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Pharmacologic P2X Purinergic Receptor Antagonism in the Treatment of Collagen-Induced Arthritis

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Objective. **To assess the therapeutic potential of a P2X purinergic receptor antagonist, namely, periodate oxidized ATP, in collagen-induced arthritis (CIA).**

Methods. **Arthritis was induced in male DBA/1J mice by immunization with type II collagen (CII). Animals showing digit inflammation and paw swelling were treated intraperitoneally with 100 μl of 3 m*M* oxidized ATP daily for 10 days. At the end of the treatment period, animals were killed and paws were removed for histologic analysis and evaluation of T cell infiltration. Humoral response to CII was analyzed, and specific serum autoantibody levels were correlated with the clinical scores observed in the different treatment groups.**

Results. **Treatment with oxidized ATP resulted in a sustained reduction in disease activity, which was associated with a significant decrease in CD3+ T cell infiltration in arthritic lesions and a significant amelioration of cartilage erosion. Peripheral Treg cells were significantly increased upon P2X blockade in mouse lymph nodes. Moreover, a marked reduction in circulating autoantibodies directed against mouse CII was detected. There was a significant correlation between serum autoantibody levels and the clinical efficacy of oxidized ATP.**

Conclusion. **Our findings indicate that P2X receptor antagonism has important therapeutic potential in chronic inflammatory rheumatic disorders. Taken together, our results underscore the value of the P2X receptor signaling pathway as a potential pharmacologic target for the modulation of adaptive immunity in CIA.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease that primarily affects the joints, causing pain, stiffness, and synovitis and leading to articular destruction. The etiology of RA is multifactorial, with genetic and environmental components that determine early immune perturbation in both the innate and adaptive compartments and subsequent chronic inflammation (1). The molecular and cellular pathogenic mechanisms that cause RA and/or determine the chronic nature of the disease are poorly understood. In RA, the synovial tissue of the periarticular spaces is infiltrated by granulocytes and a large number of mononuclear cells, including T cells, B cells, monocytes, and macrophages (2).

Immunization of DBA/1J mice with type II collagen (CII) results in a break in tolerance and generation of CII-specific T cells and anti-CII autoantibodies, with clinical signs that mimick RA (collagen-induced arthritis [CIA] model). In spite of the detection of CII-specific T cells in arthritic joints, passive transfer of CII-specific T cells to susceptible mouse strains induces only minor pathologic changes in the synovial joints of recipients (3). In contrast, consistent with the importance of autoantibodies in the immunopathogenesis of

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CIA, passive transfer of anti-CII sera from arthritic mice to naive recipients induces severe inflammation even in nonsusceptible strains (4). A defect in peripheral tolerance has been observed in RA patients as well as in animal models of RA. Several studies have indicated that quantitative and qualitative abnormalities of Treg cells contribute to the pathogenesis of CIA and RA (5). Indeed, Treg cells can actively modulate the autoimmune response in inflammatory arthritis (6).

Two major classes of molecules interacting with cellular receptors have been defined as major determinants of innate and adaptive immune system activation in chronic inflammatory and autoimmune conditions, namely, pathogen-associated molecular patterns and damage-associated molecular patterns (DAMPs) (7). Among the latter, extracellular ATP, which is massively released upon cell death, plays a crucial role in promoting a number of inflammatory immune responses. Plasma membrane receptors for extracellular ATP, termed P2 receptors, are found on virtually all cell types. They are divided into 2 families: P2X receptors, which are ATP-gated nonselective cation channels, and P2Y receptors, which are heterotrimeric G protein–coupled receptors. ATP was shown to crucially contribute to inflammasome activation through $P2X_7$ activation in cells of the innate immune system. Notably, deletion of $P2X_7$ receptor attenuates the inflammatory response in a passive model of antibody-induced arthritis (8).

In addition to the well-documented role of ATP as a DAMP for cells of the innate immune system, recent studies have demonstrated that ATP is also critically involved in determining productive T cell activation. Indeed, ATP is released by CD4+ T cells upon stimulation with cognate antigen, and consequent autocrine P2X receptor stimulation plays a crucial role in protracting T cell receptor (TCR)–initiated MAPK induction (9,10). Moreover, P2X antagonism was shown to blunt MAPK activation without affecting NF-AT nuclear translocation, thus determining T cell unresponsiveness to subsequent stimulation (anergy) (9). Finally, the activation of $P2X_7$ by ATP inhibits the suppressive potential of Treg cells, and pharmacologic antagonism of P2X receptors promotes conversion of naive CD4+ T cells into Treg cells after TCR stimulation (11). These observations suggest that ATP acts as a soluble factor regulating Treg cell immunosuppressive potential and de novo generation.

In the present study, we investigated the role of oxidized ATP as a P2X receptor antagonist in the treatment of CIA. We demonstrated a dramatic amelioration of symptoms, an increase in peripheral Treg cells, and a significant reduction in circulating anti-CII autoantibodies. These findings suggest the potential application of this class of therapeutics to modulation of the autoimmune response in RA patients.

MATERIALS AND METHODS

Induction and treatment of CIA. All animal studies were approved by the authorized veterinarian and were authorized by the Italian Ministry of Health (decree no. 124/2007-B). CIA was induced and evaluated as previously described (12). Briefly, 8-week-old male DBA/1J mice (Charles River Japan) were immunized on day 0 with an intradermal injection into the base of the tail of 0.2 ml of an emulsion containing 2 mg/ml of bovine CII (Morwell Diagnostics) in Freund's complete adjuvant (CFA; Difco) containing 2 mg/ml of *Mycobacterium tuberculosis*. Immunization with CFA and CII resulted, after ~18–20 days, in the appearance of signs of inflammation affecting 1 or more limbs. When animals showed visible clinical signs (digit inflammation and paw swelling) resulting in a clinical score of ≥1.5, treatment was started. Mice were treated with intraperitoneal injections of either phosphate buffered saline (PBS; 100 μl) or oxidized ATP (3 m*M* in 100 μl of PBS) (Sigma-Aldrich) daily for 10 consecutive days. At the end of the treatment period, animals were killed with an overdose of anesthetic. Blood samples were collected from each animal, and paws were removed for histologic analysis. Plasma anti-CII antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), according to the recommendations of the manufacturer (Morwell Diagnostics).

