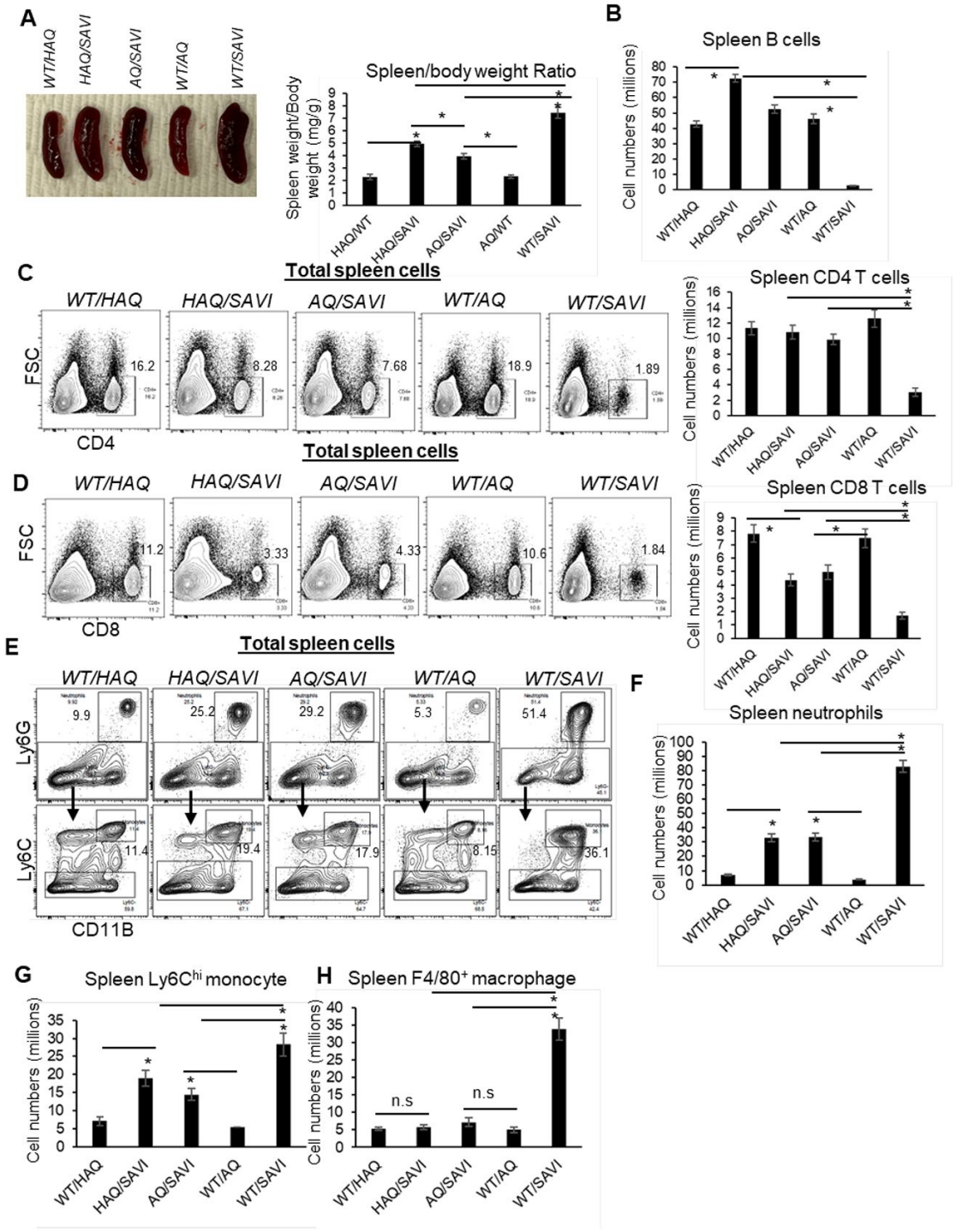

Fig. 3.

***HAQ* and *AQ* rescue the lymphopenia and suppress myeloid cell expansion in *SAVI (N153S)* mice.**

(A). The size and weight of spleens from *WT/HAQ, HAQ/SAVI, AQ/SAVI, WT/AQ, WT/SAVI*. **(B-D).** Spleen CD19^{+} B cells, CD4, CD8 T cells were determined in the indicated mice by Flow. (**E-H).** Spleen Ly6G^{+} neutrophils, Ly6C^{hi} monocytes and F4/80^{+} macrophage was determined in the indicated mice by Flow. Data are representative of three independent experiments. n=3~5 mice/group. Graphs represent the mean with error bars indication s.e.m. *P* values are determined by one-way ANOVA Tukey's multiple comparison test. * $p<0.05$, n.s: not significant.

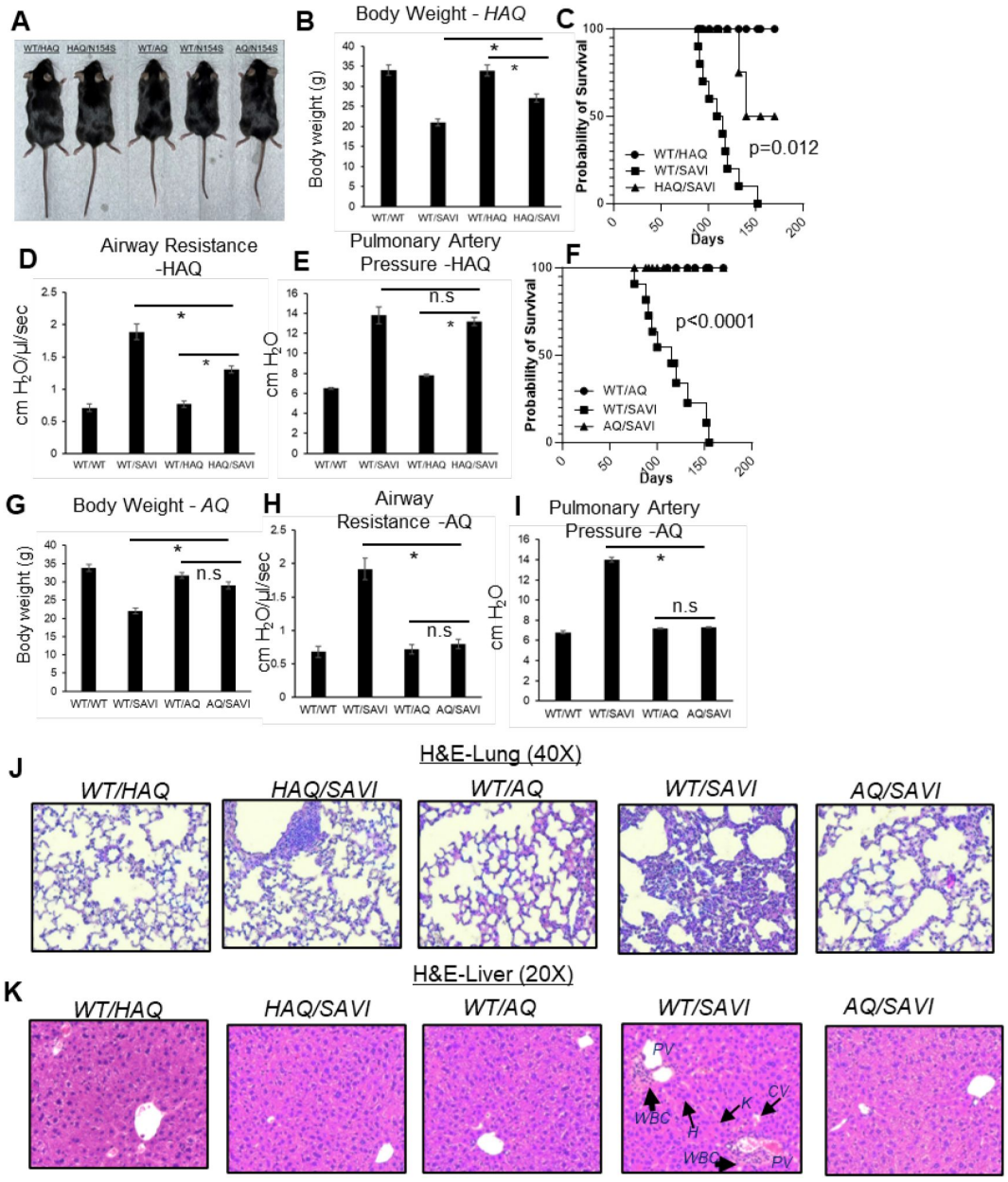

Fig. 4.

HAQ and *AQ* alleles prevent *SAVI(N153S)* disease in mice.

(A, B, G). The size and body weight of *HAQ/SAVI*, *AQ/SAVI*, *WT/SAVI* and their littermates *WT/HAQ*, *WT/AQ* mice. (**D, E, H, I**). Airway resistance, and pulmonary artery pressure were determined as described in **Materials and Methods**. **(C, F).** *HAQ/SAVI, AQ/SAVI, WT/SAVI* (10 mice/group), were monitored for survival by Kaplan-Meier. (**J, K**). Representative hematoxylin and eosin (H&E) staining of lung, liver sections from indicated mice. n=3~5 mice/group. Data are representative of 3 independent experiments. Graphs represent the mean with error bars indication s.e.m. *p* values are determined by one-way ANOVA Tukey's multiple comparison test. * $p<0.05$, ** $p<0.01$. n.s.: not significant. **WBC**: white blood cells; **H**: hepatocytes; **K**: Kupper cells; **PV**: portal vein; **CV**: central vein.

