

Impaired T cell activation and increased Th2 lineage commitment in Annexin-1-deficient T cells

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Annexin-1 is a well-known endogenous anti-inflammatory protein that modulates the activation of cells of the innate immune system such as neutrophils and macrophages. We have recently reported a positive role for the exogenous protein on T cell differentiation, however, whether such a role holds true for the endogenous protein has yet to be determined. This aspect has been investigated here finding that Annexin-1-deficient T cells display an impaired activation and proliferation in response to anti-CD3 plus anti-CD28 stimulation. Furthermore, differentiation of T cells from Annexin-1-deficient mice in Th0/Th1/Th2 or Th17 skewing conditions demonstrated an increased Th2 phenotype compared to cells from control littermates. Similar results were obtained when we analyzed the Th1/Th2 profile of lymph node cells obtained from mice immunized with keyhole limpet hemocyanin or the inflammatory infiltrate in mouse model of allergic inflammation. These results demonstrate a novel modulatory role of endogenous Annexin-1 in TCR signaling and T cell differentiation and suggest this protein might play a dual and complementary role in the innate and adaptive immune response.

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Introduction

A large body of evidence over the last few years has shown that Annexin-1 (Anx-A1) is an anti-inflammatory protein playing a homeostatic role in cells of the innate immune system such as neutrophils and macrophages [1, 2]. The very first evidence supporting this hypothesis was provided by observing the effects of human recombinant (hr)Anx-A1 and its peptides in different models of inflammation *in vitro* and *in vivo* [3–5]. Here, the protein reduced neutrophil adhesion and rolling on activated endothelial cells and inhibited the inflammatory reaction by affecting local leukocyte recruitment

[6, 7]. Consistently, analysis of the inflammatory response of Anx-A1^{-/-} mice has shown an exacerbated inflammatory cell influx as well as increased release of mediators such as TNF- α and IL-1 [8–10]. Finally, further support of an exquisite role of Anx-A1 in modulation of innate immune system has also been provided by the discovery of its capacity to bind and activate members of the formyl peptide receptor (FPR) family [7, 11], G-protein-coupled receptors used by the bacterial-derived product fMLP and an array of endogenous mediators to control leukocyte activation and trafficking [12]. By binding and activation of one member of this family, the formyl peptide receptor like-1 (FPRL-1), Anx-A1 exerts its counter-regulatory actions on neutrophil extravasation and innate immunity [13, 14].

We have recently shown that stimulation of T cells in presence of hrAnx-A1 increases T cell activation via stimulation of FPRL-1 [15]. Interestingly, this was due to an additive effect of the recombinant protein acting together with endogenous Anx-A1, the latter being released in the extracellular medium following TCR

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Abbreviations: **Anx-A1:** annexin-1 · **FPR:** formyl peptide receptor · **FPRL-1:** formyl peptide receptor like-1 · **hr:** human recombinant · **KLH:** keyhole limpet hemocyanin

stimulation. Together, these findings suggested the existence of an Anx-A1/FPRL-1 pathway that integrates with TCR signaling and modulates T cell activation. Furthermore, these results highlighted a potential role for both Anx-A1 and FPRL-1 in the adaptive immune response. In this study, we sought to verify this hypothesis and thus we investigated the T cell response in Anx-A1^{-/-} mice *in vitro* and *in vivo*. We report here that Anx-A1-deficient T cells exhibit impaired responses to TCR stimulation. Furthermore, we show that the lack of endogenous Anx-A1 favors the differentiation of CD4⁺ cells into Th2 effector cells. Thus this study, taken together with our previous observations obtained with hrAnx-A1, suggests that the Anx-A1/FPRL-1 axis is a potential therapeutic target for the Th1/Th2-mediated immune diseases.

Results

Impaired activation and proliferation of Anx-A1^{-/-} T cells

To investigate the role of endogenous Anx-A1 in T cell activation, we compared the effect of different stimuli on IL-2 production and cell proliferation in Anx-A1^{-/-} and ^{+/+} T cells. As shown in Fig. 1A and B, stimulation of T cells with anti-CD3 or anti-CD3/CD28 or PMA and

ionomycin increased IL-2 production and expression in wild-type T cells whereas activation of Anx-A1^{-/-} cells in the same conditions led to a reduced production and expression of this cytokine. Consistent with this finding, analysis of CD69 and CD25 expression, two markers of T cell activation, following stimulation with anti-CD3/CD28 showed a concentration-dependent increase in the number of CD69 positive cell in Anx-A1^{+/+} T cells and this effect was again reduced in Anx-A1-deficient cells (Fig. 1C). Similar results were obtained for CD25 expression (data not shown). IL-2 is the major cytokine produced by T cells upon activation and its function is to induce expansion and proliferation of T cells. As Anx-A1^{-/-} cells produced less IL-2 upon stimulation, we next analyzed the effects of anti-CD3 or anti-CD3/CD28 or PMA and ionomycin on T cell proliferation by measuring [³H]thymidine incorporation. Interestingly, in basal condition Anx-A1^{-/-} T cells showed a significant increase in the rate of ³H-thymidine incorporation compared to wild-type cells (Fig. 2A). However, when we stimulated the cells with either anti-CD3, anti-CD3/CD28 or PMA and ionomycin and compared the percentages of [³H]thymidine incorporation to their respective control unstimulated cells, we observed a significant decrease in proliferation in Anx-A1^{-/-} T cells compared to wild type (Fig. 2B, C and D, respectively). To demonstrate that this effect was not due to a defective IL-2 receptor signaling, we performed the same experiments in presence of

