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Deficiency of Annexin A1 in CD4$^+$ T Cells Exacerbates T Cell–Dependent Inflammation

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Annexin A1 (AnxA1) is recognized as an endogenous anti-inflammatory molecule. However, its effects on the adaptive immune response and, in particular, on T cells remain unclear. In this study, we investigated the actions of AnxA1 in three distinct models of T cell–mediated inflammation. In contact hypersensitivity, collagen-induced arthritis, and inflammation induced by OT-II TCR transgenic T cells responding to OVA, AnxA1 deficiency significantly increased Ag-induced T cell proliferation and the resultant level of inflammation. In the contact hypersensitivity model, this was associated with increased adhesion of CD4$^+$ T cells, CD8$^+$ T cells, and neutrophils in the dermal microvasculature, as well as increased T cell expression of RORγt and IL-17A. In collagen-induced arthritis, deficiency of endogenous AnxA1 increased susceptibility to arthritis and Ag-specific T cell activation. Deficiency of AnxA1 also increased OVA-induced cutaneous delayed-type hypersensitivity and IFN-γ and IL-17 release. Transfer experiments using CD4$^+$ T cells from AnxA1$^{-/-}$ mice demonstrated that the absence of AnxA1 solely in T cells resulted in increased inflammatory responses in wild-type recipients. Similarly, experiments using AnxA1$^{-/-}$ OT-II CD4$^+$ T cells demonstrated that the absence of AnxA1 in T cells was sufficient to induce increased Ag-specific CD4$^+$ T cell proliferation in vivo, augment T cell production of IFN-γ, IL-17, TNF, and IL-6, and increase Akt, ERK, and p38 activation. Together, these findings indicate that T cell–expressed AnxA1 functions to attenuate T cell–driven inflammatory responses via T cell–intrinsic effects on intracellular signaling, proliferation, and Th1/Th17 cytokine release. *The Journal of Immunology*, **2012, 190: 000–000.**

Annexin A1 (AnxA1) is a member of the annexin superfamily of calcium- and phospholipid-binding proteins and was first defined as a secondary messenger of glucocorticoids (1). Numerous studies show that recombinant AnxA1 or AnxA1-derived N-terminal peptides mediate a broad range of anti-inflammatory actions. These include cellular effects, such as inhibition of leukocyte migration and induction of apoptosis in inflammatory cells, as well as inhibition of the activity of proinflammatory proteins, such as phospholipase A2, cyclooxygenase-2, and inducible NO synthase (2). In addition, AnxA1 was shown to inhibit NF-κB activation and production of proinflammatory cytokines (3–5) and to promote expression of the anti-inflammatory cytokine IL-10 (6). In accordance with these observations, a growing body of evidence supports a role for AnxA1 as an endogenous anti-inflammatory molecule in responses driven via the innate immune system, as shown in studies of zymosan-induced peritonitis, endotoxemia, and dextran sulfate sodium–induced colitis (4, 7, 8).

In contrast, the role of AnxA1 in adaptive immunity is much less clear. AnxA1 is expressed in T cells in humans and mice but less abundantly so than in circulating neutrophils and monocytes (9–12). Several studies provide evidence that AnxA1 acts to inhibit T cell–dependent models of inflammation (13–16). In contrast, other studies suggest that AnxA1 plays a facilitatory role in T cell–mediated responses (17–19). The reasons for these divergent data are unclear, but the complexity of CD4$^+$ T cell–mediated immune responses may be a contributing factor. Naive CD4$^+$ T cells can differentiate down several distinct proinflammatory lineages (Th1, Th2, Th17) characterized by lineage-specific expression of transcription factors and proinflammatory cytokines (20–23). Emerging evidence now indicates that AnxA1 influences the process of Th cell differentiation. Studies using human PBMCs from atopic individuals revealed that exogenous AnxA1-derived peptides inhibit Th cell proliferation and differentiation, reducing Ag-stimulated release of both Th1 and Th2 cytokines (16). In contrast, studies using murine T cells indicate that exogenous AnxA1 promotes Th1 development while inhibiting development of Th2 cells (17). This was supported by in vivo studies in the Th1/Th17-mediated collagen-induced arthritis (CIA) model in mice, which was exacerbated by administration of exogenous AnxA1 (17). Conversely, AnxA1-deficient mice showed reduced inflammation in a Th1/Th17-mediated model of experimental allergic encephalomyelitis, as well as exacerbation of Th2-mediated responses (18, 19).

These conflicting data leave unresolved the question of whether endogenous AnxA1 has inhibitory or facilitatory effects on T cells

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Abbreviations used in this article: AnxA1, annexin A1; CBA, cytometric bead array; CHS, contact hypersensitivity; CIA, collagen-induced arthritis; CII, type II collagen; DC, dendritic cell; DTH, delayed-type hypersensitivity; EdU, 5-ethynyl-2′-deoxyuridine; GILZ, glucocorticoid-induced leucine zipper; LN, lymph node; MKP-1, MAPK phosphatase-1; OXA, oxazolone; WT, wild-type.

and adaptive immune responses, as well as whether this function changes under differing conditions of T cell activation. Moreover, it is unclear whether the effects of AnxA1 on T cell–mediated inflammatory responses arise from effects of AnxA1 expressed specifically by T cells. AnxA1 has been shown to affect activation of several of the myriad signaling pathways associated with T cell activation, including Akt, p38 and ERK MAPK, and NF-κB (17, 18, 24–27). However, these effects have not been observed specifically in $CD4^+$ T cells in the context of Ag-induced activation. In this study, we examined the role of AnxA1 in models of T cell activation, using experimental approaches that enabled the restriction of AnxA1 deficiency to T cells. The findings from these experiments support the hypothesis that T cell–expressed AnxA1 inhibits T cell activation in the adaptive immune response, acting as an endogenous anti-inflammatory mediator in Ag-induced inflammation.

Materials and Methods

Mice

C57BL/6 and Rag-$1^{-/-}$ (no mature B and T cells) mice were purchased from the Walter and Eliza Hall Institute (Melbourne, VIC, Australia). AnxA$1^{-/-}$ mice were generated as described (7) and backcrossed to the C57BL/6 background for at least nine generations, in-house. OT-II TCR–transgenic (C57BL/6 background) mice, with I-A^b–restricted naive $CD4^+$ T cells recognizing the $OVA_{323–339}$ peptide, expressing the αβ TCR, Vα2, Vβ5, were maintained in-house and crossed with AnxA$1^{-/-}$ mice to generate AnxA1-deficient OT-II mice. All mice were bred and maintained in a specific pathogen–free facility. All procedures involving animals were approved in advance by the Monash University Animal Ethics Committee.

Induction of contact hypersensitivity

Contact hypersensitivity (CHS) was induced as previously described (28). In brief, mice were sensitized by 50 μl 5% oxazolone (OXA) in an acetone–olive oil vehicle (4:1) applied to the shaved back. Five days later, mice were challenged with 1% OXA (50 μl) solution on the upper right flank or with vehicle alone as a control. Mice were killed 4, 24, or 48 h after allergen challenge, and inflamed skin was taken to measure tissue swelling/edema and dried over 24 h. Data for edema are shown as wet weight/dry weight. For mice undergoing a second OXA challenge, sensitized mice were challenged again on day 7 on the same skin site with 1% OXA (50 μl). Responses were examined 4 h after the second challenge.

Histological analysis

Skin biopsies from OXA-challenged areas obtained 4, 24, and 48 h after a single challenge or 4 h after a second challenge were fixed in 10% buffered formalin and embedded in paraffin, and 4-μm sections were prepared. Skin sections were stained with H&E and assessed for leukocyte infiltration using light microscopy.

Intravital microscopy of T cell and neutrophil adhesion in the dermal microvasculature

Flank skin was surgically prepared for confocal intravital microscopy as previously described (28). Mice were anesthetized (ketamine hydrochloride, 150 mg/kg; xylazine hydrochloride, 10 mg/kg). The left jugular vein was cannulated to allow the administration of labeled Abs and additional anesthetic. A midline skin incision was made, and the flank skin and associated vasculature were separated from underlying connective tissue and extended over a heated pedestal using sutures attached to the margin. Loose dermal connective tissue was removed to allow a clear view of the dermal vasculature. The exposed area was then immersed in saline and sealed with a coverslip held in place with vacuum grease. The following Abs (eBioscience) were administered i.v. to identify specific leukocyte subsets: anti–Gr-1 PE (0.8 μg/mouse) to detect neutrophils and anti-CD4 FITC (2 μg/mouse) and anti-CD8 allophycocyanin (2 μg/mouse) to detect $CD4^+$ and $CD8^+$ cells, respectively. Preparations were viewed on a Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) using a 20× 1.0 NA water-immersion objective lens. Preparations were excited using the 488-, 543-, and 633-nm laser lines. One-minute recordings were made for ≥10 venules (25–60 μm diameter) in each animal. Recordings were viewed, adjusted, and exported for figure preparation using IMARIS software (Bitplane, Saint Paul, MN). The number of adherent leukocytes (stationary for >30 s) was determined for each leukocyte subset examined and expressed as cells/mm^2 of venular surface area.

