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The Tolerogenic Function of Annexins on Apoptotic Cells Is Mediated by the Annexin Core Domain

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Immunological tolerance is constantly being maintained in the periphery by dendritic cells processing material from apoptotic cells (ACs) in the steady-state. Although research has focused on the uptake of ACs by phagocytes, tolerogenic signals exposed by the ACs are much less well defined. In this article, we show that the annexin (Anx) family members AnxA5 and AnxA13 translocate to the surface of ACs to function as redundant tolerogenic signals in vitro and in vivo. Exposure of bone marrow–derived dendritic cells to AnxA5 or AnxA13 in vitro resulted in the inhibition of both proinflammatory cytokine secretion and the upregulation of costimulatory molecules upon TLR stimulation. The highly conserved Anx core domain was sufficient to mediate these effects, whereas recognition by N-formyl peptide receptor family members was dispensable. In vivo, coinjection of OVA-expressing and Anx-expressing ACs prevented induction of Ag-specific CD8+ T cells. Moreover, mice immunized with Anx-expressing ACs became refractory to an antigenic challenge. These results suggest that several Anxs contribute to AC-induced suppression of dendritic cell activation. Therefore, manipulating Anx-mediated immunosuppression may prove beneficial for patients with cancer or autoimmune diseases and chronic inflammatory disorders. The Journal of Immunology, 2015, 194: 5233–5242.
of Ag-specific CD8 T cell responses in vivo. Importantly, mice immunized with AnxA5- or AnxA13-expressing ACs showed a significantly reduced population of CD8 T cells specific for AC-associated Ags and became refractory to an antigenic challenge. Based on these results, we propose that several Anxs contribute to AC-induced immunosuppression in a functionally redundant manner and, thus, contribute to peripheral tolerance induction.

**Materials and Methods**

**Mice**

Tlr4−/−, AnxA1−/−, wild-type (WT), OT-I, and lymphoproliferation (lpr) mice (all on a C57BL/6 background) were maintained and bred under specific pathogen–free conditions. Tlr4−/− mice were kindly provided by S. Akira (Osaka University, Osaka, Japan), S. Uematsu (Osaka University), and L. Gissmann (German Cancer Research Center). AnxA1−/− mice were purchased from B & K Universal. OT-I mice were kindly provided by N. Garbi (University of Bonn, Bonn, Germany) and G. Hämmerling (German Cancer Research Center).

**Preparation of bone marrow–derived dendritic cells**

Bone marrow (BM)-derived DCs (BMDCs) were prepared from male or female mice between 6 and 12 wk of age, as described (26, 27). In brief, BM cells were flushed from tibias and femurs, and RBCs were lysed by brief exposure to 0.168 M NH4Cl. Cells were washed twice with RPMI 1640. For differentiation of BM precursors to BMDCs using recombinant murine GM-CSF, 1 × 10^6 cells were seeded at a density of 1 × 10^6 cells/ml in RPMI 1640 complete medium (10% FCS, 10 U/ml penicillin/streptomycin, 300 mg/ml L-glutamine, 20 ng/ml GM-CSF [Immunotools]) in a 24-well plate. After 2 d, the medium was replaced by fresh medium. After 4 d, half of the medium was removed and replaced by fresh medium. Experiments were conducted 7–8 d after differentiation. For differentiation of BM precursors to BMDCs using recombinant human Flt3L, cells were seeded at a density of 3 × 10^6 cells/ml in RPMI 1640 complete medium (10% FCS, 10 U/ml penicillin/streptomycin, 300 mg/ml L-glutamine, 300 ng/ml Flt3L [eBioscience]) in a 100-mm petri dish for 8–10 d. Additional Flt3L was added to the culture at day 5 or 6. Differentiation of BM precursors to BMDCs was monitored by flow cytometric analysis of cells using mAbs against CD11c and MHC class II.

**Flow cytometry**

mAbs and reagents used for FACS were purchased from BD Biosciences (anti-CD3–FITC, anti-CD4–FITC, anti-CD8–PE, anti-CD8--allophycocyanin, anti-CD40–PE, anti-CD45R–PerCP-Cy5.5, anti-CD86–PE, anti-Thy1.1–PE, and streptavidin-allophycocyanin), eBioscience (anti-CD14–allophycocyanin, anti-CD44–PerCP–Cy5.5, anti-CD80–FITC, anti-PD-L1–PE, anti-PD-1–FITC), CalTag (anti-CD11c–PE, anti-MHC class II–FITC), Immunotools (anti-CD45R–PerCP-Cy5.5, anti-F4/80–FITC, anti-PD-L1–PE, anti-PD-1–FITC), BioLegend (anti-Ly6G–APC). Abs were generated in our laboratory and the laboratory of G. Hämmerling (11). Appropriate isotype controls (anti-CD3–FITC, anti-CD4–FITC, anti-CD8–PE, anti-CD8–allophycocyanin, anti-CD3–FITC, anti-CD4–FITC, anti-CD8–PE, anti-Thy1.1–PE, and anti-CD14–allophycocyanin, anti-CD44–PerCP–Cy5.5, anti-CD80–FITC, anti-PD-L1–PE, anti-PD-1–FITC) were purchased as follows: AnxA5 (AF399; R&D Systems), AnxA13 (AF4149; R&D Systems), pERK (E10; Cell Signaling), ERK1 (MK12; BD), p-JNK (G9; Cell Signaling), JNK1/JNK2 (Cell Signaling), p-p38 (D3P; Cell Signaling), p38α (SF11; Cell Signaling), a-tubulin (B-5-1-2; Sigma-Aldrich), and b-actin (AC-15; Abcam). Cytokine concentrations in supernatants were determined by ELISA for murine TNF-α, IL-12p40, IL-6, and IL-10 (PeproTech), according to the manufacturers’ instructions. Boc-1 and Boc-2 were purchased from MP Biomedicals. IMLF was purchased from Sigma-Aldrich.

