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Review

Molecular mechanisms underlying suppression of lymphocyte responses by nonsteroidal antiinflammatory drugs

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Abstract. Initially identified and further developed as inhibitors of cyclooxygenases, nonsteroidal antiinflammatory drugs (NSAIDs) have been more recently shown to bind to and act as agonists of the peroxisome proliferator-activated receptor family of transcription factors. Here we summarize the current knowledge on the functions of the principal targets of NSAIDs and review their role in T and B lymphocytes, with a focus on the molecular mechanisms underlying the effects of NSAIDs on lymphocyte development, activation, differentiation and death.

Key words. COX; PPAR; p38 MAP kinase; prostaglandin; transcription factor; immunosuppression; thymic development; apoptosis.

Introduction

Since the active principle responsible for the long-known antiinflammatory properties of the bark of the willow was first identified in 1829 as salicylic acid, salicylates, and later other compounds capable of interfering with the activity of cyclooxygenases (COXs), have become the drugs of choice for the treatment of inflammatory diseases. These drugs, collectively referred to as nonsteroidal antiinflammatory drugs (NSAIDs), are currently used in clinical practice not only in the treatment of acute inflammation but also for the pharmacological management of chronic inflammatory diseases such as osteoarthritis and rheumatoid arthritis. Furthermore, with a better understanding of the multiple and multifaceted functions of COXs, parallelled by the development of second-generation NSAIDs with markedly reduced side effects, the range of applications of NSAIDs has been extended to pathologies as diverse as prevention of myocardial infarction and thrombosis, and treatment and chemoprevention of colon cancer. The importance of NSAIDs in human health has stimulated an impressive amount of research on the cellular and molecular targets of these drugs, which has resulted in a wealth of information on the role of COXs and their products, prostaglandins and thromboxanes, not only in the responses of the organism to external challenge but also in key homeostatic functions. Here we shall review the activities of NSAIDs on lymphocytes and what NSAIDs have revealed about the role of COXs in the physiology of these cells.

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COXs: the molecular targets of NSAIDs

Structure and activities of COXs

COXs, also known as prostaglandin H synthases or prostaglandin endoperoxide synthases, catalyze the rate-limiting step in the biosynthesis of prostaglandins (PGs) and thromboxanes from arachidonic acid (AA), which is released from membrane phospholipids by phospholipase A_2 (PLA_2). The first reaction involves oxidation and cyclization of AA to yield the hydroperoxide endoperoxide PGG_2 (cyclooxygenase activity), followed by its reduction to PGH_2 (peroxidase activity). PGH_2 is subse-

quently converted by specific cellular synthases to biologically active products, which include the prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and the thromboxane TXA_2. The half-lives of most prostanoids are extremely short due to the activity of inactivating enzymes or to nonenzymatic catalysis, resulting in their conversion to either inactive derivatives or products with different biological activities, such as the cyclopentenone prostaglandin 15-deoxy-Δ^{12-14}-PGJ_2 (15-d-PGJ_2) (fig. 1).

There are two COX isoforms, COX-1 and COX-2, which are highly related both in structure and in enzymatic activity. Both are homodimeric heme-containing proteins, characterized by ~70-kDa monomers anchored to the lumenal side of the endoplasmic reticulum and comprising three distinct domains: a domain with homology to the epidermal growth factor at the N terminus, a central amphipathic domain responsible for tethering to the membrane and C-terminal globular catalytic domain. The COX active site lies within a narrow hydrophobic channel that also includes the binding site for AA, which is directly released from the lipid bilayer into this location through the activity of PLA_2. This channel is also the NSAID binding site. The amino acid sequences implicated in AA binding and catalysis are almost identical in COX-1 and COX-2, with the exception of two isoleucine-to-valine substitutions at positions 434 and 523 in COX-2. Because of the smaller size of valine compared with isoleucine, these substitutions result in a larger and more flexible hydrophobic channel. This property has formed the basis for the development of COX-2-selective NSAIDs, which are

