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REVIEW

Annexin-A1: a pivotal regulator of the innate and adaptive immune systems

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The glucocorticoids are the most potent anti-inflammatory drugs that we possess and are effective in a wide variety of diseases. Although their action is known to involve receptor mediated changes in gene transcription, the exact mechanisms whereby these bring about their pleiotropic action in inflammation are yet to be totally understood. Whilst many different genes are regulated by the glucocorticoids, we have identified one particular protein—annexin A1 (Anx-A1)—whose synthesis and release is strongly regulated by the glucocorticoids in many cell types. The biology of this protein, as revealed by studies using transgenic animals, peptide mimetics and neutralizing antibodies, speaks to its role as a key modulator of both of the innate and adaptive immune systems. The mechanism whereby this protein exerts its effects is likely to be through the FPR receptor family—a hitherto rather enigmatic family of G protein coupled receptors, which are increasingly implicated in the regulation of many inflammatory processes. Here we review some of the key findings that have led up to the elucidation of this key pathway in inflammatory resolution.
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Keywords: glucocorticoids; FPR; PMN; macrophages; prostaglandins; T cells; inflammation

Abbreviations: AA, arachidonic acid; ALXR, lipoxin A4 receptor; Anx-A1, annexin A1; AP-1, activator protein-1; DEX, dexamethasone; FCS, foetal calf serum; FMLP, formyl methionyl leucyl phenylalanine; FPR, formyl peptide receptor; FPRL-1/-2, formyl peptide-like receptors 1 and 2; HPA, hypothalamo-pituitary axis; hu-r-Anx-A1, human recombinant annexin A1; IL-2, interleukin-2; LTC_4, leukotriene C_4; mRNA, messenger RNA; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PGE_2, prostaglandin E_2; PMN, polymorphonuclear leukocyte; RA, rheumatoid arthritis; RF, releasing factor; ROR-γ, retinoid orphan receptor-γ; TCR, T-cell receptor; Th, T helper; Treg, regulatory T cells; TRPV-1, transient receptor potential vanilloid 1; Tx, thromboxane

Introduction

The discovery of the 37 kDa protein annexin A1 (Anx-A1; formerly macrocortin, lipocortin-1) arose from investigations by several laboratories into the mechanisms whereby anti-inflammatory glucocorticoids suppressed prostanoid synthesis by cells. In the authors' own group (Flower and Blackwell, 1979; Blackwell *et al*., 1980), it was determined that these drugs, acting through a receptor-dependent mechanism, promoted the synthesis and release of a proteinacious factor from guinea pig-isolated perfused lungs, which itself possessed glucocorticoid-like activity, in the sense that it could suppress the release of prostaglandins from lung tissue (see Figure 1). Further sources of this protein were discovered (Carnuccio *et al*., 1980, 1981; Blackwell *et al*., 1982), and it was found to possess anti-inflammatory properties. Factors with similar biological profiles (renomedullin, lipomodulin) reported by other laboratories (Russo-Marie *et al*., 1979a, b; Hirata *et al*., 1980) were, in due course, compared with this protein with the finding that all were structurally or immunologically related (Di Rosa *et al*., 1984).

Anx-A1 itself (as it is now known) was subsequently purified to homogeneity from peritoneal lavage fluid obtained from glucocorticoid-treated rats (Pepinsky *et al*., 1986), cloned and sequenced in 1986 (Wallner *et al*., 1986). A comparison of homologies between Anx-A1 and several other partially purified proteins, which had been implicated in diverse cellular functions (Kretsinger and Creutz, 1986), led to the recognition that this protein was related to a much larger family of proteins (subsequently dubbed the 'Annexin super family').

The early history of the discovery of Anx-A1 has been reviewed before in this journal (Flower, 1988) and will not be reiterated here. Instead, this paper will focus on what we

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now know about the action of Anx-A1 in the innate and adaptive immune systems today. We will not discuss the role of this protein (or other annexins) in cell biology other than as it pertains to the release of inflammatory mediators or the regulation of inflammation itself, neither will we discuss the role of Anx-A1 in the functioning of the hypothalamo-pituitary (HPA) axis. The reader is referred to other reviews (Seaton and Dedman, 1998; Gerke and Moss, 2002; Moss and Morgan, 2004; Gerke *et al.*, 2005) covering the former topic; a prior (Buckingham, 1996) and forthcoming review in this journal deals specifically with the important role of Anx-A1 in regulating glucocorticoid effects in the neuroendocrine system.

Anx-A1 structure

The annexin superfamily comprises some thousand proteins distributed throughout most eukaryotic phyla although it is absent in yeasts and prokaryotes (Moss and Morgan, 2004). The protein family is characterized by the presence of four (six in the case of Anx-A6) homologous repeating domains of approximately 70 residues (the 'core') that harbour (in most cases) 'type 2' calcium-binding domains. Each member of the family is distinguished by a unique N-terminal region. There are 12 members of the annexin family in vertebrates and these are thought to have been derived from an ancestral *Anx-A13* gene (Iglesias *et al.*, 2002). Anx-A1 itself is a monomeric amphipathic protein comprising 346 amino acids. The N-terminal domain contains several putative Ser and Thr phosphorylation sites as well as consensus sequences for glycosylation (Asn^{43}-Ser^{45}) and transglutamination. Although not apparently widespread, both glycosylated (Goulet *et al.*, 1992) and transglutaminase-linked (Pepinsky *et al.*, 1989) species have been reported in the placenta and elsewhere (Ando *et al.*, 1991).

Anx-A1 also harbours several proteolytic motifs and an N-terminal truncated moiety is a commonly found species in inflammatory fluids (Liu *et al.*, 1995; Oliani *et al.*, 2001). Proteolysis in the N-terminal domain profoundly modifies the physical and biological properties of the protein (Ando *et al.*, 1989; Chuah and Pallen, 1989; Cirino *et al.*, 1993; Porte *et al.*, 1996; Solito *et al.*, 2006).

The crystal structure of the full-length human recombinant annexin A1 (hu-r-Anx-A1) protein (Rosengarth *et al.*, 2001a) has been determined at the 1.8 Å level and reveals the protein to exist in a lenticular 'doughnut' configuration with a central pore. The N terminus is folded into the core domain on the concave surface, but in the presence of calcium, can 'flip' out. Six calcium-binding sites of differing affinities on the convex aspect mediate the binding to negatively charged phospholipids in what is probably a pH-dependent fashion (Rosengarth *et al.*, 2001b).

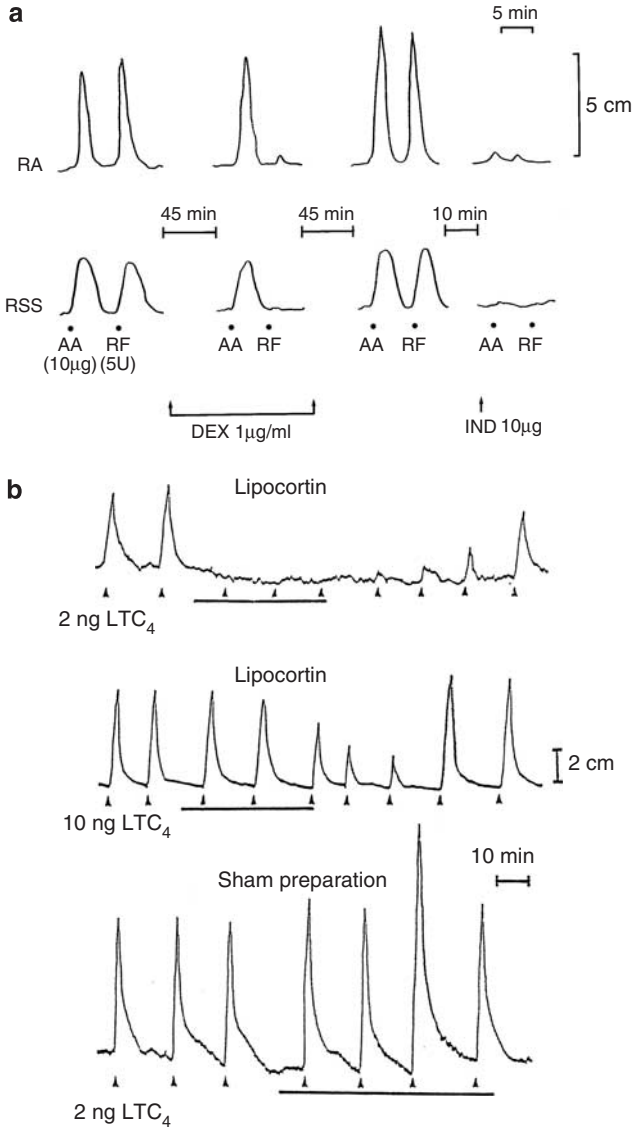


Figure 1 Detection and subsequent characterization of gluocorticoid-induced protein with eicosanoid suppressive actions. (**a**) An experiment in which the outflow from a guinea pig-isolated perfused lung was passed over a rabbit aortic (RA) or rat stomach strip (RSS) to detect the release TxA_2. Arachidonic acid (AA; 10 μg) and 'releasing factor' (RF; 5 U—later identified as a mixture of leukotrienes) both release substantial amounts of TxA_2 from this preparation. When dexamethasone (Dex; 1 μg mL^{-1}) is infused for 45 min into the perfused lung, the release of TxA_2 by RF is selectively inhibited. This effect decays about 45 min after the infusion is terminated. Administration of a cyclooxygenase inhibitor (Ind; 10 μg) blocks the generation of eicosanoids triggered by either stimulus. The experiment demonstrates that glucocorticoids do not block the cyclooxygenase directly, but rather inhibit some process leading to the liberation of the substrate arachidonic acid. The nature of the soluble factor that brought this about was subsequently identified as Anx-A1 (redrawn from Nijkamp *et al.* (1976)). (**b**) The first demonstration of the biological activity of hu-r-Anx-A1. Once again the rabbit aortic strip was used as a detecting organ to monitor the release of TxA_2 from the guinea pig-isolated perfused lung. Injections of LTC_4 were made at 30 min intervals. During the time periods indicated by the horizontal bar, human recombinant Anx-A1 (0.4 μg mL^{-1}) was infused into the preparation, blocking the release of TxA_2 in response to 2 ng (top panel) or 10 ng (middle panel) of LTC_4 in a manner strongly reminiscent of the glucocorticoids themselves. The bottom panel shows that a sham preparation, prepared from *Escherichia coli* transfected with a dummy construct for control purposes, was without effect (taken from Cirino *et al.* (1987)). Anx-A1, annexin A1; hu-r-Anx-A1, human recombinant annexin A1; LTC_4, leukotriene C_4; TxA_2, thromboxane A_2.

