

REVIEW

The danger within: endogenous danger signals, atopy and asthma

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Clinical and Experimental Allergy

Summary

In allergic asthmatics, airway inflammation is triggered by specific (inhalation of allergen such as house dust mite allergen and pollen spores) or non-specific triggers (such as air pollutants and viral infection). Most of these inhaled particles are immunologically inert. Dendritic cells (DCs) are essential for priming and T helper-2 differentiation of naïve T cells towards aeroallergens. Contamination of antigens with pattern-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), is required to activate DCs to mount an immune response. Damage-associated molecular patterns (DAMPs), such as uric acid and adenosine triphosphate (ATP), also contribute to the induction of inflammation by activation and recruitment of various inflammatory cells. Compelling evidence suggests that a tight collaboration between PAMPs and DAMPs is needed to start an immune response to allergens. Several studies have recently demonstrated an important role of endogenous danger signals at the inception and maintenance phase of allergic disease. Further research into this area should focus on the possible role of these factors in maintenance of chronic disease and induction of airway remodelling.

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Asthma

The incidence of allergic sensitization and asthma has increased dramatically in the developed countries in the last 50 years. What is even more alarming is that also the severity of the disease is still increasing. The world-wide estimate of people with asthma is 300 million [1]. Asthma is a chronic immune-mediated inflammatory disorder, which is characterized by attacks of wheezing and breathlessness due to bronchoconstriction, mucus secretion, airway hyperresponsiveness to non-specific stimuli and airway wall thickening. Asthma occurs in atopic and non-atopic settings, which differ in their type of immune responses. In allergic asthmatics, airway inflammation is triggered by specific (inhalation of allergen such as airborne house dust mite (HDM) allergen, pollen spores or animal dander) or non-specific triggers (such as air pollutants and viral infection). One third of all asthma patients have intrinsic or non-atopic asthma. Non-atopic asthma often has a later onset and a more prolonged course than atopic asthma. Patients with atopic asthma have an elevated level of total and allergen-specific IgE in serum and a positive skin prick test to common allergens. On the contrary, non-atopic asthmatics are skin test negative to common allergens and there is no evidence of increased allergen-specific or total

serum IgE [2, 3]. Non-atopic asthmatics mainly display high numbers of neutrophils, macrophages and mast cells, whereas atopic asthmatics display a predominance of eosinophils and mast cells [2, 4, 5]. Some studies indicate, however, that there are more similarities than differences in the airway abnormalities of atopic and non-atopic asthma [6–8]. There is indeed evidence to support the role of T cells, especially Th₂ cells, in atopic as well as non-atopic asthma, which maintain the features of asthma [3, 6]. However, Th₂ cells do not react directly to inhaled antigen, as their T cell receptor can only recognize antigen that is processed into peptides for presentation on major histocompatibility complex (MHC) molecules [9].

This review focuses on the role that antigen presenting dendritic cells (DCs) play in the activation of T cell responses in atopic asthma, and how various endogenous danger signals trigger DCs to mount T cell responses to inhaled allergen.

Th₂ priming by dendritic cells

DCs are essential for priming and Th₂ differentiation of naïve T cells towards aeroallergens [9]. They are considered the most powerful antigen presenting cells (APCs) and play a central role in the initiation of primary immune responses [10, 11], and in the enhancement of secondary

immune responses [12, 13]. Under basal conditions, airway DCs are situated just below the epithelial layer. Inhaled allergens are captured by these DCs through endocytosis. These allergens are processed and generally presented by MHC class II molecules to CD4 T-helper (Th) cells [14]. DCs that have captured antigen subsequently migrate to the T cell area of mediastinal lymph nodes where they report the inhaled antigen to recirculating T cells. In this process, DCs acquire a mature phenotype, meaning that they up-regulate CD80 and CD86 co-stimulatory molecules for naïve T cell activation, and acquire the capacity to stimulate an effector response in dividing T cells [15–19].

The DC becomes a reporter of its earlier microenvironment and has the potential to induce a polarized Th₁, Th₂ or Th₁₇ type of response [10, 20]. Many factors are decisive in the process of Th polarization. The type of antigen, the presence of a microbial antigen, the route of exposure and the genetic background of the host, all play an important role [15, 21, 22]. These signals are translated, at the level of the APC, into a signal that can be read by the T cells. The most critical defining factors in this translational step are the type of DCs involved in the interaction with T cells, the type of co-stimulatory molecules expressed on the surface of the DCs and the polarizing cytokine micro-environment during antigen presentation [20, 23, 24].