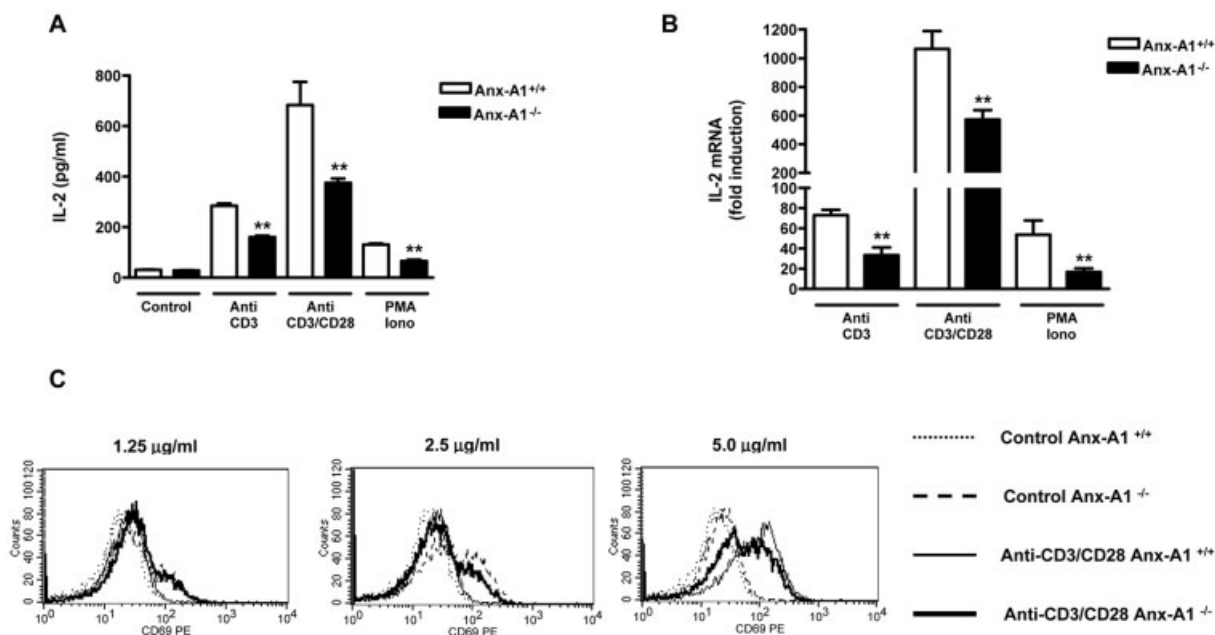


Figure 1. Endogenous Anx-A1 modulates T cell activation. (A) IL-2 production from naive lymph node T cells stimulated with anti-CD3, anti-CD3/CD28 (5.0 µg/mL) or PMA (20 ng/mL) and ionomycin (2 ng/mL) for 24 h. Values are mean ± SE of *n* = 4–5 mice. ***p* < 0.01. (B) Analysis of IL-2 expression by RT-PCR in naive lymph node T cells stimulated with anti-CD3, anti-CD3/CD28 (5.0 µg/mL) or PMA (20 ng/mL) and ionomycin (2 ng/mL) for 24 h. Values are expressed as a fold increase compared to control unstimulated cells. Values are mean ± SE of *n* = 4–5 ***p* < 0.01. (C) Murine naive lymph node T cells were stimulated with the indicated concentration of anti-CD3/CD28 for 12 h and then analyzed for CD69 expression by FACS.

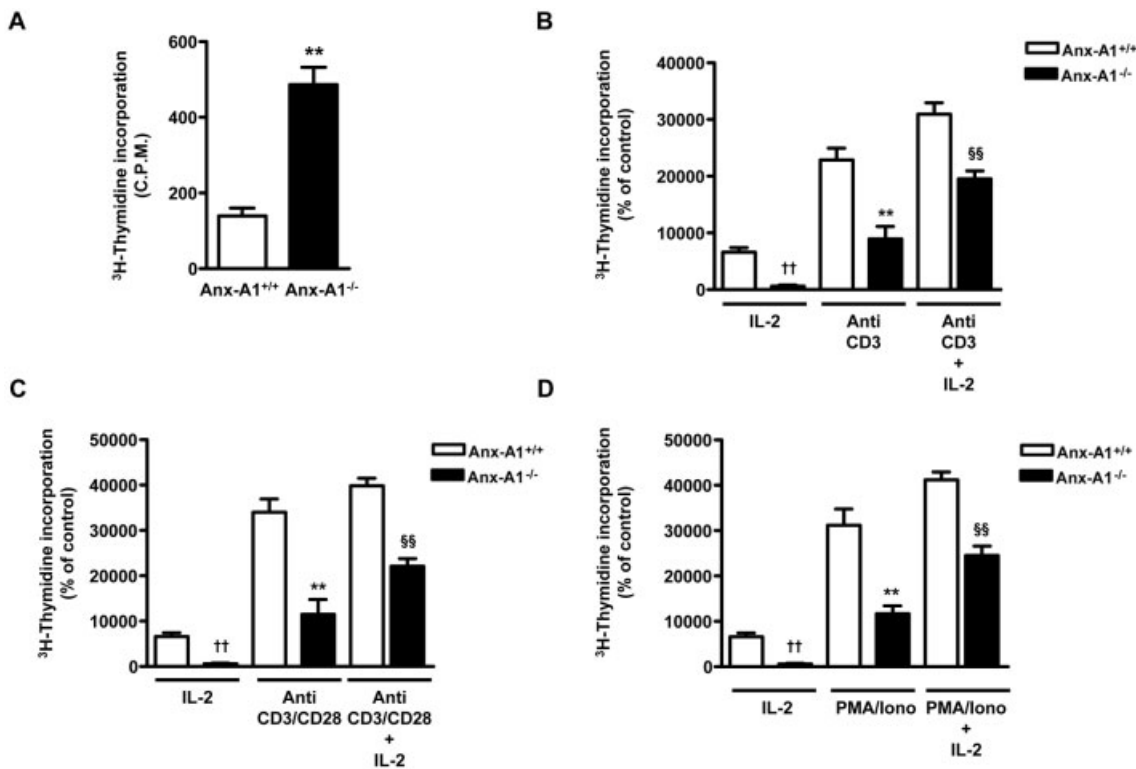


Figure 2. Endogenous Anx-A1 modulates T cell proliferation. (A) Basal incorporation (CPM) of [³H]thymidine in naive lymph node T cells from wild-type or Anx-A1-deficient mice. Values are mean $\pm$ SE of $n = 4-5$ ** $p < 0.01$. (B) Anti-CD3 (5.0 μ g/mL), (C) anti-CD3/CD28 (5.0 μ g/mL) or (D) PMA (20 ng/mL) and ionomycin (2 ng/mL) induced proliferation of wild-type and Anx-A1-deficient T cells expressed as percentage of [³H]thymidine incorporation compared to control unstimulated T cells. In some experiments, cells were also activated in presence of mouse recombinant IL-2 (20 ng/mL). Values are mean $\pm$ SE of $n = 4-5$, †† $p < 0.01$ vs. IL-2-stimulated Anx-A1^{+/+} cells; ** $p < 0.01$ vs. anti-CD3 or anti-CD3/CD28 or PMA/ionomycin-stimulated Anx-A1^{+/+} cells; §§ $p < 0.01$ vs. anti-CD3 or anti-CD3/CD28 or PMA/ionomycin-stimulated Anx-A1^{-/-} cells.

mouse recombinant IL-2. As shown in Fig. 2, addition of IL-2 to the Anx-A1-deficient T cells increased but not completely rescued the defective proliferation observed in these cells. Together, these results suggest that genetic deletion of Anx-A1 in T cells increases their basal proliferation but impaired their capacity to respond to stimulation *via* the TCR or PMA and ionomycin. This defect can be explained by their reduced capacity to produce IL-2. Addition of exogenous recombinant IL-2 to stimulated Anx-A1^{-/-} T cells overcomes the defect in proliferation but not completely (Fig. 2B, C and D); this is because of their reduced expression of the IL-2 receptor CD25.