Flow cytometric analysis. Mouse spleens and mesenteric/inguinal lymph nodes were harvested and smashed through a cell strainer; after erythrocyte lysis, cells were stained with CD11b and Gr-1 antibodies (Becton Dickinson) and analyzed by flow cytometry using a FACSCalibur instrument (Becton Dickinson). For flow cytometric analyses, monoclonal antibodies conjugated with either biotin, fluorescein isothiocyanate, phycoerythrin, CyChrome, PerCP, or allophycocyanin against the following antigens were used: CD8a (53-6.7), CD4 (L3T4), CD62L (MEL-14), CD44 (IM7), CD19 (6D5), CD21 (8D9), CD23 (B3B4) (all from eBioscience), IgM (II/41), IgD (11.26c.2a), and FoxP3 (MF23) (all from BD Biosciences). Data were analyzed with CellQuest Pro software (Becton Dickinson).

Evaluation of interleukin-10 (IL-10) production. An in vitro IL-10 secretion assay was performed as follows. Briefly, CD19+ B cells were isolated from mouse spleens by immunomagnetic beads and stimulated for 5 hours with lipopolysaccharide (LPS; 10 μg/ml), phorbol myristate acetate (1 μg/ml), and ionomycin (1 μg/ml). Intracellular staining with anti–IL-10 antibodies was performed. IL-10 production was also evaluated by ELISA in supernatants of CD19+ B cells stimulated for 4 days with CpG 1826 (2.5 μg/ml).

Histologic analysis. For histologic analysis, in each animal, the first limb to become arthritic was identified, removed, fixed in 10% neutral buffered formalin, and decalcified in formic acid solution (50%). The paw was then embedded in paraffin, and 2 sagittal sections were prepared. Tissue sections were stained with hematoxylin and eosin or Safranin O and scored as previously described (12).

Immunohistochemical analysis. For immunohistochemistry, serial formalin-fixed and paraffin-embedded sections from each animal were immunostained with primary antibodies raised against CD3, complement C3c fraction, mouse IgG, and FoxP3. Dako EnVision polymer was used to detect the immunoperoxidase reaction. Immunoreactivity was revealed by incubating the sections with 3,3′-diaminobenzidine (Vector). Sections were counterstained with Mayer's hematoxylin. For each sample, serial sections were also incubated with rabbit polyclonal anti–*Helicobacter pylori* antibodies (Dako) as a negative control. To establish the extent of CD3+ T cell infiltration in mouse arthritic lesions, each sample was scanned at 40× magnification, and 3 immunoreactive areas were selected from within articular/periarticular inflammatory lesions. Upon identification of these areas, the number of immunopositive cells with membranous CD3 expression was further defined at 200× magnification. To establish the extent of FoxP3+ Treg cells populating the arthritic lesions, synovial membranes and articular capsules present in each sample were scanned at 100× magnification for the identification of 6 immunoreactive foci. Upon identification of these foci, the number of immunopositive cells with FoxP3 nuclear expression was further defined at 200× magnification.

Statistical analysis. Clinical score variances were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni adjustment. Cartilage erosion was analyzed by Mann-Whitney test. One-way ANOVA followed by Tukey's test was used to analyze secondary lymphoid organ cellularity and granulocyte representation. Serum IgG autoantibody concentration was analyzed by Student's *t*-test.

RESULTS

In order to test whether pharmacologic P2X receptor antagonism affected arthritis development in