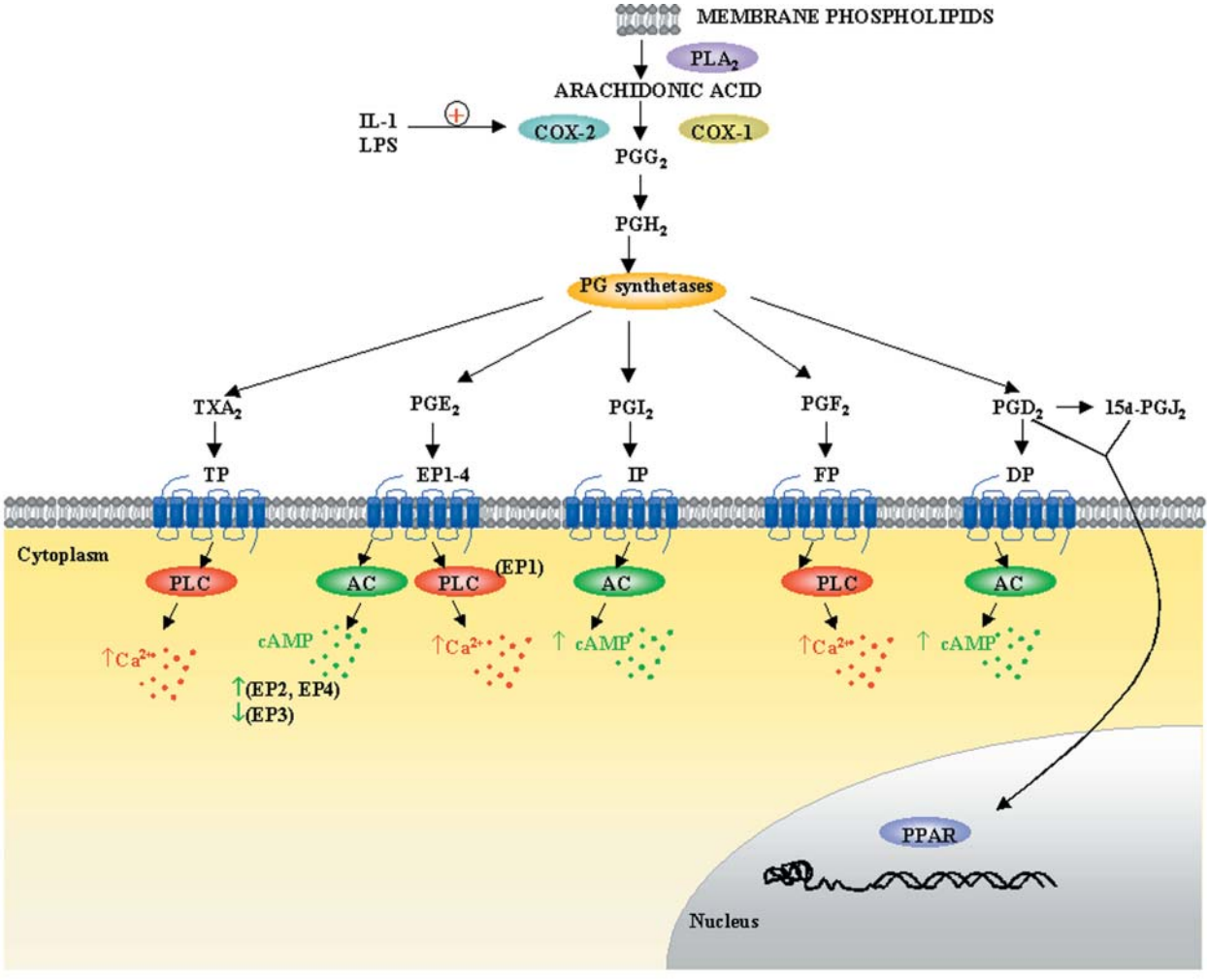

Figure 1. Biochemical pathway of PG biosynthesis and downstream targets. The pathway of prostanoid biosynthesis from membrane phospholipids is schematized on the top part of the figure. The principal proinflammatory stimuli promoting COX-2 expression are indicated. Different PG synthases are responsible for the production of TXA_2, PGE_2, PGI_2, PGF_2 and PGD_2. 15-d-PGJ_2 is the principal biologically active derivative of PGD_2. The lower part of the figure schematizes the seven-spanning membrane receptors for each class of prostanoid, as well as their coupling to the intracellular effectors, adenylate cyclase (AC) and phospholipase C (PLC). The nomenclature for the prostanoid receptors is TP (TXA_2 receptor), EP (PGE_2 receptor), IP (PGI_2 receptor), FP (PGF_2 receptor) and DP (PGD_2 receptor). Although only plasma membrane receptors are indicated, PG receptors have also been described at the nuclear membrane. In addition to binding to DP receptors, PGD_2, as well as 15-d-PGJ_2, also bind to nuclear receptors, identified as PPARs.

designed to fit preferentially the larger COX-2 channel, resulting in a remarkably effective discrimination between the two COX isozymes (reviewed in [1, 2]).

Homeostatic and disease-related functions of COX-1 and COX-2

Notwithstanding their similar activities, COXs appear to play distinct roles in cellular physiology. COX-1 is constitutively expressed in a wide variety of cell types, while COX-2 is mostly inducibly expressed in a restricted number of cell types. Specifically, with the exception of the central nervous system, the renal cortex and the mature ovum, where it is expressed constitutively, COX-2 is inducibly expressed in monocytes, macrophages and polymorphonuclear cells in response to proinflammatory stimuli. Furthermore, COX-2 is ectopically expressed at high levels in some forms of neoplasia, including colon, breast and prostate cancer (reviewed in [3]). Prolonged usage of nonselective NSAIDs results in gastric ulceration and bleeding, supporting a key role for COX-1 in the maintenance of gastric integrity and homeostasis. On the other hand, selective inhibition of COX-2 effectively dampens inflammation with significantly less gastric damage (reviewed in [4]). Furthermore, there is good evidence for beneficial effects of COX-2 inhibitors both in chemoprevention and in treatment of some forms of colorectal cancer (reviewed in [5]).

The homeostatic functions of COX-1, as opposed to the disease-related functions of COX-2, have led to the concept of 'good' and 'bad' COXs; however, more recent data describing the effects of NSAIDs on specific cellular functions, as well as the analysis of the phenotypes of mice lacking either COX isoform, have provided significant insight into the roles of each isoform. Unique functions selectively implicating individual COX isozymes include platelet aggregation and parturition for COX-1, and ovulation and implantation, as well as some aspects of neonatal development, for COX-2 (reviewed in [6]). Unexpected synergies between COX-1 and COX-2 have, however, emerged in the control of a variety of physiological and physiopathological functions, as best exemplified by inflammation and colon cancer. Although according to the current dogma COX-2 is principally responsible for inflammation, COX-1 also appears to participate in this process. The antiinflammatory effects of COX-2-selective inhibitors in carrageenin-induced paw inflammation in the rat can indeed be achieved only at doses of the drugs that inhibit COX-1 [7]. Furthermore, a decreased inflammatory response to AA was observed in COX-1-deficient mice [8]. A cooperation of COX-1 or COX-2 in promoting carcinogenesis, rather than a unique role for COX-2, has also been established. Deficiency of either COX-1 and COX-2 results in a similar reduction in polyp formation in the *Min*$^{+/-}$ mouse model of adenomatous polyposis coli [9], in agreement with the proposed sequential function of COX-1 in the early stages of tumorigenesis and of COX-2 in tumor promotion after loss of heterozygosity of the *APC* gene has occurred [10].

