Immunomodulatory impact of the A_{2A} adenosine receptor on the profile of chemokines produced by neutrophils

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Abstract

In LPS-stimulated human neutrophils, engagement of the adenosine A_{2A} receptor selectively prevented the expression and release of TNF- α , MIP-1 α /CCL3, MIP-1 β /CCL4, MIP-2 α /CXCL2, and MIP-3 α /CCL20. In mice lacking the A_{2A} receptor, granulocytes that migrated into the air pouch 4 h after LPS injection expressed higher mRNA levels of TNF- α , MIP-1 α , and MIP-1 β than PMNs from wild-type mice. In mononuclear cells present in the air pouch 72 h after LPS injection, expression of IL-1 β , TNF- α , IL-6, and MCP-2/CCL6 was higher in A_{2A}R knockout mice. In addition to highlighting neutrophils as an early and pivotal target for mediating adenosine anti-inflammatory activities, these results identify TNF- α and the MIP chemokine family as gene products whose expression is pivotally affected by activation of A_{2A}R in LPS-activated PMNs. Modulation by A_{2A}R in the production of inflammatory signals by PMNs may thus influence the evolution of an inflammatory response by reducing the activation status of inflammatory cells.

Keywords

polymorphonuclear leukocytes; experimental animal models; resolution of inflammation

Polymorphonuclear neutrophils (PMNs) are the most abundant circulating leukocytes and are usually the first to migrate toward damaged/infected tissues where they accumulate to participate in initial phases of the inflammatory response (1). Because of their early influence the progression of inflammatory and immune reactions. In response to specific stimuli, PMNs can synthesize and release an array of soluble mediators, including the eicosanoids, leukotriene B₄, thromboxane A₂, and prostaglandin E₂, as well as a number of cytokines/chemokines, such as tumor necrosis factor (TNF)- α , IL-1 β , IL-8/CXCL8, and macrophage inflammatory peptides (MIPs) (2–9).

Each of these mediators can influence host responses in distinct ways. In particular, chemokines produced by stimulated PMNs, such as IL-8/CXCL8, which specifically attracts and stimulates PMNs, and members of the MIP family, which regulate a wide range of

mononuclear cell functions, including chemotaxis, degranulation, phagocytosis, and inflammatory mediator synthesis (10), contribute to the onset and progression of inflammation, in part through recruitment and/or activation of a range of cell types (11–14).

Although frequent and severe infections seen in patients with defects in PMN function confirm their importance in host defense against infection (15), PMNs also have an enormous destructive capacity and can elicit significant tissue damage; their unchecked activation have been associated with disease states such as ischemia, gram-negative bacterial sepsis and rheumatoid arthritis (16). Elevated numbers of neutrophils in the synovial fluid of patients with arthritis support a role for these cells in joint destruction (17). Unraveling endogenous mechanisms that regulate and specifically limit PMN activation is, in turn, of interest in the context of identifying new and better therapeutic targets in the treatment of inflammatory diseases associated with unrepressed PMN activation.

Adenosine (Ado) is an extracellular messenger with a large spectrum of biological activities, including a protective role in acute inflammation and the promotion of wound healing (18). This autacoid, whose formation is increased under unfavorable conditions such as sepsis, inflammation, and hypoxia (19), can be found at high concentrations in traumatized tissues and mediates potent modulatory effects on immune cells (20). The A_{2A} Ado receptor ($A_{2A}R$) takes part in a nonredundant physiological negative feedback mechanism that limits and terminates both tissue-specific and systemic inflammatory responses. No other mechanism of down-regulation of inflammation *in vivo* appears able to fully compensate a lack of $A_{2A}R$, as was clearly demonstrated in mice deficient in the $A_{2A}R$, which developed extensive tissue damage in response to subthreshold concentrations of inflammatory stimuli (21). Accumulating evidence indicates that PMNs constitute an early and important target for the modulatory activities of Ado through activation of the $A_{2A}R$. In PMNs, $A_{2A}R$ engagement inhibits their adhesion to endothelial cells, phagocytosis, and generation of superoxide anions (22). $A_{2A}R$ also causes a shift in the profile of lipid mediators generated by PMNs by blocking, on one hand, arachidonic acid release, 5-lipoxygenase activation, leukotriene and platelet-activating factor biosynthesis (23), and by potentiating, on the other hand, the expression of the inducible isoform cyclooxygenase (COX-2), as well as the capacity of PMNs to generate PGE_2 from exogenous arachidonic acid sources (12).

Although PMNs appear to be particularly responsive to $A_{2A}R$ activation, the impact of Ado on the generation of chemokines by PMNs is largely undocumented (24). In view of unrestrained inflammatory responses and elevated serum cytokines levels observed in $A_{2A}R$ knockout mice (21), we hypothesized in the present study that unavailability of this crucial modulatory pathway may affect the profile of PMN-derived chemokines. We investigated the effect of Ado and of $A_{2A}R$ activation on the profile of chemokines and compared this with that of other cytokines produced by PMNs stimulated through LPS treatment (a toll-like receptor ligand) (24), both *in vitro* and *in vivo*. We report here that engagement of $A_{2A}R$ prevents the generation of selected chemokines, an event likely to mediate at least in part, anti-inflammatory activities resulting of $A_{2A}R$ engagement.