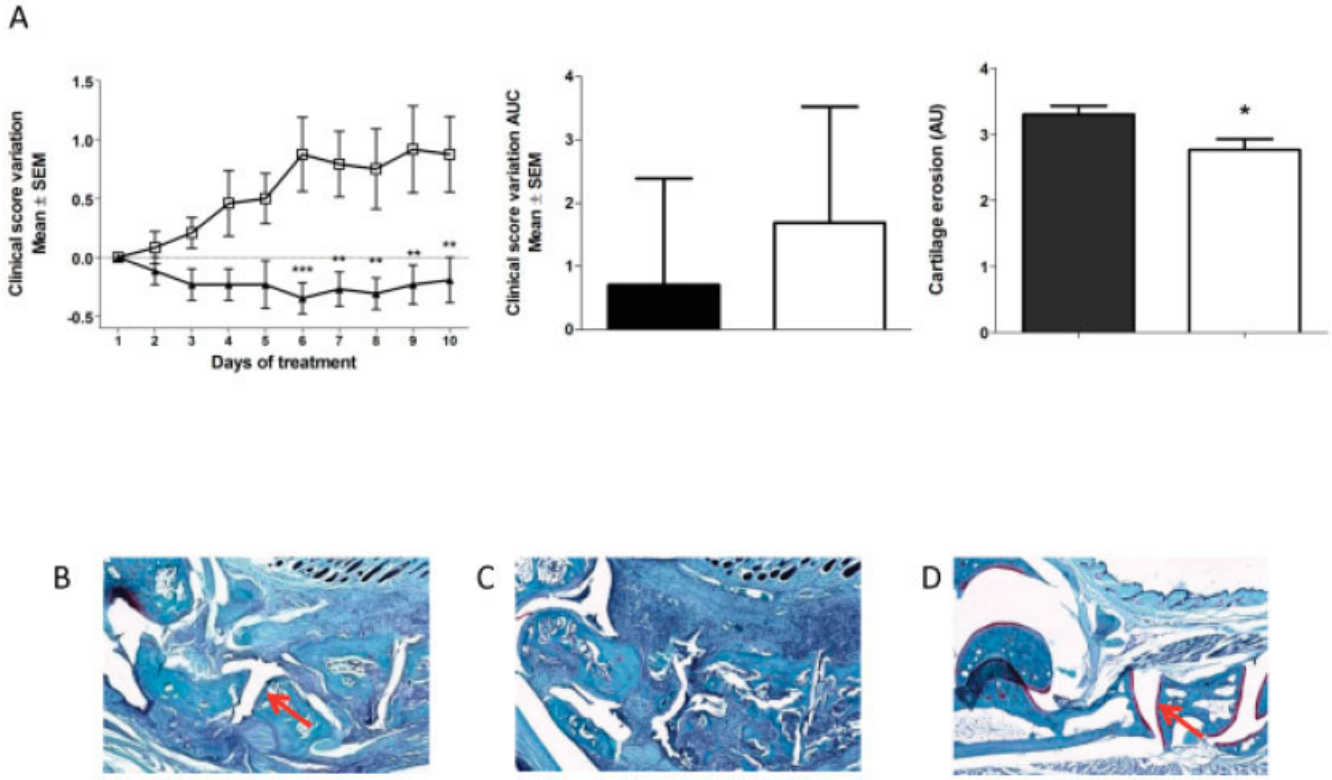

Figure 1. A, Left, Clinical scores for male DBA/1J mice with collagen-induced arthritis (CIA) treated with either phosphate buffered saline (PBS) (open symbols; n = 12) or oxidized ATP (3 m*M*) (solid symbols; n = 13) intraperitoneally. Treatment was started when clinical signs of arthritis were present (clinical score ≥1.5). The clinical score variation was calculated for each day, using as a reference the clinical score observed before the first administration of treatment. Values are the mean ± SEM of 2 independent experiments. ** = $P < 0.01$; *** = $P < 0.001$ versus PBS treatment, by two-way analysis of variance followed by Bonferroni adjustment. Middle, Time-integrated area under the curve (AUC) clinical score variations for the treatment period (days 1–10) calculated versus the first day of treatment (day 1) (solid bar) and for the recovery period (days 10–17) calculated versus the last day of treatment (day 10) (open bar). Right, Cartilage erosion. Sections from mice with CIA treated with PBS (solid bar; n = 4) or oxidized ATP (open bar; n = 6), were stained with Safranin O, and cartilage erosion was evaluated in a blinded manner. Bars show the mean ± SEM. * = $P = 0.03$ versus PBS treatment, by Mann-Whitney test. **B–D,** Histologic features of sections from a healthy control mouse (**B**), a mouse with CIA treated with PBS (**C**), and a mouse with CIA treated with oxidized ATP (**D**). **Arrows** indicate healthy cartilage. Original magnification × 50.

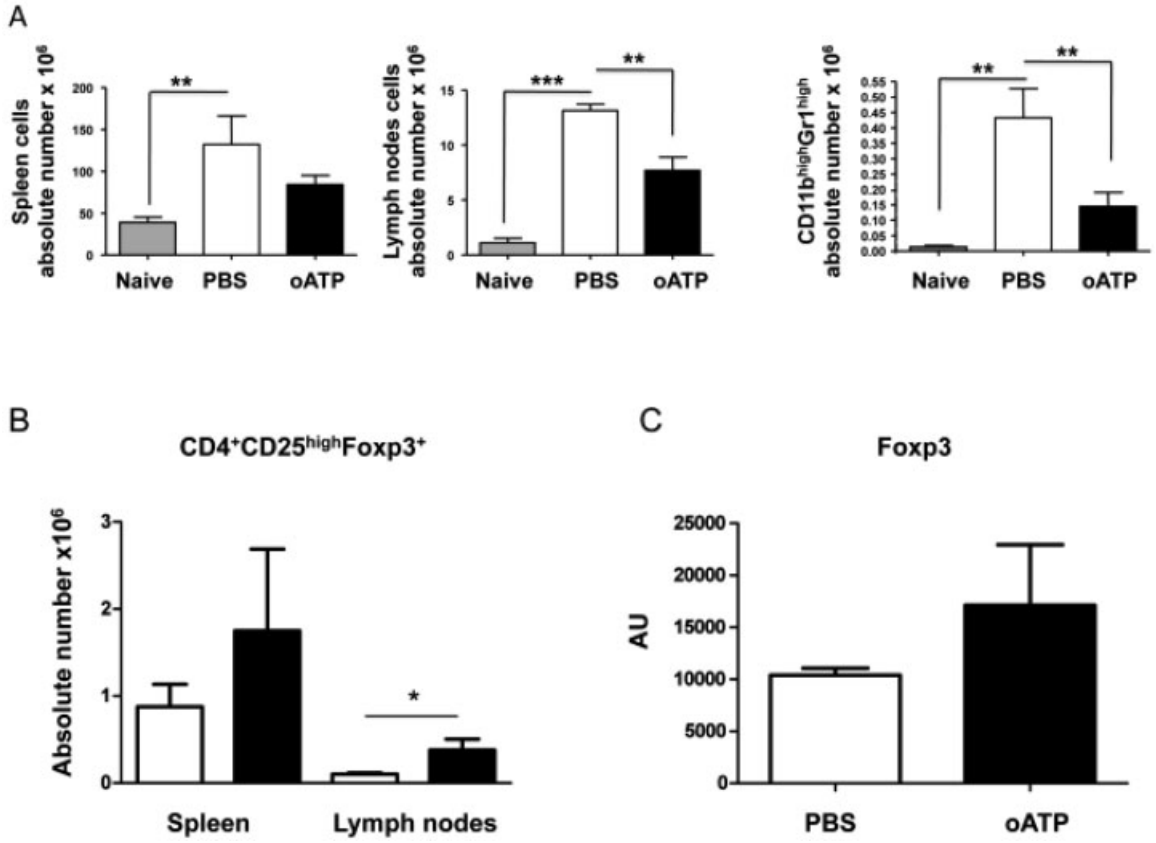

Figure 2. A, Total number of mononuclear cells in the spleens (left panel) and lymph nodes (middle panel) and number of CD11b^{high}Gr-1+ spleen cells (right panel) in mice without CIA (naive), mice with CIA treated with PBS, and mice with CIA treated with oxidized ATP (oATP). ** = $P < 0.01$; *** = $P < 0.001$, by one-way analysis of variance followed by Tukey's test. **B,** Absolute number of CD4+CD25^{high}FoxP3+ CD4 T cells in the spleens and lymph nodes of mice with CIA treated with PBS (open bars) or oxidized ATP (solid bars), enumerated by intracellular staining. * = $P = 0.0407$ by *t*-test. **C,** Results of reverse transcriptase–polymerase chain reaction analysis showing an abundance of FoxP3 transcripts in CD4+ T cells isolated from the lymph nodes of animals injected with PBS or oxidized ATP. Bars show the mean ± SEM. See Figure 1 for other definitions.