Tolerance or Th₂ immunity is controlled by dendritic cell function

Inhaled particles are immunological inert; the usual outcome of their inhalation is tolerance, and inflammation does not develop on chronic exposure. In the absence of inflammatory triggers, DCs that take up these 'clean' antigens do not properly express co-stimulatory molecules and consequently induce a T cell response that is mainly characterized by cell division, but very little differentiation to effector cells. Additionally, the majority of antigen reactive T cells are deleted after dividing, particularly when high amounts of antigen are given. Animal studies have shed some light on how this response is initiated and regulated. Tolerance is a feature of lung DCs in steady state. Tolerance is shown best for the model antigen ovalbumin (OVA). In the most common used model to sensitize mice to induce atopic asthma, OVA is administered with an adjuvant such as aluminium hydroxide [25, 26]. After challenges with OVA aerosol via the lung or droplet aspiration via the nose, tissue eosinophilia occurs, and infiltrates are formed in tissue. When OVA is administered without adjuvant to the airways of naïve mice through aerosolization, nasal droplet aspiration or intratracheal injection, it renders mice tolerant to a subsequent immunization with OVA in adjuvant and effectively inhibits the development of airway inflammation, a feature of true immunological tolerance [24, 27].

Recent studies from our group have demonstrated that tolerance is induced by particular DC subsets. Conventional DCs (cDCs) are important for generating T cell division and priming, whereas plasmacytoid DCs (pDCs) suppress T cell effector generation. Strikingly, in the absence of pDCs, exposure to harmless antigen led to Th₂ cell sensitization and to features of asthma [24]. If pDCs promote tolerance and cDCs promote immunity, it is logical to assume that the balance between both subsets is tightly controlled. In support, the administration of Flt-3 ligand, a cytokine that induces the differentiation of pDCs, to sensitized mice reduced all the features of asthma [21].

It was long enigmatic how sensitization to natural allergens occurred. One aspect that is required for priming to occur is that DCs need to be activated by a sense of danger. This danger signal could be found in the allergen itself. Hammad et al. [23] have shown that the major Der p 1 allergen from HDM has proteolytic enzymes that can directly activate DCs or epithelial cells, and in this way promote Th₂ sensitization. Mariani et al. demonstrate that water-soluble mediators from birch pollen promote their Th₂ potential and change the chemokine receptor profile of human DCs, thus favouring the attraction of Th₂ cells to the site of allergic inflammation. The water-soluble extract from birch pollen consists predominantly of E₁-phytoprostanes (lipids), which resemble the structure and function of prostaglandin E₂. These findings support the hypothesis that pollen grains themselves appear to contain important signals to pave the way towards a Th₂-dominated immune response [28, 29].

Pattern-associated molecular patterns control dendritic cell activation

Some allergens, such as the experimental allergen OVA, do not have any intrinsic activating properties, like HDM. For these antigens, additional signals from contaminating molecules or environmental exposures (respiratory viruses, air pollution or cigarette smoke) might pull the trigger on DC activation [30, 31]. In the setting of infection, microorganisms initiate a series of host events promoted by their derived products, the so-called pathogen-associated molecular-pattern (PAMP) molecules. PAMPs are recognized by membrane-bound, cytoplasmic or vesicular (endosomal) pattern-recognition receptors (PRRs), including the Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-like receptors (RLRs). Signalling through TLRs strongly activates DCs to up-regulate co-stimulatory molecules (CD80 and CD86) and to produce pro-inflammatory cytokines (TNF- α , IL-1, IL-6 and IL-12) [32, 33]. PRRs 'sense' bacterial products and activate intracellular cascades that lead to an inflammatory response [34, 35]. PAMPs, sensed by host inflammatory cells early during infection, are potent stimuli for innate

immunity and are often referred to as 'exogenous danger signals'.

Eisenbarth et al. [33] support the existence of a TLR-dependent mode of Th₂ generation. Using a murine model of asthma, characterized by airway inflammation, eosinophilia and mucus hypersecretion in response to intranasal exposure to antigen, they found that the dose of LPS that is contaminating most commercially available batches of OVA used in mouse models of asthma regulates the induction of Th₂ vs. Th₁ responses. In these studies, it was shown that intranasal antigen required concomitant low-dose LPS signalling through TLR4 to induce allergic pulmonary Th₂ responses. MyD88, a common TLR adaptor molecule, is an essential innate component in the induction of TLR4-dependent Th₂ responses to antigens, which acts by inducing the expression of inflammatory cytokines such as IL-6, IL-12 and TNF- α [33, 36–38]. The fact that endotoxin in experimental mice models promotes allergic sensitization via effects on DCs might have direct clinical relevance, because most inhaled allergens, such as allergens derived from cockroaches and HDMs, are contaminated with LPS.

