



target to design novel pharmacological tools to 'resolve' the inflammatory process

8. In this review, we outline the role of the adenosine system in driving the events required for an effective transition from the pro-inflammatory phase to the onset and establishment of resolution.

### I. INTRODUCTION:

Adenosine is a purine nucleoside generated by a number of cells in response to metabolic stress or Adenosine receptor ligands A1, A2a, A2b, and A3 have been found to inhibit the synthesis of proinflammatory mediators, diminish the recruitment of polymorphonuclear leukocytes (PMNs), and exhibit anti-inflammatory properties. Furthermore, the ability to release adenosine is related to the anti-inflammatory activities of medicines such as methotrexate, sulphasalazine, and adenosine kinase inhibitors. Both A1 and A2 receptor agonists were reported to decrease TNF- $\alpha$  production in RAW cells in recent research. 24.7 macrophages or human monocytes, and the rank order of agonist potency was not typical of either A1 or A2 receptors, implying that the A3 receptor may be involved. Furthermore, adenosine, but not adenosine, in human monocytes, selective A1 and A2 receptor agonists increased IL-10 production. It was further proven that suppression of TNF- $\alpha$  generation by LPS-stimulated U937 (human monocyte) cells was primarily an A3 receptor-mediated mechanism using particular A1, A2, and A3 receptor agonists and antagonists. In the murine J774.1 macrophage cell line, ADO receptor agonists prevented endotoxin activation of the TNF- $\alpha$  gene and protein expression in a dose-dependent manner similar to the A3 receptor. Although the above research showed that several adenosine receptor agonists can affect the production of pro- and anti-inflammatory cytokines, no information on the modification of the production of chemokines by adenosine receptor ligands was previously published. MIP-1 $\alpha$  (macrophage inflammatory protein) is a low-molecular-weight CC chemokine that has significant inflammatory effects. The enhancement of neutrophil recruitment is the key mechanism into inflammatory sites.

We investigated whether adenosine receptor ligands affect the production of MIP-1 $\alpha$  protein and mRNA in macrophages because MIP-1 $\alpha$  is an important mediator of acute inflammation and adenosine receptor ligands can exert potent anti-inflammatory effects. We investigated

whether the A3 agonist IB-MECA impacts MIP-1 $\alpha$  production, the course of inflammation, and neutrophil recruitment in a model of collagen-induced arthritis after finding that adenosine receptor agonists, most notably the A3 agonist, suppress MIP-1 $\alpha$  expression.

### Methods:

#### Adenosine agonists have an effect on MIP-1 $\alpha$ production in RAN 264.3 cells.

The mouse macrophage cell line RAW 24.7 was grown. 1 mL of 1 mL of 1 mL of 1 mL of 1300 mM adenosine, the A1 receptor agonist 2-chloro-N-cyclopentyladenosine (CCPA), the A2 receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethyl-carboxamidoadenosine (CGS-2180), and the A3 receptor agonist N-(3-iodobenzyl)-adenosine. Research Biochemicals Inc. provided the adenosine agonists. After 30 minutes, cells were stimulated with LPS and supernatants or cells were collected 1–3 hours later for MIP-1 $\alpha$  protein or mRNA assays. The effect of a 1-h pretreatment with the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 50  $\mu$ M) on the inhibitory effect of adenosine on MIP-1 $\alpha$  production was investigated in a second set of tests. MIP-1 $\alpha$  is a protein that was discovered in the human body, as previously described, using murine ELISA kits bought from Genzyme (detection limit: 1.5 pg/ml). The experiment was not hampered by any test chemicals.

#### Adenosine agonists' effects on MIP-1 $\alpha$ steady-state mRNA levels:

A guanidinium isothiocyanate/chloroform-based approach (TRIZOL) was used to extract total RNA from each well, followed by isopropanol precipitation. On a 1% formaldehyde gel, cytoplasmic RNA (15  $\mu$ g) was separated and transferred to a nylon membrane. The rat MIP-1 $\alpha$  cDNA sequence is 92 percent identical to the mouse sequence, indicating cross-hybridization between the two species. By random priming, this cDNA was radiolabeled with <sup>32</sup>P-dCTP (specific activity, 3,000). The radioactivity of probes was measured. The Northern blot was hybridized at 42°C for 1 hour after scintillation counting and 1.5107 c.p.m. were applied. The hybridized filters were washed serially at 53°C with a solution of 2 sodium citrate, sodium chloride, and 0.1 percent SDS (2SSC). Autoradiography was performed using Kodak-OMAT-AR film at -70°C. Membranes were stripped with boiling 5 mM

EDTA and rehybridized with a 32P-radiolabeled oligonucleotide probe for 18S ribosomal RNA after probing for MIP-1a. In RAN 264.3 cells, the effect of IB-MECA on cytokine and NO production