Flow cytometry

The following Abs were used for characterization of leukocytes via multicolor flow cytometric analysis: CD4–allophycocyanin–Cy7, CD8-allophycocyanin, CD45–Pacific blue, CD19–PE–Cy7, CD11c-PE, CD40-FITC, MHC class II–allophycocyanin–Cy7, CD80-FITC, and CD86–allophycocyanin–Cy7 (all from BD Biosciences, North Ryde, NSW, Australia). For detection of 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen) incorporation, a commercially available Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit was used (Invitrogen). Cells were analyzed using a BD FACSCanto (BD Biosciences).

Assessment of in vivo T cell proliferation via EdU incorporation

T cell proliferation in vivo was measured after injection of EdU (100 μg/mouse) 20 h prior to sacrifice. Single-cell suspensions were isolated from lymph node (LN), spleen, and skin. Incorporated EdU was detected by fluorescent–azide coupling reaction, according to the manufacturer's protocol (Click-iT; Invitrogen), and analyzed by flow cytometry (BD Bioscience), accompanied by Abs against CD45, CD4, CD8, and CD19.

Real-time PCR

RNA was isolated using an RNeasy kit (QIAGEN) and reverse transcribed using Superscript III and random primers or $Oligo(dT)_{20}$ (Invitrogen). Real-time PCR was performed on a Rotor Gene RG-3000 (Corbett Research, Mortlake, VIC, Australia) for measurement of T-bet, GATA3, RORγt, IFN-γ, IL-4, TNF, IL-6, MAPK phosphatase-1 (MKP-1), glucocorticoid-induced leucine zipper (GILZ), and β-actin using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific oligonucleotide primers were used and synthesized by Invitrogen, as previously described (13, 29–31). TaqMan-predesigned real-time PCR assays (Applied Biosystems) were used for detection of IL-17A and IL-10. Data are expressed as ΔΔCT (representative of fold-increase) relative to wild-type (WT) untreated samples.

Induction of CIA

CIA was induced in mice as described previously (32). Briefly, 100 μg native bovine type II collagen (CII; Chondrex, Redmond, WA) was emulsified with CFA and injected s.c. into the base of the tail in WT and AnxA$1^{-/-}$ mice (C57BL/6 background). Mice were boosted with collagen (100 μg) in IFA on day 21 and then monitored daily for clinical features of arthritis. The clinical severity of arthritis was scored in each limb on a scale from 0 to 3: 0, no erythema or swelling; 1, slight swelling and erythema in at least some digits; 2, moderate swelling and erythema involving the entire limb or multiple limbs; and 3, pronounced swelling leading to incapacitated limbs.

Measurement of T cell proliferation in vitro

LN cells (1×10^5/well) were cultured for 72 h with stimuli as follows: T cells from CIA experiments - CII (20 μg/ml) or Con A (1 μg/ml); T cells from OVA-immunized mice or OT-II mice - $OVA_{323–339}$ peptide (0.1 and 1 μg/ml). T cell proliferation was determined by measuring [^{3}H]thymidine incorporation (0.5 μCi/well) during the final 18 h, and the incorporation of [^{3}H]thymidine was detected with a liquid scintillation beta counter (Wallac 1409; Cambridge Scientific). Data are expressed as cpm.

$CD4^+$ T cell/dendritic cell coculture experiments

$CD4^+$ T cells were isolated from LN of AnxA$1^{+/+}$ and AnxA$1^{-/-}$ OT-II mice using $CD4^+$ MACS MicroBeads (Miltenyi Biotec, North Ryde, NSW, Australia). Dendritic cells (DCs) were isolated from spleens of AnxA$1^{+/+}$ or AnxA$1^{-/-}$ OT-II mice via cell sorting (MoFlo XDP; Beckman Coulter), on the basis of CD11c expression. Isolated $CD4^+$ T cells (5×10^4 cells/well) were cultured with DCs at 500 or 1000 cells/well in the presence or absence of $OVA_{323–339}$ (1 μg/ml, 72 h). T cell proliferation was determined by measuring [^{3}H]thymidine incorporation, as described above.

Cutaneous delayed-type hypersensitivity

The OVA-specific delayed-type hypersensitivity (DTH) model was induced using a modification of a previously described technique (33). In brief, mice were immunized s.c. with 10 μg OVA in CFA (100 μl). At day 10 post-immunization, 10 μg OVA in 30 μl PBS was injected into the left footpad,

and PBS was injected into the right footpad. DTH was assessed by measuring paw swelling at 24 h using calipers. Data are expressed as the difference between the OVA-injected and control (PBS-injected) footpad.

In additional experiments, a variation of this model was performed using passively transferred $CD4^+$ T cells using a modification of a previously published method (34). In this case, donor T cells were obtained from mice immunized, as described above, at day 10 and isolated and purified as above. $CD4^+$ T cells (3×10^6) from WT or AnxA1$^{-/-}$ mice were then injected i.v. into naive Rag-1$^{-/-}$ mice; on the following day, recipient mice were immunized s.c. with OVA/CFA. The DTH response was measured as above, and cytokine release from draining LN cells was measured using ELISPOT (see below).

Ag-specific $CD4^+$ T cell proliferation in vivo and ex vivo

$CD4^+$ T cells were purified from OT-II mice using $CD4^+$ MACS MicroBeads (Miltenyi Biotec) labeled with CFSE to track cell division by flow cytometry (35) and then transferred to either WT or AnxA1$^{-/-}$ recipient mice via i.v. injection (3×10^6 cells/mouse). Mice were immunized with OVA/CFA (400 μg in 100 μl/mouse) 1 d after transfer. Three days after immunization, proliferation of splenic T cells was assessed via flow cytometric evaluation of CFSE staining.

Measurement of cytokines: cytometric bead array

Concentrations of cytokines in culture supernatants were measured using a commercially available cytometric bead array (CBA; BD Bioscience). The mouse Th1/Th2/Th17 CBA kit was used to detect IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10, as described in the manufacturer's instructions.

Measurement of cytokines: ELISPOT

Mouse IFN-γ and IL-17A ELISPOT assays were performed according to the manufacturer's protocol (BD Biosciences). Briefly, 96-well MultiScreen HTS plates were coated (5 μg/ml) with capture Abs against mouse IFN-γ (BD Biosciences) and IL-17A (eBioscience). T cells (2×10^5) were stimulated and incubated for 24 h. Plates were developed with 5-bromo-4-chloro-3-indolylphosphate–NBT solution. The number of spots was counted using an ELISPOT reader (CTL ImmunoSpot S4 Analyzer).

Ig ELISA

Serum CII-specific IgGs (IgG, IgG1, and IgM) were measured by ELISA, as described previously (32).

Assessment of activation of Akt and MAPK

The functional roles of Akt, ERK MAPK, and p38 MAPK in in vitro T cell activation experiments were assessed using the following inhibitors: Akt - Akt inhibitor VIII (20 μM; Calbiochem, San Diego, CA); ERK - MEK inhibitor PD98059 (50 μM), p38 – SB203580 (5 μM) (both from Alexis Biochemicals, San Diego, CA). In these experiments, the concentrations of TNF and IL-17 in culture supernatants were measured using commercially available ELISAs from R&D Systems (Minneapolis, MN) and from eBioscience (San Diego, CA), respectively. The detection threshold of these assays is 15.6 pg/ml.

Activation of these intracellular signaling pathways was assessed via Western blotting, as previously described (36). In brief, spleen and LNs from either AnxA1$^{+/+}$ or AnxA1$^{-/-}$ OT-II mice were pooled, and erythrocytes were lysed. The prepared cells (4×10^6/well) were then treated with $OVA_{323–339}$ (1 μg/ml) for various times up to 120 min. Cell lysates were then collected using Cell Lysis Buffer (Cell Signaling Technology, Arundel, QLD, Australia) supplemented with complete mini protease inhibitor mixture (Roche). Forty micrograms of lysate protein was separated on 10% SDS-polyacrylamide electrophoresis gels and transferred to Hybond-C extra nitrocellulose membranes (Millipore, Bedford, MA). Membranes were probed with Abs against phospho-ERK, phospho-p38, phospho-Akt (all from Cell Signaling Technology), and β-actin (Sigma). Anti-mouse IgG and anti-rabbit IgG Abs conjugated to Alexa Fluor 700 (Rockland, Stepney, SA, Australia) and IRDye 800 (Rockland), respectively, were used to probe primary Abs. Protein bands were detected and quantified using the Odyssey system (Li-Cor, Surrey Hills, VIC, Australia). Densitometry data are expressed as a ratio of the phosphorylated protein to β-actin; in each experiment, data were normalized to readings from unstimulated WT cells.