A study from Braun-Fahrlander among school-aged children corresponds with the study of Eisenbarth, also showing that environmental exposure to low doses of endotoxin is associated with the development of Th₂ immunity towards ubiquitous allergens found in natural environments, whereas high dose of LPS favours tolerance [39]. These experimental and clinical observations suggest that activation of DCs by PAMPs (like TLR agonists) is a critical component of sensitization to some allergens.

Damage-associated molecular patterns also control dendritic cell activation

Not all allergens are intrinsically immunogenic or contaminated with TLR agonists. Cell stress or tissue damage can trigger inflammation even in the absence of pathogens. It is now understood that immune cells react to injury-induced release of molecules that are normally located inside the cell [40]. These molecules are part of a diffuse surveillance network devised to alert our body defences of an impending danger, and are therefore also referred to as 'endogenous danger signals' or 'damage-associated molecular patterns' (DAMPs) [41]. These DAMP molecules contribute to the induction of inflammation by recruitment of innate inflammatory cells and interact with PRRs. The actual repertoire of DAMPs in damaged tissues can vary greatly depending on the type of cell (epithelial or mesenchymal) and injured tissue. Heat shock proteins (HSP), high mobility group box 1 (HMGB-1) protein, uric acid and adenosine triphosphate (ATP) are a few examples. Many intracellular proteins secreted actively through non-classical pathways and endowed with inflammatory activity [so-called leaderless secretory proteins (LSPs)] can be released by dying cells and behave as DAMPs. HMGB-1

is a prototypical LSP, which is passively released by injured or necrotic cells, or by immune cell responses to endotoxin, which promotes tissue inflammation [42].

Interestingly, many of the receptors for DAMPs and PAMPs are shared, either expressed on the cell (e.g. TLR4) or intracellular (e.g. TLR3, TLR7–9, all NLRs and RLRs), indicating that similarities exist between pathogen-induced and non-infectious inflammatory responses [35]. Compelling evidence suggests that a tight collaboration between PAMPs and DAMPs is needed to start an immune response to allergens [43]. For example, the inflammatory cytokine IL-1 β can be produced by a cell after activation by a PAMP, but can only be released in a bio-active form by a second signal from a DAMP [44]. Figure 1 illustrates various PAMP and DAMP receptors which are present on the cell surface of DCs.

Damage-associated molecular patterns that trigger the NALP3 inflammasome trigger Th₂ responses

One recent illustration of the potential implication of endogenous danger signals to process of atopic sensitization comes from our studies on the mechanism of alum adjuvant. Alum is used in mouse models of asthma as a prototypical Th₂ adjuvant, whose mechanism of action is poorly understood. When added to DCs *in vitro*, alum poorly activates APC function [45]. *In vivo*, however, alum strongly recruits and stimulates inflammatory DCs and boosts their potential to induce Th₂ responses, dependent on the production of bio-active IL-1 β [46]. We found that alum induces the release of uric acid, an endogenous danger signal released by dying cells [47, 48]. Uric acid is

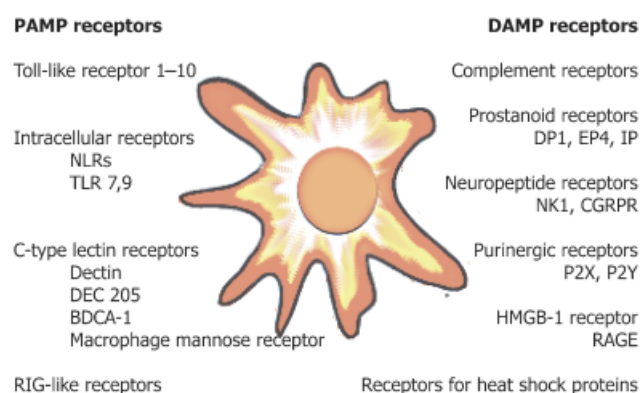


Fig. 1. Expression of 'danger' receptors by dendritic cells (DCs). DCs express the highly conserved receptors of the innate immune system also expressed by macrophages such as Toll-like receptors (TLRs), retinoic acid inducible-gene-like receptors (RLRs) and C-type selectin receptors. These receptors react to foreign pathogen-associated molecular patterns (PAMPs). In addition, DCs express numerous receptors for inflammatory mediators and necrotic debris, the so-called damage-associated molecular patterns (DAMPs) [77]. The exact receptor for heat shock proteins is not known.