DBA/1J mice, 10 daily injections of oxidized ATP (100 μl of 3 m*M* solution) were administered intraperitoneally starting when the animals began to show clinical signs of disease and had a clinical score of ≥1.5. The treatment was well tolerated, and no systemic toxicity was observed (data not shown). Treatment with oxidized ATP resulted in a significant reduction in the clinical score, which measured inflammation of the digits and paw swelling (Figure 1A). A reduction in the clinical score for oxidized ATP–treated mice compared to that for PBS-treated mice was already evident after the first injection, and this reduction reached statistical significance in the second half of the treatment period. On day 10, the treatment was stopped, and a group of animals was followed up for 7 days to assess whether the ameliorative effect of oxidized ATP was maintained after treatment suspension. The significant reduction in clinical score observed at the end of the experimental period versus treatment initiation was maintained during this recovery period; variations in clinical score were unchanged (Figure 1A).

At the end of the treatment period (day 10), animals were killed and paws were removed for histologic analysis. P2X blockade induced a significant reduction in cartilage erosion, evaluated as proteoglycan content (Figure 1A). Notably, in mice treated with oxidized ATP, cartilage surfaces were structurally conserved compared to mice treated with PBS, which showed massive cellular infiltration and cartilage profile damage (Figures 1B–D).

In response to collagen immunization, higher numbers of cells were recovered from the spleens and draining inguinal lymph nodes of immunized compared to nonimmunized mice (Figure 2A). This cellular expansion was decreased in mice treated with oxidized ATP; the decrease was statistically significant in lymph nodes

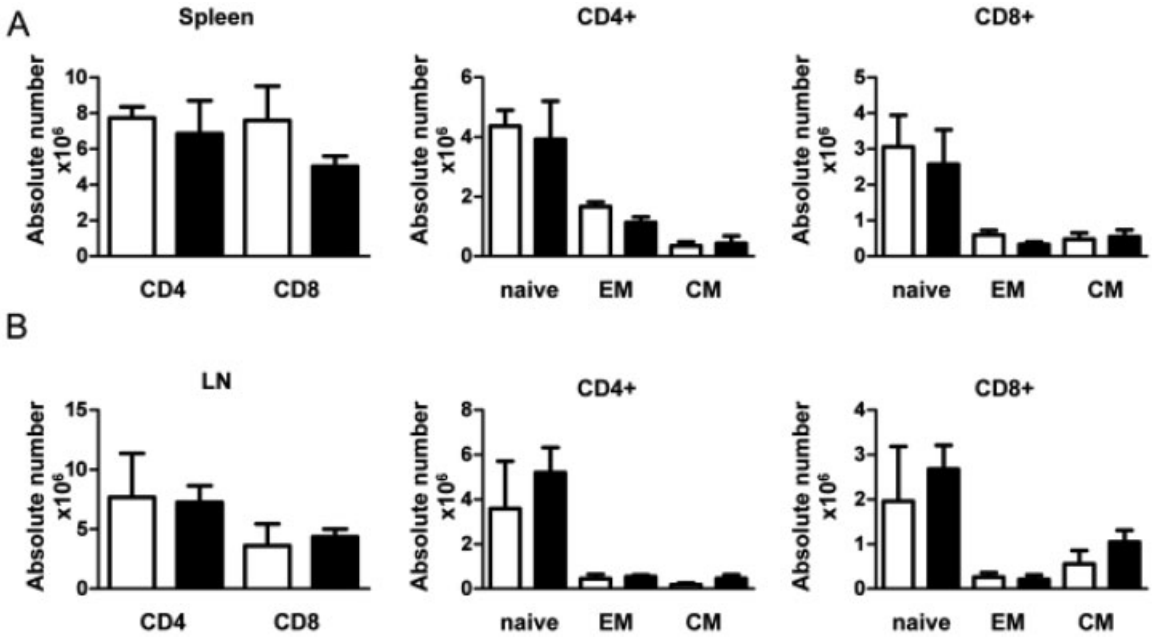

Figure 3. Absolute numbers of CD4 and CD8 T cells and of the CD4 and CD8 cell subsets CD44−CD62L+ (naive), CD44+CD62L− (effector memory [EM]), and CD44+CD62L+ (central memory [CM]) in the spleens (**A**) and lymph nodes (LNs) (**B**) of mice with CIA treated with PBS (open bars) or oxidized ATP (solid bars). Bars show the mean ± SEM. See Figure 1 for other definitions.