Sites and control of Anx-A1 synthesis

As a group, the proteins of the annexin super family are widely distributed (Fava *et al*., 1989; Gerke and Moss, 2002). However, not all annexins are co-expressed and Anx-A1, which forms the focus of this review, is found predominantly within differentiated cells. It is particularly highly expressed in cells of the haematopoietic lineage including neutrophils, monocyte-macrophages and mast cells. Polymorphonuclear leukocytes (PMNs) contain large amounts of Anx-A1, which may represent as much as 4% of the total cytosolic proteins (Ernst *et al*., 1990).

Subcellular location of the protein

The location of Anx-A1 varies between each cell type. Within neutrophils, for example, the protein is located predominantly in gelatinase granules (Perretti *et al*., 2000). Within the mast cell, it is present in, or on, α-granules (Oliani *et al*., 2000) but within the macrophage and most other cells investigated, it is chiefly located within the cytoplasm although it is also found associated with the membrane, cytoskeleton and nucleus (Peers *et al*., 1993; Seemann *et al*., 1997; Bianchi *et al*., 2003), depending upon the state of cell activation.

An important facet of Anx-A1 biology is the ability of the protein to exert its biological effects in several ways. There is ample evidence for an intracellular role of the protein (Futter *et al*., 1993; Seemann *et al*., 1997; Alldridge *et al*., 1999; Croxtall *et al*., 2000; Solito *et al*., 2006; White *et al*., 2006) but in addition it can be exported and act on target cells in an autocrine or paracrine manner. In many instances, the protein is externalized from the cell during cell activation or glucocorticoid stimulation.

The latter two pathways seem to be of particular relevance to the control of inflammation. It is not always entirely clear how Anx-A1 is exported from cells. In the case of the PMN, granule extrusion on activation leads to abundant Anx-A1 on the cell surface (Perretti *et al*., 1996, 2000; Vong *et al*., 2007) but in cells that do not store Anx-A1 in granules, secretion must be mediated through a separate mechanism. The protein does not contain a leader sequence or other secretory signal but some evidence, mainly pharmacological in nature, suggests that it is exported by the ABC-A1 cassette transporter system (Morris *et al*., 2002; Chapman *et al*., 2003; Wein *et al*., 2004). Anx-A1 has amphipathic properties and another possibility is that it, by adopting different configurations, might pass through pores or channels in the membrane but again this is speculation.

Early experiments suggested that Anx-A1 was phosphorylated (for example, Hirata *et al*., 1982) and it has become clear in recent years that phosphorylation on Ser^{27} (and possibly other Ser or Thr residues) is an important secretory signal (Croxtall *et al*., 2000; John *et al*., 2003). This post-translational modification is commonly observed before the appearance of Anx-A1 outside the cells, and a mutant protein in which Ser^{27} has been mutated to Ala^{27} is unable to accumulate at the cell membrane or be released from U937 cells *in vitro* (Solito *et al*., 2003b, 2006).

Because it is actively secreted from cells, a small amount of Anx-A1 may be found in plasma although it is likely that in the presence of physiological calcium concentrations, the protein is attached to cell surfaces. It has been observed that the concentration of the protein bound to the surface of circulating human cells correlates with circulating cortisol (Mulla *et al*., 2005).

Control of Anx-A1 synthesis

Many early investigations of Anx-A1 biology were motivated by the fact that the protein was 'induced' by glucocorticoids. In fact, it was apparent almost immediately that the situation was more complex than a straightforward stimulation of gene transcription. In many cells, glucocorticoids have a dual effect on Anx-A1 disposition and metabolism: within a few minutes of contact with glucocorticoids, existing Anx-A1 in some cell types (for example, the macrophage) is phosphorylated on serine Ser^{27} (and possibly other residues). This is followed by a secretion of the protein into the external medium. This phase, which begins within 5–10 min of contact, may persist for 30–90 min or until the internal pool of the protein is depleted. Interestingly, macrophages activated at this point are resistant to the eicosanoid-blocking action of glucocorticoids (Carnuccio *et al*., 1981). A further phase of steroid action occurs over a longer time span, beginning after approximately an hour and lasting for anything up to 18–24 h, during which an upregulation of Anx-A1 messenger RNA (mRNA) occurs leading eventually to the synthesis of new Anx-A1 protein. However, this process is cell-specific. Some cells, for example the macrophage and the mast cells, respond in a positive sense to glucocorticoid signals with an enhanced synthesis of the protein, but in T cells the reverse occurs and glucocorticoids actually decrease Anx-A1 mRNA and the synthesis of new protein (D'Acquisto *et al*., 2008a). This is the basis for a subtle regulatory action of this protein on the interaction of the innate and adaptive immune systems (see Figure 2).

The Anx-A1 promoter

The Anx-A1 promoter has been studied in some detail (Browning *et al*., 1990; Solito *et al*., 1998a, b). Unusually, for a protein that is strongly regulated by glucocorticoids in many tissues, it contains only few glucocorticoid response elements (in humans, there is one half-site in intron 1). However, numerous other potential regulatory sites have been discovered including an AP1 site, a nuclear factor interleukin-6 site and four GATA-binding protein-3 sites. Studies with mutant forms of the Anx-A1 promoter suggest that at least some of the positive glucocorticoid actions on the synthesis of this protein are accomplished through a transactivation mechanism utilizing a factor that binds to CCAT enhancer-binding protein in the upstream region.

It is clear that the regulation of this protein is subtle and that there are many factors that can influence its synthesis and disposition. Apart from glucocorticoids, the addition of cytokines, lipopolysaccharide and possibly other substances that compromise cell integrity such as heavy metal ions or thermal stress cause the upregulation of the protein. In this respect, at least in some instances, it seems to act like a

cellular 'acute phase' protein (Solito *et al*., 1998b; de Coupade *et al*., 2001).

Little is known about any process of post-transcriptional control over Anx-A1 synthesis.

The Anx-A1 receptor

Human recombinant Anx-A1 and N-terminal peptides derived from the parent molecule exert significant extracellular actions on cells involved in immune responses and mimic some of the effects of glucocorticoids both *in vivo* and *in vitro*. The first indication that there might be a cell-surface receptor came with the observation that there were discrete, saturable binding sites for human recombinant Anx-A1 on the surface of human peripheral blood monocytes and neutrophils (Goulding *et al*., 1990b) that co-precipitated with Anx-A1 from membrane extracts (Goulding *et al*., 1996). Receptor-like proteins were later described in endocrine pituitary cells (Christian *et al*., 1997). Several Anx-A1-binding proteins were subsequently isolated from A549 cells (Croxtall *et al*., 1998) although these did not seem to possess receptor functionality.

A key paper implicating the formylated peptide (formyl methionyl leucyl phenylalanine, FMLP) receptor (FPR) in the transduction of the Anx-A1 signal in leukocytes (Walther *et al*., 2000) led the field in a different direction. FPR is a member of a family of G protein-coupled receptors expressed in migratory cells and many other tissues (Panaro and Mitolo, 1999) and which, in man, includes FPRL-1 (FPR-like-1) and FPRL-2. Although FPR probably mediates the pro-inflammatory effects of the bacterial tri-peptide FMLP, FPRL-1 (also termed ALXR, lipoxin A4 receptor) is more promiscuous binding lipoxin A4, an anti-inflammatory mediator generated from arachidonic acid (Chiang *et al*., 2000), as well as serum amyloid A, a liver-derived acute-phase protein (Su *et al*., 1999).

Anx-A1, as well as the peptide Anx-A1 acetyl 2–26, induces calcium transients in human neutrophils triggering the proposal that the anti-inflammatory effects of these polypeptides are mediated through FPR itself (Walther *et al*., 2000). However, unlike the tripeptide FMLP, Anx-A1 peptides do not stimulate superoxide generation at concentrations that provoke calcium fluxes and L-selectin shedding (Walther *et al*., 2000), and there is no direct competition between FMLP and Anx-A1 binding in cells transfected with the human FPR (Walther *et al*., 2000).