known to induce the release of IL-1 β and promotes Th₂-polarizing responses by DCs [49]. The activation of IL-1 β release, an important cytokine response, requires the presence of a TLR agonist acting on APCs to promote transcription and translation of pro-IL-1 β . Subsequently, pro-IL-1 β requires cleavage by caspase-1, whose activation in turn depends on triggering of the NLR NALP3 via endogenous danger signals [50, 51]. Uric acid is the best known trigger for the NALP3 inflammasome, a complex of molecules that leads to the activation of caspase-1. We also recently obtained evidence that alum directly triggers the NALP3 inflammasome [46]. In addition to alum and uric acid, exposure of cells to high, micromolar concentrations of extracellular ATP has been known for years to activate caspase-1, and several studies have demonstrated the requirement of P2X₇ receptors (in a complex with the hemichannel pannexin-1) for ATP-induced caspase-1 activation and subsequent IL-1 β maturation [44] (Fig. 2). Apoptosis-associated speck-like protein (ASC) is an adaptor protein that is essential for the recruitment of caspase-1 to the NALP inflammasome. The generation of ASC-deficient mice demonstrated that ATP-mediated caspase-1 activation requires ASC and was therefore dependent on the activation of the NALP protein. This assumption was confirmed in studies using NALP3-deficient mice [51, 52]. These findings clearly show that uric

acid and extracellular ATP can act as a danger signal to activate the NALP3 inflammasome and promote caspase-1 activation and IL-1 β maturation.

It therefore comes as no surprise that mice deficient in NALP3 have a deficient Th₂ response to alum-adjuvanted allergens and fail to mount Th₂ mediated inflammation [46, 53]. In addition to uric acid release, there is also evidence that alum directly triggers the formation of the NALP3 inflammasome in a process that also requires the ASC protein [46, 53]. Whether uric acid and NALP3 triggering is involved in sensitization to common inhaled allergens remains to be shown. Certainly uric acid promotes the development of Th₂ responses when added to DCs *in vitro* [46, 49, 53].

Role of adenosine triphosphate and purinergic receptors in asthma

ATP is primarily known for its function as an energy source in all cells. ATP, as a DAMP, has unique features: availability in high concentrations within the cytoplasm of every cell; low, almost negligible, levels in the extracellular space in healthy tissues; quick release following cell damage; and inactivation by powerful ubiquitous ecto-ATPases [54, 55]. These properties make ATP an ideal extracellular harbinger of cell stress. ATP can be released by a variety of injurious agents: trauma, oxidants and