**Plasmid constructions, cell culture, and transfections**

The mouse (m)AnxA1, mAnxA2, mAnxA5, mAnxA9, mAnxA11, mAnxA13, mAnxA13, mAnxA5, mAnxA9, mAnxA11, mAnxA13, respectively, into a modified version of pET41a harboring a c-terminal FLAG tag, a PreScission Protease cleavage site, and a protein A tag. Recombinant proteins were expressed in the Escherichia coli strain BL21 (DE3) pLysS (Promega). Removal of LPS during protein purification was achieved by washing with TBS containing 0.1% Triton X-114 (Sigma-Aldrich). Residual LPS concentrations in the protein preparations were determined by the Limulus Amebocyte Lysate Assay (Lonza). The mAnxA1-pAc5.1 V5HisA, mAnxA5, mAnxA9, mAnxA11, mAnxA13, and mAnxA13 plasmids were generated by cloning mAnxA1, mAnxA5, and mAnxA13, respectively, into pAc5.1/V5-HisA (Life Technologies). Drosophila melanogaster Schneider 2 (S2) cells were transfected with Ca(OH)2, according to the manufacturer’s instructions (Life Technologies). To generate stably transfected S2 cell lines, cotransfection with pCoHygro was performed, and cells were selected using Hygromycin B (PAA). D. melanogaster-S2 cells were cultured in Schneider’s insect medium (Sigma-Aldrich) supplemented with 10% FCS. Human Jurkat T cells and the human T-ALL cell line CEM were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS.

**RNA isolation and quantitative RT-PCR**

RNA was isolated from cells using the RNAqueous-Micro Kit, according to the manufacturer’s instructions (Ambion). RNA was quantified by detection of SYBR Green incorporation using the ABI Prism 7500 sequence detector system (Applied Biosystems). Expression levels were normalized to GAPDH. The following primer sequences were used: Fpr1 5′-GCC TTC ATG TTC ACT GTC-3′ (fwd) and 5′-AAC CCG CAA AGG ACG CCT GG-3′ (rev), Fpr2 5′-AGC TGG TTG TTC AGA CAA AGG GAA-3′ (fwd) and 5′-TGC CCA CAA GAC CAA GGA AG-3′ (rev); Fpr3 5′-CCT TCG CCA GTT CTT ACA GGA-3′ (fwd) and 5′-CAC TAA ACT GCA TCT CTT TGA-3′ (rev); and GAPDH 5′-ACT CCA CTC AGC GCA AAT TCA-3′ (fwd) and 5′-GCC TCC CAC CAF TGT ATG TT-3′ (rev).

**Isolation of murine primary neutrophils and splenocytes**

Murine neutrophils were isolated from BM by positive selection using anti-Ly6G MicroBeads (Miltenyi Biotec), according to the manufacturer’s instructions. Neutrophils were cultured in RPMI 1640 medium supplemented with 10% FCS at a density of 2 × 10^6 cells/ml. The purity of cells was controlled by flow cytometric analysis using anti-Gr-1–FITC (Miltenyi Biotec). Cells were >90% positive for Gr-1. Murine splenocytes were isolated from spleens and filtered through a 40-μm cell strainer (BD Biosciences). Splenocytes were cultured in RPMI 1640 medium supplemented with 10% FCS.

**Preparation of AcCs**

To generate early AcCs, Jurkat T cells or CEM cells were UV-C irradiated (50 mJ/cm^2). Stratalinker 1800; Stratagene) in six-well plates at a cell density of 1 × 10^6 cells/ml. Subsequently, cells were cultured at 37°C for 2–6 h. To generate early apoptotic splenocytes, cells were UV-C irradiated (15 mJ/cm^2) in six-well plates at a cell density of 1 × 10^6 cells/ml. Subsequently, cells were cultured in media at 37°C for 3 h. Primary murine neutrophils spontaneously undergo apoptosis in cell culture and show characteristics of early AcCs after 1 d. S2 cells were irradiated with 250 μJ/cm^2 at a cell density of 1 × 10^6 cells/ml in 100-mm petri dishes and used after overnight (on) incubation.