More recently, an unanticipated role for COX-2 in the resolution of inflammation was established, based on identification of the antiinflammatory properties of cyclopentenone PGs, of which the most potent is 15-d-PGJ_2, which are produced by COX-2 late in the inflammatory response (reviewed in [11, 12]). In support of a role of COX-2 in the resolution of inflammation, selective inhibition of COX-2 results in exacerbation of inflammation in carrageenin-induced pleurisy in rats [13]. Furthermore, inflammatory responses fail to resolve in COX-2-deficient mice [7]. Of note, an antiinflammatory role has been suggested also for COX-1, as both COX-1 and COX-2 deficiencies in mice result in enhanced inflammatory responses in models of allergic asthma and inflammatory bowel disease [14, 15]. However, the mechanism underlying this activity of COX-1 remains to be elucidated.

COX-1 and COX-2: Why different outcomes from similar activities?

Differential control of enzymatic activity

The initial production of prostanoids in response to extracellular stimuli is accounted for primarily by COX-1 as the only preexisting COX isozyme, while COX-2 becomes dominant when COX-1 and COX-2 are coexpressed. During inflammation this dominance is largely related to the massive amounts of COX-2 expressed as compared with COX-1. Furthermore, the activity of COX isozymes is differentially controlled by endogenous peroxide concentrations and availability of AA. Specifically, COX-1, but not COX-2, is activated by reactive oxygen species, which are rapidly but transiently produced in response to proinflammatory stimuli or growth factors. Furthermore, COX-1 requires higher concentrations of AA than COX-2, a condition also achieved early following receptor engagement. Hence, the temporally controlled changes of endogenous peroxide and AA concentrations result in differential and sequential activation of COX-1 and COX-2 [11]. These quite subtle differences in the regulation of activity are, however, not sufficient to explain why, notwithstanding their largely overlapping features in substrate usage and catalytic properties, COX enzymes harbor dramatic differences in the panel of cellular responses evoked.

Differential coupling to PLA2 and PG synthases

With the exception of platelets and endothelial cells, where the predominant prostanoid synthases are TXA_2 and PGI_2 synthase, respectively, much remains to be

learned about the specific cellular pattern of expression of the different PG synthases, as well as about the quantitative and qualitative changes in the balance of prostanoids when COX-2 is coexpressed with COX-1. It has been proposed that COX isozymes might be differentially coupled to specific PG synthases [16–18]. For example, as opposed to the constitutively expressed cytosolic PGE_2 synthase, a membrane-bound PGE_2 synthase is inducibly expressed concomitantly with the expression of COX-2 [18], suggesting coupling of COX-2 to the production of PGE_2 by the latter synthase. A similar functional coupling has been proposed for COX-2 and the secretory form of PLA_2. While the initial production of AA by COX-1 relies on the constitutively expressed, cytosolic form of PLA_2, in the presence of sustained extracellular stimulation a second, secreted PLA_2 isozyme is expressed, which, through coupling to COX-2 and the inducible PGE synthase, would be implicated in the delayed and prolonged synthesis of PGE_2 [11].

Differential coupling to PG receptors

The pleiotropic cellular responses to prostanoids are elicited as the result of their interaction with G-protein-coupled rhodopsin-type seven-spanning membrane receptors, encoded each by a distinct transcript which, as in the case of the PGE_2 receptor, can give rise to splice variants. Coupling of these receptors to adenylate cyclase or phospholipase C results in the production of two key second messengers, cyclic AMP (cAMP) and Ca^{2+} (fig. 1). Analysis of the phenotypes of mice deficient for each of the eight types and subtypes of prostanoid receptors has underscored not only the unique roles of the different prostanoids in various physiological and pathophysiological processes but also the variety of effects that the same prostanoid can elicit within the same cell depending on the type of receptor expressed (reviewed in [19]). It is therefore not surprising that as the result of coupling to different PG synthases, COX-1 and COX-2 can induce different biological responses in target cells. Furthermore, the existence of nuclear PG receptors preferentially coupled to COX-2 has been postulated. Functional PGE_2 receptors have indeed been described on nuclear membranes [20, 21]. These receptors appear to specifically participate in a signalling pathway implicated in the regulation of cell growth. The presence of PLA_2, as well as COX-2 at the nuclear envelope [22, 23], suggests that COX-2 might be coupled both to nuclear PLA_2 and to nuclear PGE_2 receptors as the result of physical segregation to the same subcellular localization.

Differences in substrates?

Although in vitro both COX isozymes can effectively use AA and other 20-carbon polyunsaturated fatty acids as substrates, recent data showing that COX-2, but not COX-1, can use as substrates esterified fatty acids such as 2-arachidoynyl glycerol suggests the provocative possibility of different physiological substrates for the two isozymes [24].

COX-independent targets of NSAIDs

PPARs

In addition to their ability to interact with COX, NSAIDs have been recently identified as ligands of the peroxisome proliferator-activated receptor (PPAR) family of ligand-activated transcription factors [25–27]. Initially implicated in the differentiation of adipocytes, PPARs have more recently been shown to play a key role in the modulation of immune responses through suppression of proinflammatory gene expression in macrophages [tumor necrosis factor α (TNF-α), interleukin (IL)-1, inducible nitric oxide synthase (iNOS)]. Upon binding of agonist, PPARs heterodimerize with the retinoic X receptors, and as such become competent to bind to PPAR-responsive elements on the promoters of target genes. In addition to their activity as transcriptional activators, agonist-bound PPARs can also function as transcriptional repressors by interacting with other transcription factors, such as nuclear factor kappa B (NF-κB), resulting in their inactivation or sequestration (reviewed in [28, 29]). In agreement with the the immunosuppressive activities of PPARs in vitro, PPAR agonists attenuate inflammation in animal models of inflammatory diseases, while deletion of the PPARα or PPARγ gene results in exacerbation of the disease [30–33].