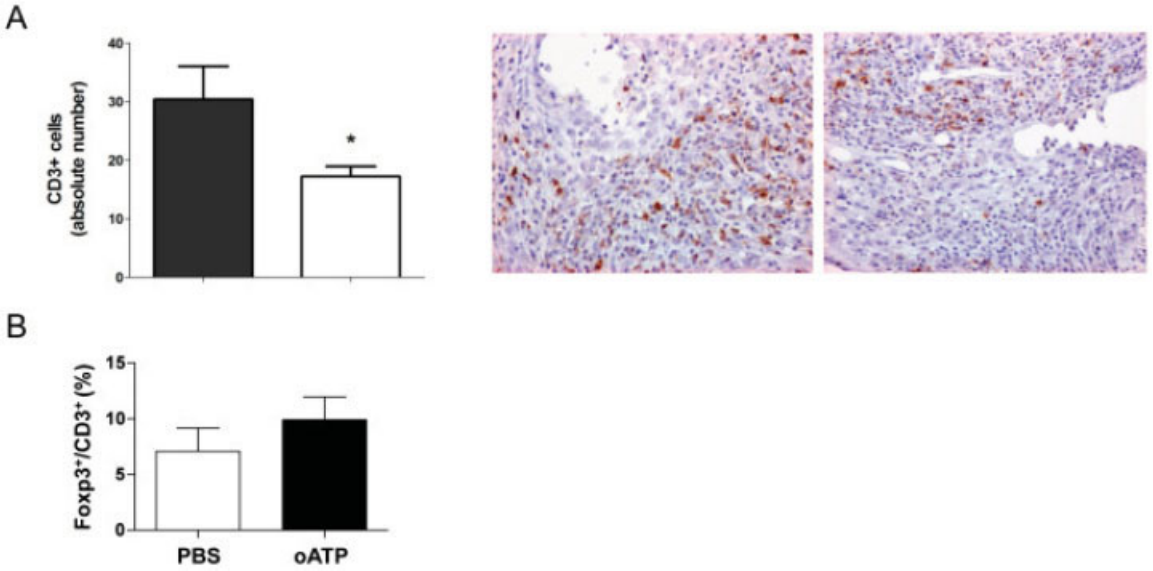

Figure 4. A, Absolute numbers of CD3+ cells in specimens from mice with CIA treated with PBS (solid bar) or oxidized ATP (oATP; open bar) (left panel), determined by CD3 immunostaining of formalin-fixed and paraffin-embedded acral portions of limbs from mice with CIA treated with PBS (middle panel) or oxidized ATP (right panel). Bars show the mean ± SEM. $* = P < 0.05$ versus PBS treatment, by one-way analysis of variance followed by Tukey's test. Original magnification × 40. **B,** Number of FoxP3+ cells relative to the total number of CD3+ cells infiltrating the joints in mice with CIA treated with PBS or oxidized ATP. For each slide, the synovial membrane and articular capsule were scanned at 100× magnification for identification of 6 immunoreactive foci. The number of FoxP3+ cells was defined at 200× magnification. Bars show the mean ± SEM. See Figure 1 for other definitions.

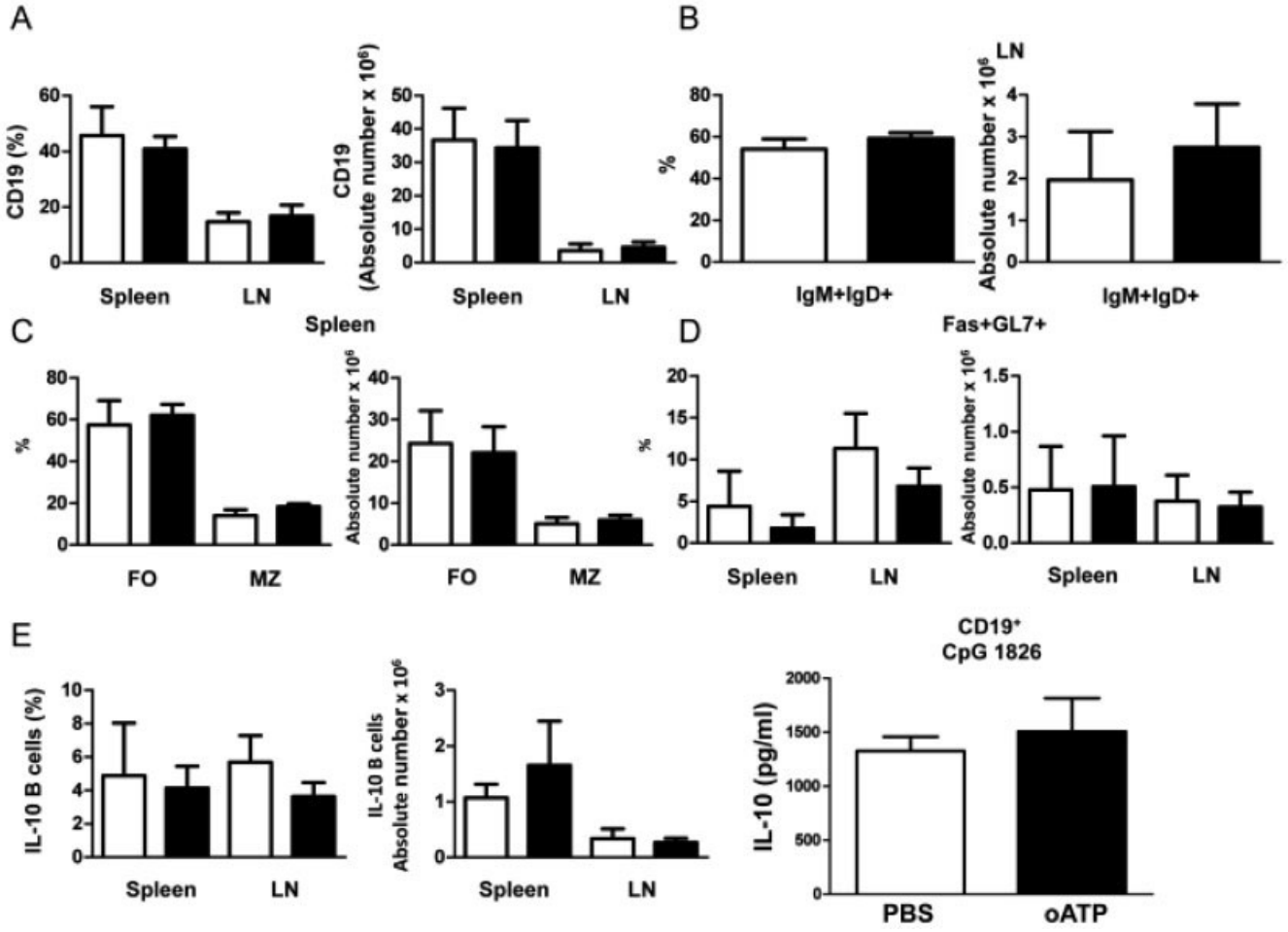

Figure 5. A–D, Percentages and absolute numbers of CD19+ B lymphocytes in the spleens and lymph nodes (LNs) (**A**), of mature (IgM+IgD+) B cells in the lymph nodes (**B**), of marginal zone (MZ; CD19+CD21+CD23−) and follicular (FO; CD19+CD21+CD23+) B cells in the spleens (**C**), and of germinal center (Fas+GL7+) B cells in the spleens and lymph nodes (**D**) of mice with CIA treated with PBS (open bars) or oxidized ATP (oATP; solid bars). **E,** Ex vivo analysis of interleukin-10 (IL-10)–secreting B cells from mice with CIA treated with PBS (open bars) or oxidized ATP (solid bars). Bars show the mean ± SEM. See Figure 1 for other definitions.

(Figure 2A). A feature of T cell–mediated immunopathology is the increase in immature and mature elements of the granulocyte lineage in the spleen due to enhanced granulopoiesis in the bone marrow, which is induced by inflammation mediators (13). Treatment with oxidized ATP resulted in a significant reduction in $CD11b^{high}$ $Gr\text{-}1^{high}$ mature granulocytes in the mouse spleen, thus suggesting the amelioration of this inflammation-related feature (Figure 2A).