'Boc' antagonists of FPR inhibit Anx-A1-induced calcium fluxes and L-selectin shedding by PMN and also prevent the Anx-A1 acetyl 2–26-induced inhibition of leukocyte adhesion and emigration in murine inflammatory models and elsewhere (Perretti *et al*., 2001; La *et al*., 2001a). However, these antagonists lack specificity and could

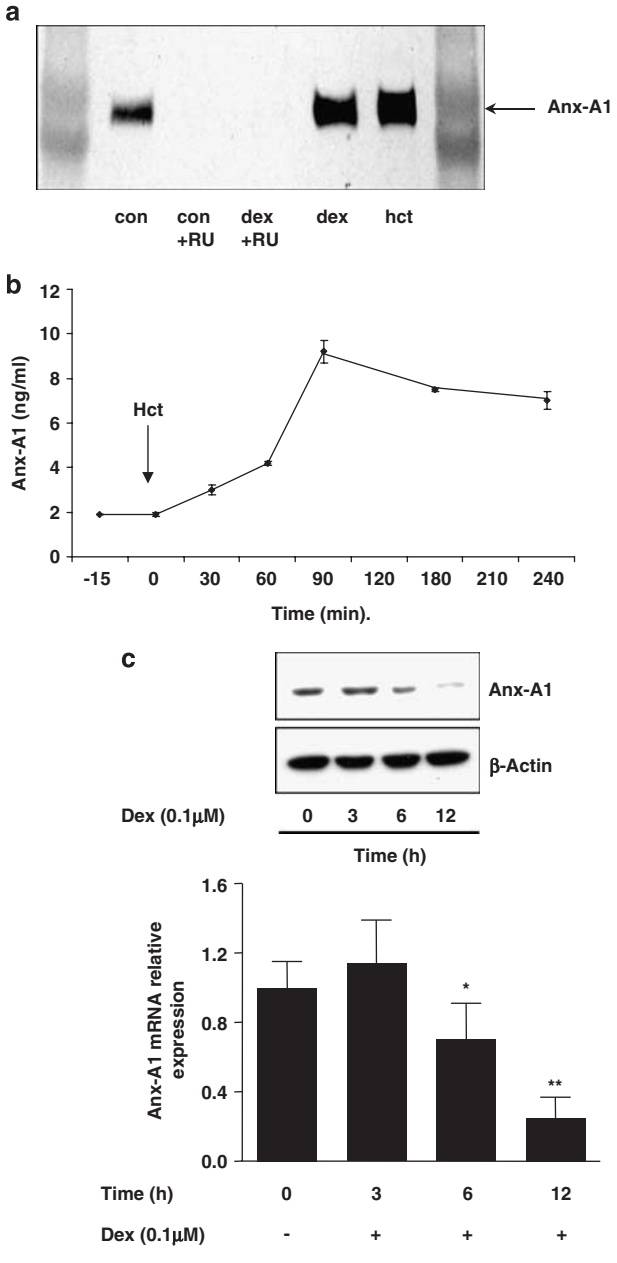


Figure 2 Regulation of Anx-A1 expression by glucocorticoids in three different systems. (**a**) This experiment shows the induction by glucocorticoids of Anx-A1 in resident rat peritoneal macrophages as assessed by western blotting. Con: injection of saline vehicle for 30 min before collection of cells. Con + RU: rats were pretreated with $2 \times 20\,mg\,kg^{-1}$ RU486 18 and 2 h before the saline vehicle. Dex + RU: $0.08\,mg\,kg^{-1}$ dexamethasone injected after pretreatment with RU486 for 1 h. Dex: $0.08\,mg\,kg^{-1}$ dexamethasone injected 30 min before collection. HCT: injection of $1\,mg\,kg^{-1}$ hydrocortisone 30 min before collection of cells. The experiment demonstrates that Anx-A1 is present in 'resting' resident peritoneal macrophages, presumably because of the endogenous glucocorticoid drive since administration of RU486, the glucocorticoid antagonist, reduces this below detectable limits. Both dexamethasone and hydrocortisone further induce Anx-A1 in these cells, but the effects are blocked by a preadministration of the glucocorticoid receptor antagonist (taken from Peers *et al*. (1993)). (**b**) The injection of 100 mg hydrocortisone into a human volunteer results, within 30 min, in a rise of Anx-A1 expression on the surface of human peripheral blood leukocytes as measured by an ELISA assay (redrawn from Goulding *et al*. (1990a)). (**c**) In T cells, unlike most other leukocytes, glucocorticoid treatment downregulates Anx-A1 gene synthesis and transcription. Upper panel shows western blot and lower panel shows real-time PCR for Anx-A1 protein or mRNA in human peripheral blood CD4 + T cells incubated for 12 h with dexamethasone for different times (taken from D'Acquisto *et al*., 2008a). Anx-A1, annexin A1; HCT, hydrocortisone. $^{*}P<0.05$; $^{**}P<0.01$.

interact with other members of the FPR family. Indeed, Anx-A1 acetyl 2–26 still retains some anti-inflammatory activity in FPR null mice, which have an intact *fprl-1* gene (Gavins *et al*., 2003). Taken together, these data suggest that while the FPR family mediates some Anx-A1 effects, the receptor responsible is unlikely to be the FPR itself, but is probably a closely related species.

Of the three products of the *FPR* gene cluster in humans, only one, FPRL-1 (ALXR), binds the anti-inflammatory lipoxin A4. This binding can be displaced by serum amyloid A, high concentrations of FMLP or the synthetic peptide mitogen-activated protein kinase homologue (Chiang *et al*., 2000). We have recently shown that Anx-A1- and Anx-A1-derived peptides also bind to FPRL-1 in human PMN and compete specifically with lipoxin A4. Endogenous Anx-A1 and FPRL-1 co-immunoprecipitate from human adherent PMN *in vitro*, and a similar species immunoprecipitates from murine extravasated PMN *in vivo* (Perretti *et al*., 2002). Like lipoxin A4, Anx-A1 acetyl 2–26 retains the ability to promote the detachment of adherent leukocytes in FPR null mice, suggesting that FPRL-1 is a candidate receptor for Anx-A1 (Gavins *et al*., 2003).

In the mouse, the *fpr* gene cluster (on chromosome 17) has undergone differential expansion, and six genes have been identified (Gao *et al*., 1998). Two of these are not expressed and another is found only in skeletal muscle. The remaining three genes (*fpr1*, *fpr-rs1* and *fpr-rs2*) are all expressed in leukocytes, spleen and lung and were therefore potential candidate receptors for Anx-A1. In mouse, however, the functions of human FPRL-1 seem to be split between fpr-rs1 and fpr-rs2. Recent studies suggest that it is likely that the mRNA for both *fpr-rs2* and *fpr-rs1* are generated from a single gene by alternative transcription.

Tools developed to study the role of Anx-A1 and its function *in vivo* and *in vitro*

Many tools have been developed to study the function and role of Anx-A1 in the functioning of the immune system. Neutralizing antibodies, antisense and small interfering RNA techniques have been applied to the problem. A transgenic mouse has been developed with the Anx-A1 gene globally deleted and another strain, which overexpresses Anx-A1, has also been used for some studies (Yona *et al*., 2007). Mice lacking the FPR receptor have also been used and more recently, a novel transgenic mouse line lacking the FPRL-1 receptor has also been generated (Dufton *et al*., 2008).

Following the discovery (Cirino *et al*., 1993) that the biological activity of Anx-A1 could be reproduced by the first 26 amino acids of the N terminus (*N*-acetyl 2–26), as well as by some shorter peptides, it has become common practice to use these instead of the full-length recombinant molecule, which, despite its greater potency, exhibits more instability in use and is difficult to prepare in a correctly folded and endotoxin-free manner. The *N*-acetyl 2–26 peptide does exhibit a slightly different affinity profile for the FPR family of receptors but nevertheless possesses very similar anti-inflammatory properties to the full-length molecule (Hayhoe *et al*., 2006).

Anx-A1 in the innate immune system

Generally, Anx-A1 has a powerful suppressive effect on the innate immune system (see Table 1), acting on PMNs, monocyte-macrophages and mast cells as well as many other cell types to inhibit cell trafficking as well as for the generation or release of mediators. We will examine the effect of this protein on these different cells in turn.

The polymorphonuclear leukocyte

Polymorphonuclear leukocytes contain abundant Anx-A1 stored within gelatinase granules. When PMNs are activated (for example, by treatment with cytokines or by 'rolling' over endothelial cells *in vitro*), the protein is immediately mobilized to the plasma membrane, where it acts in a negative regulatory fashion on the FPRL-1 receptor. The action of the protein at this site is terminated within a few minutes by the action of the membrane-bound PR3 enzyme, which cleaves Anx-A1 between residues 29 and 33 (Vong *et al*., 2007). Other proteinases, such as elastase, may also terminate the action of Anx-A1 in an analogous manner (Smith *et al*., 1990; Rescher *et al*., 2006).

PMNs isolated from the blood of Anx-A1 null transgenic mice or wild-type cells treated with neutralizing antibodies exhibit hyper-reactivity to stimuli such as chemotactic factors (Chatterjee *et al*., 2005), which manifests as an increased release of superoxide radicals (Perretti *et al*., 1995).

Several studies have suggested that Anx-A1 is involved in neutrophil apoptosis and that this may be important in limiting the inflammatory response (Perretti and Solito, 2004). Administration of the protein to human PMN *in vitro* provokes L-selectin shedding, caspase-3 activation and

Table 1 Some actions of Anx-A1 in the innate and adaptive immune systems

System	*Innate*	*Adaptive*
In vitro	*Inhibition of:* $cPLA_2$ activation Eicosanoid production Superoxide generation Phagocytosis *Promotion of:* L-selectin shedding PMN apoptosis Apoptotic PMN phagocytosis	*Promotion of:* T-cell proliferation TCR downstream signalling Th1 cell differentiation *Inhibition of:* Th2 cell differentiation
In vivo	*Inhibition of:* PMN trafficking PMN adherence Histamine release Cytokine generation Acute inflammation (some models) Chronic inflammation (some models) Hyperalgesia/nociception Fever (some pyrogens) NMDA neuronal damage	*Promotion of:* T cell-dependent inflammatory responses

Abbreviations: Anx-A1, annexin A1; PMN, polymorphonuclear leukocyte; TCR, T-cell receptor.

accelerated apoptosis (Solito *et al*., 2003a; Parente and Solito, 2004) and has also been suggested to be important in the recognition of apoptotic cells by phagocytic macrophages (Parente and Solito, 2004). Indeed, conditioned medium from apoptotic human PMN stimulates the phagocytic activity of macrophages, and the factor responsible for this is thought to be Anx-A1 (Scannell *et al*., 2007).