Statistics

One-way ANOVA with the Newman–Keuls multiple comparison post hoc test was used when more than one treatment was compared with a control. The Student *t* test was used when only two variables were compared. Differences between groups with regard to CIA incidence were assessed using the χ^2 test. Results are expressed as mean ± SEM; $p < 0.05$ was considered statistically significant.

Results

AnxA1 deficiency increases skin inflammation in CHS

We first examined the role of AnxA1 in OXA-induced dermal CHS. This model differs from other adaptive immune models in that the site of inflammation can undergo multiple antigenic challenges, with the nature of the effector response changing following repeated challenge toward a Th2-dominant phenotype (28, 37). Mice were sensitized with OXA and challenged 5 d later (first challenge). Skin edema was evaluated 0, 4, 24, and 48 h after challenge, and histological inflammation was evaluated at the 24-h time point. WT and AnxA1$^{-/-}$ mice developed comparable skin inflammation as measured by histopathology (Fig. 1A) or by skin edema (Fig. 1B), peaking 24 h after challenge in both strains. We next assessed the response to a second OXA challenge. As we recently reported, the inflammatory response after a second challenge peaks much earlier than in the first challenge (28). Therefore, mice were assessed after 4 h. At this point, both WT and AnxA1$^{-/-}$ mice showed severe leukocyte infiltration, but edema was significantly increased in AnxA1$^{-/-}$ mice compared with WT controls (Fig. 1C, 1D). These results indicate that the absence of AnxA1 results in increased inflammation in a Th2-associated repeat-challenge model of hapten-induced CHS.

AnxA1 deficiency increases adhesion of T cells and neutrophils in the inflamed dermal microvasculature

Previous studies indicated that AnxA1 regulates neutrophil–endothelial interactions in models of acute inflammation (38). However, it is unknown whether the inhibitory effect of AnxA1 on leukocyte trafficking extends to T cells. Therefore, we used subset-specific surface markers and confocal intravital microscopy to examine the adhesive interactions of $CD4^+$ T cells, $CD8^+$ T cells, and neutrophils in dermal venules following one or two hapten challenges. As previously described, 24 h after a single challenge, leukocyte adhesion was significantly elevated above basal levels,

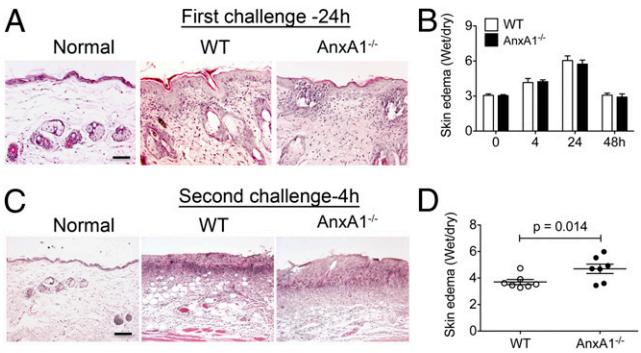

FIGURE 1. Absence of AnxA1 increases skin inflammation in CHS. WT and AnxA1$^{-/-}$ mice were sensitized with OXA on day 0 and challenged on day 5 (first challenge) or on days 5 and 7 (second challenge), on the abdominal skin, to elicit a CHS response. (**A**) Skin histology (H&E) in normal (nonsensitized) versus sensitized WT and AnxA1$^{-/-}$ mice 24 h after a single challenge. Scale bar, 20 μm. (**B**) Skin swelling in untreated skin (0), as well as at 4, 24, and 48 h after a single challenge, as determined by the wet weight:dry weight ratio. (**C**) Skin histology (H&E) in normal (nonsensitized) versus sensitized WT and AnxA1$^{-/-}$ mice 4 h after a second challenge. Scale bar, 20 μm. (**D**) Skin swelling 4 h after a second challenge, as determined by the wet weight:dry weight ratio (each symbol represents an individual animal). Data were derived from at least two independent experiments and are shown as mean ± SEM. $n = 4$ for untreated and challenge 1, 4 h. $n = 7$ for all other groups.

with the adherent leukocyte population comprising numerous neutrophils, with less frequent $CD4^+$ and $CD8^+$ T cells (Fig. 2A) (28, 39). At this time point, adhesion of $CD4^+$ T cells was significantly increased in $AnxA1^{-/-}$ mice relative to WT mice, whereas adhesion of $CD8^+$ T cells and neutrophils was comparable (Fig. 2B). Following a second challenge, $CD4^+$ T cell, $CD8^+$ T cell, and neutrophil adhesion were all significantly increased in $AnxA1^{-/-}$ mice relative to the responses in WT mice (Fig. 2C). The increase in neutrophil adhesion in $AnxA1^{-/-}$ mice in response to the second, but not first, challenge parallels the increase in edema observed in these animals at the same stage (Fig. 1). These results point to a role for AnxA1 in suppressing the intravascular adhesion of T cells and neutrophils during T cell–dependent inflammation, particularly in response to inflammation induced by a repeat-challenge model.

AnxA1 deficiency increases in vivo T cell proliferation in CHS

Numerous studies demonstrated that inflammation in CHS is dependent on Ag-specific T cell activation (40, 41). To examine whether increased T cell activation underlies the increased CHS-associated inflammation in $AnxA1^{-/-}$ mice, we compared in vivo T cell proliferation in WT and $AnxA1^{-/-}$ mice undergoing CHS, by evaluating T cell incorporation of EdU. After a single challenge, Ag exposure induced the proliferation of T cells in the draining LNs of WT mice, peaking at 48 h (Fig. 3A; for representative flow cytometry plots, see Supplemental Fig. 1). T cell proliferation was accelerated in AnxA1-deficient mice, being significantly greater than in WT mice at 4 and 24 h (Fig. 3A). Examination of $CD4^+$ and $CD8^+$ lymphocytes demonstrated that AnxA1 deficiency affected proliferation of both subsets at these time points (Fig. 3B, 3C). At this time point, no Ag-induced proliferation was observed in splenocytes of either strain, and proliferation of T cells in the skin was not detected (data not shown).

After a second challenge, proliferation of T cells was no longer apparent in the draining LN. However, examination of both the skin and spleen demonstrated that T cell proliferation was significantly increased in $AnxA1^{-/-}$ mice at these sites (Fig. 3D, 3E; for representative flow cytometry plots, see Supplemental Fig. 2). This finding indicates that, in this multiple-challenge model, the locations of Ag-induced T cell proliferation change over the extended time course examined. Increased splenic T cell proliferation in the setting of AnxA1 deficiency was seen in both $CD4^+$ and $CD8^+$ T cells (Fig. 3F). These data indicate that endogenous AnxA1 has an inhibitory effect on T cell proliferation in CHS and that this applies both in the secondary lymphoid organs and, at later stages, in the target tissue of the effector response as well.

AnxA1 deficiency increases RORγt and IL-17A mRNA expression in T cells

To address a further potential mechanism underlying the increased dermal inflammation during CHS in $AnxA1^{-/-}$ mice, we investigated the effects of AnxA1 on the expression of transcription factors associated with Th subset differentiation, as well as Th cytokine mRNA expression. Evidence supports roles for IL-17A and Th2 cytokines in the development of inflammation during OXA-induced CHS (21). This led us to hypothesize that AnxA1 regulates CHS by enhancing $CD4^+$ T cell polarization along the Th17 and Th2 pathways. To address this, expression of transcription factors relevant to Th1/Th2/Th17 lineages was compared in WT and $AnxA1^{-/-}$ mice via examination of cells from LNs draining the site of challenge. Cells were analyzed 4 h after a second challenge via quantitative PCR for expression of T-bet, GATA3, and RORγt, as well as related T cell cytokines IL-4, IL-10, IL-17A, IL-6, and IFN-γ. T-bet, a Th1-related transcription factor, was comparably expressed in control and $AnxA1^{-/-}$ mice (Fig. 4A). Similarly, there was no significant difference in IFN-γ mRNA (Fig. 4B), indicating that AnxA1-mediated regulation of CHS is independent of effects on Th1 cytokines. GATA3, a transcription factor expressed in Th2 cells, was significantly increased in $AnxA1^{-/-}$ LN cells compared with WT cells (Fig. 4C). However, there was no difference in the expression of mRNA for the Th2 cytokines IL-4 and IL-10 between WT and $AnxA1^{-/-}$ mice (Fig. 4D, 4E). In contrast, RORγt, a transcription factor that drives Th17 cell differentiation, was significantly increased in $AnxA1^{-/-}$ LN cells in comparison with WT controls, and this was accompanied by increased IL-17A mRNA (Fig. 4F, 4G). These data support the hypothesis that the increased skin inflammation in $AnxA1^{-/-}$ mice undergoing repeated hapten challenge is chiefly associated with increased Th17 activation.