MATERIALS AND METHODS

Materials

Adenosine deaminase (ADA) was purchased from Roche Applied Science (Indianapolis, IN). CGS 21680 was from Research Biochemicals International (Natick, MA). Lipopolysaccharide (LPS; from *E. coli* 0111; B4), was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). DFP (diisopropylfluorophosphate) was from Serva Electrophoresis (Heidelberg, Germany). Leupeptin and aprotinin were obtained from ICN Biomedicals (Irvin, CA).

Human leukocyte isolation

PMNs were isolated as originally described (25) with modifications (12). Briefly, venous blood collected on isocitrate anticoagulant solution from healthy volunteers was centrifuged (250 *g*, 10 min), and the resulting platelet-rich plasma was discarded. Leukocytes were obtained following erythrocytes sedimentation in 2% Dextran T-500 (Amersham Biosciences, Piscataway, NJ). PMNs were then separated from other leukocytes by centrifugation on a cushion of 10 ml lymphocyte separation medium (Wisent, St-Bruno, Quebec, Canada). Contaminating erythrocytes were removed by a 15-s hypotonic lysis, and purified granulocytes (>95% PMNs, <5% eosinophils) contained fewer than 0.2% monocytes, as determined by esterase staining. Viability was greater than 98%, as determined by trypan blue dye exclusion. The whole cell isolation procedure was carried out in a sterile environment at room temperature (RT).

Cell incubations

PMNs were resuspended in 1.5 ml Eppendorf tubes at a concentration of 5×10^6 cells/ml (25×10^6 cells/ml in experiments where RNA was to be extracted) in Hank's balanced salt solution (HBSS; 37°C) containing 10 mM HEPES pH 7.4, 1.6 mM Ca^{2+} and no Mg^{2+} and containing the following antiprotease cocktail: 0.2 µg/ml diisopropylfluorophosphate (DFP), 10 µg/ml leupeptin, 10 µg/ml aprotinin. Where mentioned, adenosine deaminase (ADA; 0.1 U/ml) was added to cell suspensions 20 min before stimulation with LPS. CGS 21680 (1 µM final concentration) was dissolved in DMSO and added to cell suspensions 10 min before stimulation. The final organic solvent concentration never exceeded 0.1% (v/v).

RNA isolation

PMN total RNA was isolated using Trizol (Gibco, Burlington, VT), according to the manufacturer's protocol, with modifications. Briefly, 25×10^6 PMNs were homogenized in 1 ml Trizol and 200 µl of chloroform were added. After mixing, samples were centrifuged at 10 000 *g* for 15 min (4°C). The upper aqueous phase was transferred in a tube containing an equal volume of isopropanol. Mixtures were thoroughly vortexed and centrifuged at 12 000 *g* for 10 min (4°C). Supernatants were discarded and the precipitated RNA pellets were washed twice using 1 ml of 75% ethanol. RNA pellets were centrifuged at 12 000 *g* for 5 min (RT). After discarding supernatants, pellets were allowed to air-dry for 2–3 min, then resuspended in DEPC-treated water. RNA was quantitated by UV absorbance at 260 nm.

Northern blot analysis

Total PMN RNA (5 µg/well) was migrated on an agarose/formaldehyde gel, and transferred onto a nylon filter (Hybond-XL), using a Vacugene XL transfer apparatus (Amersham Pharmacia Biotech, Baie d'Urfé, Qc., Canada). RNA was fixed using a UV-crosslinker, according to the manufacturer's specifications (Amersham Pharmacia Biotech). Integrity of the RNA and equal loading were assessed by hybridization of the filters with GAPDH. The COX-2 cDNA probe was generated by reverse transcriptase-polymerase chain reaction. The primers used to generate human COX-2 cDNA probe were: 5'-GCT GAC TAT GGC TAC AAA AGC TGG-3' (forward) and 5'-ATG CTC AGG GAC TTG AGG AGG GTA-3' (reverse). Primers used to generate human MIP-1α/CCL3 cDNA probe were: 5'-ATG CAG GTC TCC ACT GCT G-3' (forward) and 5'-AGC AAG TGG TGC AGA GAG GA-3' (reverse). Primers used to generate human MIP-1β/CCL4 probe were: 5'-AAG CTC TGC GTG ACT GTC CT-3' (forward) and 5'-CAG GAC TCA CTG GGA TCA GC-3' (reverse). Sequence homology between MIP-1α and MIP-1β probes did not exceed 60%. Filters were hybridized with appropriate cDNA probes labeled with [α -³²P] dCTP using Neblot Kit (New England Bio Labs, Beverly, MA). Bands were revealed and analyzed with a BAS-1800 bioimaging analyzer (Fuji Medical Systems, Stamford, CT).