The monocyte-macrophage

Most studies of Anx-A1 function in these cells have used resident peritoneal or bone marrow-derived macrophages cultured from transgenic animals.

Early studies established that rat isolated resident peritoneal macrophages contain abundant Anx-A1 that can be released from the cells following treatment with glucocorticoids and other agents (Blackwell *et al*., 1980; Carnuccio *et al*., 1980; Peers *et al*., 1993). As in the case of PMN, the model would be that the secreted protein acts at cell-surface FPRL-1 (and possibly FPRL-2) receptors to downregulate aspects of macrophage activity, including the release of eicosanoids and superoxide radicals. There are several reports implicating Anx-A1 in the process of phagocytosis, but interestingly, the effects seem to be complex (Becker and Grasso, 1988; Harricane *et al*., 1996). Early papers (for example, Becker and Grasso, 1988) suggested that a factor, subsequently believed to be Anx-A1, was liberated from glucocorticoid-treated macrophages and mimicked their inhibitory action on phagocytosis. This antiphagocytic action of Anx-A1 itself or the *N*-acetyl 2–26 peptide has subsequently been confirmed and the modest inhibitory action of the glucocorticoid hydrocortisone on IgG phagocytosis is abolished in Anx-A1 null cells (Yona *et al*., 2004, 2005; see Figure 3). Unexpectedly though, macrophages lacking the Anx-A1 gene seem to exhibit a defect in zymosan phagocytosis; the reason for this is not currently clear although it may involve secondary changes in cell-surface adhesion molecules (Yona *et al*., 2004, 2005, 2006) or the failure of intracellular vesicles to be routed correctly in the absence of the protein (Collins *et al*., 1997). The addition, to macrophages, of the full-length hu-r-Anx-A1 or the *N*-acetyl 2–26 peptide can 'rescue' the phenotype of the Anx-A1 null cell, suppress prostanoid release, superoxide generation and, paradoxically, phagocytosis.

Transfection of the Anx-A1 gene into U937 cells sensitizes the cells to the apoptotic effects of tumour necrosis factor-α or etoposide (Solito *et al*., 2001).

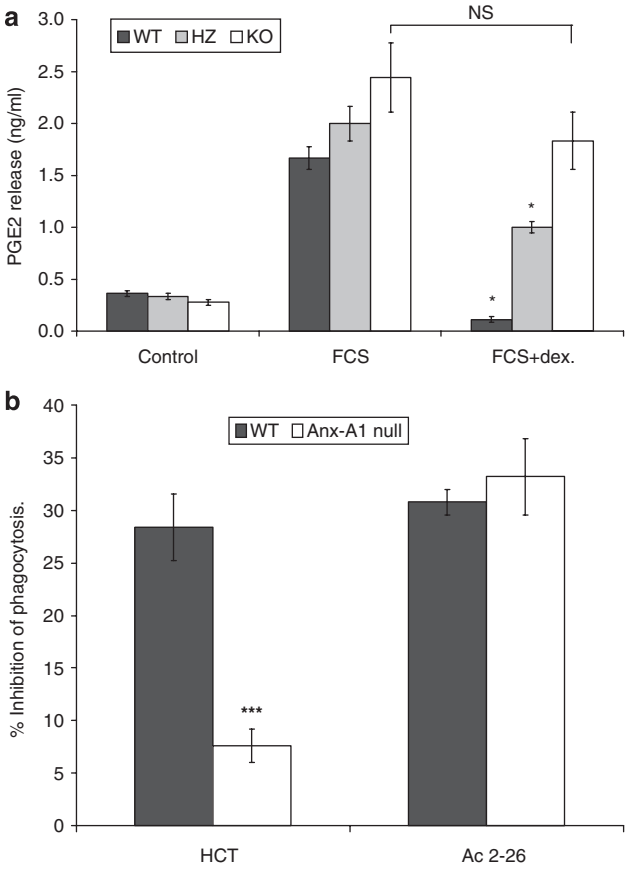


Figure 3 Attenuated effect of dexamethasone in wild-type and Anx-A1 null animals shown *in vitro* and *ex vivo*. (**a**) Lung fibroblasts obtained from Anx-A1 wild-type, heterozygote or null animals were cultured such that the generation of PGE_2 could be measured in the supernatant. The figure shows the increase in PGE_2 release after the addition of FCS and the inhibitory action of dexamethasone (1 μM). There is marked increase in PGE_2 generated by cells derived from the wild-type animals, but this is superseded in cells from both the heterozygote, and especially the Anx-A1 null, animals. Conversely, dexamethasone exerts a profound inhibition of PGE_2 release in the wild-type cells, much less in the heterozygote, and exhibits no appreciable inhibition at all in the Anx-A1 null cells (redrawn from Croxtall *et al*. (2003)). $^*P<0.05$ relative to control PGE_2 synthesis. (**b**) Inhibition of phagocytosis by hydrocortisone. In this experiment, phagocytosis of aggregated IgG was assessed in murine peritoneal macrophages using the dihydrorhodamine assay. Hydrocortisone (10 μM) exerts a moderate inhibitory effect on this process in the Anx-A1 wild-type animals, but much less in the Anx-A1 null animals. However, the N-terminal peptide *N*-acetyl 2–26 (100 μg mL^{-1}) is equally inhibitory in both phenotypes (redrawn from Yona *et al*. (2005)). $^*P<0.05$; $^{***}P<0.001$ relative to control phagocytosis. Anx-A1, annexin A1; FCS, foetal calf serum; PGE_2, prostaglandin E_2.

The mast cell

The mast cell also contains abundant Anx-A1 located in, or on, the α-granules and is also found in other compartments including the cytoplasm and the nucleus. The mast cell is a site of intense Anx-A1 synthesis (Oliani *et al*., 2000) in response to glucocorticoids and other stimuli. Treatment with these drugs causes secretion of the protein where it may engage with cell-surface FPRL-1 receptors to downregulate aspects of mast cell physiology, including the secretion of histamine and the generation of prostaglandin D_2. Mast cells in the Anx-A1 null animal exhibit histological signs of constitutive activation being partially degranulated. There are reports suggesting that Anx-A1 itself or peptides derived from the protein (Lloret and Moreno, 1994) inhibit mast cell degranulation *in vitro* or in models of allergic inflammation (Bandeira-Melo *et al*., 2005) and that Anx-A1 itself is involved in the inhibitory action of some other agents that block histamine release (Tasaka *et al*., 1994).

Vascular endothelial cells

Anx-A1 is elaborated by cells of the vascular endothelium (Raynal *et al*., 1992) and its expression may be increased

during the inflammatory response (Oliani *et al*., 2001) possibly by uptake of the protein externalized by adherent PMN. It has been suggested that Anx-A1 prevents firm adhesion of monocytes to endothelial cells possibly by binding to α-4-integrin (Solito *et al*., 2000).

The eosinophil

There are some discrepancies in the literature regarding the role of Anx-A1 in eosinophil biology. Early studies in the mouse suggested that the protein was not increased in these cells following the administration of dexamethasone and that the neutralizing anti-Anx-A1 antibody did not reverse dexamethasone-induced eosinophilia (Das *et al*., 1997; Teixeira *et al*., 1998). In the sensitized rat, however, it has been reported that the *N*-acetyl 2–26 peptide blocks ovalbumin-induced eotaxin release (Bandeira-Melo *et al*., 2005), and in human eosinophils stimulated with interleukin (IL)-5 or eotaxin, fluticasone proprionate appears to inhibit β2-integrin-induced cell adhesion and translocation of cytosolic PLA_2 to the nuclear membrane in an Anx-A1-dependent manner (Liu *et al*., 2005) as both inhibitory actions could be blocked by the administration of anti-Anx-A1 antiserum, and the effects of the glucocorticoid could be partially mimicked by the *N*-acetyl 2–26 peptide.

The synoviocyte

Anx-A1 is seen in several locations within samples of human synovial tissue (Goulding *et al*., 1995). Anx-A1-binding sites have been detected in synoviocytes taken from patients with rheumatoid arthritis (Sampey *et al*., 2000), but the actual number of sites was less than that in cells taken from patients with osteoarthritis. Administration of the *N*-acetyl 2–26 peptide to cultured synoviocytes inhibited PLA_2 activation.

Other cell types

Anx-A1 has a potent inhibitory action on mediator release by some other cells, which may be relevant to the inflammatory process. Hu-r-Anx-A1 concentrations (nM) potently inhibit the release of thromboxane A_2 from guinea pig-perfused lungs challenged with various stimuli (Cirino *et al*., 1987), and fibroblasts prepared from Anx-A1 null mice exhibit exaggerated responses to stimuli that release arachidonic acid and eicosanoids and resistance to glucocorticoid suppression (Croxtall *et al*., 2003; see Figure 3).

Anx-A1 in the adaptive immune system

Anx-A1 has a totally different distribution and function within the cells of the adaptive immune system. It was noted early that, in contrast to other peripheral blood cells, mixed lymphocytes appeared to contain little Anx-A1 under most circumstances (Morand *et al*., 1995) and did not bind Anx-A1 in the same way as monocyte/macrophages (Goulding *et al*., 1996; Kim *et al*., 1996). Little was known about the function of Anx-A1 in immune cells in general although there were suggestions that the protein could suppress antigen-specific T-cell proliferation (Gold *et al*., 1996, 1999). It was also hypothesized that abnormal post-translational modifications of the protein occurred in lymphocytes taken from patients with fragile X syndrome (Sun *et al*., 2001).

Systematic investigations into the role of Anx-A1 in T-cell biology are of comparatively recent origin yet these results have significantly changed and broadened our view of the range of biological functions exerted by this protein.