(DSP), we showed that STING in *WT/SAVI*, *AQ/SAVI* BMDM forms a similar dimer *in situ* (**Figure 5F**). Thus, the *AQ/SAVI* BMDM had similar STING degradation, TBK1, IRF3, NFκB activation, and dimerization as the *WT/SAVI* BMDM.

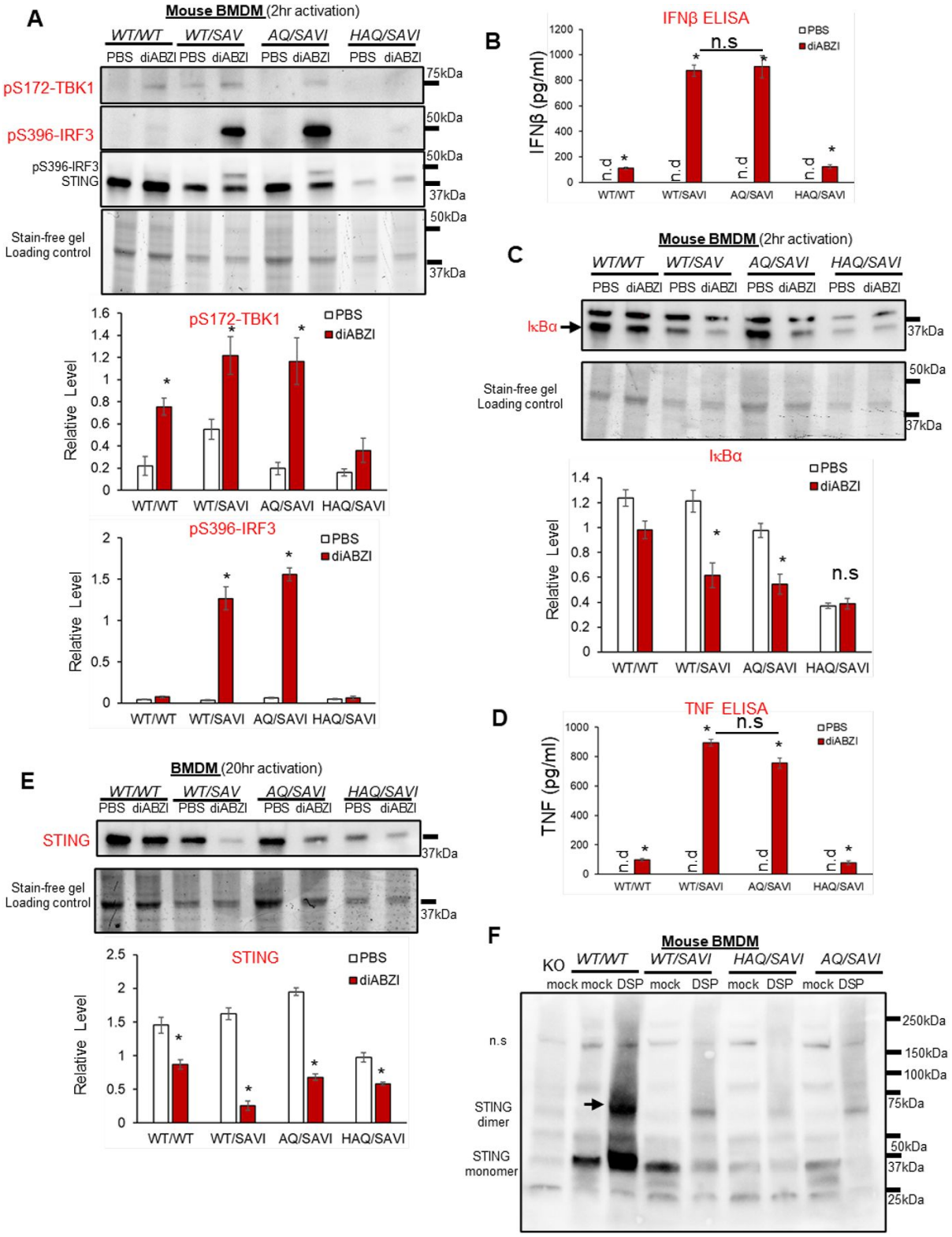

Fig. 5.

***AQ/SAVI(N153S)* cells had similar TBK1-IRF3, NFκB activation and STING degradation as the *WT/SAVI(N153S)* cells.**

(**A, C**). BMDM from *WT/WT, WT/SAVI, HAQ/SAVI* and *AQ/SAVI* were treated with 100ng/ml diABZi in culture for 2 hrs. Cells were lysed and run on a 4∼20% Mini-PROTEAN TGX Stain-Free Precast Gel. The blot was probed for phospho-Thr172-TBK1 antibody (CST, clone D52C2), phosphor-Ser 396-IRF3 (CST, clone 4D4G), STING (Proteintech, #19851-1-AP) and IκBα (CST, clone 44D4) antibody. **(E).** BMDM from *WT/WT, WT/SAVI, HAQ/SAVI* and *AQ/SAVI* were treated with 100ng/ml diABZi in culture for 24 hrs. Cells were lysed and run on a 4∼20% Mini-PROTEAN TGX Stain-Free Precast Gel. The blot was probed for STING antibody (Proteintech, 19851-1-AP). (**B, D**). IFNβ and TNF were determined by ELISA in the cell supernatant from **E**. (**F**). BMDM from *WT/WT, WT/SAVI, HAQ/SAVI* and *AQ/SAVI* were treated with 400μM cleavable chemical crosslinker DSP (Pierce™, cat no: PG82081) in PBS for 1 hr at 4°C. Cells were washed with PBS and lysed in RIPA buffer. Whole cell lysate was mixed with 4x Laemmli Sample Buffer (BioRad, cat no 1610747) containing 5% 2-mercaptoethanol, heated at 95°C for 10 min and, run on a 4∼20% Mini-PROTEAN TGX Stain-Free Precast Gel. The blot was probed for STING antibody (Proteintech, 19851-1-AP). Densitometry was determined by ImageLab 5. Data are representative of three independent experiments. Graphs represent the mean with error bars indication s.e.m. *p* values are determined by unpaired student T-test (**A-E**) or one-way ANOVA Tukey's multiple comparison test (**D, B**). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n.s.: not significant; n.d: not detected.

The HAQ allele increased, and the *AQ* allele restored T-regs in *SAVI(N153S)* mice

IFNγ was proposed to drive SAVI disease [15,23,38]. We confirmed that *WT/SAVI* CD4 T cells were enriched with IFNγ$^+$ cells (**Figure 6A**). However, *WT/SAVI* mice have CD4 T cellpenia. Thus, the total numbers of spleen IFNγ$^+$ CD4 T cells were comparable in *WT/SAVI* and *AQ/SAVI* mice (**Figure 6A**). In contrast, the *HAQ/SAVI mice* had decreased IFNγ$^+$ CD4 T cells (**Figure 6A**).

The induction of Foxp3 expression in T-reg cells during ongoing autoimmune inflammation resolved inflammation and pathology in mice [51]. CD4 T cellpenia depletes CD4 T-regs. Indeed, *WT/SAVI* mice had ∼20-fold reduction of spleen FoxP3$^+$ T-regs compared to *AQ/SAVI* or *WT/WT* littermates (**Figure 6B**). The *HAQ/SAVI* mice also had ∼10-fold more T-regs than the *WT/SAVI* littermate (**Figure 6B**).

Discussion

This study, using the *HAQ, AQ, SAVI(N153S) TMEM173* knock-in mice, reveals the *in vivo* significance and mechanism of STING-mediated CD4 T cell death. *HAQ, AQ* alleles prevent CD4 T cellpenia, and increase/restore CD4 T-regs in SAVI mice. The results are consistent with previous finding that the impaired CD4 T cell proliferation by the *SAVI(V155M)* mutant could be rescued by the addition of the *HAQ* allele *in vitro* [27]. STING has been increasingly implicated in inflammatory diseases such as nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, cardiomyopathy, obesity, diabetes, neurodegenerative diseases, aging, and kidney injury, many of which are independent of type I IFNs [2,3,9]. It is tempting to suggest that STING activation in CD4 T cells leads to CD4 T-regs depletion that break tissue tolerance and exacerbates tissue inflammation.