**In vivo experiments**

For analysis of OVA-specific, endogenous T cells, a mixture of 5 × 10^5 apoptotic, membrane-anchored OVA (mOVA)-expressing S2 cells (mOVA) together with 5 × 10^5 AnxA1- or AnxA13-expressing apoptotic S2 cells (mOVA AnxA1, mOVA AnxA13, or mOVA mock) were injected i.v. into female C57BL/6 WT mice. Six to eight days after immunization, single-cell suspensions of mesenteric lymph nodes were analyzed for OVA-specific CD8 T cells using PE-labeled Kb/SIINFEKL pentamers, according to the manufacturer’s instructions (ProImmune). For the analysis of OVA-specific CD8 T cell proliferation and mAnxA13 pAc5.1/V5-HisA plasmids were generated by cloning into S.2 cells were injected i.v. into female C57BL/6 WT mice. The next day, mice were immunized i.v. with a mixture of 0.5 × 10^5 mOVA, together with 1 × 10^6 AnxA5, 5 × 10^5 AnxA13, or mOVA mock. After 12–13 d, mice were challenged i.p. with 50 μg OVA protein (InvivoGen) in 125 μl PBS emulsified in 125 μl IFA (Sigma-Aldrich). On day 7 after challenge, splenic cell suspensions were analyzed by flow cytometry and prepared for 4′,6-diamidino-2-phenylindole (DAPI) staining. Abs were detected using a FACSCanto II (BD Biosciences). For determination of absolute OT-I T cell numbers/spleen, we analyzed an exact aliquot of each spleen using Trucount Beads (Becton Dickinson), according to the manufacturer’s instructions.
ELISPOT analysis

IFN-γ ELISPOT assays were performed with 1.5 × 10^6 splenic cells/well and 1 μM the OVA-derived, MHC class I-dependent peptide SIINFEKL (Axxora). Splenic cell suspensions were stimulated o/n, and IFN-γ ELISPOTs were developed according to the manufacturer’s instructions (BD Pharmingen).

Coculture of BMDCs and ACs or pretreatment with recombinant proteins

A total of 1 × 10^5 BMDCs was incubated with recombinant protein (125–500 nM) or with apoptotic Jurkat T cells (2–4 × 10^5 cells), apoptotic neutrophils (1 × 10^5–1 × 10^6 cells), apoptotic splenocytes (1 × 10^5–1 × 10^6 cells), or apoptotic S2 cells (5 × 10^5–1 × 10^6 cells) for 6–8 h. Depending on the mouse background, BMDCs were subsequently stimulated with LPS (Sigma-Aldrich), CpG 1668, or CpG 2395 (InvivoGen). Cytokine concentrations in the supernatants were analyzed by ELISA 12–16 h after TLR stimulation. Surface molecule expression was analyzed 48 h after TLR stimulation by flow cytometry.

Statistical analysis

Statistical analysis of data was performed by one-way ANOVA, followed by the Bonferroni posttest for multiple comparisons. If not otherwise indicated, the significance of the difference compared with CpG-stimulated cells is depicted. The p values < 0.05 were considered statistically significant.

Ethics statement

All animal studies were approved by the veterinary authorities (Regierungspräsidium Karlsruhe) of Baden-Württemberg (G-96 06, G-173/11).

Results

The tolerogenic effect of AnxA1 is mediated by its core domain

We previously showed that AnxA1 on the surface of ACs negatively regulates DC activation, as evidenced by low expression of co-stimulatory molecules and low proinflammatory cytokine secretion upon TLR stimulation (11). To investigate the extent to which known anti-inflammatory sequences of AnxA1—the N terminus and the AF-2 sequence—contribute to this tolerogenic effect, we generated recombinant full-length AnxA1 and AnxA1 mutants (Fig. 1A, Supplemental Fig. 1A–D). LPS was carefully removed during purification of recombinant proteins by addition of Triton X-114, and all in vitro experiments were performed using BMDCs from Tlr4^-/- mice to exclude effects induced by endotoxin toler-
ance. In addition, a control protein, serpin 8b, purified in an identical fashion as AnxA1, was inactive in our functional assays (11). Surprisingly, deletion of the N-terminal domain, mutation of the AF-2 sequence, or a combination of both did not affect AnxA1-induced inhibition of proinflammatory cytokine secretion upon stimulation with the TLR9 ligand CpG (Fig. 1B, Supplemental Fig. 1E). This was not due to an absence of FPR expression, because FPR family members involved in AnxA1 recognition are expressed in BMDCs on the mRNA level and on the protein level (Fig. 1C, 1D). To further study the involvement of FPR family members in AnxA1-induced DC tolerization, the pan-specific FPR antagonists Boc-1 and Boc-2 were used in subsequent experiments. The presence of Boc-1 and Boc-2 did not abrogate inhibition of TLR-induced TNF-α secretion by AnxA1 (Fig. 1E, Supplemental Fig. 1F, 1G). In line with this, the prototypic FPR ligand MLE, but not AnxA1, led to activation of MAPK (Fig. 1E, 1G). In conclusion, FPR family members do not contribute to the recognition of AnxA1 by BMDCs in the context of suppression of DC activation upon TLR stimulation. Instead, the AnxA1 core domain is sufficient to mediate DC tolerization, independent of the AF-2 sequence and the AnxA1 N-terminal domain.