Naive T cells

D'Acquisto *et al*. (2007) investigated the role of Anx-A1 in T cells *in vitro* using a very simple approach: hu-r-Anx-A1 was added to T cells stimulated with anti-CD3 and -CD28 antibodies so as to reproduce the microenvironment of an inflammatory site where the influx of neutrophils and macrophages precedes the arrival of lymphocytes. During this initial phase, neutrophils, as well as other cell types release a number of mediators including Anx-A1 into the inflammatory fluid (Smith *et al*., 1995; Van Hal *et al*., 1996; Perretti *et al*., 1999) begging the question of whether this could subsequently inhibit or downregulate the T cell-mediated immune response.

However, the anticipated inhibitory effect of hu-r-Anx-A1 on the activation of T cells was not seen—instead, the protein increased both T-cell proliferation and activation. Interestingly, Anx-A1 added alone in the absence of T-cell stimulation was without any effect, and the stimulatory action was most evident when the cells were activated with very low concentrations of anti-CD3 and anti-CD28 (D'Acquisto *et al*., 2007). These results suggested that while the protein was without effect on resting T cells, the initiation of T-cell receptor (TCR) signalling somehow rendered them responsive to the exogenous protein.

An analysis of the biochemical 'fate' of endogenous Anx-A1 within naive resting T cells revealed that although the protein was localized mainly in the cytosol, upon stimulation with anti-CD3 and anti-CD28, it accumulated at the plasma membrane and was subsequently released into the extracellular *milieu* from where it could bind and activate its receptor, FPRL-1 (D'Acquisto *et al*., 2007). Occupation of FPRL-1 activates several signalling pathways, including extracellular signal-regulated kinase and Akt, which play a key role in regulating T-cell proliferation by modulating the strength of TCR signalling (Jorritsma *et al*., 2003; Na *et al*., 2003). Addition of exogenous Anx-A1 to T cells, stimulated with anti-CD3 and anti-CD28, increased extracellular signal-regulated kinase and Akt activation, whereas these very same pathways were shown to be down-regulated in Anx-A1-deficient T cells (D'Acquisto *et al*., 2007, 2008a). These observations, taken together with those obtained with the innate immune cells, clearly suggest that Anx-A1 acts as a cytokine that is, once it is released it acts in a paracrine and autocrine fashion by controlling the cells that produce it as well as others present in the inflammatory microenvironment.

This investigation also revealed a fascinating and unanticipated insight into the role of the FPRL-1 receptor in T-cell activation. The FPRs have always been described as a class of

receptors with functions restricted to cells of the innate immune system as neutrophils, macrophages and dendritic cells (Chiang *et al*., 2006; Panaro *et al*., 2006). The above study demonstrated that stimulation of TCR induced a time- and concentration-dependent externalization of FPRL-1 onto the T-cell plasma membrane. Furthermore, this externalization occured at the same time as the release of Anx-A1, highlighting the existence of a 'coordinated' signalling pathway that we have recently named the 'Anx-A1-FPR' system. This system seems to be 'silent' in naive resting T cells but becomes active once the T cell is stimulated via the TCR. This system probably 'senses' the microenvironment and, depending on the amount of Anx-A1 present, directs T-cell activation by modulating the strength of TCR signalling.

Anx-A1-induced activation of extracellular signal-regulated kinase and Akt in T cells triggers a broad range of transcriptional effects including the activation of all the major transcriptional regulators of T-cell activation, namely activator protein-1, nuclear factor of activated T cells (NFAT) and nuclear factor-kB (D'Acquisto *et al*., 2007) and, congruently, the opposite situation obtained in Anx-A1-deficient T cells (D'Acquisto *et al*., 2008a, b). Activation of such a wide spectrum of downstream signalling targets is not very common; previously it has been described that the adaptor proteins or kinases play a specific role in single molecular pathways (Koretzky and Myung, 2001). However, this aspect of Anx-A1 signalling coincides with the view that this protein acts as a 'homoeostatic tuner' that controls many aspects of cell activation at once. From the T-cell viewpoint, the fact that Anx-A1 controls many signalling pathways at once is an attractive concept as it suggests that by modulating one single molecule it is possible to control three signalling pathways simultaneously. Interestingly, current immunomodulatory therapies, such as cyclosporin or glucocorticoids, which target T cells, modulate all the above-mentioned transcription factors (Graham, 1994; Rogatsky and Ivashkiv, 2006). Finally, the fact that Anx-A1 simultaneously activates activator protein-1, NFAT and nuclear factor-κB might also have clinical importance. In fact, T cells from patients suffering from rheumatoid arthritis express more Anx-A1 than those from healthy control volunteers (D'Acquisto *et al*., 2007). One prominent feature of these cells is a lower threshold of TCR signalling and increased activation of activator protein-1, NFAT and nuclear factor-κB upon TCR engagement (Sweeney and Firestein, 2004); a phenotype similar to that observed in T cells activated in the presence of hu-r-Anx-A1. In the light of these findings, it is possible to hypothesize that the increased expression of Anx-A1 in T cells from rheumatoid arthritis patients might explain their excessive degree of activation of these transcription factors in response to TCR stimulation.

T helper cells

One of the hallmarks of T-cell biology is the ability of some subtypes to differentiate into 'effector' cells that is cells that are able to destroy invading pathogens by coordinating the response of cells (for example, macrophages or B cells); for this reason, these cells are also called 'T helper (Th) cells'. There are several types of Th cells depending mainly on the type of stimulus that triggers their differentiation. Th1 lymphocytes produce IL-2 and interferon-γ, which induce macrophage activation, thus inhibiting intracellular replication of many pathogens. Th1 cells can also be directly cytolytic for infected cells and can promote the expansion of cytotoxic CD8T lymphocytes, which also recognize and destroy infected cells.

Th2 cells produce IL-4, IL-5, IL-10 and IL-13 and are involved in the humoral immunity against extracellular pathogens, particularly helminths. These Th2 cytokines also aid antigen-activated B cells to proliferate and differentiate into antibody-producing plasma cells and to undergo class switching from IgM to IgG, IgA and IgE production (Flavell *et al*., 1999; Glimcher, 2001).

Recently, two new classes of Th cells have been described: IL-17-producing Th cells (Th17) and regulatory T cells (Treg). A growing number of studies in the last few years has focused attention on these cells as they are believed to play a key role in the development of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis (McInnes and Schett, 2007).

Many factors are thought to control the specific differentiation of Th cells. Among the best-described are the cytokines present in the microenvironment of the inflammatory site. For instance, 'skewing' naive T cells (Th0) *in vitro* with IL-12 and anti-IL-4 leads to differentiation in Th1 cells, whereas in the presence of IL-4 and anti-interferon-γ, the cells acquire a Th2 phenotype (Murphy and Reiner, 2002). Treg and Th17 share the same 'skewing' cytokine (IL-6) but differ in that Th17 cells also require transforming growth factor-β for differentiation in mice (Weaver *et al*., 2006; Stockinger and Veldhoen, 2007) and IL-1 and tumour necrosis factor-α (Acosta-Rodriguez *et al*., 2007) for that in humans.

Besides these cytokines, other factors modulate Th differentiation especially when cells are grown in the absence of skewing cytokine for example, differentiation under 'neutral' conditions. In fact, different subtypes of Th cells can be obtained by changing the affinity of the antigen for a given TCR. Thus, antigens with low affinity generally induce the differentiation in Th2 cells, whereas antigens with strong affinity favour the differentiation in Th1 cells (Janeway and Bottomly, 1994; Leitenberg and Bottomly, 1999; Blander *et al*., 2000). This corresponds to weak or strong TCR signalling, such that low-affinity antigens induce a transient and low-level activation of TCR-downstream mitogen-activated protein kinases favouring Th2 differentiation, whereas high-affinity antigens induce a stronger and more rapid activation of these pathways thus favouring Th1 differentiation.

The fact that Anx-A1 positively modulates the strength of TCR signalling prompted an investigation into the effects of this protein on T-cell differentiation. When cells are differentiated under Th0 conditions in the presence of hu-r-Anx-A1, they acquired a Th1 phenotype, whereas differentiation of Anx-A1-deficient T cells under identical conditions produced high levels of Th2 cytokines (D'Acquisto *et al*., 2007, 2008a). These results are consistent with a model in which high levels of Anx-A1 augments TCR signalling by

activating FPRL-1, and this in turn induces differentiation to Th1 cells. Congruent with this notion is the observation that when differentiated, Anx-A1-deficient T cells acquired a marked Th2 phenotype.

Taken altogether, the above observations might seem to suggest that Anx-A1 plays a pro- rather than an anti-inflammatory role although this is an oversimplification as an efficient Th1-protective response is crucial for the elimination of certain types of host pathogens. On the other hand, many studies have also highlighted the role of Th1 and Th17 cytokines in the development of autoimmune diseases such as rheumatoid arthritis and thus, high levels of Anx-A1 might actually be detrimental in such cases. Consistent with this hypothesis is the observation that carefully timed treatment of arthritis-prone DBA mice with hu-r-Anx-A1, immediately after collagen immunization, actually exacerbated the symptoms of the disease and the production of Th1 cytokines by the T cells of the immunized mice (D'Acquisto *et al*., 2007).

Given the recent interest in the role of Th17 in rheumatoid arthritis, it is tempting to hypothesize that exogenous Anx-A1 increased Th17 cell differentiation rather than Th1 in these animals. Although these studies have not yet been completed, Anx-A1-deficient T cells show an impaired production of IL-17 during differentiation in Th17-skewing conditions and they also express significantly lower levels of Th17-specific transcription factor ROR-γ (D'Acquisto *et al*., 2008b).