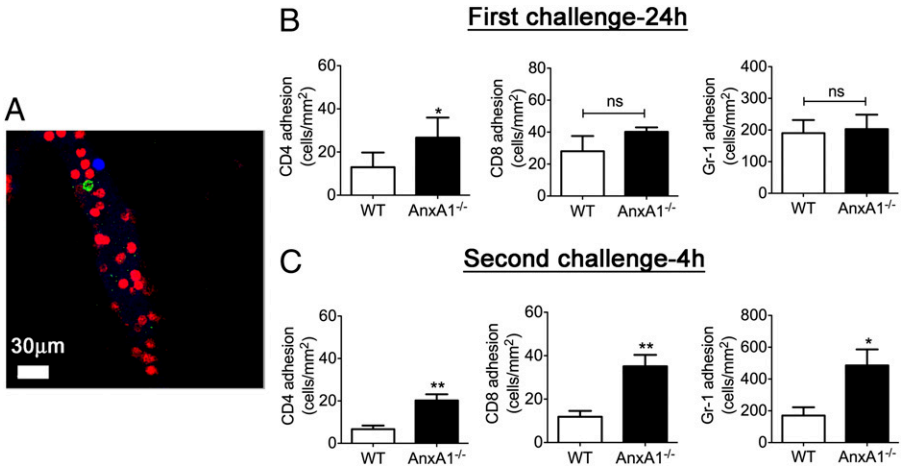

FIGURE 2. AnxA1-deficient mice display increased T cell and neutrophil adhesion in the dermal vasculature during CHS. WT and $AnxA1^{-/-}$ mice underwent CHS experiments, and the dermal microvasculature was examined via confocal intravital microscopy 24 h after the first OXA challenge, as well as 4 h after the second challenge, in separate groups of mice. $CD4^+$ T cells, $CD8^+$ T cells, and neutrophils were identified using differentially labeled subset-specific Abs administered i.v. (**A**) In vivo confocal microscopy image showing $CD4^+$ T cells (green), $CD8^+$ T cells (blue), and neutrophils (red) interacting with the endothelium of an inflamed dermal vessel during a CHS response in a WT mouse. Number of adherent $CD4^+$ T cells, $CD8^+$ T cells, and neutrophils ($Gr\text{-}1^+$ cells) in WT and $AnxA1^{-/-}$ mice 24 h after a single challenge (**B**) or 4 h after a second challenge (**C**). Data are shown as mean ± SEM (n = 4–8 mice/group from at least three separate experiments). $^*p < 0.05$, $^{**}p < 0.01$, versus WT mice. ns, Not significant.

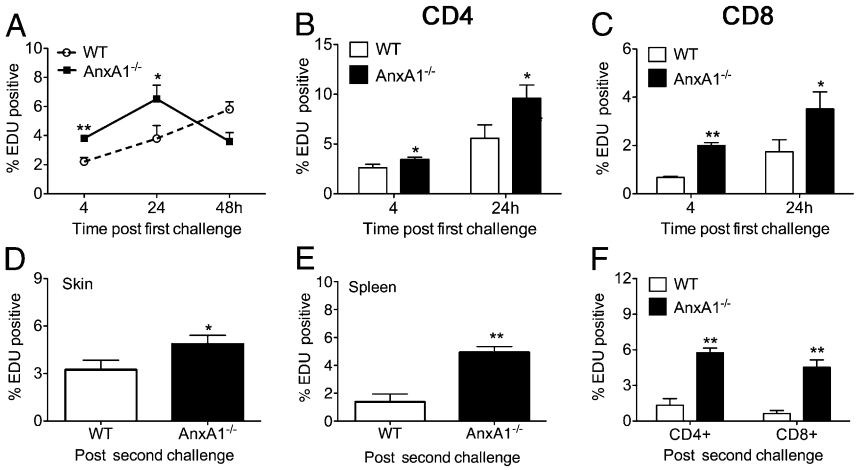

FIGURE 3. T cell proliferation in CHS is increased in the absence of AnxA1. To compare in vivo T cell proliferation in WT and AnxA1$^{-/-}$ mice, sensitized WT and AnxA1$^{-/-}$ mice were challenged with OXA on day 5 (**A**–**C**, single challenge) or on days 5 and 7 (**D**–**F**, two challenges) to elicit CHS. To assess lymphocyte proliferation in vivo, mice were injected with EdU 20 h prior to being sacrificed. Flow cytometry was used to quantitate EdU$^+$ T cells in LNs, spleen, and inflamed skin. (A) Percentage of EdU$^+$ cells (as a proportion of total CD45$^+$ CD19^{-ve} cells) in draining LNs of WT and AnxA1$^{-/-}$ mice at 4, 24, and 48 h after a single challenge. Percentage of EdU$^+$ CD4$^+$ (B) and CD8$^+$ (C) T cells in draining LNs of WT and AnxA1$^{-/-}$ mice 4 and 24 h after a single challenge. Percentage of EdU$^+$ cells (as a proportion of total CD45$^+$ CD19^{-ve} cells) in inflamed skin (D) and spleen (E) 4 h after the second challenge. (F) Percentage of EdU$^+$ CD4$^+$ and CD8$^+$ T cells in spleens of WT and AnxA1$^{-/-}$ mice 4 h after the second challenge. Data are shown as mean ± SEM of four to nine mice/group from at least two separate experiments. $*p < 0.05$, $**p < 0.01$, versus WT mice.

Absence of AnxA1 increases lymphocyte proliferation and disease incidence in CIA

We next investigated whether these inhibitory effects of AnxA1 on T cell–mediated inflammation also applied in CIA, the classical T cell–dependent model of rheumatoid arthritis in which exogenous AnxA1 administration was reported to exert amplifying effects (17). CIA is mediated by an immune response directed against CII and is dominated by Th1 and Th17 pathways (42, 43). As previously observed, WT (C57BL/6) mice were relatively resistant to the development of CIA, with <40% of mice developing arthritis (Fig. 5A). In contrast, 80% of AnxA1$^{-/-}$ mice developed disease within the 47-d experimental time course (Fig. 5A), an incidence that was significantly greater than that in WT mice. AnxA1$^{-/-}$ mice also exhibited significantly increased CII-induced splenocyte proliferation relative to WT splenocytes (Fig. 5B), in accordance with our findings in CHS. Moreover, basal and Con A–induced proliferation were also significantly increased in AnxA1$^{-/-}$ splenocytes (Fig. 5B). Circulating Ag-specific IgG, IgG1, and IgM concentrations did not differ between WT and AnxA1$^{-/-}$ mice (Fig. 5C–E), indicating that the increased arthritis incidence in AnxA1$^{-/-}$ mice was independent of changes in the humoral response.

Finally, given the consistent finding of increased inflammation in these T cell–dependent models of inflammation, we asked whether these differences were associated with alterations in T cell development in the absence of AnxA1. To address this, we compared the phenotypes of lymphocytes in LNs (Table I) and spleens (data