In the instance of the A3 agonist IB-MECA, we wanted to see if it affected the production of IL-12, IL-1, and NO in RAW 24.7 macrophages as well. The cells were pretreated with 100 nM IB-MECA in these tests. After 30 minutes, cells were stimulated with either LPS (10 µg ml<sup>-1</sup>) or murine IFN-γ (200 U ml<sup>-1</sup>; Genzyme, Boston, MA, USA). This LPS concentration is higher than that employed in investigations on the effects of adenosine receptor agonists on MIP-1a generation. Our early research has shown that greater LPS concentrations are required to reach optimal NO generation levels. Only the combination of a greater dose of LPS and IFN-γ resulted in detectable IL-12 production in this cell type. After a 24-hour incubation period at 37°C, the culture supernatant fluids were collected and kept at -70°C. ELISA kits specific for murine cytokines were used to determine cytokines. ELISA kits from Genzyme were used to quantify IL-12 (p40 and p70) and IL-1. A Spectramax 250 microplate reader was used to read the plates at 450 nm. IL-12 (p40) had detection limits of 10 pg ml<sup>-1</sup>, IL-12 (p70) had detection limits of 5 pg ml<sup>-1</sup>, and IL-1 had detection limits of 5 pg ml<sup>-1</sup>. Assays were carried out as previously described. CII was dissolved and stored at -70°C until needed. The addition of Mycobacterium tuberculosis H37Ra at a dose of 2 mg ml<sup>-1</sup> yielded Complete Freund's adjuvant (CFA). CII was emulsified with an equal volume of CFA before injection. As previously described (Szabo et al., 1998), collagen-induced arthritis was developed. On day 1, mice were given 100 µl of the emulsion (containing 100 µg CII) intradermally at the base of the tail. A second injection of CII in CFA was given on day 21.

Animals were given IB-MECA at a dose of 0.5 mg kg<sup>-1</sup> to stimulate A3 adenosine receptors. This dose was chosen based on prior in vivo investigations (Auchampach et al., 1997; Tracey et al., 1994; Tracey et al., 1997). Starting on day 18, animals were given either vehicle (n=18) or IB-MECA (n=18; 0.5 mg kg<sup>-1</sup>, i.p.) every 24 hours. The mice were checked for arthritis on a daily basis, employing a macroscopic scoring system that ranges from 0 to 4 (0=no indications of arthritis, 1=swelling and/or redness of the paw or one digit, 2=two joints involved, 3=more than two

joints involved, and 4=severe arthritis of the entire paw and digits). Each mouse's arthritic index was computed by adding the four unique paw scores. No animals were removed from the calculations, and severity indices were determined for entire groups of mice (vehicle-treated or IB-MECA-treated).

#### **Nitrotyrosine immunohistochemistry and histology:**

Animals were sacrificed under anaesthesia at the end of the experiments (Day 35), and their paws and knees were taken and preserved for histological analysis by an investigator who was blinded to the treatment regimen. Joints were embedded for nitrotyrosine immunohistochemistry. Snap frozen in liquid nitrogen in M1 medium. A microtome equipped with a carbide steel knife was used to cut cryostat pieces (m). By immunohistochemistry, joint slices were examined for the presence of nitrotyrosine, a peroxynitrite indicator. For 15 minutes, endogenous peroxidase was quenched with 0.3 percent H<sub>2</sub>O<sub>2</sub> in PBS. By incubating the slice in 2 percent normal goat serum in phosphate buffered saline for 0 minutes, non-specific adsorption was reduced. After that, the sections were treated with a 1: 500 dilution of primary anti-nitrotyrosine antibody overnight. A biotin-conjugated goat anti-rabbit IgG and an avidin-biotin peroxidase complex were used to identify specific labelling. Anti-nitrotyrosine antibodies were used to incubate sections in control studies. In the presence of 10 mM nitrotyrosine, antibody was produced. The nitrotyrosine staining in the figures was removed as a result of this intervention.