Defective FPRL-1 signaling in Anx-A1^{-/-} T cells

We have previously shown that stimulation of T cells with suboptimal concentrations of anti-CD3/CD28 in the presence of hrAnx-A1 led to increased activation of NF- κ B, NFAT and AP-1 compared to cells stimulated with anti-CD3/CD28 alone [15]. This increased activation was due to stimulation of FPRL-1 by hrAnx-A1 and led to the activation of ERK and PKB/Akt, two well-known

FPRL-1 downstream signaling pathways [16, 17] that have also been shown to increase the strength of TCR signaling [18, 19]. Given that endogenous Anx-A1 was released after anti-CD3/CD28 stimulation, we concluded that basal and “physiological” levels of Anx-A1, together with the exogenously added hrAnx-A1, would account for the increase in FPRL-1 signaling. If this holds true, one could expect Anx-A1-deficient T cells to exhibit defective FPRL-1 signaling. As predicted, stimulation of wild-type T cells with anti-CD3/CD28 led to phosphorylation of ERK and PKB/Akt whereas Anx-A1^{-/-} T cells showed a decreased ERK and Akt activation (Fig. 3A). This effect was not due to different levels of expression of the FPR(s) in wild-type and Anx-A1-deficient T cells. In fact, analysis of FPR1 and FPR-rs1 in these cells showed similar level of expression either before or after stimulation of the TCR (Fig. 3B). Finally, analysis of NF- κ B, NFAT and AP-1 activation showed that Anx-A1^{+/+} T cells responded to anti-CD3/CD28 stimulation with a robust increase in DNA-binding activity of all three transcription factors whereas a significantly reduced binding was observed in Anx-A1^{-/-} cells (Fig. 3C).

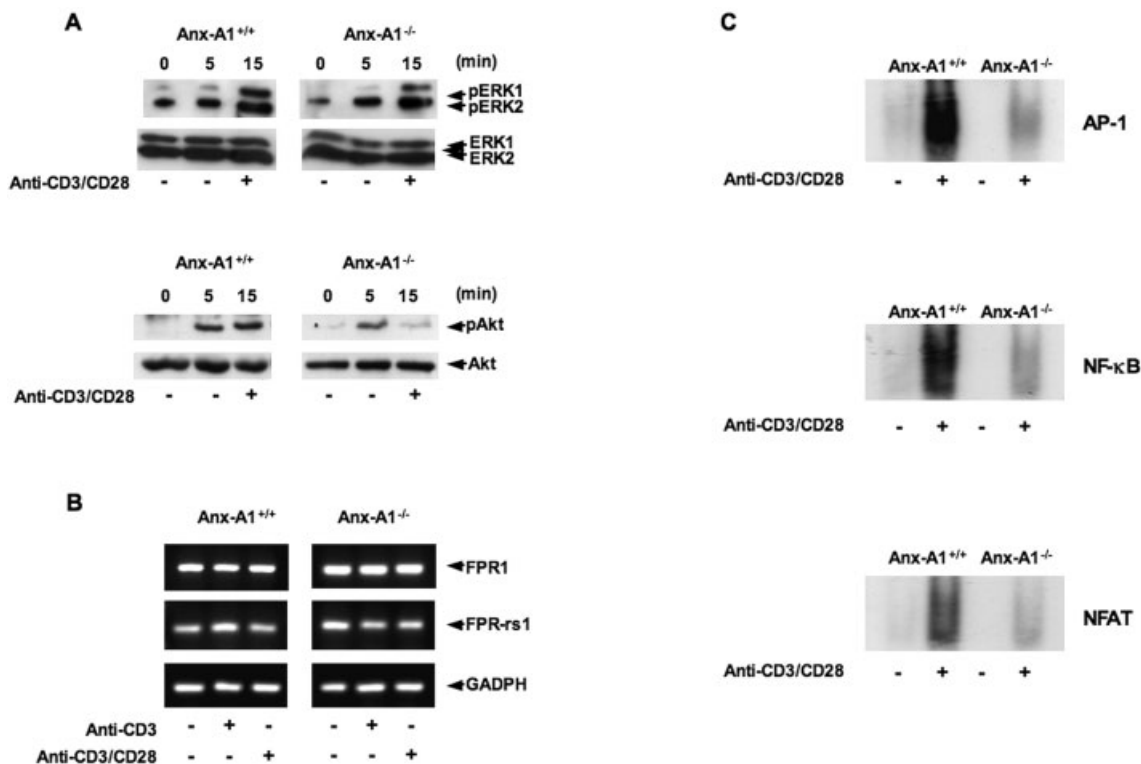


Figure 3. Endogenous Anx-A1 modulates early and late TCR signaling events. (A) Western blot analysis of total and phospho Erk and Akt of naive T cells stimulated with anti-CD3/CD28 (5.0 μ g/mL) for the indicated time. (B) Expression of FPR1 and FPR-rs1 in Anx-A1^{+/+} and Anx-A1^{-/-} T cells stimulated with anti-CD3 (5.0 μ g/mL) or anti-CD3/CD28 (5.0 μ g/mL) for 24 h. (C) Electrophoretic mobility shift assay showing AP-1, NF- κ B, and NFAT DNA-binding activity in T cells stimulated with anti-CD3/CD28 (5.0 μ g/mL) for 8–10 h. In all the experiments results are representative of three separate experiments with similar results.

Endogenous Anx-A1 modulates Th1/Th2 differentiation

Next, we tested whether the absence of endogenous Anx-A1 might determine potential changes in the balance between Th1 or Th2 cell differentiation. To test this possibility we first examined the differentiation of Anx-A1^{-/-} cells in neutral conditions (Th0; see *Materials and methods*) an experimental setting in which T cell differentiation is driven only by the strength of TCR signaling. Naive T cells from wild-type or Anx-A1^{-/-} mice were cultured in Th0 for 4 days and thereafter restimulated with anti-CD3 to stimulate the production of Th1/Th2 cytokines. Th0 Anx-A1^{-/-} produced lower levels of IL-2 and IFN- γ and high levels of IL-4 and IL-13 compared to control cells (Fig. 4A). Furthermore, analysis of the two major transcriptional switches in Th1 or Th2 differentiation, T-bet and GATA-3, respectively [20, 21], by real-time PCR supported the results obtained with the cytokine production and showed that Anx-A1-deficient T cells expressed higher levels of GATA-3 and lower levels of T-bet, compared to control cells (Fig. 4B).

We observed differences that are more marked when the cells were differentiated in Th1 or Th2 skewing

conditions. For instance skewing Anx-A1^{-/-} T cells in Th2 condition (Fig. 5, white bars) led to approximately 150% higher production of IL-4 compared to wild-type T cells against a 40% increase detected in Th0 conditions. Similarly, differentiation of Anx-A1^{-/-} T cells under Th1 conditions led to a decreased production of both IL-2 and IFN- γ (Fig. 5, black bars). Furthermore, we were able to detect significant amounts of IL-10 under Th2 skewing conditions and in this case, an increased production of this cytokine in Anx-A1^{-/-} T cells compared to wild-type was observed (Fig. 5, white columns). A growing number of studies have shown that Th17 cells play a key role in autoimmune and allergic inflammation [22–24]. Therefore, we compared IL-17 production and expression in Anx-A1^{+/+} and Anx-A1^{-/-} mice T cells differentiated under optimal Th17 conditions [25]. As shown in Fig. 6A and B, Anx-A1^{-/-} T cells produced significantly less IL-17 compared to Anx-A1^{+/+} cells after either 24 or 72 h of culture. Analysis of the expression of ROR γ , the key transcription factor involved in Th17 differentiation [26], also showed a significant decrease in Anx-A1^{+/+} Th17 cells compared to their control littermates (Fig. 6C). This difference was more marked at 24 h time after which the peak of expression of this transcription occurs [26].