Human immunodeficiency virus (HIV) primarily infects CD4 T cells and might activate the STING pathway in CD4 T cells [52–57]. The loss of CD4 T cells is the hallmark of untreated HIV infection [58,59], and the measurement of CD4 T cell count is a central part of HIV care. We found that

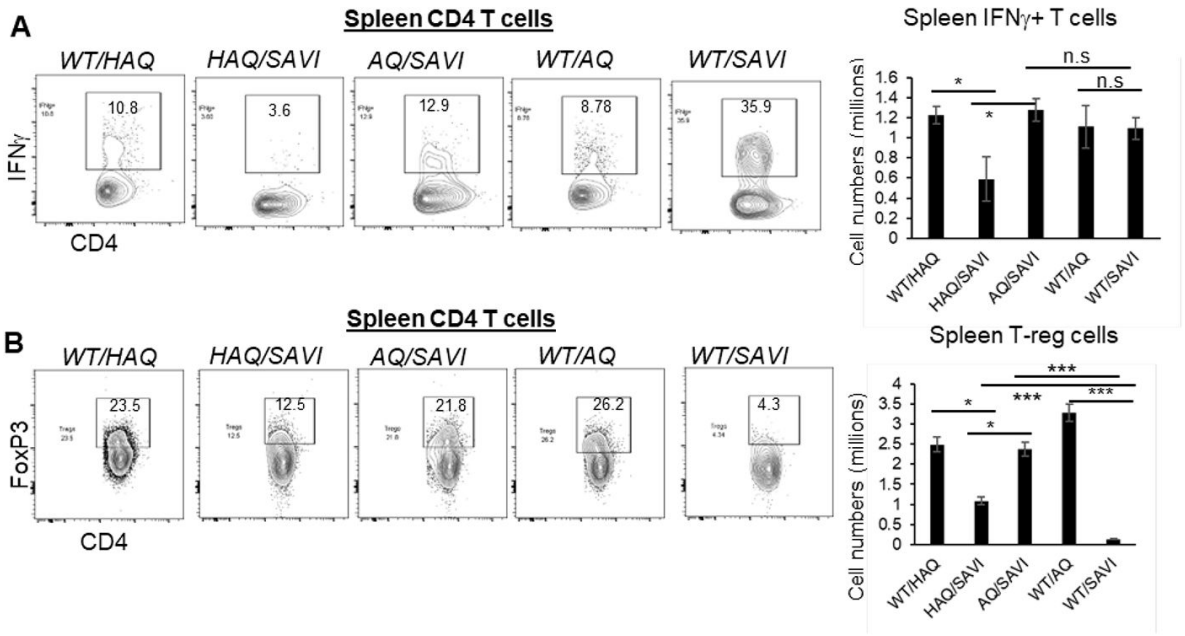

Fig. 6.

***HAQ/SAVI(N153S)* and *AQ/SAVI(N153S)* cells had 10-fold and 20-fold increased spleen T-regs compared to *WT/SAVI* mice.**

(A). Flow cytometry analysis of IFNγ producing spleen $CD4^+$ T cells from *WT/HAQ, WT/AQ, WT/SAVI, HAQ/SAVI* and *AQ/SAVI* mice. (**B**). Flow cytometry analysis of $CD4^+$ $FoxP3^+$ spleen T-regs. n=3∼5 mice/group. Data are representative of 3 independent experiments. Graphs represent the mean with error bars indication s.e.m. *p* values are determined by one-way ANOVA Tukey's multiple comparison test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n.s.: not significant.

HAQ and *AQ* CD4 T cells are resistant to STING-mediated cell death. Mogensen and colleagues reported that *HAQ/HAQ* was enriched in HIV-infected long-term nonprogressors [42]. These *HAQ/HAQ* individuals had reduced inhibition of CD4 T cell proliferation and a reduced immune response to DNA and HIV [42]. It is likely that HIV infection activates STING-cell death pathway in CD4 T cells. In *HAQ/SAVI* and *AQ/SAVI* mice, one copy of *HAQ*, *AQ* allele suppressed CD4 T cell death. *HAQ*, *AQ* carriers might have fewer HIV-induced CD4 T cell death, thus being long-term nonprogressors in HIV infection-induced Acquired immunodeficiency syndrome (AIDS)[42]. Targeting STING to prevent CD4 T cell death might be a valid therapy for AIDS. Activating the STING pathway is a promising strategy for cancer immunotherapy [60–66].

Multiple STING agonists are in clinical trials [47,48]. Recently, the safety issue emerged in some STING agonist trials [47,48]. For example, in the STING agonist, ADU-S100, clinical trial, Grade 3/4 treatment-related adverse events were reported in 12.2% of 41 pretreated patients (NCT02675439)[47,48]. The National Institutes of Health defines grade 3 as “incapacitating; unable to perform usual activities; requires absenteeism or bed rest.” In a clinical trial using STING antibody-drug-conjugate (ADC) that conjugates diABZI to anti-HER2 Ab, a Grade 5 (fatal) serious adverse event was recorded and deemed related to the STING-ADC (NCT05514717). SAVI disease, driven by overreacting STING, is often fatal[13]. *AQ*, to a less degree, *HAQ*, suppress mortality in SAVI mice. Future STING clinical trials should be based on human *TMEM173* genotype to achieve safe and effective responses.

Mechanistically, apoptosis, pyroptosis, ferroptosis, necroptosis, and PANoptosis have all been reported in STING-mediated cell death [28–34,45,46]. Different cell types and STING agonists used likely contributed to the inconsistency and complexity. Here, we focused on lymphopenia in the SAVI mice that avoids ligand-dependent, non-physiological dosage in STING-mediated cell death. *HAQ* and *AQ* alleles could prevent CD4 T cellpenia in the SAVI mice strongly indicating that residue A230 or Q293 prevent STING-mediated CD4 T cell death *in vivo*. Splenocyte from *Q293* mice were resistant to STING agonists-induced cell death *ex vivo*. Thus, it is likely that the Q293 residue is critical for STING-mediate lymphopenia. Notably, Q293 is outside the C-terminal tail (CTT) (residues 341–379 of human STING) critical for TBK1 recruitment and IRF3 phosphorylation [67] or miniCTT domain (aa343–354) [27], or the UPR motif (aa322–343) [49] important for T cells death *in vitro*. Further studies are needed to understand how the aa293 of STING mediates cell death *in vivo*. Noteworthy, *AQ/SAVI* cells had similar TBK1-IRF3, NFκB activation and STING degradation as the *WT/SAVI* cells. Yet, *AQ/SAVI* mice did not have CD4 T cellpenia as *WT/SAVI* mice suggesting that the canonical STING-TBK1-IRF3/NFκB pathway, likely STING oligomerization, is not sufficient for the induction of cell death at the physiological condition.

We used the *WT/N153S* knock-in SAVI mouse model that spontaneously develop lung inflammation, T cell cytopenia, and early mortality, mimicking pathological findings in human SAVI patients[16]. Using the *WT/N153S* SAVI mouse model and human Jurkat T cell line, it was proposed that STING activation causes chronic ER stress and unfolded protein response, leading to T cell death by apoptosis [49]. Furthermore, the study showed that crossing *WT/N153S* mice to the OT-I mice reduced ER stress and restored $CD8^+$, but not $CD4^+$, T cells [49]. The restoration of $CD8^+$T cells reduces inflammation and lung disease [49]. However, human *WT/N154S* SAVI patients have normal $CD8^+$ T cells numbers [13], and primary human $CD8^+$ T cells are largely resistant to STING-agonists-induced cell death *ex vivo* (**Figure 2A**) [28]. Thus, it is puzzling how restoring $CD8^+$ T cells can rescue SAVI phenotypes since the SAVI patients already have normal $CD8^+$ T cells numbers.

Finally, it is unexpected that both *HAQ* and *AQ* alleles are resistant to cell death. Our previous studies showed that the *HAQ* and *AQ* alleles have opposite functions [37]. AQ-STING, not HAQ-STING, responds to CDNs [35–37,39–43,68]. *AQ* mice are lean while *HAQ* mice are fat [37]. Most importantly, *HAQ* was positively selected, while *AQ* was negatively selected, in modern humans outside Africans [37]. Thus, the death pathway of STING is also distinct from the STING

function that was naturally selected. It is worth noting that the *AQ* allele does better than the *HAQ* allele in suppressing SAVI disease. Thus, besides preventing cell death, additional mechanism by the *AQ* allele, e.g. fatty acid metabolism [37,69], is involved in curing SAVI.

The limitations of the study

The poor transferability of mouse to humans is a major issue in STING research [47,48]. The present study used *AQ/SAVI* and *HAQ/SAVI* mice. Confirmation is needed in humans with the identification and evaluation of people who are *AQ/SAVI*, *HAQ/SAVI*.

Materials and Methods

Experimental Design

The study was designed to reveal (i) the *in vivo* significance of the type I IFNs-independent, STING-dependent cell death function; (ii) the interplay between common *STING* alleles *HAQ*, *AQ* and the rare, gain-of-function SAVI STING mutation; (iii) the driver for the inflammatory SAVI disease. Mouse splenocytes, primary human lung cells, human THP-1 cells and *HAQ, AQ, SAVI* knock-in mice were used to establish the *in vivo* significance and human relevance. All the repeats were biological replications that involve the same experimental procedures on different mice. Where possible, treatments were assigned blindly to the experimenter by another individual in the lab. When comparing samples from different groups, samples from each group were analyzed in concert, thereby preventing any biases that might arise from analyzing individual treatments on different days. All experiments were repeated at least twice.

Mice

WT/SAVI(N153S) mice were purchased from The Jackson Laboratory. *HAQ*, *AQ* mice were previously generated in the lab [36,37]. The *Q293* mice were generated by Cyagen Biosciences. Briefly, the linearized targeting vector was transfected into JM8A3.N1 C57BL/6N embryonic stem cells. A positive embryonic stem clone was subjected to the generation of chimera mice by injection using C57BL/6J blastocysts as the host. Successful germline transmission was confirmed by PCR sequencing. The heterozygous mice were bred to Actin-flpase mice [The Jackson Laboratory, B6.Cg-Tg (ACTFLPe)9205Dym/J] to remove the neo gene and make the *Q293* knock-in mouse. Age-and gender-matched mice (2 – 6 month old, both male and female) were used for indicated experiments. *WT/SAVI* (male) x *WT/HAQ* (female), *WT/SAVI* (male) x *WT/AQ* (female) breeders were set up to generate *HAQ/SAVI*, *AQ/SAVI* mice. Mice were housed at 22°C under a 12-h light-dark cycle with ad libitum access to water and a chow diet (3.1 kcal/g, Teklad 2018, Envigo, Sommerset, NJ) and bred under pathogen-free conditions in the Animal Research Facility at the University of Florida. Littermates of the same sex were randomly assigned to experimental groups. All mouse experiments were performed by the regulations and approval of the Institutional Animal Care and Use Committee at the University of Florida, IACUC202200000058.