The finding that Anx-A1 plays a critical role in T-cell activation and differentiation highlighted an apparent anomaly in the effect of glucocorticoids on Anx-A1 expression. These drugs inhibit early as well as late TCR-signalling events in naive T cells and favour the differentiation of Th2 cells (Franchimont, 2004). But how could this observation be reconciled with the notion that glucocorticoids increase Anx-A1 expression in cells when an increased T-cell activation (and a differentiation skewed towards a Th1 phenotype) was seen after treatment with the recombinant protein?

The unexpected explanation that finally emerged was that glucocorticoids actually inhibit rather than increase the expression of Anx-A1 mRNA and protein in human CD4+ T cells *in vitro*. These results were confirmed in RA patients treated with depomedrone for 2 days, in whom Anx-A1 expression in CD4+ cells was found to be significantly inhibited (D'Acquisto *et al*., 2008a).

Why would glucocorticoids exert such contrasting effects in cells of the innate and adaptive immune system? The reduction of Anx-A1 expression by glucocorticoids would deplete the T cells of a 'positive modulator' of TCR signalling and thus cause immunosuppression. Two lines of evidence support this hypothesis. First, Anx-A1 added to dexamethasone-treated CD4+ T cells rescued the inhibitory effect of the steroid on anti-CD3/anti-CD28-induced IL-2 production. Second, Anx-A1-deficient T cells displayed the same phenotype as dexamethasone-treated cells. Most interestingly, these experiments also provided a possible explanation for the Th2-skewing capability of glucocorticoids: when the strength of TCR signalling is low, naive T cells tend to acquire a Th2 phenotype. These data suggest that the suppressive effects of dexamethasone on Anx-A1 expression might very well bring this about by depressing the expression of the positive modulator Anx-A1, thus favouring the development of Th2 cells (Figure 4).

If we combine these observations together with those on the innate immune system, it is possible to speculate that the immunosuppressive effects of glucocorticoids might require the suppression of Anx-A1 expression in T cells whereas the anti-inflammatory actions might be attributable to the increased release or expression of this protein in cells of the innate immune arm. This hypothesis, if validated, will provide a completely novel perspective for the design of specific drugs for the treatment of immune- or non-immune-mediated diseases.

In conclusion, despite significant recent progresses in understanding the role of Anx-A1 in the adaptive immune system (see Table 1), there are still many aspects that need to be clarified. These include the role of Anx-A1 in positive and negative selection in the thymus as well as the role of this protein in CD4 versus CD8 cells or in NK and B cells or in Treg and Th17. Further exciting challenges will be to understand how and why Anx-A1 exerts a complete opposite effect on the innate and adaptive immune system.

Anx-A1 in complex models of inflammation

In vivo *studies*

The full-length recombinant human protein, as well as the N-terminal acetyl 2–26 peptide, and sometimes the long fragment Anx-A1 1–188 have been tested in many models of experimental acute and chronic inflammation with the general finding that the protein exerts a potent anti-inflammatory action on both the cellular and humoral mediators in models in which the innate immune system is activated (see Figure 5) and a potent immunomodulatory action in cases in which the adaptive system is activated.

Acute models of inflammation have been studied most frequently. I.v., s.c. or i.p. injections, into rats, rabbits or mice, of amounts (μg) of (usually) the hu-r-Anx-A1 itself or peptides containing the active N terminus suppress inflammatory hyperalgesia (Ferreira *et al*., 1997), fever (Carey *et al*., 1990; Davidson *et al*., 1991; Strijbos *et al*., 1992), carrageenin paw oedema (Miele *et al*., 1988; Cirino *et al*., 1989; Browning *et al*., 1990; Arcone *et al*., 1993), zymosan peritonitis (Getting *et al*., 1997) and cell migration (Perretti and Flower, 1993b; Perretti *et al*., 1993a, 1996; Mancuso *et al*., 1995; Allcock *et al*., 2001) or attachment to the vessel wall (Lim *et al*., 1998). Anx-A1 mitigates the outcome of NMDA-induced damage of the brain (Black *et al*., 1992) as well as ischaemia-reperfusion injury in the brain (Relton *et al*., 1991; Rothwell and Relton, 1993; Gavins *et al*., 2007), myocardium (D'Amico *et al*., 2000; La *et al*., 2001a, b; Gavins *et al*., 2005) and mesentery (Cuzzocrea *et al*., 1997). In many of these studies, the acute inhibitory actions of glucocorticoids were blunted or abolished by pretreatment with anti-Anx-A1-neutralizing antisera, providing presumptive evidence for the involvement of Anx-A1 in their action.

The anti-inflammatory effects of Anx-A1 in such models can often be traced to the potent inhibitory actions on PMN

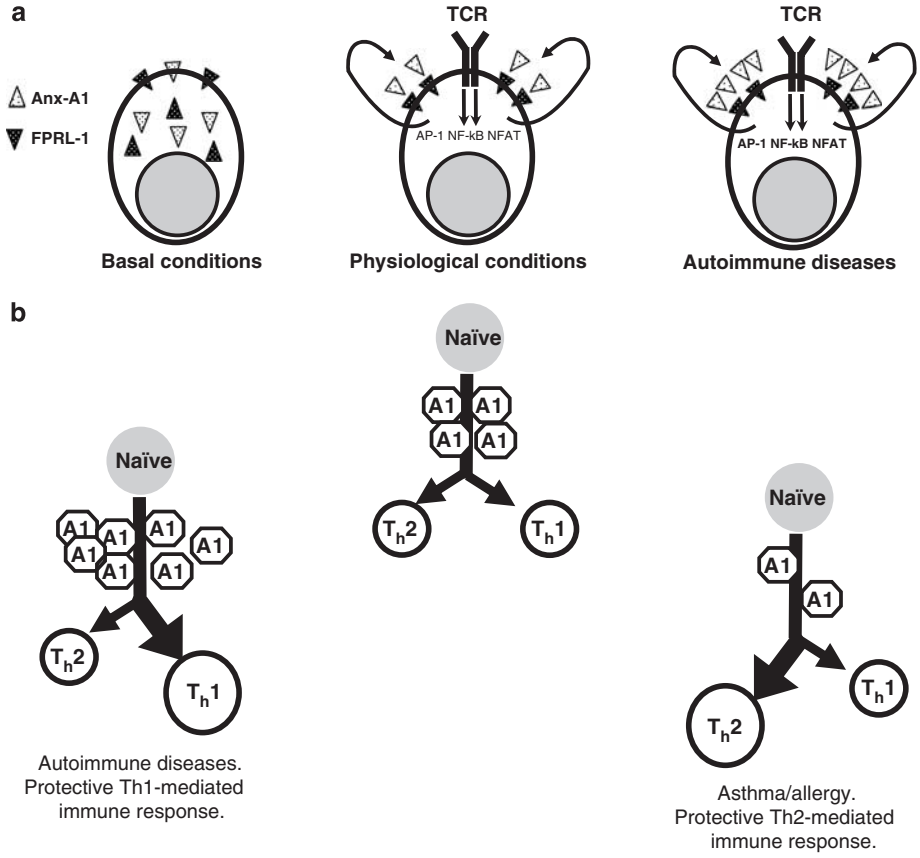


Figure 4 Schematic representation of the role of the Anx-A1/FPRL-1 system in T-cell signalling. (**a**) In basal conditions, both Anx-A1 and FPRL-1 are present at very low abundance on the membrane of naive T cells. Stimulation of T cells via the TCR leads to the externalization of FPRL-1 and the release of Anx-A1. The activation of FPRL-1 by Anx-A1 modulates the strength of TCR signalling by increasing levels of transcription factors such as AP-1, NF-κB and NFAT. In pathological conditions, such as in rheumatoid arthritis, the increased expression of endogenous Anx-A1 might contribute to the basal hyperactivated state of these cells and to the increase of transcription factors that play a key role in the regulation of the expression of several inflammatory genes. (**b**) Proposed model for the role of Anx-A1 in the differentiation of T-helper cells. In physiological conditions, naive T cells differentiate in Th1 or Th2 effector cells depending on the microenvironment in which this process occurs. The presence of high levels of Anx-A1 promotes a protective Th1 immune response or might, in a pathological context, exacerbate autoimmune diseases such as rheumatoid arthritis. When T cells express low levels of Anx-A1, they preferentially become Th2 cells and this might be responsible for the occurrence of allergic reactions or promote a protective humoral response. Glucocorticoids, by reducing Anx-A1 synthesis by these cells, promote a Th2 phenotype. Anx-A1, annexin A1; AP-1, activator protein-1; FPRL-1, formyl peptide-like receptor-1; NF-κB, nuclear factor-κB; NFAT, nuclear factor of activated T cells; TCR, T-cell receptor.

trafficking, but other Anx-A1 actions are also important. In many cases, the administration of Anx-A1 is associated with a reduction of pro-inflammatory cytokines or stimulation of anti-inflammatory cytokines (Ferlazzo *et al*., 2003; Yang *et al*., 2004; Damazo *et al*., 2005); N-terminal acetyl 2–26 also inhibits antigen-induced pleurisy (Bandeira-Melo *et al*., 2005) through an action on mast cells.

There is evidence from at least one study that the anti-inflammatory effect of Anx-A1 in intestinal ischaemia-reperfusion injury may require the production of IL-10 and that mice deficient in this cytokine fail to respond to the protein (Souza *et al*., 2007). Conversely, when Anx-A1 was neutralized (or lipoxin A4 synthesis blocked), IL-10 production in response to this type of injury was reduced.