No differences in the numbers of naive, effector memory, or central memory CD4+ or CD8+ T cells were observed in mice treated with oxidized ATP compared to mice treated with PBS (Figure 3). Also, cytokine-secreting cells were not affected by P2X blockade (data not shown). Nonetheless, we observed a significant increase in the absolute numbers of CD4+ $CD25^{high}$FoxP3+ Treg cells in the spleens as well as lymph nodes of mice treated with oxidized ATP compared to mice treated with PBS (Figure 2B). This observation was confirmed by real-time reverse transcriptase–polymerase chain reaction analysis showing an abundance of FoxP3 messenger RNA in CD4+ T cells isolated from mice treated with oxidized ATP compared to control mice injected with PBS. FoxP3 expression was significantly increased in CD4+ T cells upon P2X blockade in vivo, thus suggesting that the immunoregulatory suppressive function of Treg cells was initiated by oxidized ATP (Figure 2C).

To establish the extent of T cell infiltration in arthritic lesions, formalin-fixed and paraffin-embedded sections from mice treated with oxidized ATP or PBS were immunostained with CD3-specific antibody, and

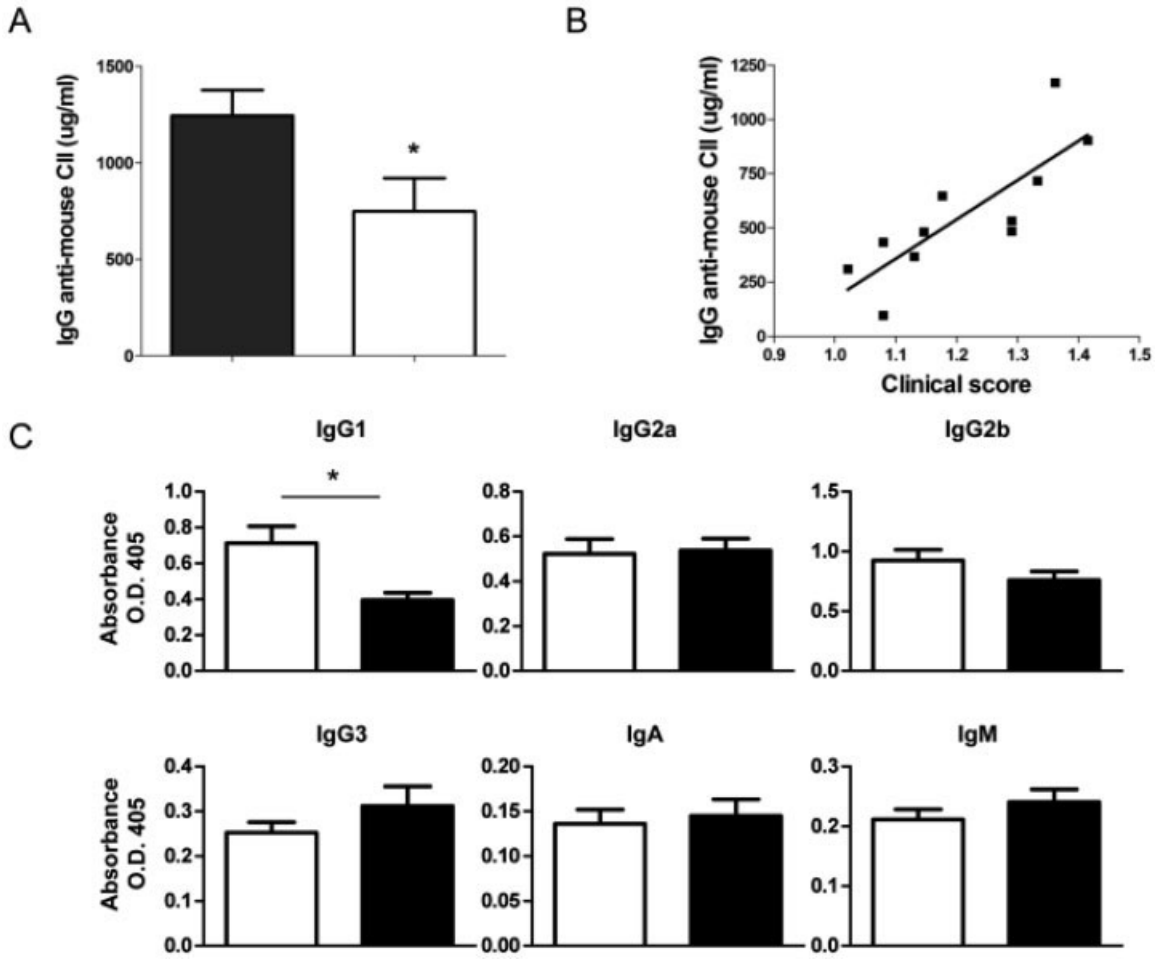

Figure 6. A, Levels of circulating anti–mouse type II collagen (CII) antibodies in mice with CIA treated with PBS (solid bar) or oxidized ATP (open bar), determined by enzyme-linked immunosorbent assay (for total IgG). After treatment with either PBS (n = 8) or oxidized ATP (n = 8), mice were killed, blood was collected, and concentrations of anti–mouse CII IgG were determined. $* = P < 0.05$ versus PBS treatment, by Student's t-test. **B,** Correlation between cumulative clinical score at the end of the treatment period and anti–mouse CII antibody titer in mice with CIA treated with oxidized ATP (n = 11) (F = 17.52; df numerator, df denominator = 1.000, 9.000; $P = 0.0024$). **C,** Absorbance for IgG subclass antibodies and IgA and IgM antibodies specific for mouse CII in mice treated with PBS (open bars) or oxidized ATP (solid bars). Sera were diluted 1:500. Bars in **A** and **C** show the mean ± SEM. $* = P = 0.04$, by Student's *t*-test. OD = optical density (see Figure 1 for other definitions).

each sample was scanned at 40× magnification; 3 immunoreactive hot spots were selected from within the articular/periarticular inflammatory lesions. For each hot spot, the number of CD3+ lymphocytes was counted. Oxidized ATP induced a significant decrease in CD3+ T cells infiltrating the synovial tissue of the periarticular space (Figure 4A). Since the frequency of Treg cells relative to conventional T cells is an important parameter for effective immunosuppression, we determined the number of FoxP3+ Treg cells in inflammatory lesions by staining with an anti-FoxP3 antibody. Interestingly, the ratio of Treg cells to total CD3 was increased by oxidized ATP, suggesting the development of more effective tissue-specific immunosuppression by P2X antagonism (Figure 4B).

