Endothelial STING controls Tcell transmigration in an IFNI dependent manner

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Abstract
The stimulator of interferon genes 1 protein senses cyclic di-nucleotides released in response to double stranded DNA, and functions as an adaptor molecule for type I interferon (IFNI) signaling by activating IFNI stimulated genes (ISG). We found impaired T cell infiltration into the peritoneum in response to TNFα in global and EC-specific STING-/- mice and discovered that T cell transendothelial migration (TEM) across mouse and human endothelial cells (EC) deficient in STING was strikingly reduced compared to control EC, whereas T cells adhesion was not impaired. STING-/- T cells showed no defect in TEM or adhesion to EC, or immobilized endothelial cell expressed molecules ICAM1 and VCAM1 compared to WT T cells. Mechanistically, CXCL10, an ISG and a chemoattractant for T cells, was dramatically reduced in TNFα-stimulated STING-/- EC and genetic loss or pharmacologic antagonism of IFN-type I interferon receptor (IFNAR) pathway reduced T cell TEM. Our data demonstrate a central role for EC STING during T cell TEM that is dependent on the ISG CXCL10 and on IFNI-IFNAR signaling.
Introduction
Mounting an inflammatory response requires a highly regulated multistep process that involves adhesive interactions of the leukocytes with the vascular endothelium. Endothelial selectins and integrin ligands adhere to leukocyte expressed selectin ligands and integrins, and the receipt of chemoattracting signals promotes leukocyte firm arrest and transendothelial migration (TEM) (2). Stimulator of interferon genes 1 is an endoplasmic reticulum protein that contributes to innate immune responses triggered by cytosolic dsDNA (3). Double stranded DNA (dsDNA) activates cytoplasmic DNA sensors that generate cGAMP that directly bind to STING and induce its phosphorylation and translocation to Golgi-endoplasmic reticulum (ERGIC). STING associates with TBK-1, IRF3, activated NFκB and other factors (4) to form a multi-molecular “signalosome” complex that promotes the transcription of interferon stimulating genes (ISG), such as CXCL10, a chemoattractant for T helper 1 cells, (5-7) and NF-κB-activated genes (8-11). In humans, gain of function mutations in TMEM173, the gene that encodes for STING, result in STING-associated vasculopathy with onset in infancy (SAVI), an inflammatory disease characterized by upregulated expression of genes associated with the type I interferon (IFNI) pathway (12). Studies in global Tmem173-/- mice (STING-/- mice) support that STING promotes inflammation in many experimental models (13-16), whereas it is critical to maintain homeostasis and prevent inflammation in other models (17,18). In endothelial cells (EC), STING has been primarily studied as an inducer of IFNI responses following viral infections (19). A recent study has demonstrated STING activation in EC occurred in a model sterile inflammation induced by free fatty acids (13). However, the cell-specific role of STING in these various inflammatory settings is poorly understood. Specifically, the role of STING in leukocyte transendothelial migration (TEM), and whether the cell-specific intrinsic actions of STING are mediated through IFNI or NF-κB activation remain to be investigated. Here, we tested the hypothesis that EC STING is required for
leukocyte TEM. We report a central role for EC STING in T cell recruitment and TEM in response to TNFα in vitro, and in vivo, that is dependent on the ISG CXCL10 and IFNAR signaling.

Results

Impaired leukocyte recruitment in response to TNF-α and thioglycollate induced peritonitis in STING-/- mice compared to WT mice. We used WT and global STING-deficient mice in two acute models of sterile inflammation to test whether STING contributes to immune cell recruitment. We first used a well-established model of peritonitis induced by TNFα to assess CD4+ T cell and Gr1+ neutrophil recruitment (20,21). After 24 hr of TNF-α stimulation we observed a three-fold increase in the recruitment of CD45+ immune cells into the peritoneal cavity of WT mice as compared to PBS treated control mice. In contrast STING-/- mice exhibited a ~50% decrease in CD45+ leukocyte recruitment (Figure 1, A and B). We further quantified CD4+ T cells and Gr1+ neutrophils within the CD45+ leukocyte gate using the gating strategy shown in Figure 1A and Supplemental Figure 1A. CD4+ T cells were abundantly recruited to the peritoneal cavity at 24h post TNF-α treatment in WT mice, whereas a 50% decrease in CD4+ T cell recruitment was observed in STING-/- mice (Figure 1C). Gr1+ neutrophil recruitment was very low and there was no significant defect in STING-/- compared to WT mice at 24h (Figure 1D). Because the recruitment of neutrophils was 10-fold less than that of CD4+ T cells at the 24h timepoint, we performed additional studies at 4h post TNFα, which coincides with robust neutrophil recruitment, as we and others have previously reported (21). As expected, neutrophil infiltration into the peritoneum was evident at 4h and significantly declined at 24h with no significant difference between WT and STING-/- mice (Figure 1, A and D). In contrast the CD4+ T cell infiltration, which was robust at 24h, was minimal at 4h with no difference in recruitment between WT and STING-/- mice (Figure 1C).
We further investigated if STING was required for thioglycollate-induced leukocyte recruitment, a well-established model of sterile inflammation that predominantly induces recruitment of monocytes and macrophages 3 days post injection (22). As expected, thioglycollate-induced significant recruitment of CD45+ cells in WT mice (Figure 1, E and F), and within the CD45+ gate, the majority of cells were CD11b+ myeloid cells (Figure 1G). Interestingly, we observed similar numbers of CD45+CD11b+ and CD4+ T cells in the peritoneal cavity of STING-/- mice treated with thioglycollate (Figure 1, E-H). Taken together, our data indicate that leukocyte recruitment in STING-/- mice is impaired in response to TNFα, but not in response to thioglycollate challenge. Our results suggest that STING regulates predominantly CD4+ T cell recruitment in response to TNFα.