Reagents

Recombinant human IFNβ (R&D, cat no. 8499-IF-010/CF), diABZI (Invivogen, cat no. 2138299-34-8), 2'3'-cGAMP (Invivogen, cat no. tlrl-nacga23-02), DMXAA (Invivogen, cat no. tlrl-dmx), H151 (Invivogen, cat no. inh-h151), RpRpSS-Cyclic di-AMP (Biolog, cat no. C118), THP1-Dual™ KO-STING Cells (Invivogen, cat no. thpd-kostg). All other chemical inhibitors are from Selleckchem. Mouse TNF alpha ELISA Ready Set Go. (eBioscience, cat no. 88-7324). Mouse IFN-Beta ELISA Kit (PBI, cat no. 42400).

Generation of THP-1 KO-STING cells stably expressing human *TMEM173* alleles

THP1-Dual™ KO-STING Cells in 6-well plate were transfected with 1µg *TMEM173* plasmid (in pcDNA 3.1 vector) with Lipofectamine LTX and Plus Reagent (Invitrogen, cat no: A12621) according to the manufacturer's instructions. *Transfecting Plasmid DNA into THP-1 Cells Using Lipofectamine LTX Reagent | Thermo Fisher Scientific - US*. 48hrs after the transfection, the cell medium was changed. G418 (1mg/ml) was added to the culture to select STING expressing THP-1 cells. The G418-resistant cells were established and expanded.

Histology

Lungs and livers were fixed in 10% formalin, paraffin-embedded, and cut into 4-µm sections. Lung, liver sections were then stained for hematoxylin-eosin. All staining procedures were performed by the histology core at the University of Florida. Briefly, tissue sectins were immersed Harris Hematoxylin for 10 seconds, then washed with tap water. Cleard sections were re-immersed in EOSIN stain for ~30 seconds. The sections were washed with tap water until clear, then dehydrate in ascending alcohol solutions (50%,70%,80%,95% x 2, 100% x 2). Afterwards, the sections werer cleared with xylene (3 - 4 x). The sections were mounted on glass slide with permount organic mounting medium for visulization.

Lung Function

Pulmonary function was evaluated using an isolated, buffer-perfused mouse lung apparatus (Hugo Sachs Elektronik, March-Huggstetten, Germany), as previously described [70]. Briefly, mice were anesthetized with ketamine and xylazine and a tracheostomy was performed, and animals were ventilated with room air at 100 breaths/min at a tidal volume of 7 µl/g body weight with a positive end-expiratory pressure of 2 cm H2O using a pressure-controlled ventilator (Hugo Sachs Elektronik, March-Huggstetten, Germany).

Isolation of lung cells

Cells were isolated from the lung as previously described [71]. The lungs were perfused with ice-cold PBS and removed. Lungs were digested in DMEM containing 200µg/ml DNase I (Roche, 10104159001), 25µg/ml Liberase TM (Roche, 05401119001) at 37°C for 2 hours. Red blood cells were then lysed and a single cell suspension was prepared by filtering through a 70-µm cell strainer.

BMDM activation

BMDMs were induced from mouse bone marrow cells cultured in RPMI 1640 (cat#11965; Invitrogen) with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 1% nonessential amino acids, 50 mM 2-ME, 1% Pen/Strep, with 20ng/ml M-CSF (Kingfisher, RP0407M) for 10 days [36]. STING agonists were added into the culture (no transfection or membrane permeabilization).

Flow cytometry

Single cell suspensions were stained with fluorescent-dye-conjugated antibodies in PBS containing 2% FBS and 1mM EDTA. Surface stains were performed at 4°C for 20 min. For intracellular cytokine or transcription factor staining of murine and human cells, cells were fixed and permeabilized with the Foxp3 staining buffer set (eBioscience, cat no 00-5523-00). Cells were washed and stained with surface markers. Cells were then fixed and permeabilized (eBioscience, cat no. 00-5523-00) for intracellular cytokine stain. Data were acquired on a BD LSRFortessa and

analyzed using the FlowJo software package (FlowJo, LLC). Cell sorting was performed on the BD FACSAriaIII Flow Cytometer and Cell Sorter. More information on the antibodies used can be found in supplementary table S1.

Human lung explants

Human lung explants were procured at the Lung Transplant Center, Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, University of Florida. Donor and patients consent was obtained for a research protocol (UF IRB201902955-Treatment with IFNβ Induces Tolerogenic Lung Dendritic Cells in Human advanced lung disease). Healthy donor lungs were surgically removed postmortem, perfused, small pieces were cut from the right middle and lower lobes for research purpose, and stored in cold Perfadex ® at 4°C for no more than 12 hrs before processing. Ex planted lungs from emphysema lung transplant patients were stored in cold Perfadex ® at 4°C for no more than 12 hrs before the process. No lung explants were procured from prisoners.

Statistical Analysis

To gain statistical power, we employ three~four mice/groups to characterize lung immunity. Ten mice/group to monitor animal health. The statistical justification for group size was calculated using the SAS program to calculate the animal numbers. The analysis was carried out using a standard error of 0.5 for immunological assays, and a power of 0.9. All data are expressed as means ± SEM. Statistical significance was evaluated using Prism 9.0 software. Comparisons between two groups were analyzed by performing an unpaired Student's *t* test. Comparisons between more than two groups were analyzed by performing a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

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Author contributions

Conceptualization: LJ

Methodology: AAT, LAS, ATP, AME

Investigation: AAT, LAS, ATP, AKS, AJB, LJ

Supervision: LJ

Writing—original draft: LJ

Writing—review & editing: LJ, AJB, AKS

Competing interests

Authors declare that they have no competing interests.

Data and materials availability

All data are available in the main text or the supplementary materials.

Supplementary Materials

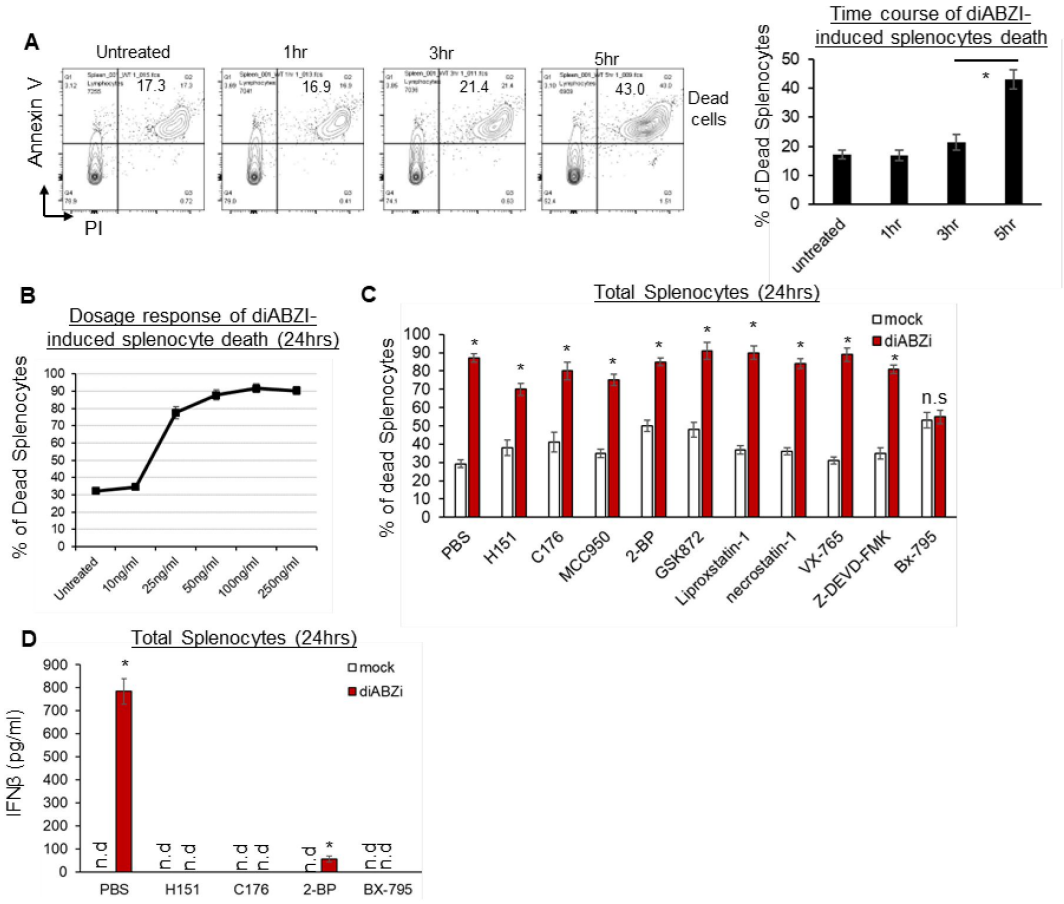

Fig. S1.

Bx-795 inhibits diABZI-induced mouse splenocyte death.