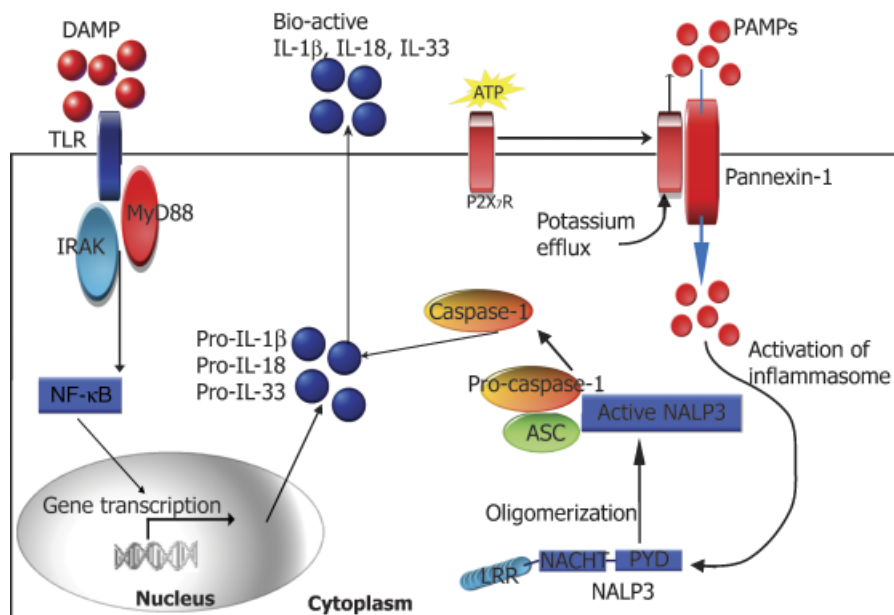


Fig. 2. Schematic representation of NALP3 inflammasome activation by lipopolysaccharide (LPS) and adenosine triphosphate (ATP) followed by release of inflammatory cytokines. Microbial molecules, such as LPS, bind to TLR4, which recruits MyD88. MyD88 will in most cases bind to IL-1 receptor-associated kinase (IRAK), which will lead to nuclear factor- κ B (NF- κ B) activation. NF- κ B is a transcription factor which is responsible for the transcription of inflammatory cytokines. Pro-IL-1 β , pro-IL-18 and pro-IL-33 are then released in the cytosol. The second signal needed to release bio-active forms of these inflammatory cytokines is provided by activation of P2X₇ receptor by ATP. Activation of P2X₇ receptor leads to large pore formation by pannexin-1. DAMPs (for example uric acid) can now enter the cytosol and activate the NALP3 inflammasome [78–80]. Activated NALP3 consists of a pyrin domain, a NACHT domain and 11 leucine-rich repeat (LRR) domains. When activated, NALP3 oligomerizes and then recruits ASC and pro-caspase-1, which results in caspase-1 processing and activation. Active caspase-1 then cleaves and thereby activates the precursor forms of IL-1 β , IL-18 and IL-33 and extracellular release of the bio-active cytokines [44, 52, 81].

pathogens. There are several sources for extracellular ATP. ATP is stored in relatively large amounts in platelets and is released during platelet activation. Similarly, ATP is stored in red blood cells from which it is released under conditions of imbalance between O₂ supply and O₂ demand. In addition, several biologic substances as well as increased blood flow can induce the release of ATP from vascular endothelial cells and smooth muscle cells. ATP is also released from neural elements as a co-transmitter and from exercising skeletal muscles [56–59].

In the late 1970s, Burnstock et al. [60] hypothesized that specific cell surface receptors, which he called purinergic receptors (P receptors), different from cholinergic and adrenergic receptors, mediate the actions of adenosine [P1 receptors (P1Rs)], and ATP [P2 receptors (P2Rs)]. It is now well established that ATP and other adenine nucleotides exert their effects by activating the P2Rs and that the P1Rs mediate the effects of extracellular adenosine, the product of ATP's enzymatic degradation [56, 61]. The P2 receptors can be divided in two families, the P2X receptors, which are the ligand-gated ion channels, and the P2Y receptors that consist of a group of G-protein-coupled receptors. P2X1 to 7 are the best known subtypes of the P2X receptor family whereas P2Y1, 2, 4, 6 and 11–14 are the best characterized P2Y receptors [62, 63]. These receptors can be activated by various nucleotides (purines and pyrimidines). Extracellular ATP recently gained attention as a mediator of intercellular communication, through the activation of these purinergic receptors [64].

Both P2X and P2Y receptors are present in the lung. The airway epithelium has been one of the most extensively studied tissues for the effect of purinergic agonists. Airway epithelium releases ATP and possesses both P2X and P2Y receptors as do endothelial cells [65, 66]. Extracellular ATP appears to be an important mediator of vascular inflammation, acting on endothelial cells to modulate vascular perfusion, to promote platelet aggregation and to regulate neutrophil function [67]. ATP could act as an autocrine regulator on epithelial cells. Although ATP is constantly released from epithelial cells, inflammatory stimuli, such as thrombin and LPS, enhance ATP release from epithelial cells [68]. Oguma et al. reported that ATP by itself did not cause airway smooth muscle (ASM) contraction *in vitro*. Pre-exposure of the ASM to ATP significantly enhanced the contraction in a reaction to methacholine. Enhanced ATP release in the lung increases the contractile ability of ASM, which may lead to airway hyperresponsiveness in patients with asthma [58]. ATP has also been shown to modify the recruitment and/or function of inflammatory cell types involved in asthma pathogenesis, including eosinophils, mast cells, lymphocytes and DCs, and it has also been found to be a potent enhancer of human lung mast cell histamine release [56, 61, 69, 70] (Fig. 3). Extracellular ATP could act as a chemoattractant for DCs via P2Y receptor activation, depending on the concentration of ATP, the maturational state and type of DC [71, 72]. Extracellular ATP may in fact be one of the factors involved in DC maturation, as micromolar ATP