**STING deficiency in EC but not in T cells results in impaired T cell TEM in response to TNFα.** We next investigated whether the impaired T cell recruitment observed in the TNFα-induced peritonitis in STING-/- mice was mediated by STING expressed in MHEC or in T cells, or in both cell types. We used a well-established *in vitro* model of TEM under flow conditions that involves primary mouse heart endothelial cells (MHEC) and Th1 cells (23,24) from both WT and STING-/- mice. STING protein was expressed in WT MHEC (Figure 2A) and its expression was similar to that reported for immune cells (25) (Supplemental Figure 2A). As expected, very few WT Th1 cells adhered to unstimulated MHEC, and the few adherent cells did not undergo TEM (Supplemental Figure 2, B and C). We did not observe any differences in the adhesion of Th1 cells to TNFα stimulated WT or STING-/- EC (Figure 2B). Strikingly, the percentage of WT Th1 cell TEM induced by TNF-α was significantly decreased in STING-/- MHEC as compared to WT MHEC (Figure 2C). This defect in TEM was not observed when STING-/- Th1 cells were perfused across WT MHEC, and, indeed, Th1 cell TEM was significantly impaired only when STING was
lacking in MHEC. (Figure 2C). WT and STING-/- Th1 cells exhibited identical levels of intracellular IFN-γ (Supplemental Figure 3), which confirms that STING deficiency does not alter in vitro Th1 cell differentiation. No further defect in TEM was observed when both Th1 cells and MHEC were lacking STING (Figure 2C). WT and STING-/- Th1 cells also showed comparable adhesion to immobilized ICAM1 and VCAM1, which are surface expressed molecules upregulated in EC by TNFα activation and are required for leukocyte adhesion and TEM (Figure 2, D-F). As a control we found that STING-/- and WT Th1 cells expressed similar levels of VLA-4 (Figure 2G) and LFA-1 (Figure 2H), ligands for VCAM1 and ICAM1, respectively. Taken together, these data demonstrate that EC STING, and not T cell STING, is central to T cell TEM under flow conditions in vitro.

Specific deletion of STING in EC results in decreased T cell recruitment in response to TNFα in vivo. To further investigate the role of EC STING in T cell recruitment in response to TNFα in vivo, we generated inducible EC-specific deficient mice (Figure 3A). To this end, we first validated EC specific recombination and found no excision band for STING in Cdh5CreERT2+/− STINGfl/fl mice in the absence of tamoxifen (an intact band was observed in a vascularized tissue such as the heart, as well as in purified MHEC). In contrast, tamoxifen-induced excision of STING in both purified and cultured EC and in the heart, but not in T cells or the splenocytes isolated from tamoxifen-treated Cdh5CreERT2+/−STINGfl/fl mice (Figure 3B). Cell specific deletion of STING was further confirmed (81.3±4.5% reduction) in STING protein expression in MHEC treated with 4OH-TMX in culture. In contrast, STING protein expression was not altered by 4OH-TMX in Th1 cells (Figure 3C and D). Moreover, when EC-STING-/- mice were challenged with TNFα i.p, we found a dramatically reduced number of CD45+ cells and CD4+ T cells in the peritoneal cavity as compared to EC-STING sufficient mice (Figure 3, E-G) at 24 h post TNFα. In contrast, Gr1+
neutrophils, which are robustly recruited to the peritoneal cavity 4h post TNFα, were similarly recruited in WT and EC-STING-/- mice at this earlier time point (Figure 3, H-J and Supplemental Figure 4). Taken together, our data demonstrate that EC STING is required for CD4+ T cell recruitment in response to TNFα.

*STING modulation of T cell TEM is dependent on the ISG CXCL10.* We further confirmed the role of EC-STING in T cell TEM using CRISPR knock down of STING (STING KD) in human umbilical vein endothelial cells (HUVEC). Using this approach, we achieved 95% KD of STING protein (Figure 4A and B). Similar to our observations in MHEC, T cell adhesion was comparable between control and STING KD HUVEC monolayers (Figure 4C), however, there was a striking reduction in TEM across STING KD HUVEC, as compared to control (Figure 4D). In contrast, neutrophil TEM was not impaired across STING KD HUVEC as compared to control HUVEC, and similar results were observed with mouse neutrophils across WT or STING-/- MHEC (Supplemental Figure 5). Given the significant reduction in T cell TEM, we hypothesized that STING deficiency lead to reduced surface expression of ICAM1 and VCAM1 in EC induced by TNFα treatment. However, we did not find differences between control and STING KD HUVEC in surface expression of ICAM1 or VCAM1 induced by TNFα, or in PECAM-1, not inducible by TNFα, and also involved in leukocyte TEM (Figure 4E) (26). Similarly, STING-/- and WT MHEC, which express high baseline levels of ICAM1 and VCAM1, as we have previously reported (23), had also similar expression of ICAM1, VCAM1 and PECAM-1 in response to TNFα stimulation (Figure 4F).