Although the identity of the physiological PPAR ligands has as yet not been clearly established, several metabolites of polyunsaturated fatty acids have been shown to bind and activate PPARs. The most potent of these are 12-hydroxyoctadecadienoic acid and 15-d-PGJ_2; however, other cyclopentenone PGs, such as PGJ_2, Δ^{12-14}-PGJ_2, PGA_2 and PGD_2, function as PPAR ligands. PPARs therefore represent a novel class of nuclear PG receptors specifically regulated by COX-2. Interestingly, COX-2-dependent 15-d-PGJ_2 production contributes to inactivation of NF-κB not only as the result of its agonistic activity on PPAR but also by direct and selective targeting of the enzymatic machinery responsible for NF-κB activation. 15-d-PGJ_2 has indeed been shown to form a covalent bond with IKKβ, one of the subunits of inhibitor of NF-κB (IκB) kinase, resulting in irreversible inactivation of the kinase and of its final target, NF-κB [34]. Participation of COX-2 in the resolution of inflammation can be accounted for primarily by these two mechanisms of inhibition of NF-κB activation by cyclopentenone PG (reviewed in [35]).

A number of nonselective and COX-selective NSAIDs function as PPAR ligands, and in fact their wide spectrum of activities, from adipocyte differentiation to immunosuppression, fully overlap the responses evoked by physiological PPAR ligands [25–27]. Most immunosuppressive activities of NSAIDs unrelated to their ability to in-

hibit the synthesis of PGs are likely to be accounted for by their capacity to act as PPAR ligands and as such inhibit the transcription of proinflammatory genes. It must to be underscored that activation of PPARs by NSAIDs and the resulting activities, including suppression of proinflammatory gene transcription, adipocyte differentiation and growth of cancer cells, are achieved at doses significantly higher than those required for inhibition of COXs [25, 27, 28, 36]. In this context, the requirement for higher doses of NSAIDs in the treatment of inflammation as compared with analgesia supports the notion that the antiinflammatory properties of NSAIDs involve additional targets distinct from COXs.

It is actually quite striking that the principal targets of NSAIDs, COXs and PPARs, are also two key modulators of inflammation. Therefore, on the one hand NSAIDs block the production of PGs, including cyclopentenone PG, through their activity on COXs, and would thereby be expected to antagonize not only inflammation but also the resolution of inflammation. On the other hand, NSAIDs bind PPARs and mimic the activity of cyclopentenone PG. This behavior implies that NSAIDs might only affect the early phase of inflammation, dominated by PGE_2 production, but not the late resolution phase marked by the production of cyclopentenone PG. While this notion is not supported by the finding that inhibition or deficiency of COXs results in exacerbation of inflammation in the carrageenin-induced pleurisy model [13] and in an allergic airway disease model [37], more work in other experimental models of inflammation is required to fully elucidate this issue.

Other targets

Additional COX-independent effects of NSAIDs have been identified; however, the mechanisms underlying these effects are as yet poorly understood. Indomethacin, but not other NSAIDs, promotes chemotaxis of Th2 cells, eosinophils and basophils by acting as an agonist of CRTH2 (chemoattractant receptor expressed on Th2 cells) [38]. Furthermore, effects elicited by aspirin, but not other NSAIDs, include inhibition of IL-4 gene transcription in T cells [39], inhibition of dendric cell maturation [40, 41], inhibition of T cell adhesion and transmigration through the endothelium [42], and VCAM-1 and E-selectin expression in monocytes [43]. The resulting defects in leukocyte adhesion are likely to contribute to the antiinflammatory activities of NSAIDs.

NSAIDs and lymphocyte physiology

Although the immunomodulatory properties of NSAIDs have been studied primarily on the cells directly implicated in inflammation, including macrophages, neutrophils, eosinophils and mast cells (reviewed in [44]), the effects of NSAIDs on lymphocytes have until recently been much less thoroughly characterized. NSAIDs are known to function as immunosuppressants on both T cells and B cells in vitro. While studies on the impact of NSAIDs on B cells are to date rather limited, significant progress has been achieved on T cells, where a highly synergistic combination of pharmacological inhibition and gene disruption has revealed fundamental and isoform-specific roles for COX isozymes in a panel of T cell functions.

NSAIDs and lymphocyte development

Thymic development is a multistep process where achievement of each principal maturation stage can be tracked by surface expression of the antigen coreceptors CD4 and CD8. Bone-marrrow-derived progenitors colonize the thymic cortex, where they can be identified as $CD4^-CD8^-$ (double negative) thymocytes. Following rearrangement of first the β and then the α chain of the T cell receptor (TCR) and expression of both CD4 and CD8, double-positive thymocytes interact with thymic stromal cells to undergo the most complex maturation phase where thymocytes expressing either self-specific or incorrectly rearranged TCRs are deleted (negative selection), while thymocytes expressing TCRs which can weakly bind self-peptides survive (positive selection). The latter further switch off either CD4 or CD8 expression, becoming single positive thymocytes. As maturation proceeds, thymocytes move to the medulla, from where single positive thymocytes eventually exit to reach the periphery as mature naive T cells (reviewed in [45]).

Both COX isoforms are expressed in the thymus, with a distinctive distribution for each isozyme. Specifically, COX-1 is expressed in developing thymocytes ($CD4^-$ $CD8^-$ and $CD4^+CD8^+$), while COX-2 expression is limited to a subset of medullary stromal cells [46]. Pharmacological inhibition of COX-1 in fetal thymus revealed a decrease in double positive thymocytes, supporting a role for COX-1 in the $CD4^-CD8^-$ to $CD4^+CD8^+$ transition. A similar analysis of thymic organ cultures treated with COX-2-selective inhibitors showed a reduction both in $CD4^-CD8^-$ thymocytes and in $CD4^+$ single positive cells [46]. Hence, COX-2 expressed by thymic stromal cells participates at two key stages of thymopoiesis, first by favoring survival and differentiation of double negative thymocytes, and subsequently by promoting maturation of the CD4 helper T cell lineage. The role of COX isozymes in thymic development has been also addressed using fetal organ cultures from mice deficient for either COX-1 or COX-2 [46]. In full agreement with the results obtained using COX-1 and COX-2 selective NSAIDs, analysis of thymocyte populations in COX-1-deficient mice showed a reduction in $CD4^+CD8^+$ cells, while COX-2-deficient mice harbored a decrease in double negative