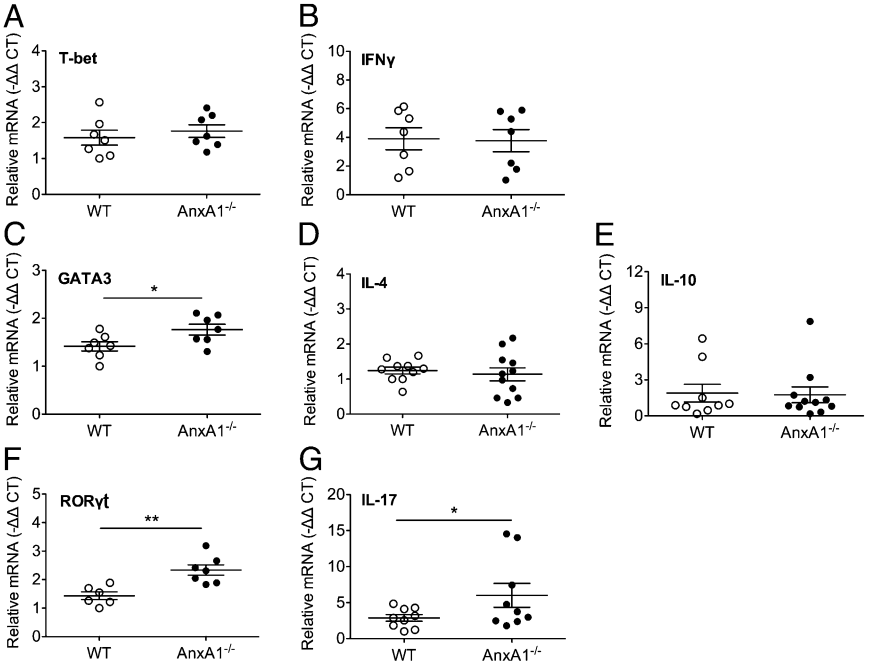

FIGURE 4. Deficiency of AnxA1 increases T cell transcription factor RORγt and IL-17A mRNA during CHS. Transcription factor and cytokine mRNA were assessed in LN cells from WT and AnxA1$^{-/-}$ mice that underwent the two-challenge CHS model. Samples were obtained 4 h after a second challenge and examined by real-time PCR. Data are shown for Th1-associated molecules T-bet (**A**) and IFN-γ (**B**); Th2-associated molecules GATA3 (**C**), IL-4 (**D**), and IL-10 (**E**); and Th17-associated molecules RORγt (**F**) and IL-17A (**G**). Each symbol represents an individual animal, and the lines/bars represent the mean ± SEM. Data were generated in at least two separate experiments. $*p < 0.05$, $**p < 0.01$.

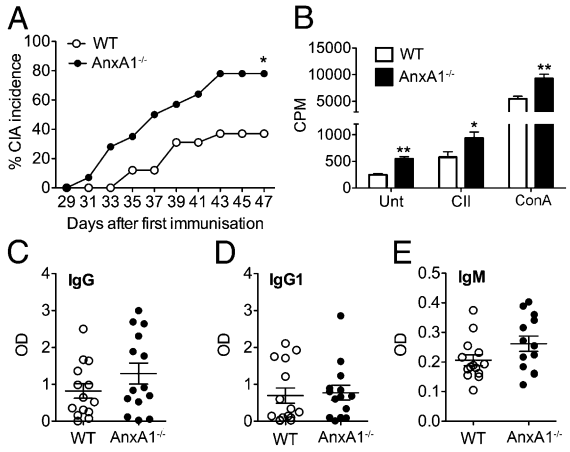

FIGURE 5. AnxA1$^{-/-}$ mice show increased disease susceptibility and lymphocyte proliferation in CIA. CIA was induced in WT (C57BL/6) and AnxA1$^{-/-}$ mice. (**A**) Cumulative arthritis incidence in WT and AnxA1$^{-/-}$ mice. *$p < 0.05$, χ^2 test. Data are derived from a total of 16 WT and 14 AnxA1$^{-/-}$ mice and were generated in two separate experiments. (**B**) Splenocyte proliferation 47 d after immunization, as determined by [^{3}H] thymidine incorporation. Results are shown for proliferation of untreated cells (Unt), Ag-specific stimulation (CII), or nonspecific stimulation (Con A). Data are mean ± SEM. Circulating serum titers of CII-specific IgG (**C**), IgG1 (**D**), and IgM (**E**) were determined in individual mice by ELISA. Each symbol represents an individual animal, and lines/bars represent the mean ± SEM. *$p < 0.05$, **$p < 0.01$, versus WT mice.

not shown) of WT and AnxA1$^{-/-}$ mice, both in the absence of stimulation and following immunization. These data show that the proportions of CD3$^+$, CD4$^+$, and CD8$^+$ T cells, as well as B cells (identified as CD19$^+$ cells), did not differ significantly between WT and AnxA1$^{-/-}$ mice under both conditions. These findings indicate that the absence of AnxA1 does not result in significant changes in T cell subset distribution.

Restriction of deficiency of AnxA1 to CD4$^+$ T cells results in exacerbation of T cell–dependent responses

Although demonstrating the importance of endogenous AnxA1 in T cell–mediated inflammation in two distinct models, the preceding studies do not define whether these effects of AnxA1 were exerted in T cells or depend on the effects of AnxA1 expressed by other cells involved in these responses. Therefore, to facilitate the assessment of the specific role of T cell–expressed AnxA1, we next examined T cell–driven inflammation in response to the model Ag OVA. We first performed experiments to determine whether the absence of AnxA1 increased adaptive immune responses to OVA, as seen in the other models used in this study. Following immunization with OVA, local injection of OVA induced a dermal DTH response, as assessed by skin swelling (Fig. 6A). OVA-induced DTH (Fig. 6B) and basal and OVA-induced proliferation of lymphocytes from the draining LNs (Fig. 6C) were all increased in Anxa1$^{-/-}$ mice relative to WT mice, consistent with our findings in CHS and CIA. Similarly, OVA-induced IFN-γ and IL-17A release were also significantly increased in AnxA1$^{-/-}$ lymphocytes (Fig. 6D–F).

Table I. Lymphocyte phenotypes of WT and AnxA1$^{-/-}$ mice before and after immunization

Lymphocyte Subset	Unstimulated		Postimmunization	
	WT (n = 3)	AnxA1$^{-/-}$ (n = 5)	WT (n = 6)	AnxA1$^{-/-}$ (n = 6)
CD3$^+$	57 ± 7	64 ± 2	52 ± 3	49 ± 5
CD4$^+$	38 ± 1	44 ± 3	31 ± 1	28 ± 1
CD8$^+$	20 ± 1	22 ± 1	15 ± 1	16 ± 1
CD19$^+$	19 ± 2	17 ± 2	29 ± 4	29 ± 4

LN cells from WT and AnxA1$^{-/-}$ mice were examined in naive mice and 14 d after immunization with BSA in CFA. Data are expressed as percentage of total CD45$^+$ cells.

We next used the same model to examine the effect of restricting AnxA1 deficiency to the T cell. This was achieved using adoptive transfer of AnxA1$^{+/+}$ or AnxA1$^{-/-}$ LN cells into T cell–deficient Rag-1$^{-/-}$ mice. Following LN cell transfer, and subsequent immunization and challenge with OVA, mice that received AnxA1$^{-/-}$ LN cells developed significantly greater DTH than those that received AnxA1$^{+/+}$ LN cells (Fig. 6G). As described for the other models, the absence of AnxA1 from T cells was associated with significantly elevated T cell proliferation, both basally and in response to cognate Ag (Fig. 6H). In addition, AnxA1$^{-/-}$ T cells isolated from skin-draining LNs in these experiments generated higher amounts of both IL-17A and IFN-γ (Fig. 6I, 6J). Together, these findings demonstrate that the absence of AnxA1 specifically in the Ag-responding T cell is sufficient to result in increased inflammation in this model of adaptive immunity.

As an alternative approach to assessing this issue, we next used OT-II mice, in which the majority of CD4$^+$ T cells recognize OVA, and generated AnxA1$^{-/-}$ OT-II mice to facilitate assessment of the role of AnxA1 in CD4$^+$ T cells. In this TCR-transgenic model, the development of Ag-specific CD4$^+$ T cells, as well as their CD69 expression in the absence of stimulation, did not differ between AnxA1$^{+/+}$ and AnxA1$^{-/-}$ OT-II cells (Supplemental Fig. 3), indicating that the absence of AnxA1 did not result in overt changes in Ag-specific CD4$^+$ T cell development or activation. In in vitro proliferation experiments, AnxA1$^{-/-}$ OT-II LN cells responded to the OT-II CD4$^+$ T cell–specific peptide OVA$_{323–339}$ to a significantly greater extent than AnxA1$^{+/+}$ OT-II LN cells (Fig. 7A). Similarly, AnxA1$^{-/-}$ OT-II LN cells produced significantly higher amounts of IL-6, TNF, IFN-γ, and IL-17A following activation with OVA$_{323–339}$ (Fig. 7B), which was accompanied by increased expression of TNF and IL-17A mRNA (Fig. 7C, 7D). IL-4 and IL-10 were undetectable in these assays (data not shown). These findings demonstrate that the proactivation effect of AnxA1 deficiency is maintained on the OT-II background. To isolate this effect specifically to AnxA1 expressed by CD4$^+$ cells, we purified CD4$^+$ T cells from AnxA1$^{+/+}$ and AnxA1$^{-/-}$ OT-II mice and assessed their proliferation in response to OVA$_{323–339}$ in coculture experiments with WT DCs. Under conditions in which the same population of DCs was responsible for Ag presentation, proliferation of AnxA1$^{-/-}$ OT-II cells was significantly greater than that of AnxA1$^{+/+}$ cells (Fig. 7E). In contrast, AnxA1$^{+/+}$ OT-II cells proliferated to a similar degree when cultured with either AnxA1$^{+/+}$ or AnxA1$^{-/-}$ DCs (Fig. 7F). Moreover, the absence of AnxA1 did not affect DC expression of maturation markers CD80, CD86, MHC class II, and CD40 (Supplemental Fig. 4). These findings indicate that the absence of AnxA1 from DCs was not responsible for the increased proliferation of AnxA1$^{-/-}$ T cells.