Finally, to extend the impact of our study to physiological conditions, we immunized Anx-A1^{-/-} mice and their control littermates with keyhole limpet hemocyanin (KLH) plus complete Freund's adjuvant

(CFA). At day 7, draining lymph node cells were collected and restimulated with KLH. Consistent with the data obtained *in vitro*, cells from Anx-A1^{-/-} mice displayed a clear Th2 profile as judged by the increased

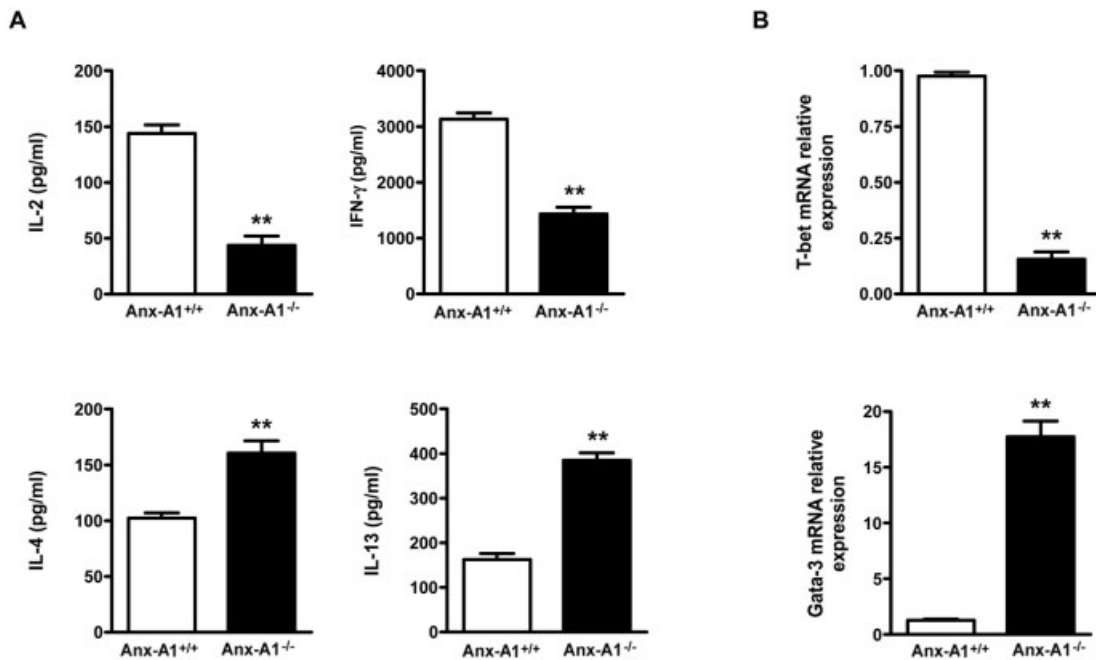


Figure 4. Endogenous Anx-A1 modulates the differentiation of naive cells in effector cells in Th0 conditions. (A) Th1/Th2 cytokine production profile of naive lymph node T cells differentiated *in vitro* in Th0 condition and then restimulated with plate-bound anti-CD3 (5.0 µg/mL) for 8 h. Values are mean ± SE of *n* = 4–5 mice. ***p* < 0.01 (B) Analysis of T-bet and GATA-3 expression by real-time PCR in cells differentiated in Th0 conditions for 4 days. Values are mean ± SE of *n* = 3–4 mice. ***p* < 0.01

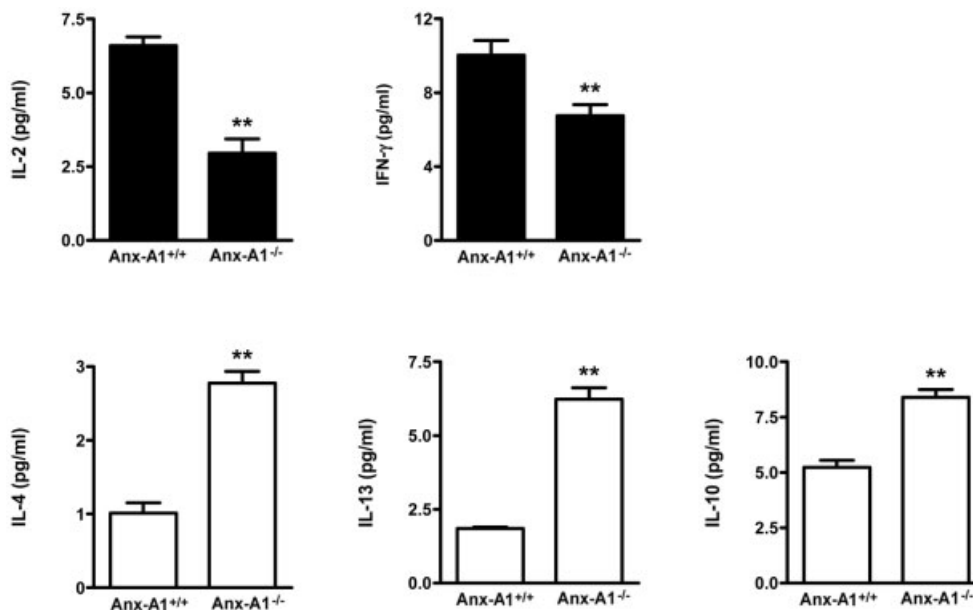


Figure 5. Endogenous Anx-A1 modulates the differentiation of naive cells in effector cells in Th1/Th2 conditions. Naive lymph node T cells were differentiated *in vitro* in Th1 (black bars) or Th2 (white bars) conditions and then restimulated with plate-bound anti-CD3 (5.0 µg/mL) for 8 h to measure Th1 (upper graphs) or Th2 (lower graphs) cytokine production. Values are mean ± SE of *n* = 4–5 mice. ***p* < 0.01

production of IL-4 and IL-13 and decreased levels of IFN- γ and IL-2 when compared to littermate control cells (Fig. 7). Analysis of IL-17 production in these cultures also confirmed the previous *in vitro* results, i.e. decreased production in Anx-A1^{-/-} cells compared to control littermates (data not shown). These data, combined with the ones obtained *in vitro*, demonstrated that Anx-A1 plays an important role in regulating Th1/Th2 and Th17 balance.