Real-time PCR and cDNA reverse-transcription

First-strand cDNA synthesis was performed using 1 µg of total RNA with Superscript II (Invitrogen Life Technology, Carlsbad, CA) in manufacturer-recommended conditions, using 500 ng of random hexamers. Amplification of PMN cDNA was carried out in a Rotor-Gene 3000 operated with Rotor Gene software version 6.0.19 (Corbett Research, Mortlake, New South Wales, Australia). Each sample consisted of: 1 µg cDNA, 1.3 mM MgCl₂, 0.2 mM dNTP, 500 nM of primers, 0.5 unit of Taq polymerase (Amersham Biosciences) and SYBR Green dye (Molecular Probe, Eugene, OR; 1:30 000 dilution) in a reaction volume of 20 µl. Amplification conditions were as follows: 95°C (20 s), 58°C (20 s), 72°C (20 s); 35 repetitions. Specificity of each reaction was ascertained by performing the Melt procedure (58–99°C; 1°C/5s) after completion of the amplification protocol, according to the manufacturer's instructions. Human and mouse primers used in real-time PCR procedures are listed in Table 1.

Determination of secreted cytokines/chemokines by ELISA

PMN suspensions were centrifuged briefly and cell-free supernatants were conserved at –20°C, until analysis for their content in cytokines/chemokines using commercially available specific ELISA kits, according to manufacturers' instructions. ELISAs for human IL-1β, IL-8/CXCL8, and TNF-α were from BioSource (Camarillo, CA). ELISAs for human MIP-1α/CCL3, MIP-1β/CCL4, MIP-2α/CXCL2 and MIP-3α/CCL20 were from R&D Systems (Minneapolis, MN).

Mouse A_{2A}R knockout

Couples of A_{2A}R heterozygotes (A_{2A}R^{+/-}) CD1 mice were bred; offsprings were genotyped in order to select A_{2A}R^{-/-} and A_{2A}R^{+/+} animals. Aggressiveness, hypoalgesia, and high blood pressure were reported in A_{2A}R^{-/-} mice; they otherwise appeared normal and bred

normally (26). Genotyping was performed as follows. Tail ends (0.5–1 cm) were cut from anesthetized animals and immersed in 500- μ l tail buffer (40 mM Tris, 200 mM NaCl, 20 mM EDTA, and 0.5% SDS) supplemented with 20 μ l of a 15 μ g/ μ l proteinase K preparation (Invitrogen Life Technology) and 2.5 μ l β -mercaptoethanol, and incubated overnight at 55°C. Saturated NaCl (250 μ l) was added to samples and incubated on ice for 15 min, then centrifuged for 60 min at 12,000 *g*. Supernatants were transferred in fresh tubes, and 750 μ l ethanol were added. After mixing, tubes were centrifuged for 4 min at 6,000 *g*, supernatants were discarded and pellets were washed with 70% ethanol then centrifuged again (5 min, 12 000 *g*). Air-dried pellets were dissolved in 100 μ l TE. PCR was performed using these conditions: an initial step of 3 min at 94°C; then 45 s at 94°C; 45 s at 58°C; 45 s at 72°C, 35 repetitions, followed by 5 min at 72°C. Primers used to detect endogenous A_{2A}R were: 5'-TGG GTA ACG TGC TTG TGT GCT-3' (forward) and 5'-AAC CAG GCT ACT CTT TTC CAT-3' (reverse). To detect knocked-out A_{2A}R gene, primers for internal Neo cassette were used: 5'-AGA GGC TAT TCG GCT ATG ACT G-3' (forward) and 5'-TTC GTC CAG ATC ATC CTG ATC-3' (reverse). Presence of the native A_{2A}R generated a PCR product of 467 bp, while that elicited from the Neo cassette was of 433 bp.

Dorsal air pouches

All experimental designs involving animals have been approved by the institution's Committee for Animal Protection. Air pouches were raised on the dorsum by subcutaneous injection of 5 ml of sterile air on day 0 and 3 ml on day 3. Experiments were conducted on day 6. Individual air pouches (one per mouse) were injected either with vehicle alone (1 ml endotoxin-free PBS) or containing 500 ng LPS. At indicated times, mice were sacrificed and individual air pouches were lavaged two times with ice-cold PBS (total of 2 ml). Leukocyte suspensions were enumerated with an automated cell counter (Model ZM, Coulter Electronics, Luton Beds, England). Identification of leukocyte subtypes was assessed by Giemsa staining.

Statistical analysis

Where applicable, statistical analysis was performed by Student's nonpaired *t* test (two-tailed), and significance (*) was considered to be attained when $P < 0.05$.

RESULTS

Dose-response and time-course experiments were first performed to obtain optimal conditions for modulation of chemokine mRNA in LPS-stimulated PMNs, as determined by Northern blot analysis (Fig. 1A–C). The result of these experiments indicated 0.1 μ g/ml of LPS for 60 min in the presence of 1.0% FCS to be optimal. Human PMNs were stimulated under these conditions with either LPS alone or in combination with adenosine deaminase (ADA; 0.1 U/ml), a condition preventing accumulation of endogenous Ado in cell suspensions (27) or with the stable and specific A_{2A}R agonist, CGS 21680 (1 μ M). Under these conditions, LPS caused an increase of MIP-1 α /CCL3 and MIP-1 β /CCL4 mRNA levels and elimination of extracellular Ado accumulation elicited a superinduction of mRNA for these two chemokines (Fig. 1D). In contrast, A_{2A}R activation with CGS 21680 markedly reduced their expression. COX-2 mRNA expression, which is potentiated with A_{2A}R

activation in PMNs, was used as an internal control for these experiments (12). Together, these results indicate that A_{2A}R activation can alter the profile of chemokines produced by PMNs.