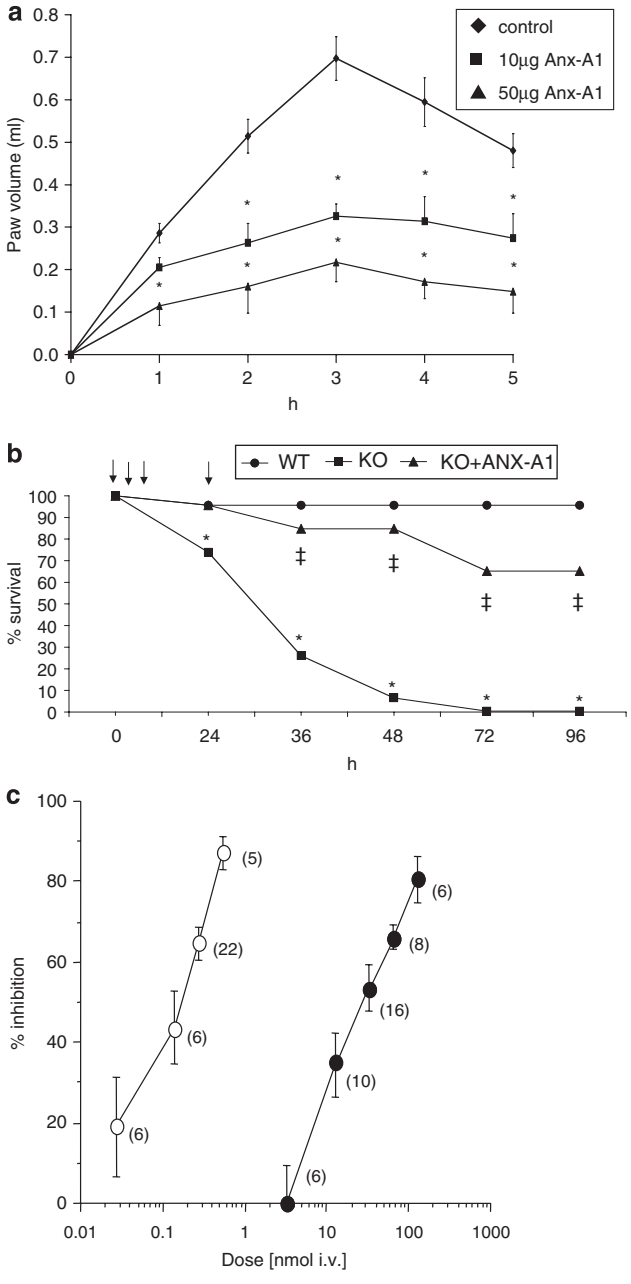
In the Anx-A1 null mouse, responses to inflammatory or nociceptive substances or procedures such as TRPV1 activation (Ayoub *et al*., 2008), ischaemia-reperfusion injury, carrageenin-induced paw oedema (Roviezzo *et al*., 2002; Hannon *et al*., 2003), zymosan peritonitis and lipopolysaccharide-induced septic shock (Damazo *et al*., 2005) are greatly enhanced compared with the Anx-A1 wild-type animal and the response to glucocorticoids reduced or abolished (see Figure 6). This phenotype can be rescued by injection of the full-length recombinant protein or the N-terminal acetyl 2–26 peptide.

In vivo, microcirculation studies have revealed that deletion of the protein leads to enhanced migration of cells at sites of local inflammation (Chatterjee *et al*., 2005; Gavins *et al*., 2007) and that the administration to normal animals of a neutralizing antibody to the protein can block the inhibitory action of dexamethasone in various models of neutrophil migration. Conversely, administration of the full-length Anx-A1 protein or the *N*-acetyl 2–26 peptide produces a very strong inhibition of PMN migration into acute inflammatory lesions.

Interestingly, there appears to be a sex difference in the neutrophilia that is seen following the administration of an

inflammatory stimulus to Anx-A1 null mice (Hannon *et al*., 2003). During zymosan-induced peritonitis, the ensuing neutrophilia in Anx-A1 null male animals quickly returns to normal, but in the female animals, this proceeds unchecked for some hours.

The involvement of the Anx-A1 system in chronic models of inflammation has also been tested. Treatment of rats with neutralizing anti-Anx-A1 monoclonal antibodies exacerbated carrageenin- or antigen-induced arthritis in rats and partially reversed the protective action of dexamethasone (Yang *et al*., 1997, 1999). Induction of the arthritis was enhanced in Anx-A1 null mice with more severe histological and biochemical markers of inflammation at day 7 (Yang *et al*., 2004). Dexamethasone also had much reduced activity in this model and in particular failed to reverse the severity



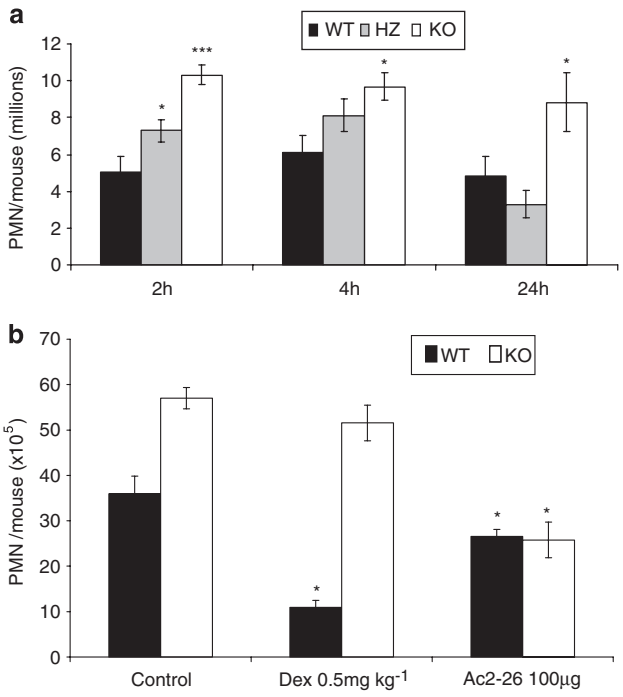


Figure 6 Exacerbation of the acute inflammatory response in Anx-A1 null mice and their lack of response to dexamethasone. (**a**) Zymosan-induced peritonitis produces a brisk neutrophil infiltration into the peritoneal cavity. In comparison to the wild-type controls, this is elevated in both the heterozygote and the homozygote Anx-A1 null animals at the 2 and 4 h time point and at 24 h in the Anx-A1 homozygote null animal (redrawn from Hannon *et al*. (2003)). *$P<0.05$ relative to control migration. (**b**) Zymosan peritonitis was again used as a measure of acute inflammation. Here, dexamethasone (0.5 mg kg^{-1}) produced a striking inhibition of the control migration, but was without any effect in the Anx-A1 null animals. However, the N-terminal peptide *N*-acetyl 2–26 (100 μg) was equi-active in both genotypes (unpublished—but see Hannon *et al*. (2003) for analogous experimental data). *$P<0.05$; ***$P<0.001$ relative to control migration. Anx-A1, annexin A1.

Figure 5 Potent anti-inflammatory effects of hu-r-Anx-A1 in three models of inflammation. (**a**) Anx-A1 or vehicle was injected together with carrageenin into the rat paw and the ensuing oedema measured over the next 5 h. Both 10 and 50 μg Anx-A1 produced a striking inhibition of all phases of the oedema (Cirino *et al*., 1989). *$P<0.05$ relative to control response. (**b**) Mortality in the Anx-A1 null mouse induced by LPS and its rescue by Anx-A1. *Escherichia coli* LPS, 10 mg kg^{-1}, was injected into the mice at time 0 h. This produced a negligible mortality in the wild-type population, but was 100% fatal in the Anx-A1 null animals within 48 h. The arrows indicate points (0, 4 and 8 h) at which hu-r-Anx-A1 (10 ng) was injected into the third group. This substantially reversed the mortality caused by the LPS allowing approximately 75% survival (Damazo *et al*., 2005). *$P<0.05$ relative to wild-type controls; $^{‡}P<0.05$ relative to Anx-A1 null mice. (**c**) Inhibition of PMN migration into the murine IL-1-induced air pouch model showing the graded inhibition produced by increasing doses of Anx-A1 (open circles) injected i.v., 1 h before the IL-1 injection, and also the striking effect of the N-terminal peptide *N*-acetyl 2–26 (filled circles). It should be noted that the dose–response curve of the latter agent is parallel to that of the intact molecule although it is clearly some two orders of magnitude less potent (Perretti *et al*., 1993a). Anx-A1, annexin A1; hu-r-Anx-A1, human recombinant annexin A1; IL, interleukin; LPS, lipopolysaccharide; PMN, polymorphonuclear leukocyte.

of the synovitis and bone damage in the Anx-A1 null animals at this time point.

In models in which the adaptive immune system is crucial, the situation is more complex. Consistent with the finding that the upregulation of Anx-A1 in T cells skews them towards a Th1 phenotype is the observation that treatment of arthritis-prone DBA mice with hu-r-Anx-A1, immediately after collagen immunization, exacerbated the symptoms of the disease and the production of Th1 cytokines by the T cells of the immunized mice (D'Acquisto *et al*., 2007).

Anx-A1 in human disease

The term 'annexinopathies' has been coined to describe diseases characterized by an abnormal synthesis, metabolism or action of members of the annexin superfamily (Rand, 1999), and there have been several suggestions that Anx-A1 itself is involved in human inflammatory and other disorders. Defects in Anx-A1 function or metabolism have been reported in familial Mediterranean fever (Shohat *et al*., 1989; Garcia-Gonzalez and Weisman, 1992), fragile X syndrome (Sun *et al*., 2001) and Weber–Christian disease (Akama *et al*., 1995). Anx-A1 also undergoes an abnormal metabolism in cystic fibrosis (Tsao *et al*., 1998; Bensalem *et al*., 2005) and other lung disorders (Smith *et al*., 1990; Mikuniya *et al*., 1998; Lindahl *et al*., 1999) and may play a role in tumour development (Ahn *et al*., 1997; Mulla *et al*., 2004), skin disorders (Kitajima *et al*., 1991; Bastian *et al*., 1993) and CNS pathology (Elderfield *et al*., 1992; Eberhard *et al*., 1994). Autoantibodies to Anx-A1 have been detected in man and may be responsible for glucocorticoid resistance in rheumatoid patients (Podgorski *et al*., 1992), associated with the pathology of systemic lupus erythematosus (Goulding *et al*., 1989) and Crohn's disease (Stevens *et al*., 1993), or could act as a diagnostic marker for some tumours (Brichory *et al*., 2001; Mulla *et al*., 2004).