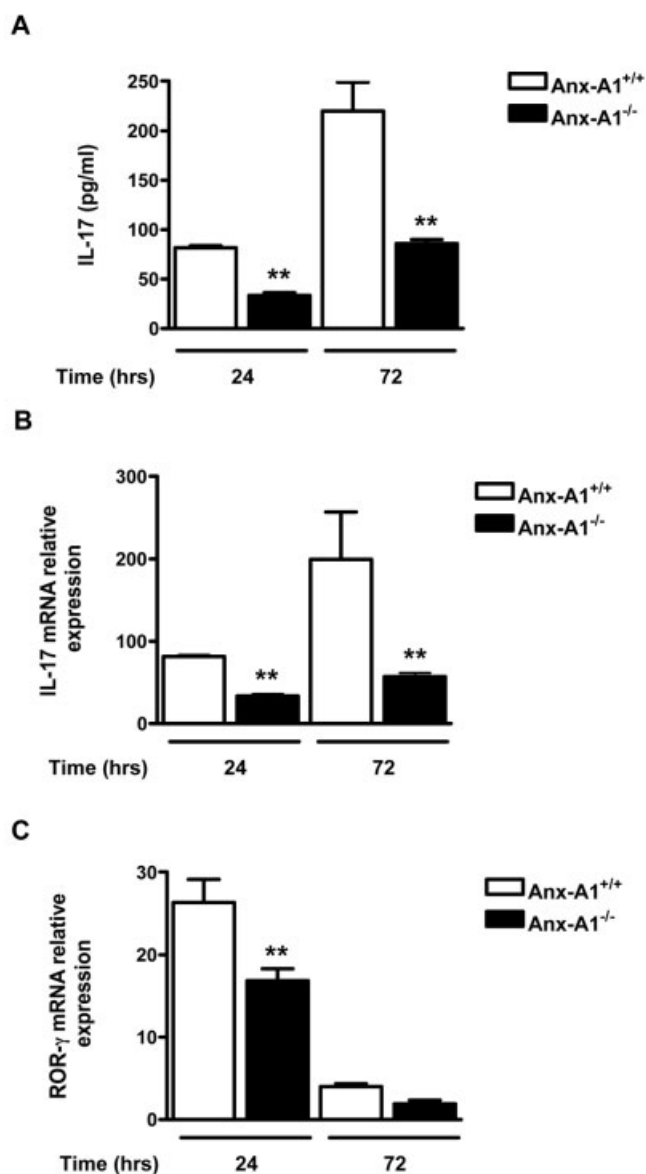


Figure 6. Endogenous Anx-A1 modulates the differentiation of naive cells in effector cells in Th17 conditions. Naive lymph node T cells were differentiated *in vitro* in Th17 conditions for the indicated time and then analyzed for their production (A) or expression (B) of IL-17 and ROR γ (C). The real-time PCR results are expressed as fold increase over cells cultured in Th0 conditions. Values are mean $\pm$ SE of $n = 4$ –5 mice. ** $p < 0.01$

Anx-A1^{-/-} mice display an exacerbated allergic response

As the results demonstrated that effector Anx-A1^{-/-} T cells showed a pronounced Th2 phenotype, we sought to investigate if Anx-A1-deficient mice would display the same phenotype *in vivo*. To this aim, we used a Th2-driven mouse model of inflammation, the ovalbumin-induced allergic peritonitis [27, 28]. Animals were sensitized to ovalbumin, and then challenged with the same antigen or PBS, as described in *Materials and methods*. Analysis of inflammatory cell recruitment in the peritoneal cavity of PBS-challenged wild-type or Anx-A1^{-/-} mice showed 48 h later a significant increase in the total cell number recovered in the peritoneal cavity of the Anx-A1^{-/-} mice compared to wild type (Fig. 8A). Phenotypic analysis of the cells recovered also showed a significant increase in the number of mononuclear cells in the Anx-A1-deficient mice compared to their control littermates (Fig. 8B). The situation was similar in ovalbumin-sensitized and -challenged animals; a clear increase was observed in total cell number, due to increases in neutrophils, lymphocytes, macrophages and especially, eosinophils. More importantly, Anx-A1^{-/-} mice showed a significant increase in the number of eosinophils compared to the wild-type littermates (Fig. 8C). Together these results confirm our hypothesis that genetic deletion of Anx-A1 causes an

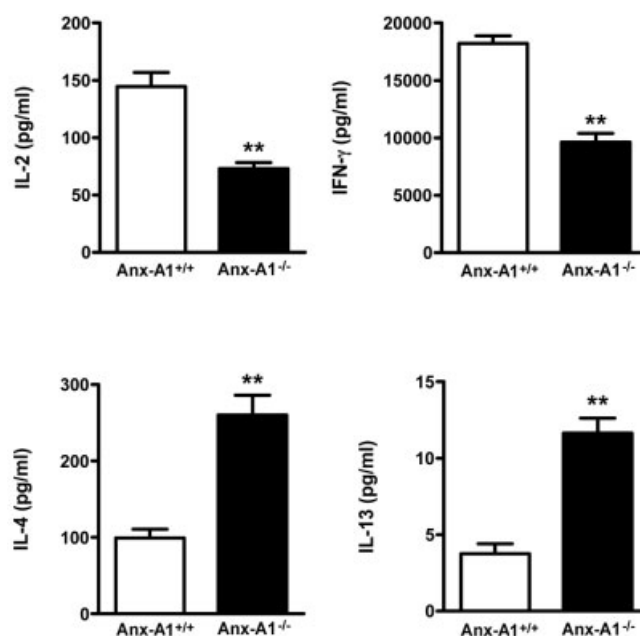


Figure 7. Endogenous Anx-A1 modulates Th1/Th2 differentiation *in vivo*. Th1/Th2 cytokine production profile of lymph node T cells obtained from mice immunized *in vivo* with KLH and restimulated *in vitro* with the same antigen for 4 days. Values are mean $\pm$ SE of $n = 3$ –4 mice; ** $p < 0.01$

exacerbated inflammatory reaction and an increased susceptibility to Th2-driven immune diseases.

Discussion

The major finding of this study is the discovery of a novel role of endogenous Anx-A1 in the adaptive immune response. Guided by its high level of expression in cells like neutrophils or macrophages, many research groups including ourselves have focused their attention on the role of this protein in inflammatory processes where these

cells play a relevant role. However, if we consider the initial observation that led to the discovery of Anx-A1 that is “a glucocorticoid-regulated protein that mediates some of the pharmacological effects of steroids” [29, 30], it is surprising that there have been so few studies of the effect of Anx-A1 on the adaptive immune response.

It is a very well-known fact that steroids modulate several aspects of the adaptive immune response including T cell response [31] and with this idea in mind, we first tested the effects of hrAnx-A1 on T cell activation. Interestingly, and contrary to our initial expectations, we found that stimulation of T cells with anti-CD3/CD28 in presence of hrAnx-A1 increases proliferation of these cells. Importantly, activation of T cells via TCR led to the release of endogenous Anx-A1 in the extracellular medium as well as at the externalization of its receptor FPRL-1 on the membrane [15]. Therefore, it seemed that the release of Anx-A1, concomitantly with the activation of its receptor FPRL-1, might serve as an autocrine/paracrine effector system involved in the regulation of T cell activation. Here, we questioned the patho-physiological impact of these pharmacological observations.

