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Guanine and inosine nucleotides/nucleosides suppress murine T cell activation

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ABSTRACT

Damaged tissues and cells release intracellular purine nucleotides, which serve as intercellular signaling factors. We previously showed that exogenously added adenine nucleotide (250 μM ATP) suppressed the activation of murine splenic T lymphocytes. Here, we examined the effects of other purine nucleotides/nucleosides on mouse T cell activation. First, we found that pretreatment of mouse spleen T cells with 250 μM GTP, GDP, GMP, guanosine, ITP, IDP, IMP or inosine significantly reduced the release of stimulus-inducible cytokine IL-2. This suppression of IL-2 release was not caused by induction of cell death. Further studies with GTP, ITP, guanosine and inosine showed that pretreatment with these nucleotides/nucleosides also suppressed release of IL-6. However, these nucleotides/nucleosides did not suppress stimulus-induced phosphorylation of ERK1/2, suggesting that the suppression of the release of inflammatory cytokines does not involve inhibition of ERK1/2 signaling. In contrast to ATP pretreatment at the same concentration, guanine or inosine nucleotides/nucleosides did not attenuate the expression of CD25. Our findings indicate that exogenous guanine or inosine nucleotides/nucleosides can suppress inflammatory cytokine release from T cells, and may be promising candidates for use as supplementary agents in the treatment of T cell-mediated immune diseases.

1. Introduction

Activation of T cells to generate an immune response is caused by the interaction of T cell receptors (TCRs) with peptide antigens bound to major histocompatibility complex (MHC) on antigen-presenting cells [1]. When TCRs are activated, calcium channels open in response to calcium depletion of the endoplasmic reticulum, leading to a persistent increase of intracellular Ca^{2+} concentration [2]. Signaling via routes such as the MAPK pathway [3] [4] activates transcription factors in the nucleus, and induces production of various cytokines [5]. Among them, the inflammatory cytokine interleukin-2 (IL-2) and the α chain of the IL-2 receptor (IL-2Rα), CD25 [6] [7], induce T cell proliferation via activation of various target genes [8].

ATP released from cells, as well as its metabolites, exert various physiological actions by binding with purinergic receptors on cell membranes. These purinergic receptors are classified into two subtypes, the ionotropic P2X1-7 receptors and the metabotropic P2Y1-14 receptors [9] [10]. Several groups, including ours, have shown that extracellular ATP is involved in T cell activation via activation of P2X7, P2X4 and P2Y6 receptors [11] [12] [13] [14] [15], and high concentrations of ATP cause cell death by binding to P2X7 receptor [16] [17]. Further, we previously showed that a lower concentration of exogenously added ATP (250 μM) inhibits the activation of T cells.

Guanine nucleotides are also involved in intracellular signal transduction and regulation of protein function. For example, guanosine decreases neuroinflammation and excitotoxicity, and has nutritional effects and neuroprotective properties on nerve cells [18]. Guanine nucleotides serve as mitogenic factors in neuronal and glial compartments. In addition, in mouse skeletal muscle cells, extracellular GTP (about 500 μM) induces intracellular calcium influx, leading to transduction of various signals [18], possibly via the P2 receptor. Also, suppression of T-cell proliferation by addition of guanosine triphosphate (GTP), guanosine or deoxyguanosine triphosphate (dGTP) has been reported, though the

Abbreviations: ConA, concanavalin A; ERK, extracellular signal-regulated kinase; IL, interleukin; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; TCR, T cell receptor.

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mechanism involved is unclear [19].

Inosine, formed by deamination of adenosine, is also released extracellularly under inflammatory conditions [20] [21]. Inosine has nutritional and neuroprotective effects in nerve cells, and induces degranulation of mast cells through activation of the A3 receptor [22]. It also has an anti-inflammatory effect, suppressing inflammatory cytokine release from mouse splenocytes and enhancing production of IL-10, an anti-inflammatory cytokine, by activating adenosine receptor. Clinical trials suggest that inosine may be a promising agent for the treatment of patients with multiple sclerosis [23]. However, little is known about the roles of inosine nucleotides (ITP, IDP, IMP) in T cells.

In this study, we show that guanine and inosine nucleotides suppress the production of IL-2 in activated T cells at the mRNA and protein levels, and we suggest that they may have potential as adjuncts to existing therapeutic agents for the treatment of immune-related diseases associated with T cell activation.

2. Materials and methods

2.1. Reagents

Anti-CD3ε monoclonal antibody (mAb) was purchased from R&D Systems (U.S.A.). Anti-CD28 mAb was from eBioscience (U.S.A.). Concanavalin A (ConA) were purchased from Sigma-Aldrich. GTP, GDP, GMP, guanosine, ITP, IDP, IMP, inosine and ATP were from Sigma (U.S.A.). ARL67156 were purchased from Sigma-Aldrich. Anti-ERK1/2 mAb and anti-phospho-ERK 1/2 (Thr^{202}/Tyr^{204}) mAb and other secondary Abs were obtained from Cell Signaling Technology (U.S.A.). All other chemicals used were of the highest purity available.