The humoral autoimmune response has pathogenic relevance in the model of CIA used in this study (14). Thus, we evaluated the effect of P2X signaling blockade on the composition of mature B cell subsets. The representation of marginal zone B cells (CD19+ CD21+CD23−) and follicular B cells (CD19+CD21+ CD23+) in the mouse spleen was not modified by treatment with oxidized ATP (Figure 5C). Accordingly, mature B cells in the lymph nodes analyzed as CD19+

IgM+IgD+ were not altered in oxidized ATP–treated mice compared to PBS-treated control mice (Figure 5B). Notably, we observed a slight, although not significant, difference in the percentages of germinal center B cells (CD19+GL7+Fas+) in the spleens and lymph nodes of oxidized ATP–treated mice (Figure 5D).

Recent studies have shown that B cells can participate in peripheral immunoregulation through the secretion of IL-10 upon Toll-like receptor (TLR) stimulation (15). These cells were shown to interfere with the development of several autoimmune diseases, including experimental autoimmune encephalomyelitis and CIA. Thus, we analyzed the frequency of IL-10–secreting B cells in the spleens and lymph nodes of animals treated with oxidized ATP. Intracellular cytokine staining was performed after B cell stimulation with LPS, phorbol myristate acetate, and ionomycin in vitro for 5 hours. No differences in the percentages or absolute numbers of IL-10–secreting B cells were observed upon P2X blockade (Figure 5E). We quantitatively evaluated IL-10 secretion by B cells stimulated with CpG 1826, a TLR-9 agonist and potent inducer of IL-10 secretion by B cells (15). CD19+ B cells were stimulated with CpG 1826 (2.5 μg/ml) in vitro for 4 days, and IL-10 secretion was measured in the culture supernatant by ELISA. B cells isolated from mice treated with oxidized ATP secreted a similar amount of IL-10 as B cells from control mice treated with PBS, further supporting the notion of a lack of induction of IL-10–secreting B cells by oxidized ATP (Figure 5E).

We next evaluated total serum IgG specific for bovine and mouse CII by ELISA. Treatment with oxidized ATP significantly reduced serum concentrations of IgG autoantibodies specific for CII (Figure 6A), while levels of anti–bovine CII antibodies were not affected (data not shown). We also analyzed the IgG subclass antibodies and IgA and IgM antibodies specific for mouse CII. Only specific antibodies of the IgG1 isotype were significantly diminished by oxidized ATP, whereas specific antibodies of the IgG2a, IgG2b, IgG3, IgA, and IgM isotypes were unaltered (Figure 6C). Notably, a significant correlation between serum IgG autoantibody concentrations and clinical outcome was observed, indicating that the reduced severity of the disease was associated with a decrease in circulating IgG autoantibody levels (Figure 6B).

DISCUSSION

TCR stimulation results in an early increase in ATP synthesis and release, which acts as a crucial autocrine costimulus in CD4 T helper cell activation (9,10). Inhibition of purinergic P2X signaling results in diminished T cell proliferation and induction of a transcription program, which leads to T cell unresponsiveness to subsequent stimulation (anergy). In addition, purinergic $P2X_7$ antagonism results in more stable expression of FoxP3 in immunosuppressive Treg cells and facilitates de novo generation of adaptive Treg cells in the periphery (11). Taken together, these observations suggest that purinergic P2X antagonism might be useful in specific down-regulation of adaptive immune responses. Indeed, treatment of murine inflammatory bowel disease (IBD) and type 1 diabetes mellitus (DM) with oxidized ATP dramatically ameliorated the outcomes of these diseases (9). In the present study, we showed that therapeutic administration of oxidized ATP as a P2X antagonist was also effective in the treatment of CIA, an animal model of RA, in which B cells and innate immune system cells are more readily involved in disease pathogenesis than in IBD and type 1 DM.

With regard to histopathologic parameters, mice treated with oxidized ATP in the present study had lower synovial inflammation and cartilage erosion scores than mice treated with PBS. This observation is consistent with the results of a previous study, which showed that $P2X_7$-deficient mice had milder arthritis after they received anticollagen antibodies (8). Given that T cell infiltration plays an essential role in the pathogenesis of CIA (11), we evaluated T cell content in the arthritic joints of the mice. We found fewer CD3+ T cells and an increased Treg cell:total CD3+ cell ratio in joints from oxidized ATP–treated mice compared to PBS-treated mice, further supporting the notion of a crucial role of effector T cells in sustaining joint inflammation in CIA. Nonetheless, the beneficial effect of oxidized ATP in inflamed joints could also be related to a direct effect of the drug on synoviocytes. Indeed, the latter are involved in joint injury by producing inflammatory cytokines such as IL-6, and it has been demonstrated that human synoviocytes isolated from RA patients express functional $P2X_7$, whose activation promotes IL-6 secretion (16).

As a general consequence of immunization with adjuvants (17), as well as during chronic inflammation (13), hematopoiesis in the bone marrow is skewed toward granulopoiesis, and mature granulocytes are increased in the spleen. Analogously, under conditions of peripheral T cell–mediated immunopathology, mature and immature granulocytes are detected in the spleen (13). Treatment with oxidized ATP resulted in a reduction in the percentage of splenic mature granulocytes, showing that purinergic P2X antagonism might

modulate this effector mechanism of T cell–mediated immunopathology. The activation and function of neutrophils were recently shown to be controlled by an autocrine loop of ATP and purinergic signaling; therefore, P2X antagonism by oxidized ATP in CIA could also exert a direct effect on granulocytes.

CIA, the most commonly used model for studying antirheumatic drugs, reproduces many of the pathogenic mechanisms detected in human RA, including increased cellular infiltration, synovial hyperplasia, pannus formation, and erosion of cartilage and bone in the distal joints (3). Participation of T and B cells is required to initiate the disease, and their relative contribution needs to be further clarified. Germinal centers are found in a significant proportion of RA synovia, and high autoantibody titers indicate a poor prognosis of the disease (2).