T cells from Anx-A1^{-/-} mice have a significant decrease in IL-2 production compared to their littermate wild-type controls. These differences were observed after receptorial or post-receptorial T cell stimulation, i.e. with either anti-CD3 alone, anti-CD3 and anti-CD28 or PMA and ionomycin, suggesting that endogenous Anx-A1 does not affect the assembly of the TCR machinery but might exert an additional control *via* its receptor FPR. Analysis of T cell proliferation in Anx-A1-deficient cells showed another interesting phenotype. In basal conditions, Anx-A1-deficient T cells show a higher rate of [³H]thymidine incorporation compared to wild-type cells. However, when we activated the cells via either TCR stimulation or PMA and ionomycin, we observed a significant decrease in proliferation. These findings are consistent with previous studies showing that cells stably transfected with Anx-A1 display a low rate of cell proliferation [32] and this might hence explain why Anx-A1-deficient T cells show a higher rate of [³H]thymidine incorporation. Most interestingly, in the same paper the authors showed that overexpression of Anx-A1 in a macrophage cell line has an anti-proliferative function through sustained activation of the ERK signaling cascade and by disruption of the actin cytoskeleton and ablation of cyclin D1 protein expression [32]. Here, we show that Anx-A1-deficient T cells display a reduced ERK activation following TCR stimulation. Therefore, there seems to be an apparent contrast on the role of Anx-A1 in regulating cell proliferation and ERK activation. However, these discrepancies can be explained by the fact that the activation of the ERK pathway can have a dual effect on proliferation [33]. In

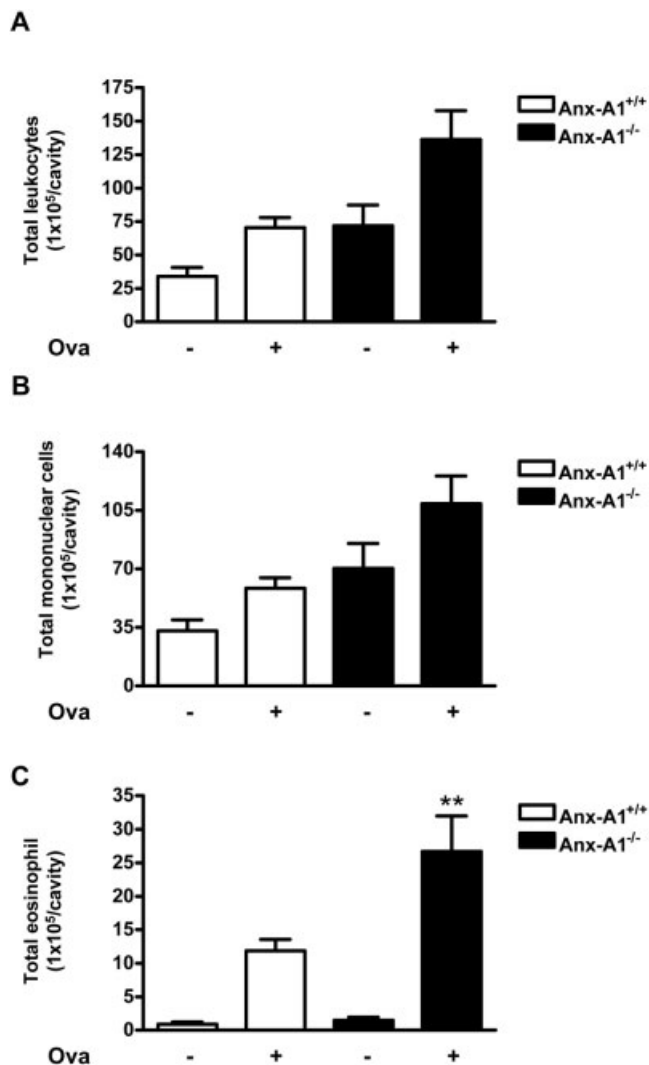


Figure 8. Anx-A1^{-/-} mice display an exacerbated allergic response. Total cell count of (A) leukocyte (B), mononuclear cells and (C) eosinophils from Anx-A1^{-/-} mice and wild-type controls immunized with 100 µg of OVA or PBS and then challenged on day 21 with either saline or 10 µg/site of OVA. Forty-eight hours after the challenge inflammatory cells were collected from the peritoneal cavity and total and differential counts performed as described in *Materials and methods*. Values are mean ± SE of *n* = 6–8 mice ***p* < 0.01.

fact, while it is known that ERK stimulate proliferation by inducing the expression of cyclin D1 and hence activation of cell cycle kinases in many cell types, it is also well known that inhibition of ERK activation in T cells leads to reduced proliferation [34]. Furthermore, these results are also consistent with our previous observation that incubation of T cells with hrAnx-A1 increases anti-CD3/CD28 induced proliferation and ERK activation [15].

Another interesting aspect of this study is that the reduced cell activation observed in Anx-A1-deficient T cells was more evident when cells were stimulated with high concentrations of anti-CD3/CD28 (see Fig. 1C). We have previously shown that the increasing effects of hrAnx-A1 on T cell activation were more evident when suboptimal concentration of anti-CD3/CD28 were used and were further augmented by increasing the concentration of recombinant Anx-A1 applied [15]. Together, these results demonstrate that Anx-A1 is not essential for T cell activation but it acts as tuner of T cell activation via stimulation of specific receptor(s). Consistent with this hypothesis, when we investigated the activation of the two main downstream signaling pathways for this family of receptors, *e.g.* ERK and Akt [12, 35], a significant decrease in the extent and duration of the phosphorylation of these two MAP kinases was found in Anx-A1^{-/-} T cells. These effects were not due to different levels of expression of either FPR1 or FPR-rs1 in Anx-A1^{-/-} and ^{+/+} T cells, as analysis by RT-PCR of the expression of these receptors did not show any significant difference.

The FPR(s) are members of the G protein-coupled receptor family that activate NF- κ B and different FPR ligands have been reported to have this effect [36]. For instance, serum amyloid (SAA), an endogenous ligand of FPRL1, stimulated matrix metalloproteinase (MMP)-9 up-regulation [37] and IL-8 [38] production by activating ERK and NF- κ B. Our previous study showed that in T cells hrAnx-A1 increases the activation of NF- κ B, as AP-1 and NFAT favoring binding to FPRL-1. Here, we confirm this finding by showing that Anx-A1-deficient T cells show an impaired activation of the same three transcription factors. These results are novel in two respects: first they show that the Anx-A1/FPRL-1 system regulate early as well as late TCR signaling events; secondly they expand the number of transcriptional regulators that are modulated by FPRL-1. However, it should be highlighted that FPRL-1 has been reported as a receptor that can either activate or inhibit proinflammatory cytokine synthesis depending on the specific ligands that bind to the receptor (for review see [12, 39]). Hence, we cannot exclude that the same Anx-A1/FPRL-1 system might exert an inhibitory effects on NF- κ B or AP-1 and NFAT in other cell types. At this regard it is interesting to note that a previous study

reported that the FPRL-1 ligands aspirin-triggered 15-epi-lipoxin A₄ (ATL), LXB₄ and its stable analog 5-R/S-methyl LXB₄ inhibit anti-CD3-mediated TNF- α secretion by human PBMC [40]. Interestingly, the effects of these lipoxins were selective, as they did not inhibit anti-CD3-induced IFN- γ release or cell proliferation. Therefore, it is possible to hypothesize that Anx-A1 and lipoxins, though sharing the same receptor, might exert different effects on T cells. In support of this hypothesis, several studies have also shown that 5-Lipoxygenase (LO)^{-/-} mice exhibited an exaggerated Th1 response (for review see [41]) while we show here that genetic deletion of Anx-A1 causes an exacerbated Th2 reaction in a mouse model of allergic inflammation.