Real-time PCR has been successfully used in our laboratory for the evaluation of mRNA levels (27). This approach was used in the present study to evaluate the impact of A_{2A}R activation on mRNA levels of a comprehensive selection of cytokines and chemokines (presented in Table 1). COX-2 mRNA levels were also assessed as a further control (27). Confirming results obtained by Northern blotting procedure, LPS-elevated COX-2 mRNA levels (Fig. 2); withdrawal of extracellular Ado decreased these levels approximately by half, while A_{2A}R activation largely reestablished the induction. mRNA levels of COX-1 and GAPDH remained essentially constant. The level of mRNA for the inflammatory cytokines IL-1 β and TNF- α mRNA were also increased after stimulation with LPS (Fig. 3A). The presence of ADA amplified the increase, whereas A_{2A}R activation significantly inhibited it, suggesting that Ado, through engagement of A_{2A}R, has a modulatory effect on the production of these important cytokines. Interestingly, the expression of the IL-1 receptor antagonist (IL-1RA), followed a similar profile to that of IL-1 β . Several other cytokines, including IL-4, IL-6, IL-10, IL-13, MCP-2, MCP-3, and IFN- γ , were assessed, but their mRNA were consistently not detected in human PMNs. ADA, and CGS 21680 were found not to affect the expression levels of any of the tested genes in unstimulated PMNs (data not shown).

Using real-time PCR, chemokine mRNAs that were detected in PMNs after exposure to LPS were, in decreasing order of abundance, IL-8/CXCL8, MIP-1 β /CCL4, MIP-1 α /CCL3, MIP-2 α /CXCL2, MIP-3 α /CCL20, and MIP-3 β /CCL19 (Table 1). IL-8 mRNA was by far the most abundant; however, its expression was only slightly increased after exposure of the cells to LPS, and A_{2A}R activation also had no detectable impact (Fig. 3B). In sharp contrast, the mRNA expression of chemokines MIP-1 α /CCL3, MIP-1 β /CCL4, MIP-2 α /CXCL2, and MIP-3 α /CCL20 each increased after LPS stimulation; elimination of Ado in the extracellular milieu amplified their expression, while A_{2A}R activation had a significant counterregulatory effect. The relative impact of A_{2A}R activation on mRNA species generated in LPS-stimulated PMNs, as determined by real-time PCR, is summarized in Fig. 3C. Together, these results confirmed the validity of the real-time PCR approach for the comparison of mRNA levels; they indicate also that Ado, via A_{2A}R activation, has a potent modulatory impact on the mRNA levels of IL-1 β , TNF- α , MIP-1 α /CCL3, MIP-1 β /CCL4, MIP-2 α /CXCL2, and MIP-3 α /CCL20 in LPS-stimulated PMNs, while not affecting IL-8/CXCL8 mRNA levels.

Cytokine production can be regulated at several levels: transcription, mRNA stabilization, translation, storage, and secretion. At this latter level, stimulation of PMNs with LPS provoked the release of TNF- α , MIP-1 α /CCL3, MIP-1 β /CCL4, MIP-2 α /CXCL2, MIP-3 α /CCL20, and IL-8/CXCL8 (Fig. 4A–G). TNF- α was secreted earliest and was detected in supernatants as early as 2 h after exposure of the cells to LPS; extracellular levels of TNF- α reached a peak at 8 hours. Accumulation of MIPs followed, all with comparable kinetics. Withdrawal of Ado during stimulation enhanced the release of TNF- α , MIP-1 α /CCL3, MIP-1 β /CCL4, MIP-2 α /CXCL2, and, to a lesser extent, MIP-3 α /CCL20. On the other hand,

activation of the A_{2A}R with CGS 21680 inhibited TNF- α secretion, which was comparable to levels found in the supernatants of saline-treated PMNs. A_{2A}R activation also had a potent inhibitory impact on the release of MIP-1 α /CCL3, MIP-1 β /CCL4, MIP-2 α /CXCL2, and MIP-3 α /CCL20; inhibitions ranged from 50% to 90% (Fig. 4H). Secretion of IL-8/CXCL8 was delayed in comparison with the other inflammatory mediators, but IL-8/CXCL8, nevertheless, was the most abundant chemokine released from PMNs. Elimination of extracellular Ado or activation of the A_{2A}R had no significant impact on the secretion of IL-8/CXCL8, at any of the time points tested. In each of the conditions tested, secretion of IL-1 β was barely detectable.

It is plausible to consider that early secretion of TNF- α contributes to the subsequent generation of MIPs and IL-8/CXCL8 by LPS-stimulated PMNs. In this context, the inhibitory impact of A_{2A}R activation on MIP expression might have been a downstream consequence of its impact on TNF- α . However, in experiments where PMNs were stimulated with LPS and ADA, in the presence or not of effective concentrations of neutralizing anti-TNF- α antibodies, expression of MIPs were not affected (data not shown), a result which does not support a significant autocrine role for PMN-derived TNF- α in up-regulating MIP mRNA levels.