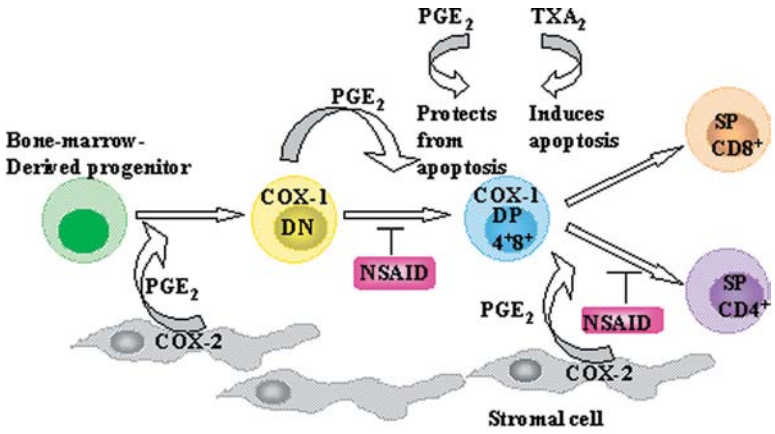

Figure 2. Targets of NSAIDs in thymic development. The developmental sequence of thymocytes from bone marrow progenitors is schematized from left to right. DN, double negative thymocytes ($CD4^-CD8^-$), DP $CD4^+CD8^+$, double positive thymocytes, SP $CD4^+$ and SP $CD8^+$, single positive thymocytes. The scheme shows that stromal cell-derived PGE_2, produced by constitutively expressed COX-2, affects two stages of thymic development, i.e. the maturation of DN and of SP $CD4^+$ thymocytes. DN and DP thymocytes constitutively express COX-1, which through the production of PGE_2 promotes the transition from the DN to the DP stage. COX-1-selective NSAIDs added to fetal thymic organ cultures block the DN-to-DP transition, while COX-2-selective NSAIDs block the maturation of SP $CD4^+$ thymocytes. PGE_2 and TXA_2 exogenously added to purified thymocytes protect from or promote apoptosis, respectively.

and in single positive $CD4^+$ cells [46]. Hence, both COX isozymes are implicated in thymic development (fig. 2).

A number of prostanoid receptors are expressed in the thymus; however, the specific role of each class of receptor on thymocyte development and selection still awaits full elucidation. TXA_2 receptors are widely expressed in the thymus, with the highest expression in double negative thymocytes followed by single positive thymocytes. Pharmacological modulation of thromboxane receptor activity in thymocytes induces apoptosis of $CD4^+CD8^+$ thymocytes, suggesting a potential role for these receptors in negative selection [47]. An opposite effect on the same thymocyte subpopulation is elicited by PGE_2 receptors. In fact, PGE_2 protects immature $CD4^+CD8^+$ thymocytes from apoptosis, an activity which appears mediated by cAMP, suggesting a role for PGE_2 receptors in positive selection [48]. Of note, notwithstanding the different pattern of PG receptor expression in thymocyte subpopulations, PGE_2, but not other PGs, could rescue the defects in thymocyte development elicited by both COX-1 and COX-2 selective NSAIDs. Furthermore, neutralization of PGE_2 by specific antibodies mimicked the effects of NSAIDs on thymocyte maturation, supporting a selective role of PGE_2 receptors [46]. While pharmacological evidence suggests a distinct role for specific PGE_2 receptor subtypes at the different stages of thymocyte development [46], it will be interesting to address the impact of ablation of individual PG receptors on this process.

NSAIDs and lymphocyte activation

The inhibitory activity of NSAIDs on lymphocyte activation in vitro is well documented; however, a better insight into the mechanisms underlying NSAID-dependent immunosuppression has been achieved only recently with a more exhaustive characterization of the molecular targets of NSAIDs. Although the role of COXs in T and B lymphocyte activation and differentiation has as yet not been addressed in vivo in COX-deficient mice, their implication in these processes is supported by the profound effects of prostanoids on these cells in vitro. Both classes of prostanoids, thromboxanes and PGs, affect lymphocyte activation; however, they harbor opposite activities, at least in vitro. TXA_2 promotes T cell activation and proliferation, as well as differentiation of effector cytolitic T cells [49]. Conversely, PGE_2 suppresses T cell activation and proliferation through a cAMP-dependent mechanism, in agreement with the agonistic activity of PGE_2 receptors on adenylate cyclase [50, 51]. PGE_2, as well as cAMP agonists, inhibit not only IL-2 and IL-2R gene expression [50–55] but also expression of JAK3, thereby contributing to defective IL-2R signalling [56]. Analysis of PGE_2 receptor-deficient mice has revealed a major role for the EP2 receptor in the immunosuppressive activity of PGE_2 [57].

PGE_2 also inhibits B cell activation, proliferation and differentiation to immunoglobulin (Ig)G-secreting cells [58–60]. Similarly, production of IgM by peritoneal B-1 cells is inhibited by PGE_2 [61]. Interestingly, the defect in B cell activation is parallelled by IgE class switching and increased production of IgE [59, 62]. As in T cells, these biological activities of PGE_2 are dependent on cAMP, in agreement with the proposed role of the EP2 and EP4 receptors in the activation and differentiation of B cells [55, 63]. Of note, opposite effects are elicited by PGE_2 when B cells are stimulated not through membrane Ig, but

through CD40. In this system PGE_2 enhances IL-4-dependent B cell proliferation and antagonizes IL-4-dependent IgE class switching [64], suggesting that different responses might be elicited by PGE_2 in different cellular microenvironments. In support of this possibility, PGB_2, a catabolite of PGE_2, has recently been shown to act as costimulator in T cell activation [65].