Several Anx family members translocate to the cell surface upon induction of apoptosis

The Anx core domain is highly conserved among Anx family members, and redundancy was reported for various functions of Anxs (12, 25, 28–31). Therefore, we hypothesized that several Anxs might act as tolerogenic signals on the surface of ACs. To test this hypothesis, we first investigated whether other Anxs, in addition to AnxA1, translocate to the cell surface upon apoptosis induction. We focused on AnxA5, because it is upregulated in different tissues of AnxAI−/− mice and might compensate for the loss of AnxA1 (32), as well as on AnxA13, which represents the founding member of the family (33). The surface translocation of Anxs was first studied by ectopic expression of murine Anxs in D. melanogaster S2 cells. Stably transfected S2 cells showed surface exposure of AnxA1, AnxA5, or AnxA13 during early apoptosis when membrane integrity was still intact (Fig. 2A, Supplemental Fig. 2). Next, we induced apoptosis in the human T cell line CEM and monitored translocation of endogenous Anxs. In line with the findings observed in stably transfected S2 cells, AnxA5 and AnxA13, in addition to AnxA1, translocated to the surface of apoptotic CEM cells (Fig. 2B, 2C). Of note, protein amounts of Anxs in the cytosolic fraction remained almost unchanged, indicating that only a minor fraction of the cytosolic pool of Anxs translocates to the cell surface during apoptosis (Fig. 2B). In summary, AnxA1, as well as AnxA5 and AnxA13, are exposed on early ACs.

**FIGURE 2.** AnxA5 and AnxA13 translocate to the cell surface upon induction of apoptosis. (A) Flow cytometric analysis of aS2. Sixteen hours after UV-C irradiation (250 mJ/cm²), mock- or AnxA1/5/13-transfected aS2 cells were early apoptotic (AnnV+/7-AAD−) and stained using biotin-conjugated anti-6xHis streptavidin-allophycocyanin. (B) Immunoblot analysis of cytosolic and membrane (EDTA wash) fractions of viable (0 h) or early UV-C–irradiated apoptotic CEM cells (50 mJ/cm², 2–6 h). (C) Quantification of cell viability of UV-C–irradiated early apoptotic CEM cells in (B) by flow cytometry using AnnV-FITC/7-AAD. AnxA5 and AnxA13 inhibit TLR-induced proinflammatory cytokine secretion by BMDCs

Because AnxA5 and AnxA13 met the prerequisite to act as a tolerogenic signal, surface translocation during early apoptosis, we next studied whether they also, like AnxA1, regulate TLR-induced DC activation. For this, either purified recombinant proteins or stably transfected S2 cells exposing the respective Anx upon apoptosis induction were used (Fig. 2A, Supplemental Fig. 3A, 3B). Incubation of BMDCs with recombinant AnxA1, AnxA5, or AnxA13 significantly reduced the secretion of the proinflammatory cytokines TNF-α, IL-12p40, and IL-6 by TLR-stimulated BMDCs in a concentration-dependent manner (Fig. 3A–D). Importantly, this effect was independent of altered cell viability of BMDCs (Supplemental Fig. 3C). Similar to the results obtained using recombinant Anxs, aS2 AnxA1 or aS2 AnxA5 reduced TLR-induced TNF-α secretion. In contrast, aS2 mock did not modulate DC activation (Fig. 3E). Flow cytometric analysis did not reveal differences in phosphatidylserine externalization, indicating that apoptosis kinetics were comparable in mock- and Anx-transfected S2 cells (Supplemental Fig. 3D). The tolerogenic effect of Anxs was concentration dependent, because the inhibition of TLR-induced proinflammatory cytokine secretion increased with the ratio of apoptotic S2 cells/BMDCs and correlated with Anx expression levels in S2 cells (Fig. 3E, 3F, Supplemental Fig. 3E). CD8α+ DCs, which can be generated via differentiation of BM precursors using Flt3L in vitro, play a critical role in the presentation of self-Ags derived from ACs (34). Addition of recombinant Anx to Flt3L-derived BMDCs reduced the TLR-induced secretion of TNF-α in a concentration-dependent manner, whereas the secretion of the anti-inflammatory cytokine IL-10 was not affected (Fig. 3G, 3H). These effects were recapitulated with Flt3L-derived BMDCs incubated with aS2 AnxA1 or aS2 AnxA5. Although the secretion of the proinflammatory cytokine TNF-α was reduced, Anxs had no influence on the secretion of IL-10 (Supplemental Fig. 3F, 3G). Taken together, our data show that AnxA5 and AnxA13 inhibit the TLR-induced secretion of proinflammatory cytokines by DCs, including CD8α+ DCs, whereas secretion of the immune-regulatory cytokine IL-10 was not affected.