Th1 cells are characterized by the expression of CXCR3, and recognition of its ligands CXCL9 and CXCL10 is necessary for efficient Th1 cell firm arrest and TEM (27-30). CXCL10 is also an ISG. Thus, we determined the amount of CXCL10 in the conditioned media from control and
TNF-α stimulated WT and STING-/- MHEC, which showed different levels of Th1 cell TEM. As expected, CXCL10 was induced in WT MHEC in response to TNFα stimulation, and, interestingly, induction of CXCL10 was not observed in STING-/- MHEC supernatants (Figure 4G). Moreover, antibody neutralization of CXCL10 in WT MHEC significantly decreased Th1 cell accumulation (Figure 4H) and almost completely inhibited TEM (Figure 4I). Taken together, these data demonstrate that STING in EC plays a critical role in T cell TEM across TNFα-activated EC and identify the decreased levels of CXCL10 resulting from EC STING deficiency as a plausible mechanism involved.

_T cell TEM is dependent on JAK/STAT and IFNI-IFNAR signaling_. STING activation can lead to IFNI and ISG transcription (19), as well as to NF-κB activation (31). Our results indicate that two NF-κB-regulated molecules in response to TNFα, ICAM1 and VCAM1, are not altered by the absence of STING. In contrast, CXCL10, an ISG downstream IFNI, required for T cell TEM, is significantly decreased in STING-/- MHEC. Thus, we hypothesized that STING modulation of TEM was through IFNI signaling. All IFNI molecules bind to the heterodimeric IFNAR and signal through JAK1, which recruits and activates STAT1 (Figure 5A) and induces the transcription and translation of ISGs (32). A heat map of RNA-sequence analysis of WT and STING-/- MHEC showed specific patterns of expression in the IFNAR-JAK/STAT-ISG axis (Figure 5B). We validated the expression of several genes in this axis by qPCR and found that, as expected, the ISGs _Irf9, Ip10_ and _M1204_, were significantly decreased in STING-/- MHEC at baseline, and not induced by TNFα. _Zbp1_, in contrast, was not changed, and the levels of _Mtap44_ were undetectable. STAT-1 was not induced by TNFα in WT endothelial cells and was also decreased in the absence of STING (Figure 5C). These data confirm that STING regulates IFNI responses in endothelial cells. These results led us to next investigate whether MHEC IFNI contributed to T cell TEM in
response to TNFα. To that end, we inhibited the JAK/STAT pathway downstream of IFNAR with Baricitinib (BAR) in MHEC and found that BAR increased accumulation of Th1 cells to MHEC (Figure 5D), but significantly inhibited TEM (Figure 5E). Furthermore, neutralizing IFNI in TNFα treated HUVEC resulted in decreased T cell TEM as compared to control (Figure 5F). Taken together, these results demonstrate that EC IFNI signaling contributes to T cell TEM in response to TNFα and suggest a mechanism for STING regulation of TEM.

**Exogenous paracrine IFNI signaling contributes to leukocyte recruitment in response to TNF-a-induced peritonitis and to T cell TEM in vitro.** We further investigated this mechanism using the TNFα induced peritonitis model in IFNAR-/- mice (Figure 6A) that are unable to initiate exogenous paracrine IFNI signaling (33). We found decreased numbers of CD45+ cells (Figure 6, A and B) and CD4+ cells (Figure 6C) were recruited into the peritoneal cavity compared to WT control mice in TNFα treated mice. Interestingly, we noticed that PBS treated IFNAR-/- mice had significantly more CD45+ cells, and subsequently more CD4+ cells at baseline compared to WT mice (Figures 6, B and C). To assess whether the impaired recruitment to TNFα in IFNAR-/- mice was due to defective IFNI signaling in EC, we performed TEM under flow conditions in vitro in TNFα treated MHEC. We found that Th1 TEM across IFNAR-/- MHEC was significantly impaired as compared to WT MHEC, and we did not observe any differences in Th1 cell adhesion (Figure 6, D and E). Taken together, our data demonstrate that exogenous paracrine IFNI signaling in EC, via the IFNAR, contributes to leukocyte recruitment and T cell TEM in response to TNF-α in vivo and in vitro.

**Discussion**

In the present study we report a central role of EC STING in T cell TEM. We demonstrate that global STING-/- mice have impaired T cell recruitment in response to a TNFα-induced model of
peritonitis compared to WT mice, and that STING deficiency in EC, but not in T cells, results in impaired TNFα-induced T cell TEM, and loss of STING has no effect on T cell adhesion. The specific role for STING in TNFα induced T cell recruitment was further corroborated in our newly generated EC-STING-/- mice. Mechanistically, we found that STING-/- EC had decreased expression of the ISG CXCL10 after TNF-α activation, and that blocking paracrine IFNI signaling in EC similarly inhibited T cell TEM in vitro and T cell recruitment in vivo (Figure 7). Given the role of IFNI in antiviral and pro-inflammatory responses, most of our knowledge in the IFNI mechanisms induced upon STING function come from innate and adaptive immune cells (5-19). Thus, our study identifies a central role for EC STING and IFNI in T cell TEM, and significantly advances our understanding of the role STING plays in the vasculature in response to sterile inflammation.