Results concerning the impact of A_{2A}R activation on secretion of TNF- α , IL-8/CXCL8, and MIPs by LPS-stimulated PMNs correlate well with those obtained for mRNA levels and assessed by real-time PCR; they also identify TNF- α and MIPs as gene products whose expression is pivotally regulated by A_{2A}R in LPS-activated human PMNs. In view of the potentially important consequences that modulation of the production of TNF- α and MIPs in inflammatory PMNs might have on immune activation, *in vivo* experiments were undertaken in order to address this issue.

The impact of A_{2A}R activation on the production of chemokines by PMNs *in vivo* was assessed with the murine air pouch model of inflammation and leukocyte recruitment (28), using wild-type (A_{2A}R^{+/+}) and knockout (A_{2A}R^{-/-}) CD1 mice (26). Dorsal air pouches were injected with LPS for 4 h, which increased the recruitment of leukocytes, predominantly PMNs; >75% as determined by Giemsa staining. Although females had a tendency to bear higher number of migrated leukocytes in the air pouch (Fig. 5A, insert), no significant difference was observed between A_{2A}R^{+/+} and A_{2A}R^{-/-} animals with respect to the number of recruited PMNs (Fig. 5A). mRNA expression of a selection of key inflammatory genes, cytokines and chemokines were compared in migrated leukocytes from A_{2A}R^{+/+} and A_{2A}R^{-/-} mice, by real-time PCR (Table 1). As presented in Fig. 5B, absence of a functional A_{2A}R was accompanied by a significant decrease in COX-2 mRNA expression, as observed previously (27), whereas that of TNF- α , MIP-1 α /CCL3, and MIP-1 β /CCL4 were increased, results are in line with data obtained *in vitro* with human PMNs. No significant difference between A_{2A}R^{+/+} and A_{2A}R^{-/-} mice was observed in lining tissues of the air cavity for any of the genes tested (data not shown). Secreted protein levels of TNF- α , MIP-1 α /CCL3, and MIP-1 β /CCL4 were assessed in cell-free exudates. As can be appreciated in Fig. 5C, concentrations of each of these inflammatory mediators were significantly increased in samples elicited from LPS-treated A_{2A}R^{-/-} animals, approximately twice that observed in wild-type mice. Basal levels (PBS-treated) of these inflammatory mediators were

comparable in $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice. These results strongly support a role in vivo for $A_{2A}R$ in regulating the expression of important inflammatory cytokines and chemokines from inflammatory PMNs, particularly that of TNF- α , MIP-1 α /CCL3, and MIP-1 β /CCL4.

As is the normal course of inflammation and wound-healing process, PMNs are the first cell type to migrate into the air pouch model after injection of LPS. Within days, mononuclear cells replace PMN and become the most numerous leukocytes present in the exudates (28, 29). We analyzed cell migration and gene expression at longer time points, up to 96 h after LPS injection. As early as 48 h after LPS injection, the vast majority of cells harvested from exudates was mononuclear cells, as assessed by Giemsa staining. As was the case at 4 h poststimulation, females had a tendency to bear higher cell counts than males (Fig. 6A, inset). Although with some degree of variation, total cell counts remained relatively stable during this time course (Fig. 6A). Real-time PCR comparison of mRNA levels (Table 1) in leukocytes collected from the air pouch from $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice revealed the increased expression of TNF- α , IL-6, MCP-2, IL-1 β and, to a lesser extent, IL-1RA (Fig. 6B). As observed at 4 h poststimulation, expression in lining tissues was not different for any of the analyzed genes (data not shown).

DISCUSSION

Immunomodulatory effects of Ado on PMNs are numerous. Via occupancy of $A_{2A}R$ on PMNs, Ado inhibits their adherence to endothelial cells, phagocytosis, the generation of superoxide anions, and production of TNF- α (22, 30). Moreover, $A_{2A}R$ activation inhibits arachidonic acid release, 5-lipoxygenase activation, leukotriene, and platelet-activating factor biosynthesis (23), whereas it potentiates the expression of the inducible isoform cyclooxygenase (COX-2) (12). Therefore, in addition to bringing further evidence to the concept that PMNs constitute an early cellular mediator for the anti-inflammatory activities of Ado, results presented herein identify MIP-1 α /CCL3, MIP-1 β /CCL4, and MIP-2 α /CXCL2 as being among the main chemokines to be modulated by $A_{2A}R$ activation.