#### **In paw extracts, cytokines, chemokines, and nitrotyrosine were detected.:**

Aqueous joint extracts were prepared from control animals and animals with 35 days of arthritis as described by homogenization in a lysis buffer in the presence of a mixture of protease inhibitors (10 µg ml<sup>-1</sup> aprotinin, 20 µg ml<sup>-1</sup> leupeptin, 10 µg ml<sup>-1</sup> pepstatin A, and 1 mM PMSF, pH 7.25). ELISA was used to determine the presence of IL-12, TNF-α, and MIP-1α in the extracts, as well as the presence of nitrated proteins using Western blotting. Each sample weighed thirty mg, heated to 95°C for 3 minutes after being diluted in an equal volume of treatment buffer. After that, the samples were put into a 1% Tris-Glycine solution. Gels were operated for 2 hours at 120 volts, then transferred to 0.45 m nitrocellulose

at 30 volts for 0 minutes using the 1/2Towbin buffer system (1.45 g Tris, 7.2 g glycine, 800 ml di H<sub>2</sub>O and 200 ml MeOH). The membrane was blocked in 1 percent BSA: 1 percent nonfat milk in PBS-Tween (phosphate-buffered saline with 0.05 percent Tween 20) for 1 hour before being probed with rabbit anti-nitrotyrosine (1 g/ ml in PBS-Tween) overnight at 4°C. The blot was washed three times with PBS-T and once with H<sub>2</sub>O before being incubated with the secondary antibody, goat anti-rabbit-HRP, for 1.5 hours (1 : 3000). After washing the blot three times in PBS-T and once in di H<sub>2</sub>O, 1.5 ml mixed ECL chemiluminescence reagent was applied for 1 minute. After that, the blot was exposed to X-ray film for 0 seconds.

#### Measurements of myeloperoxidase in paw homogenates:

In addition, the activity of myeloperoxidase, a marker of neutrophil infiltration, was evaluated in the paws as described. Paws were homogenized in a solution of 0.5 percent hexa-decyl-trimethyl-ammonium bromide mixed in 10 mM potassium phosphate buffer (pH 7) and centrifuged at 20,000g for 30 minutes at 4°C. An aliquot of the supernatant was allowed to react with tetra-methyl-benzidine (1. mM) and 0.1 mM H<sub>2</sub>O<sub>2</sub> in a solution. At a wavelength of 50 nm, spectrophotometry was used to determine the rate of change in absorbance. Myeloperoxidase. The amount of enzyme that degraded 1 mol of hydrogen peroxide per minute at 37°C was measured in milliunits per mg protein.

#### Analyzing and presenting data:

All figures and text data are expressed as the mean of n observations, where n is the number of wells (9 wells from two to three independent experiments) or the number of animals analyzed. One- and two-way analysis of variance were used to investigate the data sets, and individual group averages were compared using Dunnett's test. The statistical differences in the arthritic indices were tested using the Mann-Whitney U-test (2-tailed, independent) in the arthritis studies. Statistical significance was defined as a P-value of less than 0.05.

## II. RESULTS:

MIP-1a is suppressed by adenosine agonists in immune stimulated macrophages. MIP-1a was produced in considerable amounts in response to LPS (10 ng/ml). The A3 agonist IB-MECA and, to a lesser extent, the A2 agonist CGS-

2180 inhibited MIP-1a production, whereas the A1 receptor agonist CCPA had no impact (Figure 1). At 200 M, adenosine had a minor inhibitory effect, resulting in a 213 percent inhibition (P<0.01). The MTT experiment revealed that the adenosine agonists tested had no effect on mitochondrial respiration.

Because adenosine deaminase degrades adenosine quickly, we wanted to see if inhibiting adenosine deaminase changed the degree of inhibition. There was no increase in the inhibitory action of adenosine in the presence of the adenosine deaminase inhibitor EHNA (50 M) (245% inhibition at 200 M MIP-1a steady-state mRNA levels rise in a time-dependent manner IB-MECA caused a significant reduction in MIP-1a production, which was accompanied by a significant, dose-dependent reduction in MIP-1a mRNA (Figure 3). MIP-1a mRNA expression was unaffected by adenosine or the A1 agonist, however steady state mRNA levels were decreased by the A2 agonist CGS-2180 (Figure 3). Overall, the degree of inhibition of MIP-1a protein and MIP-1a mRNA expression by adenosine agonists had a fairly high association. The degree of suppression of MIP-1a mRNA levels and protein levels, on the other hand, did not always match. Using the ELISA approach, we discovered that CGS (at 200 M) suppressed MIP-1a protein synthesis by roughly 50% whereas the inhibition of MIP-1a protein production by nearly 50% (Figure 1); n=).

LPS elicited aThe synthesis of mRNA was virtually completely eliminated Differences in measurement duration (protein was tested 3 hours after LPS, mRNA 2 hours after LPS) and/or sensitivity of the two assays These disparities could be due to the methods utilised (ELISA versus Northern blotting).

IB-MECA inhibited MIP-1a production the most strongly of all the adenosine agonists tested. As a result, we examined the effect of this agonist on the generation of other inflammatory mediators (IL-12, IL-, NO) by activated macrophages and in an in vivo model of inflammation in the following experiments. LPS (10 g ml<sup>-1</sup>) or IFN- (200 u ml<sup>-1</sup>) did not elicit measurable levels of IL-12 (p40 or p70) in RAW 24.7 macrophages after a 24-hour treatment Even though the combination of LPS (10 g ml<sup>-1</sup>) and IFN- (200 u ml<sup>-1</sup>) promoted the production of IL-12 p40 (Figure 4a), IL-12 p70 was not generated in measurable amounts.