We selected TNFα as a pro-inflammatory stimulus, common to diverse inflammatory settings, to induce peritonitis in mice, and found that T cells were abundantly recruited by 24h in WT mice as previously reported (20-21), but were strikingly reduced in global STING-/- mice. Notably, when STING-/- mice were challenged with thioglycollate, we did not find significant differences between WT and STING-/- mice in recruitment of myeloid cells. Consistent with no defect in myeloid cell recruitment, previous studies of peritoneal macrophages from STING-/- mice have not reported differences in yields between WT and STING-/- mice. Moreover, 3 days of thioglycollate peritonitis does not induce local increases in TNFα (34), in line with our data showing no induction of T cell recruitment, and no differences in T cell numbers between WT and STING-/- mice in this model, in contrast to the TNFα peritonitis model of inflammation. Our mechanistic studies using the combinations of STING-/- T cells and STING-/- MHEC, human primary T cells, and control and knock-down of STING in HUVEC, combined with our results in
EC-STING-/- mice, further support a specific role for EC STING in T cell recruitment and TEM in response to TNFα.

We further teased out the mechanisms downstream of STING that participated in T cell TEM, and investigated the NF-κB and IRF3-dependent genes, such as ICAM1 and VCAM1, and IFNI and ISGs, respectively (35). We used a well-documented model system of T cell adhesion and TEM to investigate the role of EC STING and these two downstream pathways (21,24). Somewhat surprisingly, we found that TNFα activated STING-/- EC expressed similar levels of ICAM1 and VCAM1, as their WT counterparts, given that these molecules are not only regulated by NF-κB in response to TNFα, but also play a central role in T cell adhesion and TEM (2). The expression of PECAM-1, which was not altered by TNFα but was necessary for leukocyte TEM (36), also was not affected by deletion of STING. We and others have previously reported differential expression of ICAM1 and VCAM1 in mouse and human EC, with higher constitutive expression in MHEC as compared to HUVEC, and in both cell types, further upregulated by TNFα stimulation (37). Our data demonstrate that STING deficiency did not alter inducible adhesion molecule expression in either human or mouse EC models and supports our conclusion that regulation of TEM by STING did not involve these classic components of TEM. In contrast, the ISG chemokine CXCL10 was significantly decreased in TNFα treated STING-/- MHEC. Its receptor, CXCR3, is highly expressed on Th1 cells and is regulated by T-bet, the Th1 cell signature transcription factor (38). Rapid engagement of CXCR3 by CXCL10 and CXCL9, results in T cell adhesion to and TEM of EC monolayers as we and others have previously reported (27,28,30). CXCL9 was below detectable levels in all conditions tested (not shown). Our results support the conclusion that STING regulates IFNI signaling required for CXCL10 production that promotes T cell TEM. In line with these data, we did not observe defective neutrophil recruitment or TEM in the absence
of STING, supporting the conclusion that CXL10, which is not required for neutrophil recruitment, is specifically modulated by STING in EC and is required for T cell TEM (Figure 4). Interestingly, IFNI have therapeutic benefits in certain autoimmune inflammatory diseases, and have been shown to prevent effector T cell migration to sites of inflammation through downregulation of CXCR3 (39).

STING activation leads to IFNI induction, which in turn signals through IFNAR to activate downstream JAK/STAT resulting in transcription of several ISGs in a well described signaling feedback loop as previously reported in myeloid cells (9). How this plays out in TEM regulated by STING remains to be investigated, however, our RNAseq analysis of MHEC from WT and STING-/- MHEC provides some insight. Our data indicate that STING does not regulate the expression levels of IFNAR or its downstream adaptor molecules STAT1 and STAT2, however, STING does significantly regulate the expression of several other ISG, in addition to CXCL10 at baseline, and also in response to TNFα. Interestingly, in STING-/- MHEC not all ISG are equally inhibited upon TNFα stimulation, suggesting TNFα interferes with the IFNI-IFNAR signaling feedback loop regulated by STING. Our data using the JAK/STAT inhibitors, IFNAR blockade or IFNAR-/- EC supported a critical role for IFNI downstream of STING in triggering T cell TEM. These data are in line with reports showing that IFNI signaling and STING activation in EC enhances vascular inflammation (13), and with the suppression of inflammation in vasculitis by JAK/STAT inhibitors (40). Our findings demonstrating that IFNAR-/- mice have decreased T cell recruitment in TNFα induced peritonitis further support this mechanism. Our results also reveal significantly more leukocytes were present in the peritoneal cavity of IFNAR-/- at baseline that could be indicative of either a homing defect or defective tonic IFNI signaling. This tonic signal,
reported to be central to immune cell homeostasis (41,42), may likely be critical for vascular homeostasis by preventing leukocyte infiltration into tissues in resting conditions.