We next used adoptive-transfer experiments to investigate whether the CD4$^+$ T cell–restricted proactivation phenotype of AnxA1 deficiency was maintained in the in vivo setting. Following transfer to WT mice that subsequently underwent OVA immunization, AnxA1$^{-/-}$ OT-II cells displayed significantly greater in vivo proliferation than did AnxA1$^{+/+}$ OT-II cells undergoing the identical protocol (Fig. 7G, 7H). In contrast, AnxA1$^{+/+}$ OT-II cells transferred into either WT or AnxA1$^{-/-}$ recipients showed comparable proliferation (Fig. 7I, 7J). Together, these findings provide further evidence that the absence of AnxA1 in CD4$^+$ T cells alone is sufficient to confer increased proliferation and activation.

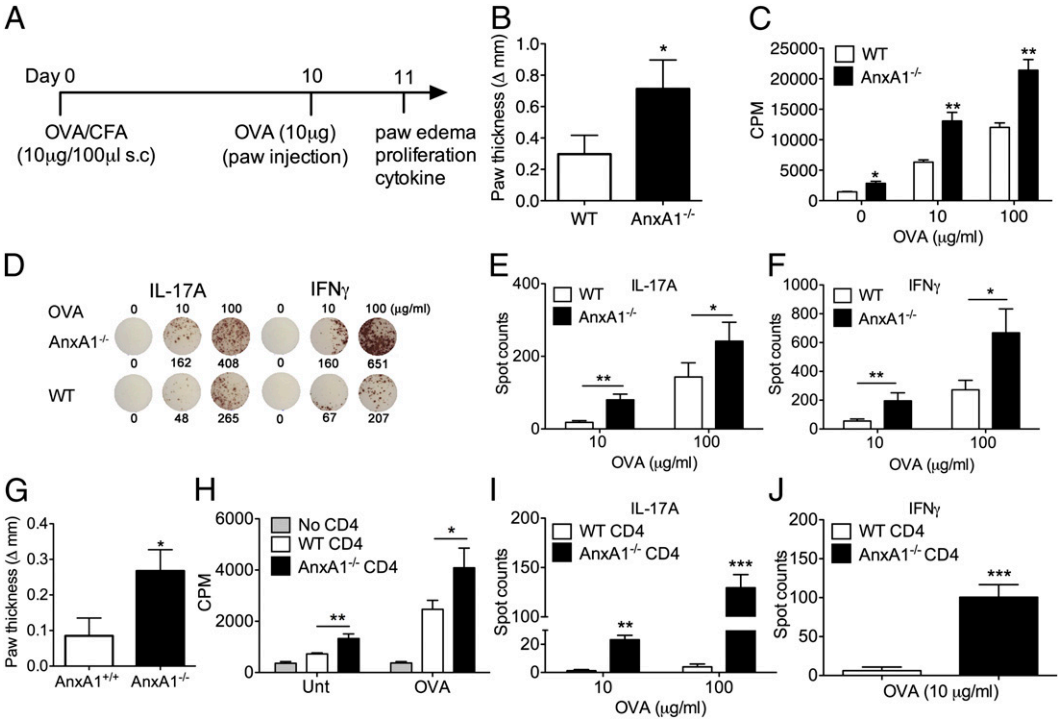

FIGURE 6. Deficiency of AnxA1 increases DTH and Ag-specific T cell responses in vivo and in vitro. (**A**–**F**) Comparison of OVA-induced DTH, T cell proliferation, and cytokine production in WT and AnxA1$^{-/-}$ mice. DTH was induced by immunizing mice with OVA in CFA and subsequently challenging the left footpad with OVA/PBS [timeline shown in (A)]. (B) Skin swelling (change in footpad thickness relative to PBS-injected control footpad) at 24 h. (C) Ag-specific proliferation of T cells from draining LNs 72 h after challenge, as assessed by [^{3}H]thymidine incorporation at two different OVA concentrations and in the absence of stimulation (n = 8/group). (D–F) OVA-induced IL-17 and IFN-γ release from draining LNs 24 h after treatment, as detected by ELISPOT. (D) Representative ELISPOT experiments following restimulation with OVA at various concentrations. Number of IL-17A–secreting cells (E) and IFN-γ–secreting cells (F) in WT and AnxA1$^{-/-}$ mice. (**G**–**J**) T cell transfer experiments comparing actions of WT and AnxA1$^{-/-}$ T cells. CD4$^+$ T cells were isolated from OVA-immunized WT and AnxA1$^{-/-}$ mice and adoptively transferred into Rag-1$^{-/-}$ mice. On the following day, the recipient mice were immunized with OVA/CFA and subsequently underwent challenge on day 10 [as per the timeline in (A)]. (G) DTH response 24 h after challenge. Data are shown for transfer of either WT or AnxA1$^{-/-}$ CD4$^+$ T cells. (H) Ag-specific T cell proliferation, as measured by [^{3}H]thymidine incorporation ex vivo. Data are shown for transfer of either WT or AnxA1$^{-/-}$ CD4$^+$ T cells (or no cells) into Rag-1$^{-/-}$ mice, which were subsequently left untreated (Unt) or immunized with OVA (OVA). Ag-induced secretion of IL-17A (I) and IFN-γ (J) by draining LN T cells from Rag-1$^{-/-}$ mice that received either WT or AnxA1$^{-/-}$ CD4$^+$ T cells, as measured by ELISPOT. Data represent mean ± SEM (n = 4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, versus WT control.

Absence of AnxA1 results in increased activation of intracellular signaling associated with TCR activation

To investigate the mechanism of the T cell–intrinsic inhibitory effect of AnxA1 on T cell activation, we investigated signaling pathways downstream of TCR activation. The PI3K/Akt and MAPK pathways are activated rapidly in response to T cell Ag recognition and are central to the subsequent T cell response (44, 45). Consistent with this, inhibition of Akt (via Akt inhibitor VIII) prevented OVA$_{323–329}$-induced proliferation of OT-II of both AnxA1$^{+/+}$ and AnxA1$^{-/-}$ T cells (Fig. 8A). Similarly, inhibition of activation of ERK and p38 MAPK significantly reduced OT-II cell proliferation, causing a 50% reduction in both AnxA1$^{+/+}$ and AnxA1$^{-/-}$ cells relative to untreated cells of the same genotype (Fig. 8A). In parallel, these inhibitors also significantly reduced OVA$_{323–329}$-induced IL-17 and IFN-γ production in T cells of both genotypes (Fig. 8B, 8C). These findings indicate that these pathways contribute to T cell activation in both the presence and absence of AnxA1.

We next compared activation of these signaling pathways in the presence and absence of AnxA1 by assessing phosphorylation of Akt, ERK, and p38 in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ splenocytes and LN cells, following stimulation with the OT-II CD4$^+$ T cell cognate OVA$_{323–339}$ peptide. In AnxA1$^{+/+}$ cells, a minimal change in Akt phosphorylation was observed over a 2-h time course (Fig. 8D, 8E). In contrast, T cells lacking AnxA1 showed significant increases in Akt phosphorylation 60 and 120 min after OVA stimulation. In addition, although ERK phosphorylation steadily increased in AnxA1$^{+/+}$ cells after OVA stimulation, ERK phosphorylation increased to a significantly greater degree in AnxA1$^{-/-}$ cells 60 and 120 min after activation (Fig. 8D, 8F). Assessment of p38 phosphorylation revealed a different phenotype; in the absence of stimulation, this parameter was significantly elevated in AnxA1$^{-/-}$ cells versus AnxA1$^{+/+}$ cells (Fig. 8D, 8G). Although not induced further by OVA stimulation, p38 phosphorylation remained significantly elevated in AnxA1$^{-/-}$ cells throughout the 2 h following exposure to OVA$_{323–339}$ (Fig. 8D, 8G). Together, these data indicate that the proactivation effects of AnxA1 deficiency in T cells are associated with increased activation of Akt, ERK, and p38 MAPK. In our previous studies of murine fibroblasts and macrophages, AnxA1 suppressed inflammatory cytokine production via upregulation of MKP-1 and GILZ in the respective cell types (3, 24). Therefore, we examined mRNA expression of these anti-inflammatory molecules in T cells in the presence and absence of AnxA1. Anxa1$^{+/+}$ and AnxA1$^{-/-}$ OT-II CD4$^+$ T cells expressed equivalent amounts of MKP-1 and GILZ mRNA, both basally and in response to antigenic (OVA) stimulation (data not shown). These findings indicate that the action of AnxA1 in CD4$^+$ T cells, unlike that in fibroblasts and macrophages, does not involve altered expression of MKP-1 or GILZ.