2.2. Animals

Male BALB/c mice were purchased from Sankyo Labo Service (Japan) and used at 6–8 weeks of age. They were housed in plastic cages with paper chip bedding and bred in rooms kept at a temperature of 23 ± 2 °C and a relative humidity of 55 ± 10% under a 12 h light–dark cycle. They were allowed free access to tap water and normal diet, CE-2 (CLEA Co). The mice were treated and handled according to the Tokyo University of Science's institutional ethical guidelines for animal experiments and with the approval of Tokyo University of Science's Institutional Animal Care and Use Committee (permit number S17007).

2.3. Preparation of splenic lymphocytes

Splenic lymphocytes were prepared as described previously [24]. In brief, splenocytes were isolated from spleen of BALB/c mice, and were purified by means of hemolysis. Cells were washed twice with complete RPMI1640 medium and re-suspended in RPMI1640-based buffer [17]. To remove adherent cells such as macrophages and dendritic cells, splenocytes were incubated for 1.5 h in a plastic cell-culture plate in an atmosphere of 5% CO_2/95% air at 37 °C. Non-adherent cells, mainly lymphocytes, were collected and washed once with RPMI1640-based buffer.

2.4. Cytokine production

We prepared culture supernatants of splenic lymphocytes and measured the concentrations of IL-2 and IL-6 by ELISA as described previously [24].

2.5. Evaluation of cell damage

Cell damage was quantified in terms of released lactate dehydrogenase (LDH) activity, as described previously [17] [24]. Splenocytes were incubated with adenine nucleotides for 6 h, and the culture supernatant were collected. Release of LDH into the cell culture supernatant was quantified with a Cytotoxicity Detection Kit (Roche Applied Science, Penzberg, Germany), according to the supplied instructions. LDH release is expressed as a percentage of the total content determined after lysing an equal amount of cells with 1% Triton X-100.

2.6. Flow cytometry

Expression of CD25 in CD4 T cells was analyzed by flow cytometry based on our previous report [24]. In brief, splenocytes (6.0×10^6 cells/well) were stimulated with 5 μg/mL plate-bound anti CD3ε mAb and soluble 5 μg/mL anti CD28 mAb in a 24-well plate in RPMI 1640 medium containing 10% heat-inactivated FBS, 100 units/ml of penicillin, and 100 μg/mL of streptomycin in an atmosphere of 5% CO_2, 95% air at 37 °C for 24 h. Splenocytes (3×10^5 cells) were collected by centrifugation (4 °C at 300 × g) and the supernatant was discarded. The cell pellet was washed twice with RPMI1640-based buffer and re-suspended in the same buffer. PECy5-conjugated rat anti-mouse CD4 (1 μL) (BD Pharmingen) and PE-conjugated anti-mouse CD25 (1 μL) (eBioscience) were added to 50 μL of the cell suspension and the mixture was incubated at room temperature for 30 min in the dark. The cells were then washed twice in RPMI1640-based buffer and immediately subjected to flow cytometry (FACSCaliber cytometer, Becton, Dickinson and Co. U.S.A.). The data were analyzed by FlowJo software (FlowJo, LCC).

2.7. Immunoblotting

Phosphorylation of ERK1/2 after stimulation of lymphocytes with ConA was analyzed by immunoblotting as described previously [24].

2.8. Measurement of IL-2 mRNA expression

Expression of IL-2 mRNA after stimulation of lymphocytes was analyzed by real-time RT-PCR as described previously [24].

2.9. Statistics

Results are expressed as mean ± SE. The statistical significance of differences between control and other groups was calculated by using Dunnett's test with the Instat version 3.0 statistical package (GraphPad Software, San Diego CA). The criterion of significance was $P < 0.05$.

3. Results and discussion

Inflammatory diseases generally involve massive release of cytokines; for example, IL-2 released from T cells contributes to the pathology of rheumatoid arthritis and multiple sclerosis. In this work, we used a BALB/c mouse splenocyte model stimulated with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb for 24 h to examine the effects of exogenously added purine nucleotides/nucleosides on cytokine release.

First, we examined the dose-dependence of the effect of guanosine and inosine nucleotides/nucleosides on IL-2 release into the supernatant of the splenocytes. Pretreatment with 100–500 μM GTP, GDP, GMP, guanosine, ITP, IDP, IMP or inosine significantly and concentration-dependently inhibited IL-2 production (Fig. 1A–H,