(A). Splenocytes from C57BL/6N mice were treated with 100ng/ml diABZI in culture for the indicated time. Dead cells were determined by PI and Annexin V staining. (**B).** Splenocytes from C57BL/6N mice were treated with indicated dose of diABZi in culture for 24hrs. Dead cells were determined by PI staining. **(C)**. Splenocytes from C57BL/6N mice were pre-treated with indicated small molecules, H151 (10μg/ml), C176 (1μM), MCC950 (1.25μM), 2-BP (20μM), GSK872 (312.5nM), Liproxstatin-1 (1μM), necrostatin-1 (10μM), VX-765 (1μM), Z-DEVD-FMK (25μM), Bx-795 (0.5μM) for 2hrs. diABZi (100ng/ml) was added in culture for another 24hrs. Dead cells were determined by PI staining. (**D**). IFNβ was determined in the cell supernatant from **C** by ELISA. Data are representative of three independent experiments. Graphs represent the mean with error bars indication s.e.m. n=3~5 mice/group. *p* values are determined by one-way ANOVA Tukey's multiple comparison test (**A, D**) or unpaired student T-test (**C**). * p<0.05. n.s: not significant. n.d: not detected.

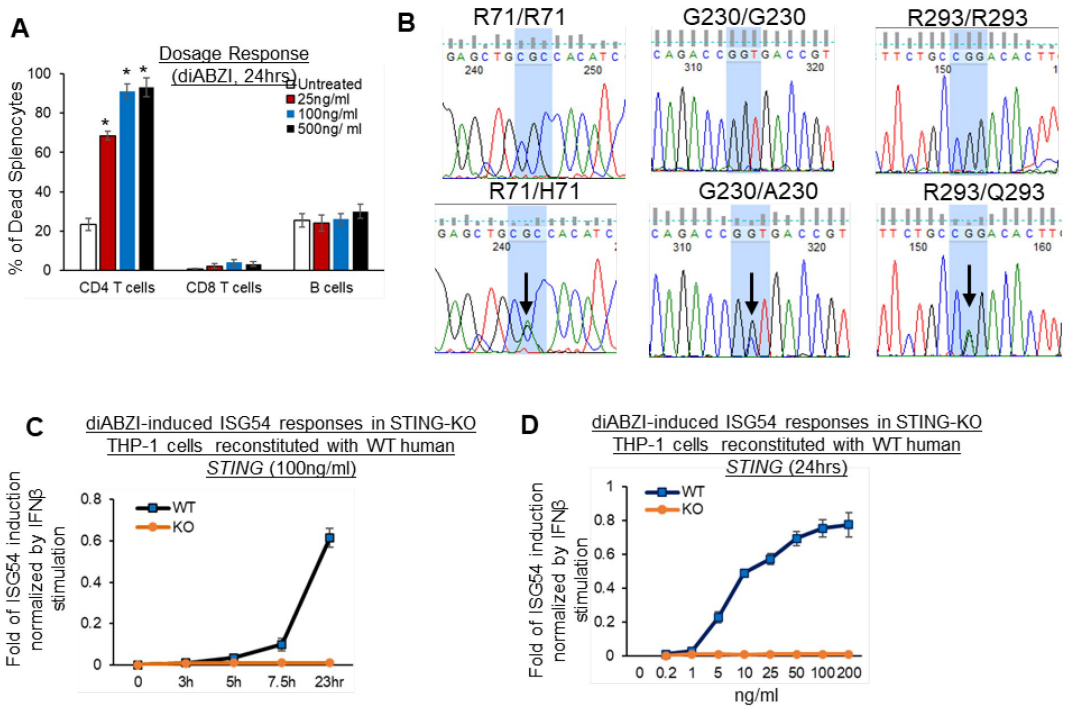

Fig. S2.

STING activation in primary human cells and THP-1 cells reconstituted with *WT* human *STING*.

(**A**). Total human Lung cells from *WT/WT* individuals were treated with indicated dose of diABZi for 24hrs. Cell death in CD4, CD8 T cells and CD19 B cells were determined by PI staining. (**B).** Sequencing results of human *TMEM173* gene in *WT/WT* and *WT/HAQ* individuals. (**C-D)**. STING-KO THP-1 cells stably reconstituted with human *WT (R232)* were treated with indicated dose of diABZI for indicated time in culture. ISG-54 reporter luciferase activity was determined in cell supernatant and normalized to 10ng/ml IFNβ-stimulated ISG-54 luciferase activity. Data are representative of at least two independent experiments. Graphs represent the mean with error bars indication s.e.m. *p* values determined by one-way ANOVA Tukey's multiple comparison test. * $p<0.05$, n.s: not significant.

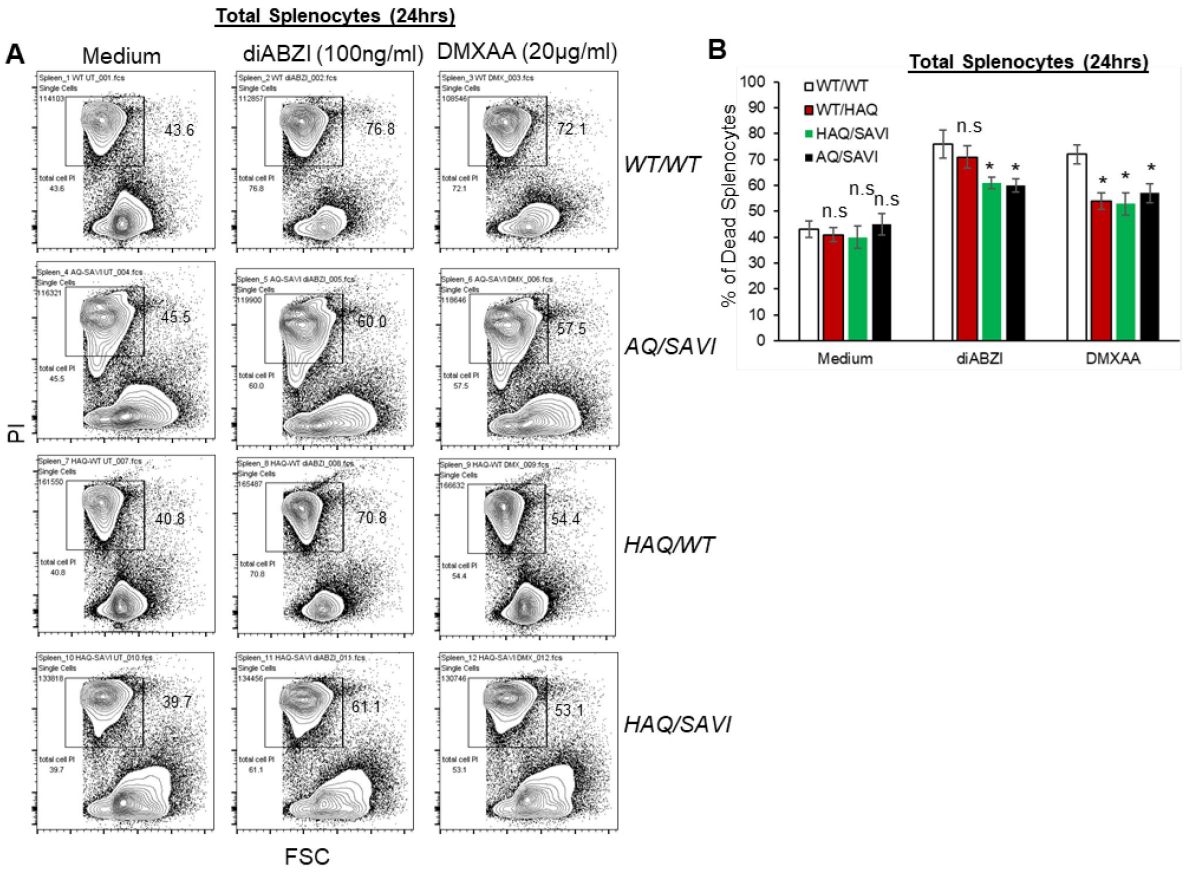

Fig. S3.

Splenocytes from HAQ/SAVI, AQ/SAVI partially resist to STING-activation-induced cell death ex vivo.

(A-B). Flow cytometry of *HAQ/SAVI*, *AQ/SAVI*, *WT/WT* or *WT/HAQ* splenocytes treated with diABZI (100ng/ml) or DMXAA (20µg/ml) for 24hrs. Cell death was determined by PI staining. Data are representative of three independent experiments. Graphs represent the mean with error bars indication s.e.m. *p* values are determined by one-way ANOVA Tukey's multiple comparison test. * p<0.05. n.s: not significant.