Considerable evidence now indicates that $A_{2A}R$ engagement as profound inhibitory consequences on PMN functions; its impact on cell movement appears, however, marginal. Indeed, results obtained herein showed that adenosine does not significantly affect IL-8—the main chemokine produced by PMNs—be that at the expression or release levels. In a series of in vitro chemotaxis experiments in which fMLP, IL-8, and leukotriene B₄ were used as chemotactic agents, neither Ado nor $A_{2A}R$ activation affected PMNs movement toward these factors (M. Pouliot; unpublished observations). In the present study, the number of PMNs recruited in the air pouches was comparable in $A_{2A}R$ -knockout and wild-type mice, in line with a previous study that reported that, in carrageenan-injected air pouches, absence of a functional $A_{2A}R$ did not result in higher cell numbers (18). The number of mononuclear cells observed at longer time points was also similar between knockout and wild-type mice, with only slightly higher cell counts in knockout animals. Given the profound effect of $A_{2A}R$ engagement observed on PMN-derived chemokines, its lack of effect on cell migration appears surprising. However, an explanation could be related to the fact that, in the murine air pouch model of inflammation, resident cells—and not migrating leukocytes—have been shown to serve as the major mechanism for leukocyte recruitment during local

inflammation (28). Of interest, in our study, none of the genes assessed in lining tissues were found affected by the absence of a functional A_{2A}R. It is likely that the chemokines elicited from lining tissue cells, which are functionally linked with vasculature, reach in a more efficient fashion, the leukocytes circulating in venous capillaries, than chemokines produced in the cavity. Nonetheless, additional experiments will be required in order to fully elucidate this aspect.

Thus, Ado appears to exert its anti-inflammatory functions predominantly by modulating the profile of inflammatory mediators generated by leukocytes present at sites of injury rather than by affecting their numbers. Cytokines derived from PMNs differentially contribute to the regulation of the inflammatory response, in part through activation of distinct leukocytes subsets. IL-8/CXCL8, for example, is a potent agonist for PMNs but not for monocytes, while MIP-1 α selectively influences lymphocytes and monocytes. TNF- α , on the other hand, is a pleiotropic cytokine, which can affect most inflammatory cell types. An alteration in the production profile of TNF- α and MIPs by inflammatory PMNs is, in turn, likely to modify the host's response to infection. Along these lines, our *in vivo* observations made with A_{2A}R knockout mice further identified TNF- α and MIPs as prominent targets of A_{2A}R signaling in PMNs.

Inhibition of PMN-derived TNF- α and MIPs may, in turn, affect the activation status of mononuclear cells, which are attracted to the site of injury. In leukocytes elicited from murine air pouches 72 h after LPS injection, the profile of pivotal cytokine/chemokine genes expressed by mononuclear cells, particularly those of TNF- α , IL-6, MCP-2, and IL-1 β , were exacerbated in the absence of a functional A_{2A}R signaling pathway. Whether or not this situation is a direct consequence of the affected production profile of PMN-derived inflammatory soluble factors remains to be established. However, in view of the fact that gene expression from lining tissues was not affected in the absence of a functional A_{2A}R, results presented herein are consistent with the possibility that inflammatory PMNs can have an influence on the activation status of incoming mononuclear cells. The demonstrated role of A_{2A}R in down-regulation of inflammation and protection from tissue damage could thus include the modulation of PMN-derived chemokines, and the consequent modulation of mononuclear cell activation (21).

In summary, our results confirm that real-time PCR is an efficient and reliable approach for comparing mRNA expression in human PMNs and present further evidence that contribute to the identification of the neutrophil as an early and pivotal mediator of Ado's anti-inflammatory actions. Results presented also identify the MIP family of chemokines as being specifically regulated by A_{2A}R; alteration of the PMN-derived profile of inflammatory mediators may, in turn, modify that of mononuclear cells and, as a consequence, limit the amplitude of an inflammatory reaction. Modulation by A_{2A}R in the production of inflammatory signals by neutrophils may thus influence the evolution of an inflammatory response by reducing the activation status of inflammatory cells.

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Figure 1. LPS-activation of neutrophils stimulates mRNA expression of chemokines: impact of A_{2A}R engagement. **A–C)** Human polymorphonuclear neutrophils (PMNs) were stimulated with LPS at indicated concentrations and for indicated times; mRNA levels for MIP-1 α and β were determined by Northern blots using specific cDNA probes for each isoform, as described in the Materials and Methods section. **D)** PMNs were stimulated with 100 ng/ml LPS for 1h (1% FCS), alone or in the presence of ADA and CGS 21680. Membranes were also hybridized for the determination of GAPDH and COX-2 mRNA levels, as internal controls of sample integrity status and stimulatory status. For each panel, results shown are from one experiment, representative of 3 identical experiments performed with PMNs from different donors.

Figure 2.

Real-time PCR approach for the comparison of mRNA levels. PMNs were stimulated with 100 ng/ml LPS for 1 h (1% FCS), alone or in the presence of ADA and CGS 21680. Samples were processed for the determination of GAPDH, COX-1, and COX-2 mRNA expression by real-time PCR, as described in the Materials and Methods section. Shown are integrated results from $n = 3$ ($\pm$ SEM) separate experiments performed in identical conditions with different donors. *Statistically different from nontreated cells. **Statistically different from nontreated cells, from LPS-treated cells and from LPS+ADA+CGS 21680-treated cells.

Figure 3.