Whether or not there are common mutations or single nucleotide polymorphisms in the Anx-A1 gene, which would alter its function in the innate or adaptive immune systems, is a subject that is under active research by several laboratories including our own.

How does Anx-A1 function during the inflammatory response?

There is now overwhelming evidence that Anx-A1 plays a key role in the modulation of both the innate and the adaptive immune systems—but how does it function during an integrated physiological response such as inflammation with all its inherent complexity? One unifying factor that may help to explain how the Anx-A1 system operates in these situations arises from the original observation that glucocorticoids can stimulate both the synthesis and the release of the protein in cells of the innate immune system and the more recent observation that these hormone/drugs have the reverse action on T-cell Anx-A1 synthesis.

Munck *et al*. (1984) have described the glucocorticoids as 'anti-defensive' hormones that are released during any situation that threatens to compromise the homoeostatic functioning of the body. According to this idea, their predominant function is to restore the balance between the homoeostatic and the immune and other defence systems that are activated to ensure survival. The correctly timed release of these hormones is essential to the organism's ability to respond to, and recover from, episodes of stress, inflammation, injury and infection. Adrenalectomy has long been known to exacerbate, prolong inflammatory responses, to delay recovery (Leme and Wilhelm, 1975; Flower *et al*., 1986; Moraes *et al*., 1987; Bertini *et al*., 1988; MacPhee *et al*., 1989; Masferrer *et al*., 1992, 1994) and to result in excessive cytokine production during the inflammatory response (Goujon *et al*., 1996). Removal of the adrenals from rodents challenged with even mild, sublethal inflammatory stimuli can have fatal consequences in adrenalectomized rodents.

There is evidence from both animal and human studies that a timely and appropriate response of the HPA axis is essential in the recovery from various experimental insults or surgery. Defects in the HPA axis of some rodent strains render the animals prone to some types of inflammation, whereas other strains with normal HPA functioning are not susceptible (Sternberg *et al*., 1989a, b, 1992; Aksentijevich *et al*., 1992). The development of chronic inflammation such as rheumatoid arthritis and Sjogren's syndrome in humans (Wilder and Sternberg, 1990; Chikanza *et al*., 1992; Templ *et al*., 1996; Johnson *et al*., 1998; Demir *et al*., 1999; Mukai *et al*., 2000) has also been attributed to a lack of appropriate HPA activity as has the decline in immune function associated with age (immunosenescence).

The acute rise in cortisol (or in rodents, corticosterone) then, which accompanies virtually any inflammatory insult, might be expected to have several effects on the Anx-A1 system, all of which have been confirmed experimentally: acutely, there will be a release of preformed protein from cells such as the PMNs, macrophages or mast cells, which will have an immediate inhibitory action on neutrophil emigration and macrophage and mast cell-mediator production. This would provide a rapid counter-regulatory 'brake' on the development of the inflammatory response that would prevent it from escalating inappropriately. Little effect is seen on the disposition of Anx-A1 in the adaptive immune system after such short exposures to glucocorticoids.

A more prolonged enhancement of glucocorticoids will increase the synthesis of Anx-A1 in cells of the innate immune system and the release of the protein will be enhanced over a longer period providing more extended anti-inflammatory cover. Within T cells, however, the more prolonged exposure to glucocorticoids will drive down the synthesis of Anx-A1 such that a Th2 response is favoured over a Th1 response. The Th2 phenotype is generally associated with a humoral immune response that is characterized by the production of IL-4, IL-5, IL-10 and IL-13, a cytokine pattern that has been linked to protective 'anti-inflammatory' effects in various animal models of Th1-driven autoimmune disease (Figure 7).

Another manner by which glucocorticoids can modulate function of the Anx-A1 pathway is through induction of the Anx-A1 receptor, ALX/FPRL1. Incubation of human monocytes with dexamethasone and other synthetic glucocorti-

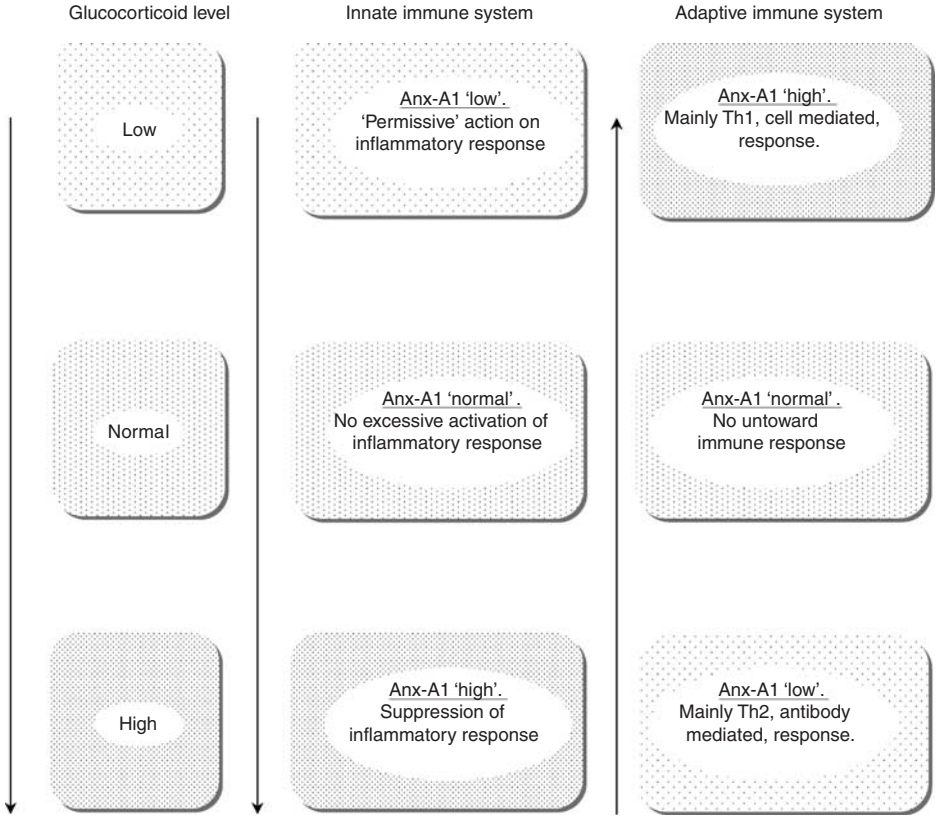


Figure 7 A schematic diagram showing the integration of glucocorticoid 'tone' with the Anx-A1 system. The diagram shows three glucocorticoid 'states'—when levels are low, normal or high, and the ensuing events in the innate and adaptive immune systems. It illustrates that, as glucocorticoids rise, the increasing amounts of Anx-A1 within the innate immune system suppress the activation of cells such as PMN, monocytes and mast cells, while decreasing levels of Anx-A1 within the adaptive immune system tend skewing any T-cell responses towards a Th2 'anti-inflammatory' phenotype. Anx-A1, annexin A1; PMN, polymorphonuclear leukocyte.

coids induced *de novo* synthesis of ALX/FPRL1, with an early increase in mRNA levels subsequently followed by enhanced protein expression (Sawmynaden and Perretti, 2006). This effect was also evident in differentiated HL-60 cells, used as a surrogate for human PMN. An investigation focusing predominantly on the biology of ALX/FPRL-1, in relation to the actions of lipoxin A_4, has confirmed the link between glucocorticoids and expression of this receptor in human neutrophils (Hashimoto *et al.*, 2007). Finally, in an unbiased microarray study, prolonged incubation of human monocytes with fluticasone revealed an upregulation in the levels of *FPR1* mRNA (Ehrchen *et al.*, 2007), a receptor structurally related to ALX/FPRL-1, as described above.

In conclusion, a common scenario is emerging, in which glucocorticoids impact on the course of inflammation and its resolution through the modulation of Anx-A1 synthesis and the upregulation of ALX/FPRL-1, and possibly other receptors of this family, thus favouring the homoeostatic actions of AnxA1 and lipoxins.

In cases in which the Anx-A1 system malfunctions, the progression of these responses may be profoundly altered. This is observed experimentally; for example, gene deletion or immunoneutralization of Anx-A1 leads to enhanced activity of the innate immune system during episodes of acute and chronic inflammation and a changed adaptive response. Reduced blood glucocorticoids also result in less Anx-A1 being generated. Within the adaptive immune system, the effects of excess Anx-A1, perhaps secondary to a defective HPA response or to some defect in Anx-A1 metabolism, would be to augment the development of the aggressive cell-mediated Th1 response. In human rheumatoid arthritis, $CD4^+$ cells exhibit increased amounts of Anx-A1 consistent with this 'switch,' whereas in the Anx-A1 null mouse, there is a predominant Th2 phenotype.

Whatever may happen at a systemic level, it is at the level of the inflammatory microenvironment that Anx-A1 is likely to have its greatest effect. The question of how locally released Anx-A1 interacts with the cells of the innate and adaptive immune systems to produce the carefully coordinated response to injury and infection—and how these malfunctions are modified by treatment with glucocorticoid or other drugs—is one of the major challenges of this exciting area.

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Conflict of interest

RJF reports consultancies with Antibe Pharmaceuticals, Morria Biopharmaceuticals, Sosei RD Ltd and Palau Pharma SA and has received fees for legal work from Pfizer. MP reports consultancies with Antibe Pharmaceuticals and NicOx SA. Part of the research activities in our lab are funded by Unigene Corportation Inc. (Fairfield, NJ, USA).

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