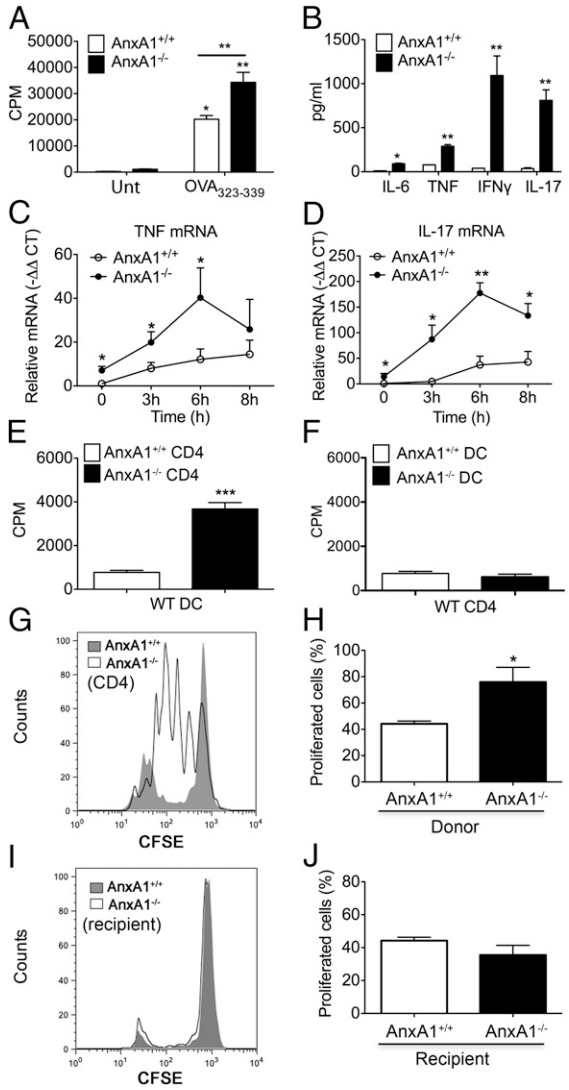

FIGURE 7. Absence of AnxA1 from T cells is sufficient to cause increased Ag-specific inflammation, T cell proliferation, and cytokine production in vivo. (**A**–**D**) Responses of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ OT-II CD4$^+$ T cells were compared following in vitro stimulation with $OVA_{323–339}$. (A) Basal and $OVA_{323–329}$-stimulated T cell proliferation assessed by [^{3}H] thymidine incorporation. (B) Cytokine production (IL-6, TNF, IFN-γ, IL-17A) by LN cells assessed via CBA. Expression of mRNA for TNF (C) and IL-17 (D) assessed by real-time PCR (n = 8, two separate experiments). (**E** and **F**) Role of AnxA1 expressed by either CD4$^+$ T cells or DCs in Ag-induced proliferation. (E) $OVA_{323–339}$-induced in vitro proliferation of purified AnxA1$^{+/+}$ or AnxA1$^{-/-}$ OT-II CD4$^+$ T cells in coculture experiments with WT DCs. (F) $OVA_{323–339}$-induced in vitro proliferation of purified AnxA1$^{+/+}$ OT-II CD4$^+$ T cells in coculture experiments with WT or AnxA1$^{-/-}$ DCs. For both experiments, n = 8, and two separate experiments were performed. (**G**–**J**) Role of T cell and non-T cell AnxA1 in Ag-specific T cell proliferation in vivo. CFSE-labeled naive CD4$^+$ T cells from AnxA1$^{+/+}$ or AnxA1$^{-/-}$ OT-II mice were transferred into AnxA1$^{+/+}$ and/or AnxA1$^{-/-}$ recipients that were then immunized with OVA. CD4$^+$ cell proliferation was measured in splenocytes by flow cytometric assessment of CFSE dilution 3 d after immunization. (G and H) Proliferation of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ OT-II cells in WT recipients (n = 8, two separate experiments). (I and J) AnxA1$^{+/+}$ OT-II cell proliferation in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ recipients (n = 8, two separate experiments). (G and I) Representative FACS plots of CD4$^+$ T cell CFSE staining. (H and J) Percentage of proliferated CD4$^+$ T cells. Data are mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, versus AnxA1$^{+/+}$.

Discussion

AnxA1 mediates a broad range of effects within the immune system. However, its role in the regulation of adaptive immune responses and T cell activation is poorly understood. We and other investigators demonstrated that AnxA1 exerts inhibitory effects in in vivo models of the adaptive immune response (13, 14) and in in vitro studies of Ag-specific T cell activation (15, 16). In contrast, other studies revealed that AnxA1 promotes Th1 responses while inhibiting Th2 responses (17–19). To further illuminate the complex actions of physiological AnxA1 in adaptive immunity, we focused on the effects of endogenous AnxA1 on adaptive immune responses in several diverse models of T cell–driven inflammation, with a particular emphasis on investigating the T cell–intrinsic role of AnxA1. Our findings indicate that, in these models, which are characterized by involvement of Th1, Th2, and Th17 responses and include loss of tolerance to an autoantigen (CIA), an immune response against an exogenous protein (OVA), and a hapten-induced immune response (CHS), endogenous AnxA1 exerts a consistent inhibitory effect on T cell activation. The CIA model is predominantly Th1/Th17 dependent. Similarly, the major products of activated OT-II cells in the present experiments were Th1 and Th17 cytokines. In contrast, the multiple-challenge CHS model features a greater contribution from the Th2 phenotype. Despite the diversity of these models, the absence of AnxA1 resulted uniformly in increased CD4$^+$ T cell activation and end-organ inflammation. Furthermore, via experiments in which AnxA1 deficiency was restricted to the Ag-responding T cell, we found that the absence of AnxA1 in CD4$^+$ T cells is associated with amplification of Ag-induced intracellular signaling in these cells, demonstrating that these actions of AnxA1 are intrinsic to the T cell. These findings are consistent with the hypothesis that, rather than differentially favoring the activation of one specific Th cell phenotype, endogenous AnxA1 acts to inhibit the activation of all of the proinflammatory CD4$^+$ lineages. Whether AnxA1 has effects on the regulatory T cells that may contribute to the anti-inflammatory actions of this protein is an additional possibility that remains to be investigated.

One of the consistent effects found in the current study was the increase in Ag-induced T cell proliferation in the absence of AnxA1. This was observed in secondary lymphoid organs in all three models examined. In addition, in the CHS model during the second challenge, CD4$^+$ cells present in the skin also exhibited greater proliferation than their WT counterparts. Despite the fact that AnxA1 is a widely expressed protein, our studies using transferred AnxA1$^{+/+}$ or AnxA1$^{-/-}$ OT-II cells showed that the absence of T cell–expressed AnxA1 was sufficient to mediate this phenomenon in an OVA-induced DTH model. Similar observations were made in relation to T cell cytokine production; in the latter model, AnxA1$^{-/-}$ T cells showed significantly greater production of IFN-γ and IL-17A when AnxA1 deficiency was restricted to the T cell. Together, these findings indicate that the regulatory effects of T cell–intrinsic AnxA1 on T cell proliferation and proinflammatory cytokine production are major factors underlying the ability of physiological AnxA1 to limit T cell–induced inflammation.