Real-time PCR-based assessment of the impact of $A_{2A}R$ activation on the mRNA expression of cytokines and chemokines in neutrophils. PMNs were stimulated with LPS for 1 h, alone or in the presence of ADA and CGS 21680. Samples were processed for the determination of mRNA expression by real-time PCR of (A) TNF- α , IL-1 β , and IL-1 β receptor antagonist (IL-1RA), and (B) MIP-1 α , MIP-1 β , MIP-2 α , MIP-3 α , and IL-8. In each panel, data show the relative expression of each indicated gene relative to that observed in unstimulated PMNs. Results are presented as the mean ($\pm$ SEM) of at least $n = 3$ experiments performed in identical conditions with different donors. (C) The impact of $A_{2A}R$ activation on the mRNA expression of indicated genes is summarized. For each gene of interest, mRNA expression from CGS 21680-incubated PMNs is compared with that observed in LPS+ADA-treated cells (mean $\pm$ SEM, $n=3$). *Statistically different from LPS+ADA-treated cells.

Figure 4.

A_{2A}R activation prevents the release of TNF-α and MIPs from neutrophils. **A–G)** PMNs were stimulated with LPS for up to 20 h, alone or in the presence of ADA and CGS 21680. Cell-free samples were processed for the determination of indicated cytokines/chemokines by specific ELISAs. In each panel, results represent the mean (±SEM) of at least $n=3$ experiments performed in identical conditions with different donors. **H)** The impact of A_{2A}R engagement on the release of the indicated soluble factors at $t=20$ h is summarized and expressed as percentage of inhibition (mean±SEM, $n=3$). *Statistically different from LPS +ADA-treated cells.

Figure 5.

Absence of a functional A_{2A}R exacerbates the expression of selected chemokines from neutrophils in vivo. **A**) Dorsal air pouches from wild type (+/+) or homozygote A_{2A}R knockout (-/-) CD1 mice were injected with PBS, alone or containing 500 ng LPS. Four hours after LPS injection, migrated leukocytes were collected, enumerated, and identified by Giemsa staining (>75% neutrophils). Results are expressed as the mean $\pm$ SEM, with values between brackets being the number (*n*) of animals for each experimental condition. **Inset:** Values obtained for females and males are presented separately, in order to show the relatively higher number of leukocytes harvested from females. *Statistically different from PBS-injected mice. **B**) Harvested leukocyte samples were equalized by cell numbers and processed for determination of mRNA expression of the genes list in Table 1, by real-time PCR. Genes for which statistically significant results were obtained are presented.

Expression in (-/-) mice is presented relative to that from (+/+) animals; results show the mean $\pm$ SEM from at least 15 animals by group. *Statistically different from (+/+) mice. **C)** Cell-free samples were processed for the determination of indicated cytokines/chemokines by specific ELISAs. Results show the mean $\pm$ SEM from at least 15 animals by group. *Statistically different from PBS-injected mice. **Statistically different from (+/+) mice.

Figure 6.

Absence of a functional $A_{2A}R$ exacerbates the expression of proinflammatory cytokines/chemokines from mononuclear leukocytes in vivo. **A)** Dorsal air pouches from wild-type (+/+) or homozygote knockout mice (-/-) CD1 were injected with PBS alone or containing 500 ng LPS. At the indicated time points, migrated leukocytes were collected, enumerated, and identified by Giemsa staining (essentially 100% mononuclear cells). Results are expressed as the mean $\pm$ SEM with values between brackets being the number (*n*) of animals for each experimental condition. *Inset:* Values obtained for females and males are presented separately, in order to show the relatively higher number of leukocytes harvested from females. **B)** Harvested leukocyte samples were equalized by cell numbers and processed for determination of mRNA expression of genes list in Table 1, by real-time PCR. In addition to COX-2, genes for which statistically significant results were obtained, are presented. Expression in (-/-) mice is presented relative to that from (+/+) animals; results show the mean $\pm$ SEM from at least 15 animals by group. *Statistically different from (+/+) mice.