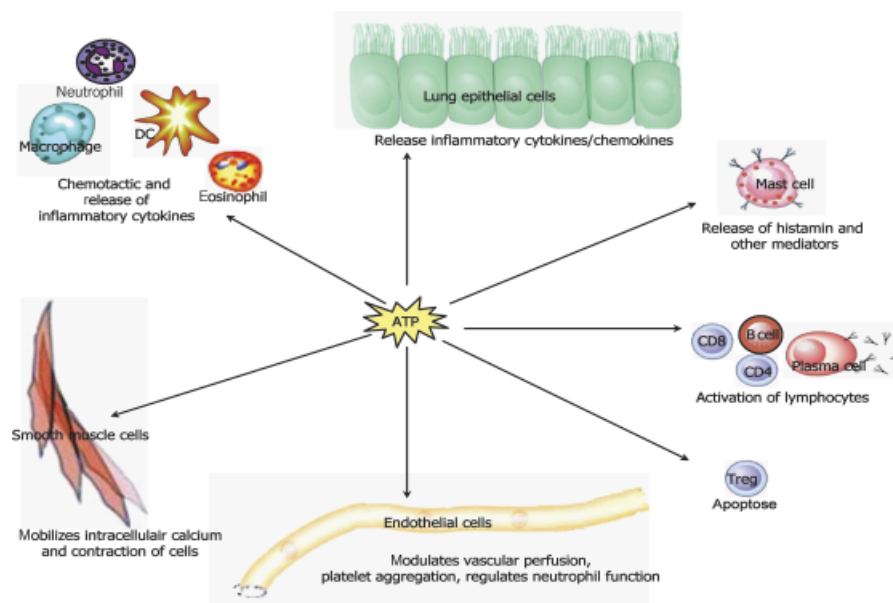


Fig. 3. Effect of extracellular adenosine triphosphate (ATP) on various cells involved in asthma. ATP can modulate vascular perfusion by endothelial cells, and regulates immune cells migration to the site of inflammation. ATP has been shown to be chemoattractive to neutrophils, eosinophils, DCs and other inflammatory cells [56, 61, 70, 82, 83]. Activation of Tregs by ATP via the P2X₇ receptor induces apoptosis, thereby promoting the inflammatory response mediated by the conventional T cells [75]. On airway smooth muscle cells, ATP works as an adjuvant in response to methacholine [58].

concentrations have been shown to induce up-regulation of maturation markers (i.e. CD80, CD83, CD86, CD54 and MHC-II) in human peripheral blood mononuclear cell (PBMC)-derived immature DCs [61, 71]. There is recently evidence that matured regulatory T cells (Treg cells) control asthmatic inflammation by interaction with cDCs. Their main feature is to reduce T cell proliferation [73, 74]. Extracellular ATP can induce rapid and efficient death in CD4⁺CD25⁺ Treg cells via action on P2X₇Rs. Conventional T cells are relatively resistant, resulting in preferential elimination of Tregs, thereby promoting increased responses of conventional T cells [75].

We have recently reported on *in vivo* evidence for a functional role of ATP in asthmatic airway inflammation. In the bronchoalveolar lavage (BAL) of patients, there was an increase in ATP after allergen challenge. Also in mice, ATP was found in the BAL after allergen challenge, whereas sham-immunized mice did not show an increase in ATP in BAL. In another experiment, we show that a non-degradable form of ATP was able to break inhalation tolerance to OVA. Most importantly, Th₂ sensitization to inhaled OVA and cardinal features of asthma such as eosinophilic airway inflammation and bronchial hyperreactivity were abolished when ATP signalling was blocked using the broad spectrum P2X and P2Y receptor antagonist suramin or when the extracellular ATP was neutralized by using the enzyme apyrase, illustrating that ATP functionally contributes to all features of asthma [76]. Exactly which P2X or P2Y receptor is involved in mediating pro-inflammatory effects of DAMPs is unresolved at present.

Conclusion

Danger signals affect a broad range of inflammatory cells. They affect DCs by changing their maturation state and are also able to activate or attract several other inflammatory cells, such as mast cells and lymphocytes, which are involved in atopic as well as non-atopic asthma. Several recent studies have demonstrated that endogenous danger signals such as uric acid and ATP might play an important role at the inception and maintenance phase of allergic disease. Further research into this area should focus on the role of DAMPs in atopic vs. non-atopic asthma and on the possible role for these factors in maintenance of chronic disease and induction of airway remodelling.

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