In the present study, we showed that treatment with oxidized ATP resulted in a significant decrease in collagen-specific IgG autoantibodies. Notably, collagen-specific IgG levels correlated with the clinical scores of mice treated with oxidized ATP, suggesting a direct involvement of B cells as autoreactive plasma cells in the pathogenesis of CIA. It remains to be elucidated whether the effect reported on serum antibody levels is due to a direct effect of oxidized ATP on B cells or to a limited availability of T cell help. $P2X_7$ expression has been described in the human B cell line (18), and the role of B cells in CIA pathogenesis has already been hypothesized. The $\mu MT^{-}/^{-}$ mice, which lack B cells due to the disruption of the μ heavy chain transmembrane exon, are resistant to CIA induction (19). Furthermore, transfer of immune sera from arthritic mice induces severe inflammation (4). Finally, as proof of principle of the pathogenetic role of B cells in RA, treatment with rituximab, a chimeric B cell–depleting anti-CD20 antibody, has demonstrated that the depletion of B cells can substantially improve signs and symptoms as well as physical function in RA patients whose disease is not responsive to tumor necrosis factor α–blocking agents (20). Therefore, it can be assumed that B cells, as antibody-producing as well as antigen-presenting and cytokine-secreting cells, represent major contributors to RA pathogenesis.

In conclusion, we have shown that pharmacologic inhibition of P2X signaling can effectively ameliorate CIA by inhibiting peripheral inflammatory tissue destruction and the autoreactive humoral response. This effect was associated with the expansion of tissue-specific Treg cells. Therefore, pharmacologic targeting of the P2X signaling pathway may dampen excessive inflammation and cure inflammatory autoimmune diseases such as RA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Ardissone and Traggiai had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ardissone, Zaratin, Ladel, Martini, Grassi, Traggiai.

Acquisition of data. Ardissone, Radaelli, Ardizzone, Traggiai.

Analysis and interpretation of data. Ardissone, Radaelli, Gattorno, Grassi, Traggiai.

ROLE OF THE STUDY SPONSOR

Merck Serono SA was involved in the study design, the collection, analysis, and interpretation of the data, and the writing of the manuscript. Publication of this article was approved by Merck Serono SA. Drs. Ardissone and Zaratin are former employees of Merck Serono SA.

REFERENCES

1. Asquit DL, Miller AM, McInnes IB, Liew FY. Autoimmune disease: rheumatoid arthritis. Animal models of rheumatoid arthritis. Eur J Immunol 2009;39:2040–4.
2. Humby F, Bombardieri M, Manzo A, Kelly S, Blades MC, Kirkham B, et al. Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium. PLoS Med 2009;6:e1.
3. Holmdahl R, Klareskog L, Rubin K, Larsson E, Wigzell H. T lymphocytes in collagen II-induced arthritis in mice: characterization of arthritogenic collagen II-specific T-cell lines and clones. Scand J Immunol 1985;22:295–306.
4. Watson WC, Brown PS, Pitcock JA, Townes AS. Passive transfer studies with type II collagen antibody in B10.D2/old and new line and C57Bl/6 normal and beige (Chediak-Higashi) strains: evidence of important roles for C5 and multiple inflammatory cell types in the development of erosive arthritis. Arthritis Rheum 1987;30:460–5.
5. Oh S, Rankin AL, Caton AJ. $CD4^{+}CD25^{+}$ regulatory T cells in autoimmune arthritis. Immunol Rev 2010;233:97–111.
6. Nguyen LT, Jacobs J, Mathis D, Benoist C. Where FoxP3-dependent regulatory T cells impinge on the development of inflammatory arthritis. Arthritis Rheum 2007;56:509–20.
7. Drexler SK, Foxwell BM. The role of Toll-like receptors in chronic inflammation. Int J Biochem Cell Biol 2010;42:506–18.
8. Labasi JM, Petrushova N, Donovan C, McCurdy S, Lira P, Payette MM, et al. Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. J Immunol 2002;168:6436–45.
9. Schenk U, Westendorf AM, Radaelli E, Casati A, Ferro M, Fumagalli M, et al. Purinergic control of T-cell activation by ATP released through pannexin-1 hemichannels. Sci Signal 2008;1:ra6.
10. Yip L, Woehrle T, Corriden R, Hirsh M, Chen Y, Inoue Y, et al. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. FASEB J 2009;23:1685–93.
11. Schenk U, Frascoli M, Proietti M, Geffers R, Traggiai E, Buer J, et al. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. Sci Signal 2011;4:ra12.
12. Camps M, Ruckle T, Ji H, Ardissone V, Rintelen F, Shaw J, et al.

Blockade of PI3Kγ suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. Nat Med 2005;11:936–43.
13. Traggiai E, Casati A, Frascoli M, Porcellini S, Ponzoni M, Sanvito F, et al. Selective preservation of bone marrow mature recirculating but not marginal zone B-cells in murine models of chronic inflammation. PLoS One 2010;5:e11262.
14. Luross JA, Williams NA. The genetic and immunopathological processes underlying collagen-induced arthritis. Immunology 2001;103:407–16.
15. Lampropoulou V, Hoehlig K, Roch T, Neves P, Calderon Gomez E, Sweenie CH, et al. TLR-activated B cells suppress T cell-mediated autoimmunity. J Immunol 2008;180:4763–73.
16. Caporali F, Capecchi PL, Gamberucci A, Lazzerini PE, Pompella G, Natale M, et al. Human rheumatoid synoviocytes express functional P2X7 receptors. J Mol Med 2008;86:937–49.
17. Ueda Y, Kondo M, Kelsoe G. Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow. J Exp Med 2005;201:1771–80.
18. Lee DH, Park KS, Kong ID, Kim JW, Han BG. Expression of P2 receptors in human B-cells and Epstein-Barr virus-transformed lymphoblastoid cell lines. BMC Immunol 2006;7:22.
19. Svensson L, Jirholt J, Holmdahl R, Jansson L. B-cell-deficient mice do not develop type II collagen-induced arthritis (CIA). Clin Exp Immunol 1998;111:521–6.
20. Edwards JC, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nature Rev 2006;6:394–403.