Table 1

Human and mouse primer sequences for mRNA analysis by real-time PCR

Gene	CT	Primers (human)	CT	Primers (mouse)
COX-2	12	S: 5'-TGCATTCTTTGCCAGCACT-3'; AS: 5'-AAAGGCGCAGTTTACGCTGT-3'	15	S: 5'-GGCCATGGAGTGGACTTAAA-3'; AS: 5'-AAGTGGTAACCGCTCAGGTG-3'
GAPDH	15	S: 5'-CGAGATCCCTCCAAAATCAA-3'; AS: 5'-TTCACACCCATGACGAACAT-3'	14	S: 5'-AACTTTGGCATTGTAGAAGG-3'; AS: 5'-ACACATTGGGGTTAGGAACA-3'
COX-1	20	S: 5'-TGCCAGCTCCTGGCCGCGG-3'; AS: 5'-GTGCATCAACACAGGCGCCTCTTC-3'	20	S: 5'-CCTCCGACCTACAACCTCAGC-3'; AS: 5'-GCCTAAGGCCTTGGTAAAGC-3'
IL-8/CXCL8	8	S: 5'-GTGCAGTTTGGCAAGGAGT-3'; AS: 5'-CTCTGCACCCAGTTTCTTC-3'	na	*
IL-1 β	10	S: 5'-GGACAAGCTGAGGAAGATGC-3'; AS: 5'-TCGTTATCCCATGTGTCGAA-3'	11	S: 5'-TCACAGCAGCACATCAACA-3'; AS: 5'-TGTCCTCATCTCGGAAGGTC-3'
MIP-1 β /CCL4	12	S: 5'-AAGCTCTGCGTGACTGTCCT-3'; AS: 5'-GCTTGCTTCTTTTGGTTTG-3'	13	S: 5'-TGTCTGCCCTCTCTCTCCTC-3'; AS: 5'-GAGCAAGGACGCTTCTCAGT-3'
IL-1RA	14	S: 5'-GCGAGAACAGAAAGCAGGAC-3'; AS: 5'-CCTTCGTCAGGCATATTGGT-3'	10	S: 5'-TTGTGCCAAGTCTGGAGATG-3'; AS: 5'-TTCTCAGAGCGGATGAAGGT-3'
TNF- α	18	S: 5'-AGCCCATGTTGTAGCAAACC-3'; AS: 5'-TGAGGTACAGGCCCTCTGAT-3'	14	S: 5'-TAGCCAGGAGGGAGAACAGA-3'; AS: 5'-TTTCTGGAGGGAGATGTGG-3'
MIP-2 α /CXCL2	18	S: 5'-TGCAGGGAATTCACCTCAAG-3'; AS: 5'-TGAGACAAGCTTCTGCCCA-3'	14	#S: 5'-AGTGAAGTGCCTGTCAATG-3'; AS: 5'-TTAGCCTTGCCCTTGTTCAG-3'
MIP-1 α /CCL3	18	S: 5'-CTCTGCAACCAGGTCTCTC-3'; AS: 5'-TTTCTGGACCCACTCCTCAC-3'	13	S: 5'-ACCATGACACTCTGCAACCA-3'; AS: 5'-CCCAGGTCTCTTTGGAGTCA-3'
MIP-3 α /CCL20	19	S: 5'-GCAAGCACTTTGACTGCTG-3'; AS: 5'-ATTTGCGCACACAGACAAC-3'	20	S: 5'-CGACTGTTGCCTCTCGTACA-3'; AS: 5'-AGGAGGTTACAGCCCTTTT-3'
MCP-1/CCL2	23	S: 5'-AGCAGCAAGTGTCCAAAGA-3'; AS: 5'-TTGGGTTTGCTTGTCAGGT-3'	na	
MIP-3 β /CCL19	23	S: 5'-GGTGCCTGCTGTAGTGTTC-3'; AS: 5'-GCTTCATCTTGGCTGAGGTC-3'	nd	S: 5'-AGACTGCTGCCTGTCTGTGA-3'; AS: 5'-GCCTTTGTTCTTGGCAGAAG-3'
IL-4	nd	S: 5'-ACTGCACAGCAGTTCACAG-3'; AS: 5'-GTTGGCTTCCCTCACAGGAC-3'	nd	S: 5'-TCAACCCCCAGCTAGTTGTC-3'; AS: 5'-TGTTCTTCGTTGCTGTGAGG-3'
IL-6	nd	S: 5'-CACAGACAGCCACTCACCTC-3'; AS: 5'-TTTTCTGCCAGTGCCTCTTT-3'	21	S: 5'-AGTTGCCTTCTTGGGACTGA-3'; AS: 5'-TCCACGATTTCCAGAGAAC-3'
IL-10	nd	S: 5'-TGCCTTCAGCAGAGTGAAGA-3'; AS: 5'-GGTCTTGGTTCTCAGCTTGG-3'	nd	S: 5'-CCAAGCCTTATCGGAAATGA-3'; AS: 5'-TTTTACAGGGGAGAAATCG-3'
IL-13	nd	S: 5'-GTACTGTGCAGCCCTGGAAT-3'; AS: 5'-TTTACAACTGGGCCACCTC-3'	nd	S: 5'-CAGCATGGTATGGAGTGTGG-3'; AS: 5'-AGGCCATGCAATATCCTCTG-3'
MCP-2/CCL8	nd	S: 5'-TCCATTCCAATCACCTGCTGCT-3'; AS: 5'-ACAGTCTCCTTGGGACATTGGA-3'	21	S: 5'-TAAGGCTCCAGTCACTGCT-3'; AS: 5'-TCTGGAAAACACAGCTTCC-3'
MCP-3/CCL7	nd	S: 5'-TTGCTCAGCCAGTTGGGATT-3'; AS: 5'-AAAGTCCTGGACCACTTCTGT-3'	na	
IFN- γ	nd	S: 5'-TTCAGCTCTGCATCGTTTGTG-3'; AS: 5'-TCTTTTGGATGCTCTGGTCA-3'	na	

Sequences designed for the detection of indicated gene products by real-time PCR are presented. For internal controls and cytokines/chemokines, gene products are presented in decreasing order of abundance, as determined by the CT value obtained from LPS-stimulated human neutrophils.

* Absent in the mouse.

Mouse MIP-2 has similar sequence relatedness to human GRO α /CXCL1, GRO β /CXCL2, and GRO γ /CXCL3 {Murphy, 2000 #35}.

nd, = not detected; na, not analyzed.