The issue is further complicated by the finding that cyclopentenone PGs, which appear to play opposite roles to PGE_2 in inflammation, harbor in lymphocytes immunosuppressive activities overlappable with those elicited by PGE_2, including suppression of T cell activation and proliferation, and inhibition of IL-4-dependent IgE class switching [66, 67]. This apparent discrepancy is likely to be accounted for by the PG receptor-independent activity of cyclopentenone PGs both as inhibitors of IκB kinase [34] and as coactivators of PPAR family transcription factors [26, 68, 69]. PPARα and PPARγ are expressed both in T and in B cells [70–73], where they function as negative regulators of transcription factors crucial for T cell activation, including NF-κB, AP-1 and STAT [68, 71, 74–77]. Furthermore, agonist-bound PPARγ interacts with nuclear factor of activated T cells (NF-AT), resulting in defective binding of this transcription factor to target promoter sequences [78]. Of note, although present in resting cells, PPARγ is transcriptionally upregulated in response to mitogenic stimuli [70], suggesting that PPARγ might function at a late stage of T cell activation as part of a regulatory feedback loop. This possibility is supported by the finding that PPARα target genes can be activated by PPAR agonists only in the presence of histone deacetylase inhibitors [71], suggesting gene reactivation by chromatin remodelling. Furthermore, PGE_2 production by COX appears to precede the production of cyclopentenone PGs, implicating PGE_2 receptors and PPARs in temporally distinct phases of T cell activation.

The profound impact of PPARs on lymphocyte activation explains at least in part the immunosuppressive activities of NSAIDs. In fact, notwithstanding their capacity to block PG production, and thereby potentially relieve the block in lymphocyte activation by PGE_2, NSAIDs strongly suppress lymphocyte activation in vitro. Recent data show that both nonselective and isoform-selective NSAIDs bind PPARs and as such modulate their transcriptional activity. Specifically, NSAIDs function as coactivators of both PPARα and PPARγ [25–27], thereby mimicking the activity of cyclopentenone PGs. Hence, NSAID-mediated immunosuppression is likely to be partly accounted for by their activity on PPARs. Nevertheless, data suggest that inhibition of COXs by NSAIDs might also be implicated. PLA_2 has been shown to function as a key regulator of lymphocyte proliferation [79], suggesting a role for AA and its metabolites in this process. COX-1 is constitutively expressed in lymphocytes, and the gene encoding COX-2 is inducibly expressed following TCR engagement, identifying it as an early response gene potentially implicated in the program of T cell activation [80]. In support of this possibility, NSAID-dependent inhibition of NF-AT can be partly recovered by PGE_2 [81]. COX-2 gene expression is strictly dependent on p38 MAP kinase, which participates both in transcriptional regulation of COX-2 gene expression and in posttranscriptional stabilization of COX-2 messenger RNA (mRNA) [82, 83]. p38 MAP kinase is also implicated in a complex serine-threonine kinase network triggered through a tyrosine kinase-dependent pathway by antigen receptor engagement and resulting in activation of gene expression, as well as rearrangement of cortical actin [84, 85] (fig. 3). COX-1 inhibitors completely and selectively block the activation of p38 MAP kinase induced both by T cell and B cell antigen receptor engagement [81]. This effect appears to be COX dependent, as activation of p38 MAP kinase can be elicited by PGE_2. Furthermore, a selective COX-1 inhibitor blocks COX-2 expression, an effect which can be reversed by exogenous PGE_2 [81]. These data suggest that COX-1 participates in the T cell and B cell antigen receptor signalling cascades leading to p38 MAP kinase activation, a process which will eventually promote activation of COX-2 gene expression (fig. 3). Further work is needed to specifically track the contributions of COXs and PPARs in the physiological process of lymphocyte activation. In this respect, the existence of mice deficient in either COX isoform, as well as of PPARα and PPARγ, will provide a powerful tool to understand to what extent COXs and PPARs contribute to the immunosuppressive activities of NSAIDs.

NSAIDs and T cell polarization

In addition to their role in lymphocyte activation, COX enzymes have been implicated in the shaping of immune responses by affecting T cell polarization. The distinction of helper T cells as Th1 or Th2 is based on the specific pattern of cytokines produced by these cells, and the polarization of T helper cells towards either phenotype is dictated by the cytokines present in the microenvironment (reviewed in [86]). PGs have been found to profoundly affect T helper cell polarization. PGE_2 specifically suppresses the expression of Th1-related cytokines (IL-12, IFNγ) while not affecting the expression of Th2-related cytokines (IL-4, IL-10) [87, 88], an activity which has been proposed to result from inhibition of IL-2 gene expression by PGE_2 since exogenous IL-2 can overcome the effects of PGE_2 [89]. In addition to its direct effect on T cells, PGE_2 also antagonizes the expression of cytokines controlling Th1 polarization in B cells, macrophages and dendritic cells, while promoting expression of cytokines controlling Th2 polarization [90-92], thereby also contributing indirectly to development of Th2 cells. More recently, PGD_2 has been suggested as primarily re-