Our data provide to the best of our knowledge the first experimental evidence that EC STING is central to CD4+ T cell TEM using novel mechanisms that do not involve downregulation of the transcriptionally regulated EC classic adhesion molecules involved in TEM. We suggest that STING regulation of TEM in response to TNFα is predominantly biased towards the IFNI signaling branch and less so through NF-κB, and that the decreased levels of CXCL10 in STING-/- EC is the likely explanation for impaired T cell TEM. However, while STING deficiency in EC inhibits TEM but not the preceding adhesion step, CXCL10 neutralization inhibits both steps, suggesting an additional mechanism(s) modulated by STING may be required for TEM. Moreover, how TNFα activates STING to induce IFNI in EC still remains to be defined. The CXCL10 promoter has interferon stimulated response elements (ISRE) which are bound by Interferon regulatory factors (IRF). Thus, it is likely that TNFα activation of STING transcriptionally regulates CXCL10 gene expression through IFNI dependent mechanisms (43). The CXCL10 promoter also contains NFκB binding sites, and given that TNFα induces NFκB, CXCL10 could also be activated this way. Additional studies also are needed to pinpoint the EC molecular signals in diapedesis that are dependent upon STING. One possibility is that STING deficiency alters calcium signaling through modulation of TRIPC channels, which were recently shown to contribute to leukocyte diapedesis (44). In addition, our RNA-seq analysis and subsequent validation of genes in the IFNAR-ISG axis, identified differences between WT and STING-/- EC that are unrelated to TEM and may involve other functions of endothelial cell STING beyond leukocyte TEM and inflammation, which are beyond the scope of these studies. Based on these findings, future studies using the newly generated Cad5ERTCre2+/−STINGfl/fl may focus on how
EC STING regulates other aspects of endothelial function, such as blood pressure regulation or responses to endothelial cell injury. Lastly, while we define a central role for STING in T cell recruitment, how TNFα and STING drive sustained inflammation in EC in a chronic setting has not been investigated here. CXCL10, for instance, is critical in leukocyte recruitment that occurs in heart failure (30), and IFNI signals downstream of STING have been reported to fuel cardiac damage in response to myocardial ischemia (15).

In conclusion, our study supports a novel role for EC STING in T cell TEM and provides mechanistic insight into how to modulate STING, or its downstream IFNI pathway to control undesired T cell tissue and organ infiltration during inflammation.

Methods

Reagents. Recombinant human and mTNFα and rmIL12, rmIL2, rmIL4, and carrier free human or mSDF-1α were purchased from Peprotech (Rocky Hill, NJ). Thioglycollate broth (REMEL # R064702) was purchased from ThermoFisher. Recombinant mouse E-selectin, P-selectin, ICAM1, VCAM1 Fc-chimeras and mouse CXCL10/IP-10/CRG-2 antibody (AF-466-NA) were purchased from R&D Systems (Minneapolis, MN). Antibodies to mouse cytokines and adhesion molecules were as follows: IL-2 (clone JES6-1A12), CD4 (clone GK 1.5), CD3 (clone145-2C11), CD28 (clone 37.51), LFA-1, VLA-4, PECAM-1, ICAM1 and VCAM1 as well as immune cell marker conjugated fluorophores: CD45-BV711, CD45-PE, CD3-APC/Cy7, CD4-FITC, CD11b-PerCP, GR1-APC, CD11c-PE and β-Actin antibody were purchased from Biolegend (San Diego, CA). Antibodies to human ICAM1 (Hu5/3) and VCAM1 (E1/6) have been reported previously (45) and PECAM-1 (P2B1, Developmental Studies Hybridoma Bank, Iowa, IA) were used as hybridoma conditioned media for flow cytometry studies. Baricitinib (16707) was purchased from Cayman Chemical (Ann Arbor, MI). IFNAR blockade (MAR1-5A3) was purchased from BD Pharminogen.
(Woburn, MA). Tamoxifen (Free Base) and 4-Hydroxytamoxifen were purchased from Millipore Sigma (Burlington, MA). STING/TMEM173 and β-Actin antibody were purchased from Cell Signaling Technologies, (Danvers MA). RosetteSep Human T Cell Enrichment Cocktail was purchased from STEMCELL Technologies (Tukwila, WA).

**Mice.** C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). STING/- mice were donated by Dr. Alexander Poltorak (25). IFNAR-/- and STING$^{fl/fl}$ mice were provided by Dr. Shruti Sharma. Cdhs$^{CreERT2^{-/-}}$STING$^{fl/fl}$ mice were generated by crossing mice expressing vascular cadherin promoter-driven inducible Cre (Cdhs$^{CreERT2^{-/-}}$), donated by Dr. Hong Chen (Boston Children’s Hospital, Boston MA) (46), with STING$^{fl/fl}$ mice, to generate the EC-STING mice, and EC-STING/- mice after tamoxifen administration. All mice were bred and housed in the Tufts University School facilities.

Mice were sacrificed at 8-12 weeks of age for harvest of naïve T cells or used between 8-12 weeks of age for peritonitis experiments and for tissue and blood harvest. The efficiency of genomic recombination after tamoxifen treatment in mice was determined by PCR analysis of DNA isolated from the heart (H), splenocytes (Sp), purified endothelial cells (EC) and T cells using primers P1 5’→3’ ACACGCTCTGTATTACTGACCTC, P2 5’→3’GGGGGAAAGGAGAAGACTGAC. To determine the efficiency of cell specific STING knock out after Cre recombinase, CD31+ and CD4+, cells were isolated from 3 Cdhs$^{CreERT2^{-/-}}$STING$^{fl/fl}$. CD31+ cells were grown in 12 well plates and CD4+ cells were grown in 24 well plates for 3 days and were further cultured in presence or absence of 4 hydroxy TMX (10 μmol/L) for 24h. Cells were washed and cultured for extra 24h. Treated cells from each well were lysed in 50 μl of loading buffer and 20 μl were loaded to run western blot.
In vivo TNFα and thioglycollate induced peritonitis models of leukocyte recruitment. 8-12-week-old age-matched WT, STING-/-, IFNAR-/- and EC-STING female mice were injected intraperitoneally (i.p) with either PBS or PBS containing TNFα (100ng/mouse) and 4h and 24h later cells recruited to the peritoneal cavity were harvested, counted and stained for the indicated immune cell markers and analyzed by flow cytometry. For peritonitis experiments using EC-STING mice, mice were treated for 3 consecutive days with i.p. injections of Tamoxifen (75 mg/kg dissolved in sunflower oil + 10% ethanol) followed by 2 days of rest prior to TNFα stimulation. In thioglycollate induced peritonitis, 8-12-week-old age-matched WT and STING-/- mice were injected i.p with PBS or thioglycollate (3ml of 3% aged thioglycollate broth), and 72h later cells were harvested by peritoneal lavage, counted, stained for the indicated immune cell markers and analyzed by flow cytometry.