Another interesting aspect of this study is the discovery of endogenous Anx-A1 as a potential master switch in Th1/Th2 differentiation. Several studies over the past few years have highlighted the importance of skewing cytokines, such as IL-4 and IL-12, and their transcription factors, such as GATA-3, c-maf, and T-bet in the regulation of Th2/Th1 lineage commitment (for review see [42, 43]). Interestingly, however, there have been few examples of factors that control or synergize with these cytokine signals and hence might influence Th2/Th1 divergence. Our results show that differentiation of Anx-A1-deficient T cells in Th0 condition, *i.e.* in the absence of exogenous sources of IL-4 or IL-12, led to effector cells with Th2 phenotype. This was also observed *ex vivo* by analyzing the Th1/Th2 profile of lymph node T cells obtained from Anx-A1^{-/-} mice immunized with KLH.

Ample evidence has recently shown that another type of effector cells, the Th17 cells, are potent inducers of autoimmunity. These cells developed in completely different way compared to Th1 or Th2 cells since they require the presence of TGF- β and IL-6 [23, 44]. Furthermore, it has been shown that the presence of either IFN- γ or IL-4 during Th17 differentiation potently inhibited Th17 development [45–47] and similarly skewing T cells with concurrent IFN- γ and IL-4 neutralization significantly increase the development of IL-17-producing cells [46–48]. These results strongly suggested that the development of Th17 and Th1/Th2 effector cells is mutually exclusive.

Analysis of IL-17 production and ROR γ expression from Anx-A1^{-/-} T cells differentiated under optimal Th17 conditions demonstrated a marked decrease compared to control littermate T cells. These results suggest that the increased Th2 phenotype (and hence the increased production of Th2 cytokines such as IL-4 and IL-13) of the Anx-A1^{-/-} T cells might be responsible for the decreased differentiation in Th17 cells. These results resemble a recent study where it has been shown that the type 2 responses promoted by IL-25 controls Th17 differentiation via IL-13 and IL-4 [49].

Most interestingly, using mouse model of ovalbumin-induced allergic reaction another study has shown that IL-17 plays a key role in allergic asthma. In fact, administration of exogenous IL-17 reduced pulmonary eosinophil recruitment and bronchial hyperreactivity whereas neutralization of IL-17 augmented eosinophil infiltration in sensitized mice [50]. In this study, we observed consistent results using another model of allergic reaction, the ovalbumin-induced allergic peritonitis. In this model, ovalbumin immunization and challenge cause an allergic response characterized by increased infiltration of eosinophils to the peritoneal cavity due to increased release of Th2 cytokines and eotaxin. As expected and consistent with the *in vitro* data, analysis of the peritoneal inflammatory infiltrate in the Anx-A1-deficient mice demonstrated an increase in the total inflammatory cell number and specifically an increase of eosinophils. Thus, it is possible to hypothesize that the increased levels of Th2 cytokine in the Anx-A1-deficient mice might reduce IL-17 production and thus cause an increased eosinophil accumulation.

Furthermore, it is noteworthy that the total basal number of mononuclear cells (mainly macrophages) present in the peritoneal cavity of the Anx-A1^{-/-} mice was higher compared to wild type. This might reflect the lack of inhibitory and homeostatic role of endogenous Anx-A1 on the innate immune system and is consistent with our previous studies where exacerbated innate immune responses were observed in these mice [8]. These data, together with our previous results showing the release of endogenous Anx-A1 upon TCR stimulation [15], suggest that Anx-A1 is an early TCR-induced signaling molecule that regulates later events responsible for lineage commitment. In support of this hypothesis stimulation of naive T cells with anti-CD3/CD28 in presence of ERK inhibitors resulted in increased Th2 cytokine synthesis [18]. It is also well known that weak ERK activation, as observed in Anx-A1^{-/-} T cells, allows naive CD4⁺ T cells to express GATA-3 and to respond to endogenous IL-2, both of which are required for ensuing IL-4 production [51, 52]. Endogenous production of IL-4 is required for priming CD4⁺ T cells to develop into high-rate IL-4-producing (Th2) cells. By contrast, intense and sustained ERK activation, as observed with recombinant Anx-A1, inhibits early GATA-3 expression and transiently desensitizes the IL-2R; the net result would be failure of naive CD4⁺ T cells to produce early IL-4 and undergo subsequent Th2 differentiation [51, 52].

In conclusion, the results here presented make us to suggest that strategies aiming at inhibiting Anx-A1 functions might favor Th2 differentiation, so that these new drugs will have therapeutic potential in the treatment of Th1-mediated diseases. This is important especially if we consider that T cells from patients with

rheumatoid arthritis expressed higher level of Anx-A1 [15] and that few studies have reported increased levels of Anx-A1 in inflammatory fluids and lymphocytes during immune-mediated inflammatory reactions [53–55].

Materials and methods

Reagents

Anti-mouse CD3 (clone 145–2C11), anti-mouse CD28 (clone 37.51), PE-conjugated anti-CD69 (clone H1.2F3), murine IL-2, IL-4, IFN- γ , IL-12, anti-IL4 (clone 11B11) and anti-IFN- γ (clone XMG1.2) were purchased from eBioscience. Antibodies against both phosphorylated and total ERK were purchased from Santa Cruz Biotechnology whereas antibodies against Akt were from Cell Signaling. Unless otherwise specified, all the other reagents were from Sigma-Aldrich.

Mice

BALB/C male mice were obtained from the Charles River. Annexin 1 null mice on BALB/C were generated in our lab and bred in pathogen-free conditions in our animal facilities. All mice used in these studies were aged between 6 and 8 weeks. Animal work was performed according to United Kingdom Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act 1986) and of the European Union directives.

Cell culture

Primary murine T cells were prepared from lymph nodes by negative selection. Briefly, axillary, inguinal and mesenteric lymph nodes were teased apart to make a single-cell suspension, then washed and layered over Ficoll. The buffy coat was washed twice and then incubated with the antibody mix and the magnetic beads following the manufacturer's instructions (DynaMouse T cell negative isolation kit). In some experiments, cells were further purified to obtain naive CD62L⁺ CD4⁺ T cells by using Miltenyi Biotec CD62L⁺ CD4⁺ T cell isolation kit. Th0 conditions were created by culturing T cells for 4 days in 6-well plates precoated with anti-CD3 (5 μ g/mL) and anti-CD28 (5 μ g/mL) in complete RPMI medium (10% FCS, 2 mM L-glutamine, and 100 units/mL gentamycin) containing murine IL-2 (20 units/mL). Th1 conditions were created with murine IL-12 (3.4 ng/mL) (eBioscience), IL-2 (20 units/mL) (eBioscience) and anti-IL4 (clone 11B11; 2 μ g/mL). Th2 conditions were created with IL-4 (3000 units/mL) (Peprotech), IL-2 (20 units/mL) and anti-IFN- γ (clone XMG1.2; 2 μ g/mL). Optimal Th17 conditions were obtained as previously described using IL-6 (10 ng/mL, R&D Systems), TGF- β 1 (5 ng/mL, R&D Systems), anti-IFN- γ , and anti-IL-4 [25].