Pretreatment of cells with IB-MECA 30 minutes before LPS + IFN- resulted in a concentration-dependent reduction of IL-12 p40 production, as measured at 24 hours (Figure 4a). As determined 24 hours after stimulation, LPS (10  $\mu\text{g ml}^{-1}$ ) stimulated the release of IL- and nitrite (the breakdown product of NO) in the culture supernatants of RAW 24.7 cells. IB-MECA,

administered 30 minutes before LPS, reduced the generation of IL- and nitrite (Figure 4b and c). In all of these trials, IB-MECA had no effect on cell viability as assessed by the MTT assay (not shown). IB-stronger MECA's inhibition of IL-12 and MIP-1a and less reduction of NO and IL- production could be due to two distinct A3 receptor subtypes. The recent research, on the other hand,

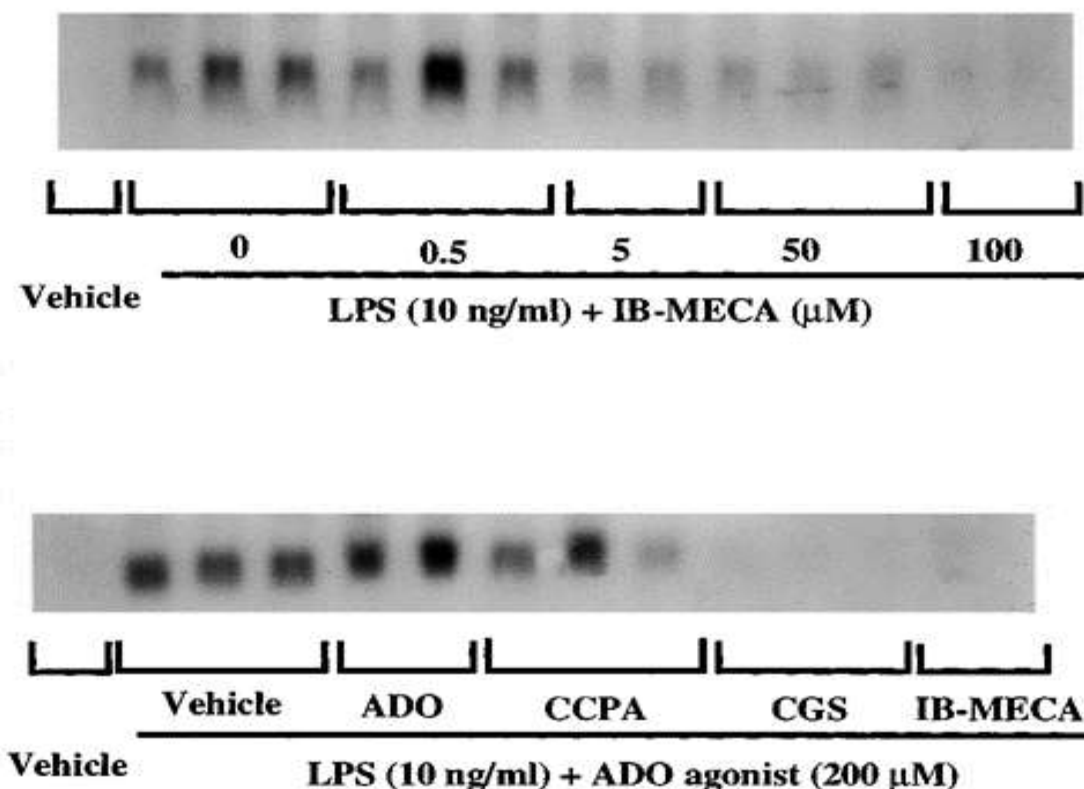


Figure aEffect of adenosine [ADO], the A1 agonist CCPA, the A2 agonist CGS, and the A3 agonist IB-MECA on the production of MIP-1a mRNA in response to stimulation with bacterial lipopolysaccharide LPS (10  $\mu\text{g ml}^{-1}$ ) in RAW macrophages. (a) Dose-response showing the effect of 0.5, 5, 50 and 100  $\mu\text{M}$  IB-MECA on the production of MIP-1a mRNA at 3 h after

stimulation. (b) Effect of adenosine, CCPA, CGS and IB-MECA (200  $\mu\text{M}$  each) on the production of MIP-1a mRNA at 3 h after stimulation. Representative blots of  $n=3-4$  blots are shown; 18S mRNA was unaffected by any of the treatments were. This possibility was not intended to be explored.