Preparation of effector T cells and mouse neutrophil isolation. Naïve CD4+ T cells were isolated from spleen cell suspensions of WT or STING-/- mice, by positive selection using magnetic beads (Miltenyi Biotec, Carlsbad, CA). Isolated CD4+ T cells were differentiated into Th1 T cells as described by stimulation with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) in the presence of IL-12 (10 ng/ml), IL-2 (25U/ml), and anti–IL-4 (50 ng/ml). Three days following stimulation, Th1 cultures were split 1:1 with medium containing IL-2 (25 U/ml). Differentiated T cells were harvested on day 4, counted, resuspended in fresh medium, and immediately used for experiments. Neutrophils were isolated from bone marrow suspensions by negative selection using the EasySep kit (Stem Cell Technologies, Vancouver, BC) (48).

Evaluation of T cell adhesion and TEM under defined flow conditions in vitro. Mouse heart endothelial cells (MHEC) were generated from WT and STING-/- mice and grown to confluence as previously described (47). Briefly, T cell adhesion and TEM were determined under defined
laminar flow conditions in a parallel plate apparatus using video microscopy (20x objective) and the Nikon Elements NIS software as previously described (37). Briefly, MHEC monolayers were treated with TNFα (125 ng/ml) for 4h and with stromal cell-derived factor-1α (SDF-1α) (250 ng/100 µl) for 15 min prior to perfusing 2×10⁶ Th1 cells in the flow chamber. MHEC were treated with 10µM Baricitinib, a JAK1/2 inhibitor, 20 min prior to Th1 perfusion. MHEC were cultured with CXCL10 blocking antibody (0.5 µg/ml) for 4h prior to Th1 perfusion. Percent (%) TEM is represented as (TEM cells ÷ total accumulated) × 100. T cell interactions with immobilized ICAM1 (20µg/ml) and VCAM1 (40 µg/ml) were evaluated using the same videomicroscopy system determining accumulated cells after perfusion of PMA treated T cells (50ng/ml, 5 min, to induce integrin activation) at a concentration of 1 × 10⁶ cells/ml at an estimated shear stress of 1 dyne/cm² (ICAM1) and 0.5 dynes/cm² (VCAM1). Adherent cells were quantified in 5-6 different fields of view. In studies involving mouse neutrophils, neutrophils were isolated from the bone marrow extracted from the femur and tibias of WT mice using an EasySep kit from Stem Cell Technologies (Vancouver, BC) according to the manufacturer’s instructions as described (48). Neutrophil purity was >90% as determined by flow cytometry for CD11b / Ly6G double-positive cells.

**STING Knock down in HUVEC and TEM assays.** The Broad Institute GPP sgRNA Designer was used to select CRISPR guide sequences. The human STING targeting lentivirus was generated using the vector LentiCRISPR v2 (a gift from Feng Zhang, Addgene plasmid # 52961) expressing the guide sequence GCTGGGACTGCTGTTAAACG. HUVEC (sc-2) were transduced with lentivirus, and 24 hr later selected with 0.5 µg/ml puromycin in culture media for 5-6 days. Cells were then plated on fibronectin-coated glass coverslips 2 days prior to flow assays. Human CD3⁺ T-cells (>95% purity) were isolated by negative selection (STEMCELLTechnologies) from
anticoagulated whole blood obtained from healthy volunteers. Blood was obtained from volunteer donors according to Brigham and Women’s Hospital IRB approved protocols for protection of human subjects, and all volunteer subjects gave informed consent, in accordance with the Declaration of Helsinki. Isolated T cells were cultured overnight in RPMI containing 10% FCS, Glutamax (Life Technologies), Penicillin/Streptomycin and 10 ng/ml IL-2 (Peprotech). TEM assays were performed on HUVEC monolayers treated for 4h with 10 ng/ml TNFα, and 50 ng/ml SDF-1α for 20 min prior to the start of the assay. A bolus of 2 x 10^6 T cells at a concentration of 10^7/ml was drawn across the monolayers in a parallel-plate flow chamber maintained at 37°C and allowed to adhere, then a single field of view was recorded using MetaMorph software for 10 min at a shear of 1.5 dynes/cm². In studies involving neutrophils, human polymorphonuclear cells (PMNs; > 95% pure) were isolated from whole blood drawn from healthy volunteers, kept at 8°C, and used immediately as described (49).

*Flow cytometry.* Flow cytometry was performed to corroborate the differentiation of Th1 cells as described, the expression levels of VCAM1 and ICAM1 ligands on Th1 cells, and the recruitment of immune cells into the peritoneal cavity *in vivo* using the antibodies listed in the Reagents section. The data was acquired on a FACS LSRII flow cytometer and analyzed using FlowJo software (Ashland, OR).