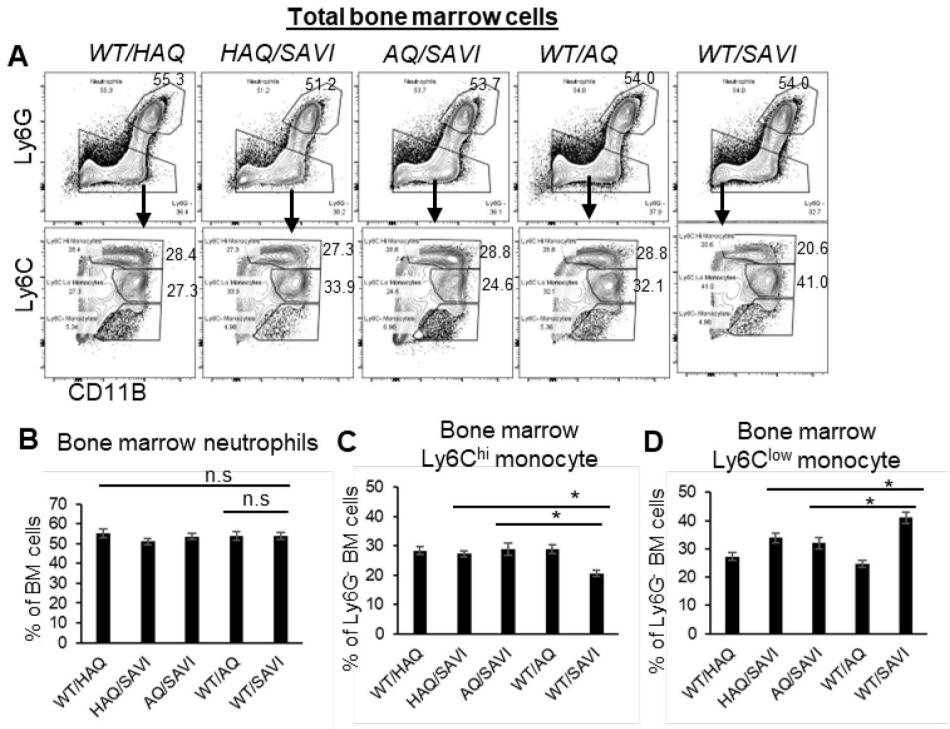

Fig. S4.

***HAQ* and *AQ* restore bone marrow monocytes in *SAVI (N153S)* mice.**

(**A-D**). Flow analysis of $Ly6G^{+}CD11B^{+}$ neutrophils and $Ly6G^{-}Ly6C^{+}CD11B^{+}$ bone marrow monocytes from HAQ/SAVI, AQ/SAVI, WT/SAVI and their littermates. n=3~5 mice/group. Data are representative of three independent experiments. Graphs represent the mean with error bars indication s.e.m. *P* values are determined by one-way ANOVA Tukey's multiple comparison test. * $p<0.05$, n.s: not significant.

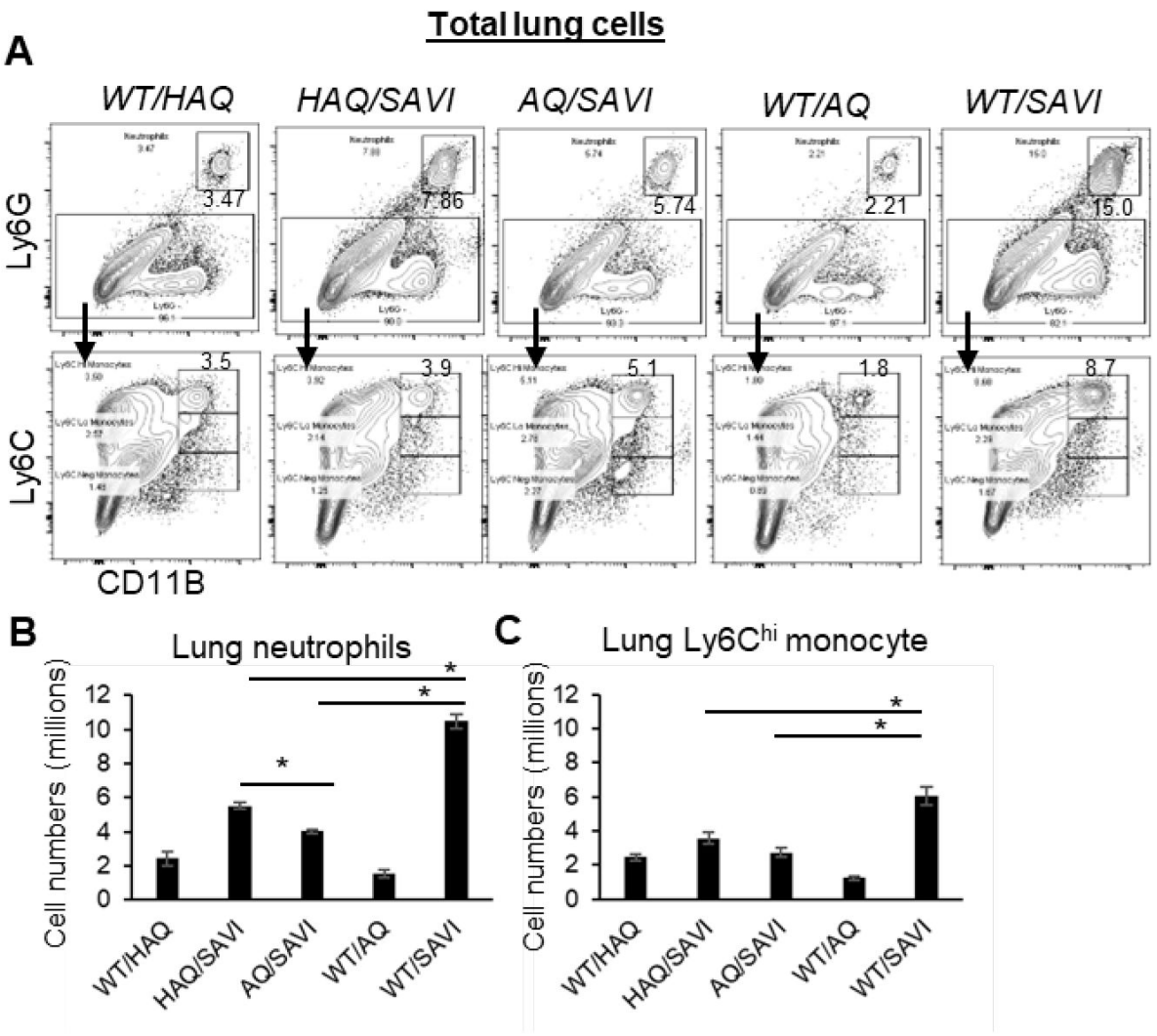

Fig. S5.

***HAQ, AQ* suppress lung myeloid cells infiltration in *SAVI(N153S)* mice.**

(A-C). Flow analysis of lung $Ly6G^{+}CD11B^{+}$ neutrophils and $Ly6G^{-}Ly6C^{+}CD11B^{+}$ monocytes from *HAQ/SAVI, AQ/SAVI, WT/SAVI and their littermates WT/HAQ, WT/AQ* mice. n=3~5 mice/group. Data are representative of three independent experiments. Graphs represent the mean with error bars indication s.e.m. *P* values are determined by one-way ANOVA Tukey's multiple comparison test. * $p<0.05$.

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Editors

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Reviewer #1 (Public Review):

Summary:

This manuscript by Aybar-Torres et al investigated the effect of common human STING1 variants on STING-mediated T cell phenotypes in mice. The authors previously made knock-

in mice expressing human STING1 alleles HAQ or AQ, and here they established a new knock-in line Q293. The authors stimulated cells isolated from these mice with STING agonists and found that all three human mutant alleles resist cell death, leading to the conclusion that R293 residue is essential for STING-mediated cell death (there are several caveats with this conclusion, more below). The authors also bred HAQ and AQ alleles to the mouse Sting1-N153S SAVI mouse and observed varying levels of rescue of disease phenotypes with the AQ allele showing more complete rescue than the HAQ allele. The Q293 allele was not tested in the SAVI model. They conclude that the human common variants such as HAQ and AQ have a dominant negative effect over the gain-of-function SAVI mutants.

Strengths:

The authors and Dr. Jin's group previously made important observations of common human STING1 variants, and these knock-in mouse models are essential for understanding the physiological function of these alleles.

Weaknesses:

However, although some of the observations reported here are interesting, the data collectively does not support a unified model. The authors seem to be drawing two sets of conclusions from in vitro and in vivo experiments, and neither mechanism is clear. Several experiments need better controls, and these knock-in mice need more comprehensive functional characterization.

https://doi.org/10.7554/eLife.96790.2.sa2

Reviewer #2 (Public Review):

Aybar-Torres and colleagues utilize common human STING alleles to dissect the mechanism of SAVI inflammatory disease. The authors demonstrate that these common alleles alleviate SAVI pathology in mice, and perhaps more importantly use the differing functionality of these alleles to provide insight into requirements of SAVI disease induction. Their findings suggest that it is residue A230 and/or Q293 that are required for SAVI induction, while the ability to induce an interferon-dependent inflammatory response is not. This is nicely exemplified by the AQ/SAVI mice that have an intact inflammatory response to STING activation, yet minimal disease progression. As both mutants seem to be resistant STING-dependent cell death, this manuscript also alludes to the importance of STING-dependent cell death, rather than STING-dependent inflammation, in the progression of SAVI pathology. I believe this manuscript makes some important connections between STING pathology mouse models and human genetics that would contribute to the field.

https://doi.org/10.7554/eLife.96790.2.sa1

Author response:

The following is the authors' response to the original reviews.