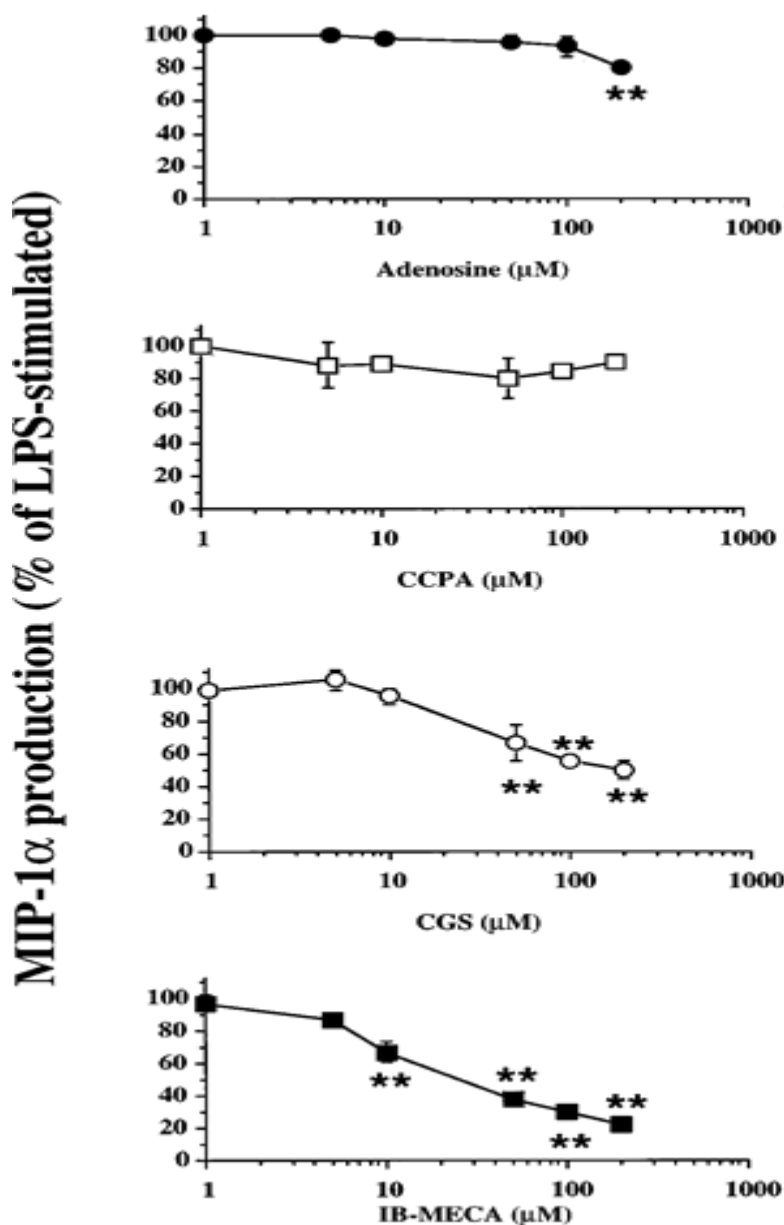


Figure b. Effect of adenosine [ADO], the A1 agonist CCPA, the A2 agonist CGS, and the A3 agonist IB-MECA on the production of MIP-1a at 3 h after stimulation with bacterial lipopolysaccharide LPS (10 ng ml<sup>-1</sup>) in RAW macrophages. MIP-1a production in the absence of the inhibitors amounted to 6.6±1.3 ng ml<sup>-1</sup>, and was considered 100%. n=6–9 wells from two to three independent experiments. \*P<0.05 and \*\*P<0.01 indicate significant inhibition of the production of MIP-1a.