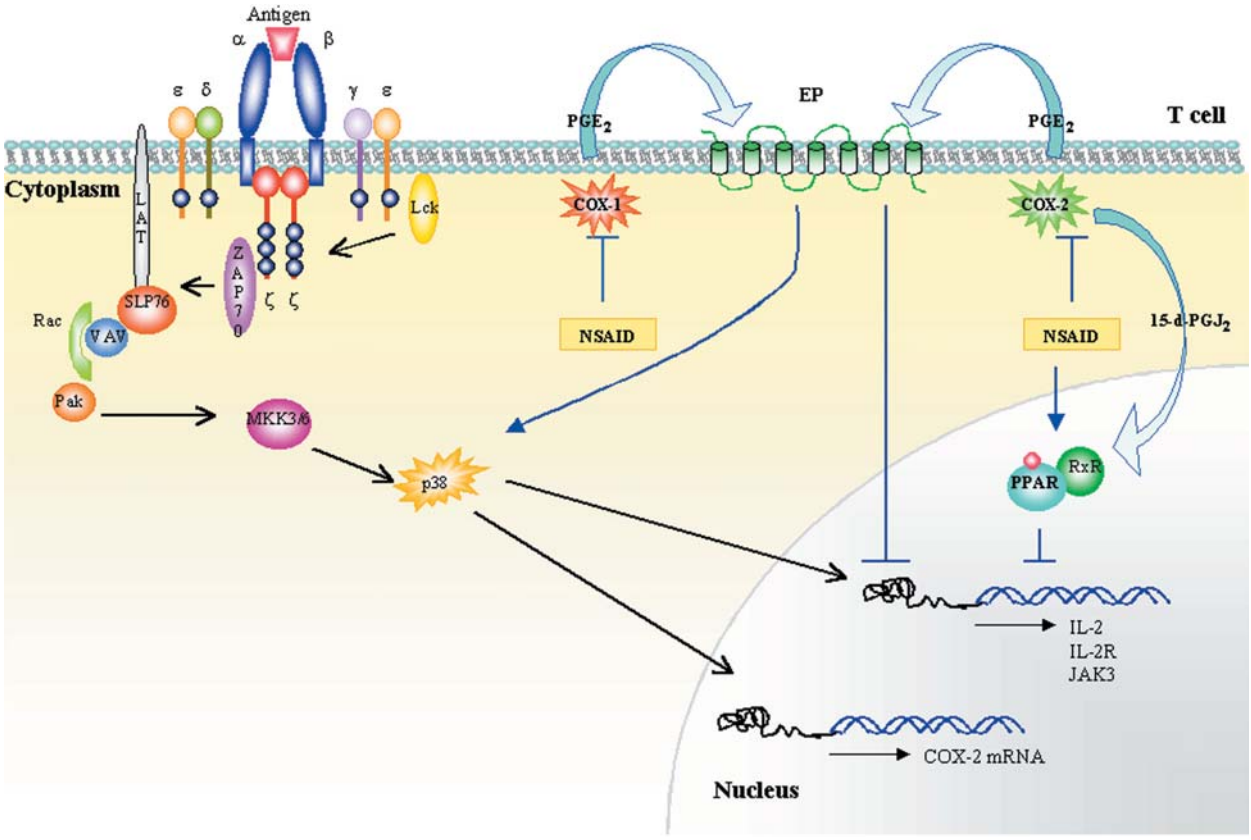

Figure 3. Targets of NSAIDs in T-cell activation. The T-cell antigen receptor and the EP (PGE_2) receptor at the T cell surface are schematized. The TCR is shown as an $\alpha\beta$ dimer, associated with the $\gamma\varepsilon$, $\delta\varepsilon$ and $\zeta\zeta$ dimers of the CD3 complex. The phosphorylated tyrosine-based activation motifs (ITAM) of the CD3 complex subunits are shown as grey circles. The Src kinase Lck is believed to be principally responsible for their phosphorylation. Bound to the ζ chain ITAMs is the tyrosine kinase ZAP-70, which in turn forms a complex with a number of proteins (LAT, SLP-76, Vav, Rac, Pak) implicated in the activation of the MKK3/6-p38 stress kinase pathway. Activated (phosphorylated) p38 translocates to the nucleus, where it participates in the transcriptional activation of genes crucially required for T cell activation (such as IL-2), as well as of the gene encoding COX-2. NSAIDs block TCR-dependent p38 activation and thereby contribute to immunosuppression. This activity of NSAIDs depends on their inhibitory activity on COX, as p38 can be activated by exogenous PGE_2. In addition, NSAIDs bind and activate PPARs, which antagonize the activity of key transcription factors responsible for T cell activation (IL-2, IL-2R, JAK3).

sponsible for Th2 polarization. PGD synthase is preferentially expressed in Th2 cells, and PGD synthase-producing $CD4^+$ T cells harbor a Th2 profile of cytokine expression [93]. Furthermore, overexpression of a PGD synthase transgene in mouse results in the selective development of Th2 cells [94].

Surprisingly, as with lymphocyte activation, also in this case the effects of NSAIDs overlap rather than antagonize the activities of PGE_2, as would be expected from their activity as COX inhibitors, suggesting an alternative mechanism of inhibition potentially involving PPARs. Indeed, NSAIDs antagonize Th1 cell development by blocking IL-12 expression both by T cells and by antigen-presenting cells [41, 95]. Expression of IL-4, which promotes Th2 cell development, was enhanced by indomethacin in concanavalin A-stimulated murine splenocytes [96]. Moreover, increased levels of the Th2-related cytokines IL-5 and IL-13 were increased in vivo in indomethacin-treated mice [37]. On the other hand, in a mouse model of *Leishmania* infection, characterized by the selective development of Th2 cells, in vivo administration of indomethacin results in upregulation of IL-12 expression and Th1 cell development, as well as increased resistance to infection [97, 98]. Furthermore, aspirin, as well as the related weak COX inhibitor salicylic acid, have been reported to inhibit IL-4 gene expression [39]. Although further studies are required to clarify this issue, the data suggest the challenging possibility that NSAIDs might be used as therapeutics in diseases characterized by an altered Th1/Th2 balance.

PGs are also likely to be implicated in Th2 cell chemotaxis, thereby contributing in the recruitment of these cells to the sites of inflammation. Th2 cells preferentially express the PGD_2 receptor CRTH2, which is also found in basophils and eosinophils, and PGD_2 induces chemotaxis of these cells through this receptor [99]. CRTH2-mediated chemotaxis is also induced by indomethacin; however, other NSAIDs do not elicit the same effect, sug-

gesting that COX-dependent production of PGD_2 is not responsible for this activity of indomethacin [38].