AnxA5- and AnxA13-treated BMDCs show impaired upregulation of costimulatory molecules upon TLR-induced DC activation

ACs and AnxA1 also regulate surface expression of costimulatory molecules on DCs (11, 35, 36). Therefore, the effect of recombinant AnxA5 and AnxA13 on the expression of coregulatory and MHC molecules was investigated 2 d after TLR stimulation. Incubation of BMDCs with AnxA1, AnxA5, or AnxA13 did not influence the
surface expression of MHC class I molecules, but it slightly increased the expression of MHC class II molecules (Fig. 4A, 4B). Remarkably, AnxA1, AnxA5, and AnxA13 inhibited upregulation of the costimulatory molecules CD40, CD80, and CD86 upon TLR stimulation (Fig. 4C–E). In contrast, surface expression of the coinhibitory molecules programmed cell death ligand (PD-L)1 and PD-L2, as well as the cell viability of BMDCs, were not affected (Fig. 4F–H). In summary, AnxA1, AnxA5, and AnxA13 impair TLR-induced upregulation of costimulatory molecules without affecting surface expression of coinhibitory and MHC molecules.

FPR family members are not required for DC tolerization by AnxA5 and AnxA13

The phenotypes of BMDCs incubated with AnxA5 or AnxA13 resemble the phenotype induced by AnxA1. Therefore, we next tested whether recognition of the AnxA5, AnxA13, or the AnxA1 core domain is independent of FPR family members. Indeed, analysis of MAPK activation revealed that MAPK was not activated in BMDCs after incubation with AnxA5, AnxA13, or the AnxA1 core domain (Fig. 5A). Furthermore, the pan-specific FPR antagonist Boc-2 did not abrogate inhibition of TLR-induced proinflammatory cytokine secretion by AnxA5, AnxA13, or the AnxA1 core domain (Fig. 5B). In conclusion, AnxA1, AnxA5, and AnxA13 induce the development of DCs with a tolerogenic phenotype, as characterized by low expression of costimulatory molecules and low proinflammatory cytokine secretion independent of FPR signaling.

AnxA5 and AnxA13 suppress CD8+ T cell immune responses in vivo

Several Anxs translocate to the surface of ACs to promote the development of tolerogenic DCs, and Anx single-knockout mice lack a severe phenotype. Therefore, we hypothesized that Anxs are redundant tolerogenic signals in vivo. Thus, deletion of AnxA1 should neither impair suppressive effects of ACs on AC-treated BMDCs nor provoke autoimmunity against self-Ags derived from ACs. In fact, the absence of AnxA1 does not lead to an altered phenotype of BMDCs after AC engulfment. Apoptotic neutrophils or splenocytes from WT and AnxA1−/− mice suppressed TLR-induced TNF-α secretion by BMDCs to a comparable extent.
The level of suppression increased with an elevated ratio of ACs/BMDCs, as seen with apoptotic Jurkat T cells, which served as a positive control (Fig. 6A, Supplemental Fig. 4A, 4B). Importantly, neutrophils and splenocytes from WT and AnxA12/2 mice showed no difference in apoptosis kinetics upon irradiation with UV-C (Supplemental Fig. 4C).

Next, AnxA12/2 mice were analyzed for signs of autoimmunity. CD62L/CD44 surface expression of different splenic T cell populations was comparable between WT and AnxA12/2 mice, indicating that there was no change in the activation status of peripheral T cells. In contrast, lpr mice, which served as a positive control for the development of systemic autoimmunity, clearly showed enhanced activation of splenic T cells (Supplemental Fig. 4D). Furthermore, splenomegaly and lymphadenopathy were absent in 5–6-mo-old AnxA12/2 mice (Supplemental Fig. 4E). For the abovementioned investigations (Supplemental Fig. 4D, 4E), cells...
from female and male mice were used, and no gender-specific differences were noticed. In conclusion, the lack of autoimmunity in AnxA1<sup>2/2</sup> mice and the unaltered suppressive capacity of ACs from AnxA1<sup>2/2</sup> mice indicated that AnxA5 and AnxA13 might substitute for the loss of AnxA1 and might act as tolerogenic signals in vivo.