*Western Blots.* WT, STING-/- cells were washed in cold PBS, lysed in RIPA buffer with protease inhibitors. Protein concentrations were determined with the BCA kit (ThermoFisher Scientific, Rockford, IL). ~50ug of lysate were run in an SDS-PAGE gel. The blots were incubated with STING/TMEM173 antibody (1∶500) or β-actin antibody (1∶5000). In the experiments involving Cdh5CreERT2+/− STING^fl/fl^ mice, MHEC were cultured in 12 well plates and treated with 10 μmol/L
of 4OH-TMX or vehicle for 24h. Cells were lysed directly in 2X laemmlı sample buffer (BioRad, Hercules, CA).

**RNA sequencing.** Total RNA was isolated from unstimulated and TNFα (125ng/ml) stimulated MHEC from WT and STING-/- mice using TRIzol. A directional cDNA library using TrueSeq kit was prepared and sequenced using MiSeq (Illumina). The data was analyzed using the Tuxedo tools (Bowtie, Tophat, Cufflinks/Cuffdiff/Cuffmerge/CuffCompare and CummbeRbund). The resulted fastq files were first mapped to the mouse reference genome (NCBI/mm9) using Tophat (V2.0.0). The normalization, quantification and different expression analysis of transcript level was determined using Cufflinks and Cuffdiff. The data were then visualized using Integrated Genome Viewer (from BROAD Institution) as well as CummbeRbund. Raw and processed sequencing data have been deposited in GEO (Gene Expression Omnibus) repository under accession number GSE178200.

**CXCL10 quantification.** CXCL10 was evaluated in supernatants of WT and STING-/- MHEC treated with TNFα (125µg/ml, 4h) using Eve Technologies Corporation (Calgary, CA) Discovery assays.

**Statistics.** Data were analyzed using GraphPad Prism software and presented as the mean ± SEM. Statistical analyses were done by Mann-Whitney nonparametric unpaired or Student t test when comparing 2 groups. Multiple group comparisons were performed by 1-way ANOVA with Bonferroni post hoc test where indicated. Differences were considered statistically significant at *P ≤ 0.05.

**Study approval.** All mice used were bred in accordance with the guidelines of the committee of Animal research at Tufts University School of Medicine, Tufts Medical Center and the NIH
Animal research guidelines. All animal studies were approved by the Tufts University Institutional Animal Care and Use Committee.

Author contributions

MA performed all the *in vivo* and the *in vitro* experiments involving mouse endothelial cells, analyzed the data and drafted the manuscript. GN performed all the experiments involving HUVEC. KK, FJCS and SAS participated in the experiments involving EC-STING-/- mice characterization, 4h peritonitis experiments, and in the generation of MHEC. ALB and SAS performed qPCR studies to validate RNAseq genes. VI and AP performed the RNAseq study, and SS provided intellectual input and ideas for the IFNAR studies. FWL oversaw and designed the studies in HUVEC and provided intellectual feedback in the manuscript writing, and PA designed the experiments and wrote the manuscript. All authors participated in revising the writing of the manuscript and intellectually contributed.
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Figure 1. Impaired leukocyte recruitment in response to TNF-α induced peritonitis in STING-/- mice compared to WT mice. WT and STING-/- mice received PBS, TNFα 4h, or TNFα 24h (A-D) or Thioglycollate 3 days (E-H) i.p. and the peritoneal lavage was analyzed by FACS (A) Representative flow cytometric panels of CD45+CD4+ cells recruited to the peritoneal cavity at 24h and of CD45+Gr1+ at 4h. (B-D) Quantification of total CD45+ (B), CD45+CD4+ (C) and CD45+Gr1+ (D) recruited to the peritoneal cavity. n= 3 (control); n= 12 (TNFα 24h); n=3-4 (TNFα 4h). (E) Representative flow cytometric panels of CD45+CD11b+ cells recruited to the peritoneal cavity in response to thioglycolate of three independent experiments. Quantification of CD45+ (F), CD45+CD11b+ (G) and CD45+CD4+ (H) recruited cells to the peritoneal cavity. n= 3 (control); n=8 (Thioglycollate). Data are shown as mean ± SEM values. *p<0.05, **p<0.01 and ***p< 0.001. 2-way ANOVA (B-D) and 1-way ANOVA (F-H).
Figure 2. STING deficiency in EC but not in T cells results in impaired TEM in response to TNFα.