Summary Responses: Besides the *WT* allele, equivalent to the mouse *TMEM173* gene, the human *TMEM173* gene has two common alleles: the *HAQ* and *AQ* alleles carried by billions of people. The main conclusions and interpretation, summarized in the Title and Abstract, are i) Different from the *WT TMEM173* allele, the *HAQ* or *AQ* alleles are resistant to STING activation-induced cell death; ii) STING residue 293 is critical for cell death; iii) *HAQ, AQ* alleles are dominant to the *SAVI* allele; iv) One copy of the *AQ* allele rescues the SAVI disease in mice. We propose that STING research and STING-targeting immunotherapy should consider human *TMEM173* heterogeneity. These interpretations and conclusions were based

on Data and Logic. We welcome alternative, logical interpretations and collaborations to advance the human *TMEM173* research.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *This manuscript by Aybar-Torres et al investigated the effect of common human STING1 variants on STING-mediated T cell phenotypes in mice. The authors previously made knock-in mice expressing human STING1 alleles HAQ or AQ, and here they established a new knock-in line Q293. The authors stimulated cells isolated from these mice with STING agonists and found that all three human mutant alleles resist cell death, leading to the conclusion that R293 residue is essential for STING-mediated cell death (there are several caveats with this conclusion, more below). The authors also bred HAQ and AQ alleles to the mouse Sting1-N153S SAVI mouse and observed varying levels of rescue of disease phenotypes with the AQ allele showing more complete rescue than the HAQ allele. The Q293 allele was not tested in the SAVI model. They conclude that the human common variants such as HAQ and AQ have a dominant negative effect over the gain-of-function SAVI mutants.*
>
> *Strengths:*
>
> *The authors and Dr. Jin's group previously made important observations of common human STING1 variants, and these knock-in mouse models are essential for understanding the physiological function of these alleles.*
>
> *Weaknesses:*
>
> *However, although some of the observations reported here are interesting, the data collectively does not support a unified model. The authors seem to be drawing two sets of conclusions from in vitro and in vivo experiments, and neither mechanism is clear. Several experiments need better controls, and these knock-in mice need more comprehensive functional characterization.*
>
> *(1) In Figure 1, the authors are trying to show that STING agonist-induced splenocytes cell death is blocked by HAQ, AQ and Q alleles. The conclusion at line 134 should be splenocytes, not lymphocytes. Most experiments in this figure were done with mixed population that may involve cell-to-cell communication. Although TBK1-dependence is likely, a single inhibitor treatment of a mixed population is not sufficient to reach this conclusion.*

We greatly appreciate Reviewer 1's insights. We changed the “lymphocytes” to “splenocytes” (line 133) as suggested. We respectfully disagree with Reviewer 1’s comments on TBK1. First, we used two different TBK1 inhibitors: BX795 and GSK8612. Second, because BX795 also inhibits PDK1, we used a PDK1 inhibitor GSK2334470; Third, both BX795 and GSK8612 completely inhibited diABZI-induced splenocyte cell death (Figure 1B) (lines 128 – 133). The logical conclusion is “*TBK1 activation is required for STING-mediated mouse spleen cell death ex vivo*”. (line 117).

Our discovery that the common human *TMEM173* alleles are resistant to STING activation-induced cell death is a substantial finding. It further strengthens the argument that the *HAQ* and *AQ* alleles are functionally distinct from the *WT* allele 1-3. We wish to underscore the crucial message of this study-that *'STING research and STING-targeting immunotherapy should consider TMEM173 heterogeneity in humans*' (line 37), which has been largely overlooked in current STING clinical trials 4.

Regarding STING-Cell death, as we stated in the Introduction (lines 65-77). i) STING-mediated cell death is cell type-dependent 5-7 and type I IFNs-independent 5,7,8. ii) The *in vivo* biological significance of STING-mediated cell death is not clear 7,8. iii) The mechanisms of STING-Cell death remain controversial. Multiple cell death pathways, i.e., apoptosis, necroptosis, pyroptosis, ferroptosis, and PANoptosis, are proposed 7,9,10. *SAVI/HAQ*, *SAVI/AQ* prevented lymphopenia and alleviated SAVI disease in mice. Thus, the manuscript provides some answers to the biological significance of STING-cell death *in vivo*, which is new. Regarding the molecular mechanism, splenocytes from *Q293/Q293* mice are resistant to STING cell death. The logical conclusion is that the amino acid 293 is critical for STING cell death (line 29).

Extensive studies are needed, beyond the scope of this manuscript, on how aa293 and TBK1 mediates STING-Cell death to resolve the controversies in the STING-cell death fields (*e.g.* apoptosis, necroptosis, pyroptosis, ferroptosis, and PANoptosis).

> *(2) Q293 knock-in mouse needs to be characterized and compared to HAQ and AQ. Is this mutant expressed in tissues? Does this mutant still produce IFN and other STING activities? Does the protein expression level altered on Western blot? Is the mutant protein trafficking affected? In the authors' previous publications and some of the Western blot here, expression levels of each of these human STING1 protein in mice are drastically different. HAQ and AQ also have different effects on metabolism (pmid: 36261171), which could complicate interoperation of the T cell phenotypes.*

These are very important questions that require rigorous investigations that are beyond the scope of this manuscript. This manuscript, titled "*The common TMEM173 HAQ, AQ alleles rescue CD4 T cellpenia, restore T-regs, and prevent SAVI (N153S) inflammatory disease in mice*" does not focus on Q293 mice. We have been investigating the common human *TMEM173* alleles since 2011 from the discovery 11 , mouse model 1,3, human clinical trial 2, and human genetics studies 3. This manuscript is another step towards understanding these common human *TMEM173* alleles with the new discovery that *HAQ*, *AQ* alleles are resistant to STING cell death.

> *(3) HAQ/WT and AQ/WT splenocytes are protected from STING agonist-induced cell death equally well (Figure 1G). HAQ/SAVI shows less rescue compared to AQ/SAVI. These are interesting observations, but mechanism is unclear and not clearly discussed. E.g., how does AQ protect disease pathology better than HAQ (that contains AQ)? Does Q293 allele also fully rescue SAVI?*

In this manuscript, Figure 6 shows *AQ/SAVI* had more T-regs than *HAQ/SAVI* (lines 251 – 261). In our previous publication on *HAQ*, *AQ* knockin mice, we showed that *AQ* T-regs have more IL-10 than *HAQ* T-regs 3. Thus, increased IL-10+ Tregs in *AQ* mice may contribute to an improved phenotype in *AQ/SAVI* compared to *HAQ/SAVI*. However, we are not excluding other contributions (e.g. metabolic difference) (lines 332-335). We are exploring these possibilities.

> *(4) Figure 2 feels out of place. First of all, why are the authors using human explant lung tissues? PBMCs should be a better source for lymphocytes. In untreated conditions, both CD4 and B cells show ~30% dying cells, but CD8 cells show 0% dying cells. This calls for technical concerns on the CD8 T cell property or gating strategy because in the mouse experiment (Figure 1A) all primary lymphocytes show ~30% cell death at steady-state. Second, Figure 2C, these type of partial effect needs multiple human donors to confirm. Three, the reconstitution of THP1 cells seems out of place. STING-mediated cell death mechanism in myeloid and lymphoid cells are likely different. If the authors want to demonstrate cell death in myeloid cells using THP1, then these reconstituted cell lines*

> *need to be better validated. Expression, IFN signaling, etc. The parental THP1 cells is HAQ/HAQ, how does that compare to the reconstitutions? There are published studies showing THP1-STING-KO cells reconstituted with human variants do not respond to STING agonists as expected. The authors need to be scientifically rigorous on validation and caution on their interpretations.*

Figure 2 is necessary because it reveals the difference between mouse and human STING cell death, which is critical to understand STING in human health and diseases (lines 160-161). Figure 2A-2B showed that STING activation killed human CD4 T cells, but not human CD8 T cells or B cells. This observation is different from Figure 1A, where STING activation killed mouse CD4, CD8 T cells, and CD19 B cells, revealing the species-specific STING cell death responses. Regarding human CD8 T cells, as we stated in the Discussion (lines 323-325), human CD8 T cells (PBMC) are not as susceptible as the CD4 T cells to STING-induced cell death 8. We used lung lymphocytes that showed similar observations (Figure 2A). For Figure 2C, we used 2 WT/HAQ and 3 WT/WT individuals (lines 738-739). We generate HAQ, AQ THP-1 cells in STING-KO THP-1 cells (Invivogen,, cat no. thpd-kostg) (lines 380-387).

A recent study found that a new STING agonist SHR1032 induces cell death in STING-KO THP-1 cells expressing WT(R232) human STING 10 (line 182). SHR1032 suppressed THP1-STING-WT(R232) cell growth at GI50: 23 nM while in the parental THP1-STING-HAQ cells, the GI50 of SHR1032 was >103 nM 10. Cytarabine was used as an internal control where SHR1032 killed more robustly than cytarabine in the THP1-STING-WT(R232) cells but much less efficiently than cytarabine in the THP-1-STING-HAQ cells 10.

Our manuscript rigorously uses mouse splenocytes, human lung lymphocytes, THP-1 reconstituted with HAQ, AQ, and HAQ/SAVI, AQ/SAVI mice, to demonstrate that the common human HAQ, AQ alleles are resistant to STING cell death in vitro and in vivo.

We agree with Reviewer 1 that STING-mediated cell death mechanisms in myeloid and lymphoid cells may be different and likely contribute to the different mechanisms proposed in STING cell death research 7,9,10. Our study focuses on the *in vivo* STING-mediated T cellpenia.