Recently, our group showed that AnxA1 restrains the function and reduces the frequency of Ag-specific CD8<sup>+</sup> T cells upon immunization of mice with xenogeneic ACs ectopically expressing AnxA1 (11). To investigate whether AnxA5 and AnxA13 also regulate the induction of Ag-specific T cell responses in vivo, aS2 mOVA were injected into mice, and the anti-OVA T cell response was monitored. Injection of aS2 mOVA induced a cell-mediated immune response and led to the development of OVA-specific CD8<sup>+</sup> T cells (Fig. 6B, Supplemental Fig. 4F). Notably, coinjection of apoptotic S2 cells overexpressing AnxA1, AnxA5, or AnxA13 strongly inhibited the development of OVA-specific CD8<sup>+</sup> T cells (Fig. 6B). We further analyzed the effect of Anxs on the surface of ACs with regard to the stimulation and fate of Ag-specific CD8<sup>+</sup> T cells in a transfer experiment. We injected CFSE-labeled OVA-specific OT-I T cells into C57BL/6 WT mice and followed proliferation and expansion of this population with the help of the congenic marker Thy-1.1. We detected no difference in proliferation or OT-I population size 6 d after immunization (data not shown). However, when challenging with OVA protein 12 d after the primary immunization, we detected a significant reduction in the absolute number of OT-I cells in mice coimmunized with aS2 AnxA5 or aS2 AnxA13 (Fig. 6C). Importantly, the immune response in mice coimmunized with Anx-overexpressing apoptotic S2 cells was also functionally impaired, as evidenced by a severely reduced number of IFN-γ–secreting CD8<sup>+</sup> T cells (Fig. 6D). Similar results were obtained by ELISA (data not shown). Thus, AnxA5 and AnxA13 negatively regulate the induction of Ag-specific CD8<sup>+</sup> T cell responses and, therefore, act as tolerogenic signals on ACs in vivo.

**Discussion**

Uptake and processing of self-Ags derived from ACs by DCs in the steady-state and subsequent presentation of self-peptides on MHC class I and MHC class II by tolerogenic DCs are important mechanisms to induce CD4<sup>+</sup> and CD8<sup>+</sup> T cell tolerance in the periphery (36, 37). Immunosuppression of phagocytes by ACs is...
mediated by tolerogenic signals on the surface of ACs, including growth arrest-specific gene 6, inactivated complement component 3b, thrombospondin 1, and AnxA1 (10, 11, 38–40). In lymphoid organs, the subsequent interaction of tolerogenic DCs and autoreactive T cells induces anergy or deletion, or it may trigger conversion of naive CD4⁺ T cells into regulatory T cells (41–46). Because the knowledge of tolerogenic signals on the surface of ACs is limited, and functional redundancy has been reported for different properties of Anxs, this study aimed at investigating whether Anxs act as redundant tolerogenic signals on ACs.

Upon induction of apoptosis, AnxA1 translocates to the surface of ACs and is recognized by phagocytosing DCs (11). Using a leukemia cell line and stably transfected apoptotic S2 cells, we showed that translocation upon induction of apoptosis is a common feature of AnxA1, AnxA5, and AnxA13 (Fig. 2). To our knowledge, this is the first report showing translocation of AnxA13 to the surface of ACs, whereas translocation of AnxA5 to the surface of rat cardiomyocytes upon treatment with staurosporine or H₂O₂ was reported previously (47). Externalization of AnxA5 also takes place under pathophysiological conditions like acute myocardial infarction or glomerulonephritis (48, 49). In both cases, passive release from necrotic cells cannot be ruled out. However, this study shows that Anxs are externalized at an early stage of apoptosis preceding the loss of membrane integrity (Fig. 2).

AnxA5 and AnxA13 induce the development of a tolerogenic DC phenotype, as we showed previously for AnxA1 (11). Soluble and membrane-bound AnxA5 and AnxA13 inhibit the TLR-induced secretion of proinflammatory cytokines (i.e., IL-12p40, IL-6, and TNF-α), whereas the secretion of IL-10 is not affected (Fig. 3B–H). TLR-induced upregulation of the costimulatory molecules CD40, CD80, and CD86 was inhibited by Anx pretreatment, whereas surface levels of MHC class I and II molecules and coinhibitory molecules were not altered (Fig. 4A–G). Thus, the phenotype of DCs obtained after Anx incubation resembles the one of DCs after uptake of ACs or upon incubation with AC-derived tolerogenic signals (10, 38, 39, 50–52). Unaltered expression of MHC molecules on DCs is a prerequisite for efficient presentation of self-peptides and, thus, is required for deletion or tolerization of autoreactive T cells in the draining lymph nodes (2). In addition, low expression of costimulatory molecules, especially CD40, was described as a decisive feature of tolerogenic DCs (53, 54). Inhibition of TLR-induced upregulation of costimulatory molecules was observed after incubation of BMDCs with the tolerogenic signals growth arrest-specific gene 6 or inactivated complement component 3b or upon ligation of receptors that recognize tolerogenic signals, including Mer tyrosine kinase, complement receptor 3, or CD36 (10, 38, 39, 51). Importantly, deletion and functional inactivation of autoreactive CD8⁺ T cells require the absence of CD40L-induced signaling, as well as the expression of PD-1 ligands PD-L1 and PD-L2, neither of which was affected by Anx pretreatment of BMDCs (46).

Similar to AC uptake or ligation of Mer tyrosine kinase, CD36, or complement receptor 3, preincubation of BMDCs with Anxs results in inhibition of TLR-induced secretion of proinflammatory cytokines, including TNF-α, IL-6, and IL-12p40 (Fig. 3B–H) (10, 38, 39, 50). The low secretion of the Th1-promoting cytokines TNF-α and IL-12p40, as well as of the Th17-promoting cytokine IL-6, implies that Th1 and Th17 priming of naive CD4⁺ T cells by Anx-treated BMDCs is unlikely.