NSAIDs and lymphocyte apoptosis

The role of COX enzymes, and particularly of COX-2, in cell apoptosis, and the effects of NSAIDs on this process, have been principally established in colon carcinoma cells. As opposed to their normal counterparts, these cells constitutively express high levels of COX-2. A causal relationship between COX-2 expression and failure of cancer cells to undergo apoptosis has been established. Remarkably, NSAIDs not only induce apoptosis of colon carcinoma cells in vitro, but also reduce colonic adenomas in vivo. The apoptogenic activity of NSAIDs, which has been found to extend to other cancers, has been correlated to their capacity to stimulate the expression of proapoptotic genes while blocking the pathways controlling proliferation and survival (reviewed in [5]).

PGE_2 has been reported both to promote and suppress apoptosis of lymphoid cells in vitro [100–103]. Differences in the specific stage of maturation, in the state of activation or in neoplastic vs. normal phenotype of T and B cells have been suggested to underlie this dual activity of PGE_2. For example, PGE_2 suppresses apoptosis in $CD4^+CD8^+$ thymocytes, while promoting apoptosis of isolated $CD4^-CD8^-$ thymocytes in vitro [48, 104]. Both the proapoptotic and antiapoptotic activities of PGE_2 have been correlated with a modulation of cAMP levels and the expression of antiapoptotic or proapoptotic genes, such as Bcl-2 family proteins and Fas ligand [56, 103, 105, 106], thereby implicating adenyl cyclase-coupled PGE_2 receptors in lymphocyte apoptosis.

As opposed to the opposite effects of PGE_2 on apoptosis, cyclopentenone PGs have been consistently shown to promote apoptosis of both T and B cells through their interaction with PPARs, although there is as yet no consensus whether the cellular targets of the apoptogenic activity of PPAR agonists are only neoplastic or also normal lymphocytes [73, 107–109]. As with PGE_2, the apoptogenic activity of PPAR agonists can be correlated to a change in the balance of apoptotic and antiapoptotic proteins or in downregulation of proteins implicated in cell survival, such as c-myc [110].

In agreement with their capacity to both inhibit COX activity and function as PPAR coactivators, NSAIDs promote apoptotic death both of normal and leukemic cells in vitro; however, transformed cells appear more sensitive to the apoptogenic activity of NSAIDs. For example, the COX-2 inhibitor NS-398 promotes apoptotic death of human T lymphotropic virus (HTLV-1) transformed cells, which are characterized by constitutive COX-2 expression, by downregulating the expression of Bcl-2 and Bcl-xL [111]. Sodium salicylate blocks spontaneous proliferation of splenocytes of HTLV-1 Tax transgenic mice [112]. Furthermore, aspirin has been reported to induce apoptosis of chronic lymphocytic leukemia B cells, albeit in a COX-independent fashion [113]. Of note, inhibition of NF-κB, a target both of COX and of PPAR, has been shown to promote apoptosis of B cells [114, 115], suggesting that the apoptogenic activity of NSAIDs might be largely dependent on their capacity to inhibit NF-κB. Whichever the mechanism, the data suggest that NSAIDs

Table 1. Summary of the activities of NSAIDs and prostanoids on lymphocytes.

	Thymocyte development	T/B cell activation	T cell polarization	T/B cell apoptosis
NSAIDs[a]				
nonselective[a]	↓ DN to DP [46]	↓ [80, 81, 116, 117]	↓ Th1/↑ Th2 [37, 41, 95, 96] ↑ Th1/↓ Th2 [39, 97, 98]	↑ [112, 113]
COX-1[b]	↓ DN to DP [46]	↓ [81]		
COX-2[c]	↓ SP CD4 [46]	↓ [80, 81]		↑ [111]
Prostanoids				
TXA_2	↑ neg. selection [47]	↑ [49]	nd	↑ [47]
PGE_2	↑ survival DP [48]	↑ [50–63]	↓ Th1/↑ Th2 [87–92]	↑ [100–102, 104] ↓ [48, 103, 106]
PGD_2	nd	nd	↑ Th2 [93, 94]	nd
PDB_2	nd	↑ [65]	nd	nd
15-d-PGJ_2/ PPAR ligands[d, e]	nd	↓ [66, 67, 70, 72, 76, 78]	nd	↑ [73, 107–110]

[a] NSAIDs used in the work cited in the references (in brackets).
[b] Aspirin, indomethacin, sulindac, piroxicam and the proprionic acid derivatives ibuprofen, naxoprofen, ketoprofen.
[c] Resveratrol, L-759,700.
[d] NS-398, celecoxib, L-745,337.
[e] PPAR ligands: -thiazolidinediones (PPARγ agonists: ciglitazone, troglitazone), -fibrates (PPARγ agonist: WY14,643; PPARα agonist GW7,647).
[e] The following nonselective NSAIDs have also been shown to be PPAR ligands: indomethacin, sulindac, ibuprofen, naxoprofen, fenoprofen, ibuprofen, flufenamic acid.

might be included in the design of novel strategies in the pharmacological treatment of lymphoproliferative disorders. The effects of NSAIDs and prostanoids on lymphocyte development, differentiation and death are summarized in table 1.

Conclusions

The successful combination of pharmacological and genetic approaches has answered a number of questions regarding the physiological and physiopathological functions of COXs and their products in lymphocyte biology, and has clarified to a large extent the mechanisms underlying the activities of NSAIDs in these cells; however, the picture is far from complete. Significant insight into the role of each COX isoform, as well as of the individual membrane PG receptors, is expected to be gained from the analysis of lymphocyte maturation, differentiation and death in the existing knockout mice. Furthermore, significant progress in the functional characterization of the different PGs, and particularly the identification of PPARs as nuclear receptors of cyclopentenone PGs and NSAIDs, has set the prioritary goal of understanding the specific contribution of membrane PG receptors and PPARs to the biological effects of NSAIDs. Clarifying these crucial issues is likely to result in the design of NSAID-based therapeutical strategies based on the immunosuppressive or apoptogenic activities of these drugs for the treatment of inflammatory pathologies initiated or sustained by T lymphocytes, such as rheumatoid arthritis, inflammatory demyelinating diseases and asthma, as well as lymphoproliferative diseases.

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