(A) Cultured MHEC from WT and STING-/- mice were lysed and analyzed by immunoblot to evaluate STING expression and β-actin, used as a loading control. (B and C) Quantification of accumulation and %TEM respectively of WT and STING-/- Th1 cells perfused across WT and STING-/- MHEC (for WT Th1 groups: n= 3 independent experiments with WT and STING-/- MHEC preparations and Th1 preparations, in duplicate or triplicate coverslips (D). Representative images of WT and STING-/- Th1 cell adhesion on ICAM1 and VCAM1-coated coverslips following perfusion under flow conditions. Scale bar is 100 μm, (D), and quantification of Th1 adhesion on ICAM1 (n=3 independent experiments, triplicate coverslips). (E) and on VCAM1 (n=3 independent experiments) (F). Representative flow cytometry histograms and quantification of VLA-4 (G) and of LFA-1 (H) from WT and STING-/- Th1 cells (n=3 independent Th1 cell preparations) (G-H). Data are shown as mean ± SEM values. *p<0.05, **p<0.01 and ***p< 0.001. 1-way ANOVA was used for (B and C) and t test (E and H).
Figure 3. Decreased T cell recruitment into the peritoneal cavity of EC-STING-/- mice in response to TNFα (A) Schematic gene-targeting map of STING gene showing STING floxed and STING conditional alleles before and after Tamoxifen (TMX) treatment (75mg/kg body weight) and primer (P1 and P2) binding sites, with orange arrows pointing at the LoxP sites. (B) Primer pair P1 and P2 were used to detect unexcised and excised STING alleles in the heart (H), Splenocytes (Sp), MHEC (EC) and T cells (T) purified from Cad5 ERTcre2+/−STINGfl/fl treated with vehicle or TMX. (C) Quantification of STING protein expression in cultured MHEC from Cad5 ERTcre2+/−STINGfl/fl and WT Th1 cells treated with vehicle or 4OH-TMX for 24h. (D) Western blotting images of 4OH-TMX treated cell lysate. Each line is an independent cell preparation (n=3) (E) Representative flow cytometric panels of CD45+CD4+ cells recruited to the peritoneal cavity 24h after TNFα. Quantification of total CD45+ (F) and CD4+ (G) cells recruited cells to the peritoneal cavity 24h post TNFα. (H) Representative flow cytometric panels of CD45+Gr1+ cells recruited to the peritoneal cavity 4h after TNFα. Quantification of total CD45+ (I) and Gr1+ (J) cells recruited to the peritoneal cavity 4h post TNFα. Data represent n=2 independent experiments; n=3 control and n=4 TMX-treated mice per experiment (24h TNFα); n=6 animals per group (4h TNFα). *p<0.05, **p<0.01 and ***p<0.001. t test.
Figure 4. STING modulation of T cell TEM is independent of NF-κB-inducible adhesion molecules and dependent on the ISG CXCL10. (A and B) Immunoblot (A) and quantification from 3 independent experiments (B) of STING and β-actin expression in control and STING KD HUVEC monolayers using CRISPR. (C-D) CD3+ T cells isolated from human blood were perfused under flow conditions across WT and STING KD HUVEC stimulated for 4h with TNFα and adhesion (C) and %TEM (D) were quantified. n=8 Ctl and n=10 TNFα coverslips from three independent experiments (E and F) Representative histograms of surface PECAM-1, ICAM1 and VCAM1 per cell fluorescent intensity on HUVEC (E) and MHEC (F). (G) Quantification of CXCL10 in supernatants collected from WT and STING-/- MHEC at baseline and in response to 4h TNFα stimulation from n=3 cell preparations. (H and I) Quantification of accumulation (H) and %TEM (I) of Th1 cells perfused across control and anti-CXCL10-treated MHEC from n=3 independent experiments. Data are shown as mean ± SEM values. *p<0.05, **p<0.01 and ***p< 0.001. t test.
Figure 5. T cell TEM is dependent of EC JAK/STAT and IFN. (A) Schematic of IFNα and IFNβ molecules binding to the heterodimeric IFNAR and signal through JAK1 to recruit STAT1. (B) Representative heat map corresponding to Log2 values from RNAseq of WT and STING-/- control and 4h TNFα-stimulated MHEC (n=3 mice/group were pulled for RNAseq). (C) qPCR validation of STAT-1 and the indicated ISGs in WT and STING-/- control and 4h TNFα-stimulated MHEC (n=3 independent MHEC preparations, n=2 replicates per condition.) (D) Quantification of WT Th1 T cell accumulation; and (E) % WT Th1 T cell TEM across 4h TNFα-stimulated MHEC treated with JAK1 inhibitor, BAR. n= 3 independent experiments (duplicate coverslips for control). (F) Quantification of % human CD3+ T cell TEM across 4h TNFα-stimulated HUVEC with IFNAR blockade, n=4 independent experiments, triplicate coverslips. Data are mean ± SEM. *p<0.05. t test.
Figure 6

A

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B

![Graph showing CD45+ cells recruited (x10^9)]

C

![Graph showing CD4+ cells recruited (x10^9)]

D

![Graph showing accumulation (cells/mm^2)]

E

![Graph showing % TEM]
Figure 6. Exogenous Paracrine IFNI signaling contributes to leukocyte recruitment in response to TNF-ɑ induced peritonitis and TEM in vitro. (A-C) WT and IFNAR-/- mice received PBS or TNFα i.p and the peritoneal lavage was harvested and analyzed by FACS. (A) Representative flow cytometric panels of CD45+CD4+ cells recruited to the peritoneal cavity. Quantification of total CD45+ (B) and CD4+ (C) cells recruited cells to the peritoneal cavity (n=4-7 mice/group). (D-E) WT Th1 cells were perfused across TNF-ɑ-stimulated MHEC monolayers and adhesion (D) and % WT TEM (E) were quantified from n=3 independent experiments, duplicate or triplicate coverslips. Data are mean ± SEM. *p<0.05 and **p<0.01. 1-way ANOVA (B-C) and t-test (D-E).
Figure 7. Schematic representation of the role of endothelial cell Stimulator of IFN genes 1 in T cell transendothelial migration (TEM) and recruitment to sites of TNFα-mediated inflammation. The absence of endothelial STING impairs TEM in a type I interferon (IFNI) manner via the IFNAR and impairment of the IFNI induced gene CXCL10, a chemoattractant for T cells.