Tolerance induction by AnxA1, AnxA5, and AnxA13 is independent of DC apoptosis, recognition of Anxs by FPR family members, or inhibition of phospholipase A₂ activity. cPLA₂ was described to be the key enzyme for signal transduction of inflammation (55, 56). Inhibition of cPLA₂ is mediated by specific interaction of cPLA₂ and aa 275–346 of AnxA1. Other Anxs, including AnxA5, do not inhibit cPLA₂ activity in vitro (57). Because AnxA5 and AnxA13 promote the development of tolerogenic DCs (Figs. 3B–H, 4A–G), inhibition of cPLA₂ is not required for suppression of DC activation by Anxs. High amounts of AnxA1 released into the inflammatory fluid accelerate apoptosis of neutrophils and monocytes, which contributes to the resolution of inflammation (58–60). AnxA5 can have proapoptotic or antiapoptotic effects, depending on the cell type investigated (47, 61). Importantly, preincubation of BMDCs with AnxA1, AnxA5, or AnxA13 did not influence apoptosis progression, ruling out DC apoptosis as a mechanism for tolerance induction by Anxs (Fig. 4H, Supplemental Fig. 3C).

N-terminal peptides and the AF-2 sequence in the core domain were implicated in mediating anti-inflammatory effects of AnxA1 (15, 21, 62, 63). However, several points make it unlikely that recognition by FPR family members contributes to the tolerogenic effect of Anxs on BMDCs. First, the Anx core domain, which is highly conserved among Anx family members, is sufficient to inhibit TLR-induced cytokine secretion of BMDCs (Fig. 1B). Second, mutation of the AF-2 sequence, the only other peptide sequence binding to FPR family members, does not abrogate the suppressive effect elicited by Anxs (Figs. 1E, 5B). Fourth, pan-specific antagonists of FPR family members do not abrogate the suppressive effect elicited by Anxs (Figs. 1E, 5B). Finally, phosphorylation of ERK, induced upon binding of N-terminal peptides of AnxA1 to FPR family members, could not be detected in BMDCs upon treatment with AnxA1, AnxA5, or AnxA13 (Figs. 1F, 5A). The lower levels of FPR family members expressed on DCs and/or different signaling thresholds of DCs compared to neutrophils may explain the differential effects noted for the individual domains of Anxs, depending on the cell type or biological setting (Fig. 1C). The data presented in this article support the existence of a different, hitherto unknown receptor involved in the recognition of Anxs on DCs.

Our results show that several Anx family members translocate to the surface of ACs and induce tolerogenic signaling upon recognition by their cognate receptor on DCs. The absence of severe phenotypes in mice deficient in individual Anx family members and almost identical tissue-specific expression patterns of most similar Anx genes suggest that Anxs have redundant functions in vivo (12, 25, 28–31). In fact, redundant functions for different Anxs have been reported in the context of membrane trafficking, inhibition of phospholipase activity, and blood coagulation in vitro (12). In a noninflammatory or noninfectious setting and on a mouse background not prone to develop autoimmunity, AnxA1⁻/⁻ mice lack symptoms of autoimmune or chronic inflammatory diseases (Fig. 6). This is likely due to upregulation of other, equally suppressive Anx family members in AnxA1⁻/⁻ mice (Fig. 6) (32). By analyzing AnxA1, AnxA5, and AnxA13 we chose representative Anxs that are distributed equally through the phylogenetic tree of vertebrate Anxs, including AnxA13 as the founding member of this protein family (13). As typical representatives of the Anx family, the core domains of murine AnxA1, AnxA5, and AnxA13 show a comparatively high mean amino acid similarity of 55–60% to all other Anx core domains. Therefore, we hypothesize that the immune-suppressive activity residing in the Anx core domain, as identified in our study, is likely shared by more, if not most, members of the Anx family. If so, the ubiquitous expression of the Anx family as a whole (13) could afford protection from autoimmunity to virtually any tissue of the organism.
By analyzing the fate of transferred Ag-specific T cells, we observed that AnxA5 and AnxA13 exert a tolerogenic effect on the surface of ACs, which leads to a significant reduction in the population size and activity of Ag-specific CD8+ T cells. Regarding CD8+ T cell activity, AnxA5 and AnxA13 recapitulate the effect of AnxA1, as analyzed in our previous study (11). Moreover, these results further elucidate the Anx-mediated mechanism of immune suppression in vivo and suggest a mechanism similar to deletional tolerance, as described by Steinman and colleagues (64).