> *(5) Figure 2G, H, I are confusing. AQ is more active in producing IFN signaling than HAQ and Q is the least active. How to explain this?*

We stated in the Introduction that "*AQ* responds to CDNs and produce type I IFNs *in vivo* and *in vitro* 3,12,13 "(line 92-93). We reported that the *AQ* knock in mice responded to STING activation 3. We previously showed that there was a negative natural selection on the *AQ* allele in individuals outside of Africa 3. 28% of Africans are *WT/AQ* but only 0.6% East Asians are *WT/AQ* 3. In contrast, the *HAQ* allele was positively selected in non-Africans 3. Investigation to understand the mechanisms and biological significance of these naturally selected human *TMEM173* alleles has been ongoing in the lab.

> *(6) The overall model is unclear. If HAQ, AQ and Q are loss-of-function alleles and Q is the key residue for STING-mediated cell death, then why AQ is the most active in producing IFN signaling and AQ/SAVI rescues disease most completely? If these human variants act as dominant negatives, which would be consistent with the WT/het data, then how do you explain AQ is more dominant negative than HAQ?*

In this manuscript, Figure 6 shows *AQ/SAVI* had more T-regs than *HAQ/SAVI* (lines 251 – 261). In our previous publication on *HAQ*, *AQ* knockin mice, we showed that *AQ* T-regs have more IL-10 and mitochondria activity than *HAQ* T-regs 3. Nevertheless, we are not excluding other contributions (e.g. metabolic difference) by the *AQ* allele (lines 332-335). Last, we used modern human evolution to discover the dominance of these common human STING alleles.

In modern humans outside Africans, *HAQ* was positively selected while *AQ* was negatively selected 3. However, *AQ* is likely dominant to *HAQ* because there is no *HAQ/AQ* individuals outside Africa. The genetic dominance of common human *TMEM173* allele is a new concept. More investigation is ongoing.

> *(7) As a general note, SAVI disease phenotypes involve multiple cell types. Lymphocyte cell death is only one of them. The authors' characterization of SAVI pathology is limited and did not analyze immunopathology of the lung.*

Both radioresistant parenchymal and/or stromal cells and hematopoietic cells influence SAVI pathology in mice 14,15. Nevertheless, the lack of CD 4 T cells, including the anti-inflammatory T-regs, likely contributes to the inflammation in SAVI mice and patients 16. We characterized lung function, lung inflammation (Figure 4), lung neutrophils, and inflammatory monocyte infiltration (Figure S5) (lines 232-235).

> *(8) Line 281, the discussion on HIV T cell death mechanism is not relevant and over-stretching. This study did not evaluate viral infection in T cells at all. The original finding of HAQ/HAQ enrichment in HIV/AIDS was 2/11 in LTNP vs 0/11 in control, arguably not the strongest statistics.*

Several publications have linked STING to HIV pathogenesis 17-22 (line 271). CD4 T cellpenia is a hallmark of AIDS. The manuscript studies STING activation-induced T cellpenia in vivo. It is not stretching to ask, for example, does preventing STING T cell death (e.g *HAQ, AQ* alleles) can restore CD4 T cell counts and improve care for AIDS patients?

> ***Reviewer #2 (Public Review):***
>
> *Aybar-Torres and colleagues utilize common human STING alleles to dissect the mechanism of SAVI inflammatory disease. The authors demonstrate that these common alleles alleviate SAVI pathology in mice, and perhaps more importantly use the differing functionality of these alleles to provide insight into requirements of SAVI disease induction. Their findings suggest that it is residue A230 and/or Q293 that are required for SAVI induction, while the ability to induce an interferon-dependent inflammatory response is not. This is nicely exemplified by the AQ/SAVI mice that have an intact inflammatory response to STING activation, yet minimal disease progression. As both mutants seem to be resistant STING-dependent cell death, this manuscript also alludes to the importance of STING-dependent cell death, rather than STING-dependent inflammation, in the progression of SAVI pathology. While I have some concerns, I believe this manuscript makes some important connections between STING pathology mouse models and human genetics that would contribute to the field.*
>
> *Some points to consider:*
>
> *(1) While the CD4+ T cell counts from HAQ/SAVI and AQ/SAVI mice suggest that these T cells are protected from STING-dependent cell death, an assay that explores this more directly would strengthen the manuscript. This is also supported by Fig 2C, but I believe a strength of this manuscript is the comparison between the two alleles. Therefore, if possible, I would recommend the isolation of T cells from these mice and direct stimulation with diABZI or other STING agonist with a cell death readout.*

Please see the new Figure S3 for cell death by diABZI, DMXAA in Splenocytes from *WT/WT, WT/HAQ, HAQ/SAVI, AQ/SAVI* mice. The *HAQ/SAVI* and *AQ/SAVI* splenocytes showed similar partial resistance to STING activation-induced cell death (lines 214-216).

> *(2) Related to the above point - further exemplifying that the Q293 locus is essential to disease, even in human cells, would also strengthen the paper. It seems that CD4 T cell loss is a major component of human SAVI. While not co_mpletely necessary, repeating the THP1 cell death experiments from Fig 2 with a human T cell line would round out the study nicely._*

We examined *HAQ, AQ* mouse splenocytes, *HAQ* human lung lymphocytes, THP-1 reconstituted with *HAQ, AQ,* and *HAQ/SAVI, AQ/SAVI* mice, to demonstrate that the common human *HAQ, AQ* alleles are resistant to STING cell death in vitro and in vivo. Additional human T cell line work does not add too much. We hope to conduct more human PBMC or lung lymphocytes STING cell death experiments from *HAQ, AQ* individuals as we continue the human *STING* alleles investigation.

> *(3) While I found the myeloid cell counts and BMDM data interesting, I think some more context is needed to fully loop this data into the story. Is myeloid cell expansion exemplified by SAVI patients? Do we know if myeloid cells are the major contributors to the inflammation these patients experience? Why should the SAVI community care about the Q293 locus in myeloid cells?*

This is likely a misunderstanding. We use BMDM for the purpose of comparing STING signaling (TBK1, IRF3, NFkB, STING activation) by *WT/SAVI*, *HAQ/SAVI*, *AQ/SAVI*. Ideally, we would like to compare STING signaling in CD4 T cells from *WT/SAVI to HAQ/SAVI, AQ/SAVI* mice. However, *WT/SAVI* has no CD4 T cells. Doing so, we are making the assumption that the basic STING signaling (TBK1, IRF3, NFkB, STING activation) is conserved between T cells and macrophages.

> *(4) The functional assays in Figure 4 are exciting and really connect the alleles to disease progression. To strengthen the manuscript and connect all the data, I would recommend additional readouts from these mice that address the inflammatory phenotype shown in vitro in Figure 5. For example, measuring cytokines from these mice via ELISA or perhaps even Western blots looking for NFkB or STING activation would be supportive of the story. This would also allow for some tissue specificity. I believe looking for evidence of inflammation and STING activation in the lungs of these mice, for example, would further connect the data to human SAVI pathology.*

Reviewer 2 suggests looking for evidence of inflammation and STING activation in the lungs of *HAQ/SAVI, AQ/SAVI*. We would like to elaborate further. First, anti-inflammatory treatments, e.g. steroids, DMARDs, IVIG, Etanercept (TNF), rituximab, Nifedipine, amlodipine, et al., all failed in SAVI patients 23. JAK inhibitors on SAVI had mixed outcomes (lines 55-58). Second, Figure S5 examined lung neutrophils and inflammatory monocyte infiltration. Interestingly, while *AQ/SAVI* mice had a better lung function than *HAQ/SAVI* mice (Figure 4D, 4E vs 4H, 4I), *HAQ/SAVI* and *AQ/SAVI* lungs had comparable neutrophils and inflammatory monocyte infiltration (Figure S5). Last, SAVI is classified as type I interferonopathy 23, but the lung diseases of SAVI are mainly independent of type I IFNs 24-27. The *AQ* allele suppresses SAVI *in vivo*. Understanding the mechanisms by which *AQ* rescues SAVI may lead to curative care for SAVI patients.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations For The Authors):***
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> *One suggestion is to streamline this study by focusing on STING-mediated cell death only in CD4 T cells. The authors can use in vitro PBMC isolated human T cells, ex vivo T cells from the knock-in mice, and in vivo T cells from the SAVI breeding. The current*

> *manuscript includes myeloid cell death, Tregs, complex SAVI disease pathology, which is too confusing and too complex to explain with the varying effect from the three human STING1 variants.*

We sincerely appreciate Reviewer 1's suggestion. The goal of our human *STING* alleles research has always been translational, i.e. improving human health. Even as a monogenetic disease, the SAVI pathology is still complex. For example, thought as a type I Interferonopathy, SAVI is largely independent of type I IFNs. Similarly, STING-activation-induced cell death, while contribute to SAVI, is not the whole story, as the Reviewer pointed out in the Comment 3 & 6 &7. *HAQ/SAVI* mice still died early and had lung dysfunction (Figure 4). In contrast, *AQ/SAVI* mice restore lifespan and lung function. We had Figure 6 show different T-regs between *AQ/SAVI* and *HAQ/SAVI* mice. In addition, *AQ* mice had more IL-10+ T-regs than *HAQ* mice 3. Therefore, we are excited about developing AQ-based curative therapy for SAVI patients (preventing cell death and inducing immune tolerance). Again, we thank the Reviewer for the suggestion. Additional research is ongoing.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Minor points*
>
> *(1) Generation of THP1 cells with the human STING alleles is missing from methods.*

We added the protocol in the methods (lines 380-387). THP-1 KO line stable expressing WT STING was first described by Weikang Tao's group 10.

> *(2) Some abbreviations are not expanded (CDA).*

CDA is expanded as cyclic di-AMP (e.g. line 375).

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