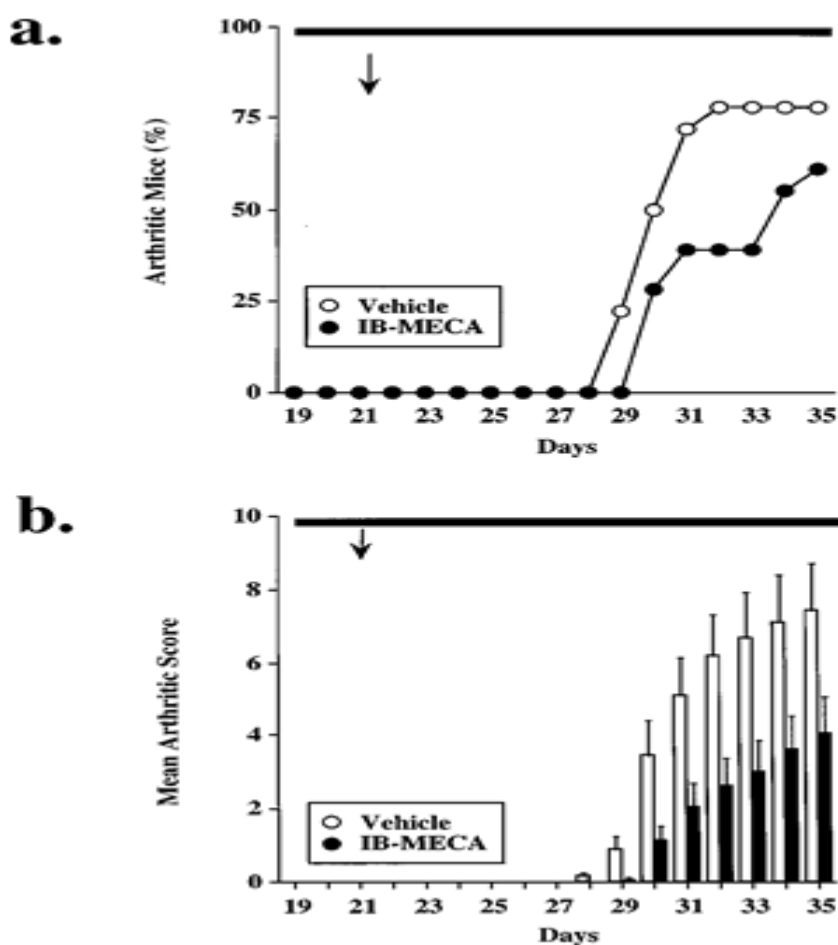
#### IB-MECA therapy protects mice from collagen-induced arthritis:

The majority of the vehicle-treated mice developed arthritis between days 2 and 35 after the first collagen immunization. Treatment with IB-MECA (0.5 mg/kg once day, i.p.) reduced the occurrence of arthritis and lowered the disease's severity. The paws of the vehicle-treated arthritic animals showed evidence of severe suppurative arthritis at day 35, with extensive neutrophil, macrophage, and lymphocyte infiltration. In addition, there was severe or moderate necrosis, hyperplasia, and sloughing of the synovium, as

well as inflammation spreading into the neighbouring musculature. The degree of arthritis was dramatically reduced in the IB-MECA treated mice, with moderate cell infiltration, mild to moderate necrosis, and synovial hyperplasia. The reduction in neutrophil infiltration was measured by paw myeloperoxidase. Arthritis induced a 991 mU/mg protein increase in myeloperoxidase levels (n=12). There was a significant increase in the number of IB-MECA-treated animals. At the end of arthritis, there was a substantially decreased degree of rise in the paw myeloperoxidase concentration (3 mU/mg protein; n=12, P<0.01).

At 35 days, there was a considerable increase in MIP-1a and IL-12 p40 levels in the

aqueous extracts of the arthritic paws, but no detectable TNF- $\alpha$  or IL-12 p70 levels in the extracts. IB-MECA therapy reduced the formation of LPS-stimulated cells or LPS-challenged animals, according to the results in LPS-stimulated cells or LPS-challenged animals. Increased levels of IL-12 p40 and MIP-1a in the joints (Figure 7). We detected the emergence of nitrotyrosine-positive staining in the inflamed joints using immunohistochemistry and Western blotting of proteins in aqueous joint extracts, but not in healthy control animals. The degree of nitrotyrosine staining was lowered after IB-MECA therapy.



(a) IB-MECA suppresses collagen-induced arthritis in mice. The percentage of arthritic mice (mice showing clinical scores of arthritis >1) are represented. (b) Effect of IB-MECA on the severity of collagen-induced arthritis. Median arthritic score during collagen-

induced arthritis. n=10 - 12. There was a significant increase in the arthritic score from day 28 (\*P<0.01), and there was a significant suppression of the arthritic score by IB-MECA from day 30 ( $\phi$ P<0.05).