Flow cytometric analysis

Purified lymph node T cells were stimulated with plate-bound anti-CD3 and anti-CD28 as indicated in the figures. After 16 h,

the cells were stained with PE-conjugated anti-CD69 (clone H1.2F3) diluted in FACS buffer (PBS containing 1% FCS and 0.02% NaN_2). Intact cells were gated by using forward and side scatter and analyzed with the CellQuest program (Becton Dickinson) on a FACScan flow cytometer. For the allergic peritonitis, samples of 10^6 cells were suspended and incubated in PBS containing 0.1% sodium azide, 10% rat serum (PBS-S) and Fc γ IIR block mAb (CD16/CD32) for 30 min at 4°C, to avoid unspecific background staining. After the blocking step, cells were labeled with the appropriate concentration of anti-CD4 PE- and anti-CD3 PerCP-Cy5-conjugated mAb diluted in PBS-S for another 30 min at 4°C. After labeling, cells were washed and analyzed. Forward and side scatters were set to exclude erythrocytes and dead cells, and at least 10^4 lymphocytes were analyzed per sample. Granulocytes were gated based in the FSC/SSC distribution and lymphocytes were gated on FSC/SSC and positive staining with CD3, using the CellQuest™ software. Determination of positive and negative populations was performed based on the control staining with an irrelevant IgG isotype labeled with PE or PerCP-Cy5. Once determined, quadrants were maintained for all analyses.

Cell proliferation assay

Purified lymph node T cells (10^5 cells/mL) were stimulated by plate-bound anti-CD3 and anti-CD28 for 24 h in 96-well plates. After 18 h, cultures were pulsed for 8 h with 1 μCi of [^3H]thymidine (Amersham Pharmacia Biotech) and incorporated radioactivity was measured by automated scintillation counter (Packard).

Cytokine ELISA

For Th1/Th2 cytokine production analysis, Th0/Th1/Th2 cells (10^6 /mL) obtained after 4-day differentiation in skewing conditions and 1 day of resting in complete RPMI medium, were stimulated with plate-bound anti-CD3 (5 $\mu\text{g}/\text{mL}$) for 8 h in 24-well plates. Culture supernatants were collected and analyzed for IFN- γ , IL-2, IL-4 and IL-10 content by using Th1/Th2 panel ELISA kit (eBioscience). The IL-13 ELISA kit was also purchased from eBioscience.

In vivo immunization with KLH

Five 6- to 8-week-old female Anx-A1 $^{-/-}$ mice and five wild-type littermates were immunized by injection of 50 $\mu\text{g}/\text{mouse}$ of KLH in CFA at the base of the tail. At day 8 after injection, cells were collected from the draining lymph nodes and stimulated *in vitro* with KLH at 50 $\mu\text{g}/\text{mL}$ for 1 and 4 days. The IL-2 concentration in supernatants collected from cells (10^6 /mL) cultured for 24 h and IFN- γ and IL-4 concentrations in supernatants collected from cells cultured for 96 h were measured by ELISA as described above.

Ovalbumin-induced allergic peritonitis

Mice were sensitized by a subcutaneous injection of 100 μg of OVA (Sigma) adsorbed in 3.3 mg of aluminum hydroxide gel (Rehydragel low viscosity – REHEIS; USA) in sterile saline in a final volume of 0.4 mL on days 1 and 14. On day 21, the mice

were injected intraperitoneally with 0.2 mL of either vehicle (sterile saline) or 10 $\mu\text{g}/\text{site}$ OVA, 48 h post-OVA challenge, and the animals were killed by CO_2 asphyxiation. The peritoneal cavities were flushed with 3 mL of sterile PBS containing 3 mM EDTA and 25 U/mL of heparin. Total leukocyte counts were performed in a hemacytometer and a cytospin smear was performed and stained, by a hematoxylin-eosin-based staining kit (Hemacolor; Merck), for differential leukocyte counts under light microscopy (100x). For flow cytometric analysis, recovered cells were processed as described below.

Electrophoretic mobility shift assays

Nuclear extracts were harvested from 3 to 5×10^6 cells according to previously described protocols [18]. Nuclear extracts (3 to 5 μg) were incubated with 1 μg (for NFAT) or 2 μg (for NF- κB and AP-1) of poly (dI:dC) in 20 μL of binding buffer with ^{32}P end-labeled, double-stranded oligonucleotide probes (5×10^5 cpm), and fractionated on a 6% polyacrylamide gel (29:1 cross-linking ratio) in 0.5% TBE for 2.5 h at 150 volts. The NF- κB and AP-1 binding buffer (10X) was 100 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM EDTA, 50% glycerol, 10 mg/mL albumin, 30 mM GTP, and 10 mM DTT. The NFAT-binding buffer (10X) was 100 mM Hepes, pH 7.9, 500 mM KCl, 1 mM EDTA, 1 mM EGTA, 50% glycerol, 5 mg/mL albumin, 1% Nonidet P-40, 10 mM DTT. The NF- κB and AP-1 double-stranded oligonucleotide probes were from Promega whereas the NFAT was from S. Cruz Biotechnology.

RT-PCR analysis

Total RNA was extracted from treated or differentiated cells with Quiaquick mini spin columns (Qiagen) according to manufacturer's protocol and were reverse transcribed using 2 μg oligo(dT)15 primer, 10 units AMV reverse transcriptase, 40 units RNase inhibitor (Promega), and 1.25 mM each dNTP (all reagents from Promega) for 45 min at 42°C. The resultant cDNA was used for PCR using murine FPR1 (563 bp), FPR-rs1 (422 bp), or GAPDH (363 bp) primers. The primers for FPR1, FPR-rs1 and GAPDH and the PCR running conditions have been previously reported [56]. Real-time PCR was carried out by using TaqMan Universal PCR master mix and fluorescent primers obtained from Applied Biosystem web site (Assay-on-demand Gene Expression products). Cycling conditions were set according to manufacturer's instructions. Sequence-specific fluorescent signal was detected by an ABI Prism 7700 Sequence Detector System. mRNA data was normalized relative to GAPDH or 18S RNA and then used to calculate expression levels. We used the comparative Ct method [57] to measure the gene transcription in samples. The results are expressed as relative units based on calculation of $2^{-\Delta\Delta\text{Ct}}$, which gives the relative amount of gene normalized to endogenous control (GAPDH) and to the sample with the lowest expression set as one.

Western blotting analysis

Lymph node T cells were incubated as indicated in the figures. After incubation at 37°C for various time-periods, cells were

lysed in ice-cold lysis buffer (1% NP-40, 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl₂, mM EGTA, 0.5 mM PMSF, 1 μ M Aprotinin, 1 μ M Leupeptin, 1 μ M Pepstatin, 50 mM NaF, 10 mM Na₄P₂O₇, and 1 mM NaVO₄, 1 mM β -glycerophosphate). The cell lysates were centrifuged at 14 000 rpm for 5 min at 4°C and the supernatants collected and subjected to electrophoresis on SDS-10% polyacrylamide gel. After transfer, the membranes were incubated overnight with antibodies diluted in Tris-buffered saline solution containing Tween-20 (TTBS: 0.13 M NaCl; 2.68 mM KCl; 0.019 M Tris-HCl; 0.001% v/v Tween-20; pH 7.4) with 5% non-fat dry milk at 4°C. For the experiments with anti-pERK1/2 and anti-Akt, the TTBS buffer was supplemented with 50 mM NaF and BSA (5%) was used instead of milk. For each condition, extract equivalents obtained from the same number of cells were used. Immunoblotting and visualization of proteins by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) were performed according to manufacturer's instructions.

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