The multiple-challenge form of CHS is associated with greater Th2 and Th17 Th responses relative to Th1 responses (46). For example, although Th1 cells are observed in inflamed skin, IFN-γ–deficient mice develop normal CHS responses, suggesting that Th1 cells are not essential in CHS (47). In contrast, mice deficient in STAT-6, which is essential for IL-4 and IL-13 signal transduction, exhibit attenuated CHS responses (48). In this study, we found that increased T cell activation in CHS in the absence of AnxA1 was associated with increased expression of the Th17

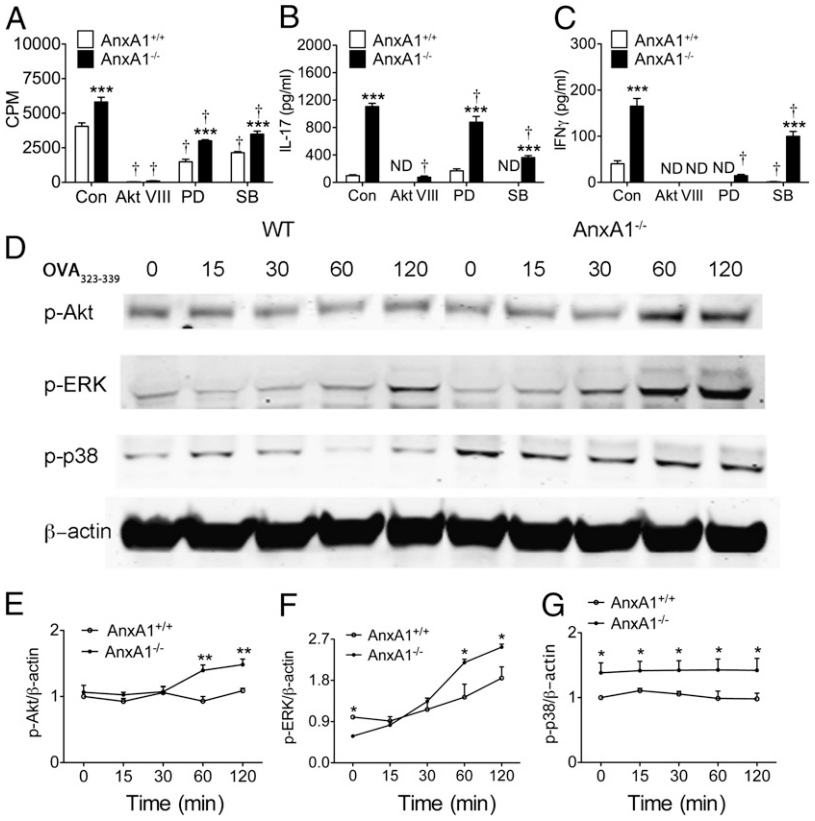

FIGURE 8. Absence of AnxA1 from T cells increases Ag-induced signaling. (**A**–**C**) Effect of inhibition of Akt, ERK MAPK (via MEK inhibition), and p38 MAPK on Ag-induced T cell responses in WT and AnxA1$^{-/-}$ mice. LN cells from AnxA1$^{+/+}$ and AnxA1$^{-/-}$ OT-II mice were stimulated with $OVA_{323-339}$ and then either left untreated (Con) or treated with Akt inhibitor VIII (Akt VIII), MEK inhibitor PD98059 (PD), or the p38 inhibitor SB203580 (SB). (A) T cell proliferation assessed by [^{3}H]thymidine incorporation. Production of IL-17 (B) and IFN-γ (C) assessed by ELISA. Data are mean ± SEM of $n = 8$ (two separate experiments). (**D**–**G**) Comparison of Ag-induced signaling in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ T cells. Naive splenocytes and LN cells of AnxA1$^{+/+}$ or AnxA1$^{-/-}$ OT-II mice were stimulated with $OVA_{323-339}$ for 15–120 min, and activated Akt, ERK, and p38 were analyzed by Western blot. (D) Representative Western blot. Densitometric analysis of ratio of p-Akt (E), p-ERK (F), and p-p38 (G) to β-actin. Data are mean ± SEM ($n = 4$ experiments). *$p < 0.05$, versus WT at the same time point, **$p < 0.01$, ***$p < 0.001$, versus WT, $^{\dagger}p < 0.05$, versus untreated cells of the same genotype. ND, Not detected.

transcription factor RORγt and IL-17A, supporting the concept that alterations in Th17 activity underlie the contribution of AnxA1 to regulation of CHS. Deficiency of IL-17 reduces CHS responses to the haptens 2,4-dinitrofluorobenzene, trinitrochlorobenzene, and OXA (49, 50), and IL-17 was reported to be involved in the regulation of immune-mediated human skin diseases (51–54). To our knowledge, our studies are the first to report suppression of IL-17A production by endogenous AnxA1. This effect of endogenous AnxA1 is supported by our observations in another Th17-dependent in vivo model, CIA, in which deficiency of endogenous AnxA1 increased susceptibility to CIA and CII-specific T cell activation.

Nevertheless, it should be noted that these findings contrast with those reported in experimental autoimmune encephalomyelitis, in which expression of IL-17 was reduced in AnxA1$^{-/-}$ LN cells (19). The difference between these results may reflect subtle differences in the requirement for Th17 responses between the two models. For example, although Th17 cytokines are associated with experimental autoimmune encephalomyelitis, IL-17$^{-/-}$ mice are capable of developing significant inflammation after myelin oligodendrocyte glycoprotein immunization (55). In human contact dermatitis, IL-17 is produced by Th0, Th1, and Th2 cells in response to hapten exposure, suggesting that this cytokine is central to many forms of hapten-induced skin inflammation (54).

One of the consistent observations in the current study was the increased inflammation in the target organ in the absence of AnxA1. These results could simply reflect the proinflammatory effects of increased T cell proliferation and cytokine production associated with AnxA1 deficiency. However, one of the intriguing characteristics of the biology of AnxA1 is its capacity to affect many aspects of the inflammatory response. For example, AnxA1 is well recognized for its ability to restrict neutrophil adhesion in acute inflammatory models. The present findings extend these observations in neutrophils to include CD4$^+$ and CD8$^+$ T cells among the leukocytes whose adhesive function is inhibited by endogenous AnxA1. As such, the degree of end-organ inflammation seen in AnxA1$^{-/-}$ mice may also reflect actions of AnxA1 on non–T cell elements of the effector response. Despite this, in the current study, increased end-organ inflammation was observed in mice in which AnxA1 deficiency was restricted to CD4$^+$ T cells. This finding demonstrates that additional actions of AnxA1 on innate aspects of the effector response are not necessary for exacerbation of inflammation in the context of T cell AnxA1 deficiency.

Leukocyte adhesion in the inflamed microvasculature represents a pivotal step in the delivery of T cells and other leukocytes to the inflamed skin (56). Previous studies of acute neutrophil recruitment induced by platelet-activating factor showed that, in AnxA1$^{-/-}$ mice, neutrophils show increased transmigration but not adhesion (38). In contrast, in our CHS model we observed that adhesion of CD4$^+$ T cells (during the first challenge) and CD4$^+$ and CD8$^+$ T cells, as well as neutrophils (during the second challenge), were all increased in the absence of AnxA1. These findings suggest that the role of AnxA1 in controlling leukocyte–endothelial cell interactions is more prominent in this T cell–dependent model of inflammation. The mechanisms underlying these increases in adhesion are unclear. It is possible that they stem from direct effects of AnxA1 on the adhesion molecules expressed by leukocytes and/or endothelial cells. An alternative explanation, given the increased level of T cell activation observed in this study in the absence of AnxA1, is that these effects are downstream of increased Ag-induced T cell activity in the hapten-challenged tissues.

To investigate the mechanism of the T cell–intrinsic actions of AnxA1, we examined activation of the Akt and ERK/p38 MAPK pathways. We previously observed that AnxA1 inhibits phosphorylation of ERK in human fibroblasts (25) and p38 and ERK phosphorylation in murine fibroblast lines (24), findings that

raised the possibility that AnxA1 may mediate similar effects in T cells. This hypothesis was confirmed by observations of increased ERK/p38 activation in AnxA1$^{-/-}$ T cells in the current study. In parallel, we observed increased activation of Akt in T cells lacking AnxA1. These findings are in direct contrast to a previous analysis of the actions of AnxA1 in T cells, in which reduced phosphorylation of Akt and ERK MAPK in activated AnxA1-deficient T cells was observed (18). The reasons for these discrepancies are unclear. However, in the previous study the investigators used nonantigenic stimuli, such as anti-CD3, anti-CD3/CD28, and PMA/ionomycin, to stimulate T cell activation. It is likely that the process of T cell activation induced by these stimuli differs considerably from that induced by cognate Ag, as used in the present studies. Alternatively, these discrepancies may stem from differences in the Th lineages of the T cells examined. Nevertheless, our observation of the susceptibility of T cell activation to inhibition via inhibitors of these pathways, together with the increased activation of both of the signaling pathways and T cells, per se, in the absence of AnxA1, supports the contention that the effect of endogenous AnxA1 on T cell signaling during antigenic responses is inhibitory.

In summary, our data indicate that endogenous AnxA1 plays an important role in determining the intensity of adaptive immune responses, T cell activation, and T cell–dependent inflammation in three distinct settings: hapten-induced CHS, autoimmunity (CIA), and immunity to a foreign Ag (OVA). Moreover, the key finding that emerges from this work is that T cell–intrinsic AnxA1 is sufficient for these effects, in that T cell–expressed AnxA1 regulates the activation of several T cell–signaling pathways downstream of TCR activation. Our data support the hypothesis that the anti-inflammatory effects of endogenous AnxA1, well established in innate immunity, extend to several forms of T cell–driven inflammation. These findings raise the possibility that modulating T cell expression of AnxA1 could represent a strategy for therapeutic intervention in the setting of T cell–dependent inflammatory and autoimmune diseases.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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