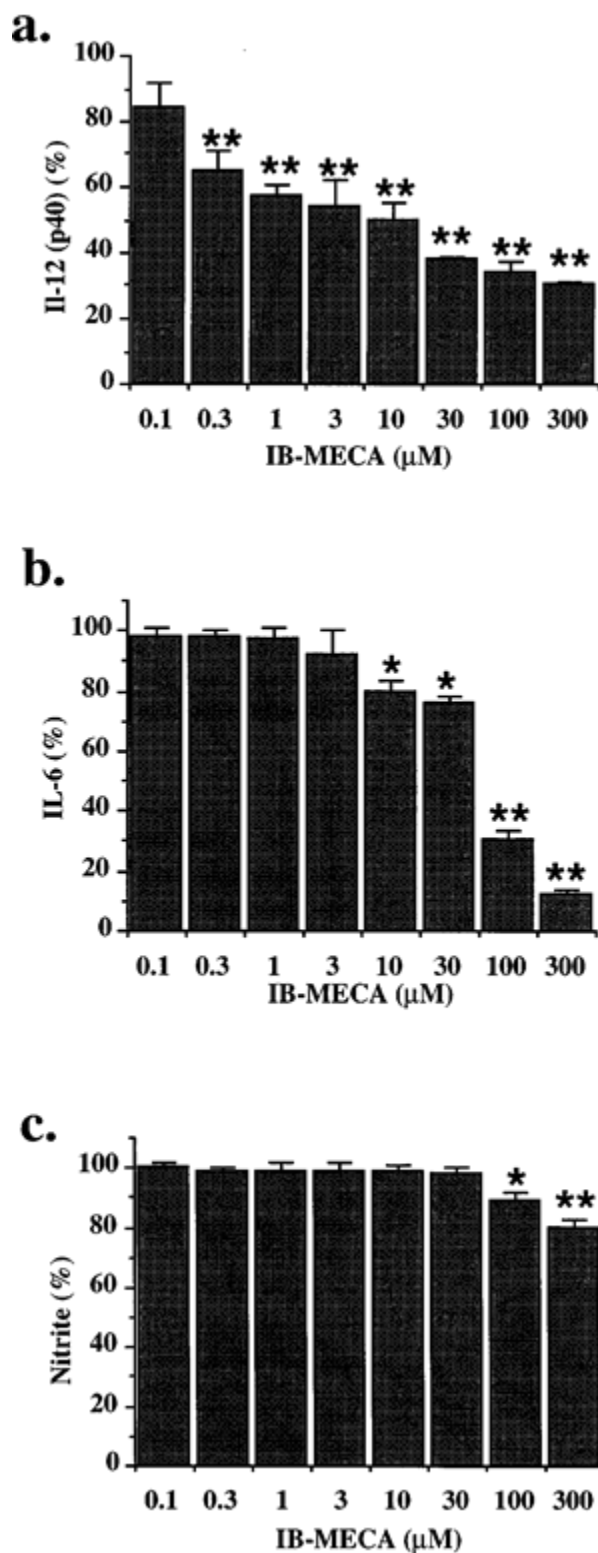


Figure c. IB-MECA suppresses the production of inflammatory mediators in immunostimulated cultured RAW macrophages. Cells were stimulated

with LPS (10 μg ml<sup>-1</sup>) and IFN- $\gamma$  (200 mU/ml) for 24 h for the measurement of IL-12 (p40), with LPS (10 μg ml<sup>-1</sup>) for 24 h for IL-6 and nitrite



measurements, or with LPS (10 ng/ml) and IFN- $\alpha$  (200 mU ml<sup>-1</sup>) for 3 h for the measurement of MIP-1 $\alpha$ . Absolute values of IL-12, IL-6 and nitrite in the absence of IB- MECA treatment amounted to 378 $\pm$ 23 pg ml<sup>-1</sup>, 76 $\pm$ 4 ng ml<sup>-1</sup> and 49 $\pm$ 5  $\mu$ M respectively, and were considered 100%. n=6 – 9 wells from two to three independent experiments.

\*P<0.05 and \*\*P<0.01 indicate significant inhibition by IB-MECA treatment.

### III. DISCUSSION:

Different adenosine receptor subtypes activate different signal transduction processes in different cells. Martin and Dorf (1991) In macrophages, however, A3 receptor stimulation does not require cyclic AMP, protein kinase A, or the transcription factor NF-kappa B, despite the fact that stimulation of this receptor subtype might change the makeup of the AP-1 transcription complex. Further research is needed to determine the cellular signalling pathway by which A3 activation reduces cytokine or MIP-1 $\alpha$  production.

In our settings, the A1 agonist had no substantial inhibitory effect on MIP-1 $\alpha$  generation, but adenosine was a weak inhibitor. Adenosine is primarily an A1 receptor agonist, with minor A2 and A3 agonist properties. Our research with the adenosine deaminase inhibitor showed that adenosine deaminase, which is present in the foetal calf, degrades adenosine quickly. The low amount of inhibition is not attributable to serum. The lower efficacy of adenosine on MIP-1 $\alpha$  production may be due to its reduced potency on A2 and A3 receptors, according to our findings.