A better understanding of tolerogenic signals on ACs provides new opportunities to interfere with peripheral tolerance induction for the treatment of autoimmune diseases, chronic inflammatory diseases, and cancer. The clearance of apoptotic tumor cells by tumor-associated macrophages or DCs is associated with the release of anti-inflammatory cytokines and tolerance induction to tumor Ags (65). The immunoregulatory environment established by the tumor and the presentation of tumor Ags in the absence of danger signals may account for the failure of most anticaner chemotherapies to promote curative T cell immunity (66). One powerful approach to counteract the tolerogenic properties of the tumor is to induce immunogenic cell death in which alarmins are powerful approach to counteract the tolerogenic properties of the tumor (66). One powerful approach to counteract the tolerogenic properties of the tumor is to induce immunogenic cell death in which alarmins are released during apoptosis (67). However, the release of alarmins has severe side effects, including promotion of angiogenesis, resistance to chemotherapeutics, chronic inflammation, development of gout, and renal failure (65). Our data can guide the development of an alternative approach: interference with the presentation of tolerogenic signals, such as Anxes, or blockade of those on dying tumor cells with the goal to maximize the impact of cancer immunotherapy.

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Disclosures

The authors have no financial conflicts of interest.

References


Figure S1

Figure S1 The tolerogenic effect of AnxA1 is mediated by its core domain

Data here are related to Fig. 1. (A) Immunoblot and silver-stained SDS-PAGE gel of the purification of recombinant AnxA1 from *E. coli*. Lane 1 = soluble fraction of noninduced bacteria, lane 2 = soluble fraction of IPTG-treated bacteria, lane 3 = soluble fraction after incubation with IgG sepharose beads, lane 4 = membrane fraction of IPTG-treated bacteria, lane 5 = TBS-Tx114 wash fraction, 6 = TBS-Tween 20 wash fraction, 7 = IgG sepharose beads after cleavage using PreScission Protease, lane 8 = dialysed, PreScission Protease-depleted recombinant protein. Asterisk and arrow indicate recombinant full length Protein A-tagged and PreScission Protease-cleaved protein, respectively. (B-D) Immunoblot analysis and silver-stained SDS-PAGE gel of the purification of AnxA1ΔN (B), AnxA1 mAF-2 (C) and AnxA1ΔN mAF-2 (D) from *E. coli*. (E) Quantification of cell viability of BMDCs of one representative experiment from Fig. 1B by flow cytometry using AnxV-FITC/7-AAD. (F) Quantification of cell viability of BMDCs of one representative experiment from Fig. 1E by flow cytometry using AnxV-FITC/7-AAD. (G) Functionality of FPR antagonists was controlled by blocking CD62L shedding on NΦ induced by fMLF in the presence of Boc-2. Surface expression of CD62L was detected by flow cytometry.
Figure S2 Upon induction of apoptosis AnxA5 and AnxA13 translocate to the surface of apoptotic cells

Data here are related to Fig. 2. Immunoblot analysis of S2 cell lysates either transfected with the empty vector pAc5.1 V5/HisA (mock) or a vector encoding mAnxA1 (AnxA1), mAnxA5 (AnxA5) or mAnxA13 (AnxA13).
Figure S3 AnxA5 and AnxA13 inhibit TLR-induced DC activation

Data here are related to Fig. 3. (A-B) Immunoblot analysis and silver-stained SDS-PAGE gel of the purification of AnxA5 (A) and AnxA13 (B) from E. coli. (C) Quantification of cell viability of BMDCs from one representative experiment of Fig. 3B by flow cytometry using AnxV-FITC/7-AAD. (D) Quantification of cell viability of viable or apoptotic S2 cells of Fig. 3E by flow cytometry using AnxV-FITC/7-AAD. (E) Immunoblot analysis of S2 cell lysates either transfected with the empty vector pAc5.1 V5/HisA or a vector encoding mAnxA1 (S2 AnxA1). (F-G) Flt3L-differentiated BMDCs from Tlr4−/− mice were incubated with the indicated ratio of aJ or aS2 cells. 4-6 h later, cells were stimulated with 40 nM CpG o/n. TNF-α (F) or IL-10 (G) concentrations in the supernatants were determined by ELISA. Data are mean ±SEM of three independent experiments.
Figure S4

AnxA1-/− mice do not show symptoms of autoimmunity

Data here are related to Fig. 6. (A) GM-CSF-differentiated BMDCs from WT mice were incubated with the indicated ratio of aJ or apoptotic splenocytes (a spl, UV-C irradiation 15 mJ/cm², 3h) from WT or AnxA1-/− mice. 4 h later, cells were stimulated with 1 ng/ml LPS o/n. TNF-α concentrations in the supernatants were determined by ELISA. Data are mean ± SEM of three independent experiments. (B) Immunoblot analysis of cell lysates of neutrophils (NΦ) or splenocytes (spl) from WT or AnxA1-/− mice. (C) Quantification of cell viability of neutrophils and splenocytes used in Fig. 6A and Fig. S4A by flow cytometric analysis using AnxV-FITC/7-AAD. (D) Flow-cytometric analysis of the activation status of naïve (CD62L+) vs. activated/memory T cells (CD44⁺CD62L−, CD44⁺CD62L+) in spleens of 6-8-week-old WT, AnxA1-/− and lpr mice. Data are mean ± SD of n = 3-5. (E) Representative photograph of spleen and lymph nodes of 5-6-month-old WT and AnxA1-/− mice.