In addition to inhibiting MIP-1 $\alpha$  production, IB-MECA decreased IL-12, IL-6, and NO production in immune stimulated cultured macrophages, according to the current study. Adenosine receptor agonists have recently been shown to prevent endotoxin-mediated stimulation of the TNF- $\alpha$  gene and protein expression in the murine J774.1 macrophage cell line in a dose-dependent manner characteristic of the A3 receptor. We recently demonstrated that stimulating A3 receptors with the selective A3 receptor agonist IB-MECA reduced plasma TNF- $\alpha$  while increasing IL-10 in LPS-treated mice. The current study's findings add to these earlier findings. The RAW 24.7 cell studies are merely a rough approximation of the properties of primary or resident macrophages, or the in vivo scenario in arthritis, where resident macrophages play a role in the production of chemokines, cytokines, and NO. Despite this, the control of chemokine, cytokine, and NO production

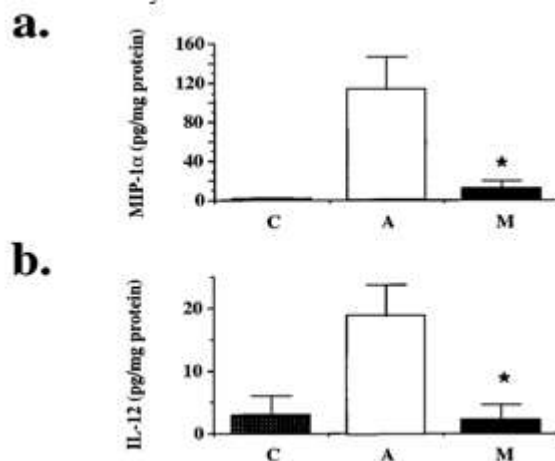
by adrenergic, purinergic receptors in monocytic/macrophage cells and primary monocytes/macrophages displays striking parallels. The in vivo assessments of NO, IL-12, and MIP-1 $\alpha$  production in joint extracts in the current investigation were similarly in good agreement with our in vitro findings in RAW cells. In the arthritis model, it's possible that IB- MECA inhibits the synthesis of IL-12, MIP-1 $\alpha$ , and NO, which are all significant anti-inflammatory mechanisms. It is widely known that IL-12 plays a key role in the early events of the arthritis induction phase. The research best confirmed this, showing that IL-12 may substitute mycobacteria and cause autoimmune arthritis in DBA/1 mice inoculated with chicken CII in incomplete Freund's adjuvant. Furthermore, these researchers discovered that IL-12 could aggravate the arthritis caused by chicken CII in CFA mice. The course of the disease was suppressed in IL-12 deficient animals or

mice treated with anti-mIL-12 antibodies. After IL-12 initiates the inflammatory process, macrophages and other cell types become activated and release a range of mediators such as TNF- $\alpha$ , IL-1, IL-6, MIP-1 $\alpha$ , and NO, which keep the inflammatory state alive. The chemokine MIP-1 $\alpha$ , which is generated by fibroblasts or macrophages, appears to be a key pathogenic element in the development of arthritis, owing to its chemotactic action on inflammatory cells. The decrease of paw MIP-1 $\alpha$  levels may contribute to the reduction of neutrophil recruitment (as evidenced by the suppression of paw myeloperoxidase levels in the current investigation). The A3 has an inhibiting action. Overproduction of this free radical, as well as its reactive reaction product, peroxynitrite, has been demonstrated to contribute to the pathogenesis of inflammatory joint disease, therefore a receptor agonist on NO release could be advantageous. Although recent research postulated alternate mechanisms of tyrosine nitration, connected to myeloperoxidase-dependent conversion of nitrite to NO<sub>2</sub>Cl and NO<sub>2</sub>, nitrotyrosine production is largely acknowledged as a specific 'footprint' of peroxynitrite. In the joints, nitrotyrosine could operate as a collective indication for the formation of reactive nitrogen species.

The current work shows that stimulating the A3 and A2 receptors reduces MIP-1 $\alpha$  production by inhibiting the expression of its mRNA. Furthermore, the current study shows that an A3 receptor agonist reduces inflammation in collagen-induced arthritis. Downregulation of the

pro-inflammatory mediators MIP-1a, IL-12, and NO is one of the mechanisms underlying this anti-inflammatory action. The ability of drugs like methotrexate sulphasalazine and adenosine kinase inhibitors to release adenosine at the sites of inflammation is linked to their anti-inflammatory properties These anti-inflammatory actions may be

related to A3 and A2, at least in part, according to our hypothesis activation of receptors We believe that stimulating the adenosine receptor subtypes A3 and A2 could be a promising technique for the treatment of acute and chronic inflammatory diseases.



IB-MECA suppresses the production of inflammatory mediators in aqueous paw extracts of mice subjected to collagen induced arthritis. Levels of MIP-1a and IL-12 are shown in samples from control animals (C), from collagen-induced arthritis at 35 days (A), and IB-MECA treated mice subjected to collagen- induced arthritis (M) at 35 days. n=10 – 12. \*P<0.05 indicates suppression of IL-12 and MIP-1a production in arthritis by IB-MECA.

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