Fall in oxygen tension of culture medium stimulates the adenosinergic signalling of a human T cell line

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Received: 14 November 2011 / Accepted: 1 February 2012 / Published online: 14 February 2012
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Abstract We examined the short-course expression of various parameters involved in the adenosinergic signalling of a human T cell line during in vitro decrease of the medium culture oxygen tension mimicking in vivo hypoxia. Fall of 92 mmHg in oxygen tension of culture medium induced in CEM, a CD4⁺ human T cell line, a continuous production of hypoxia-inducing factor-1 α with a plateau value at 9 h, a rapid increase in adenosine production peaking at 3 h and a decrease in adenosine deaminase peaking at 6 h. The adenosine A_{2A} receptor (A_{2A}R) protein level of CEM cells was enhanced with a peak at 6 h. Intracellular 3',5'-cyclic adenosine monophosphate accumulated in CEM cells with a maximal level at 9 h. These results show that a human-cultured T cells line can upregulate its own adenosine production and A_{2A}R expression during exposure to acute hypoxia. Hypoxia-increased stimulation of the adenosinergic signalling of T cells may have immunosuppressive properties and, consequently, A_{2A}R agonists may have therapeutic relevance.

Keywords Hypoxia · HIF-1 α · Adenosine · A_{2A}R · T cells

Introduction

Oxygen deprivation, ranging from hypoxia to anoxia, is a physiologic stimulus which occurs transiently in vivo within inflamed sites and growing tumours [1]. According to a previously proposed model [2], inflammatory damage to

blood vessels induces low oxygen supply and hypoxia-driven increase of extracellular adenosine which accumulates because of altered expression of several key enzymes involved in the adenosine triphosphate (ATP) metabolism [3]. Adenosine, an endogenous purine nucleoside that is produced intra- or extracellularly by injured tissue cells, binds to four types of receptor, namely A₁, A_{2A}, A_{2B} and A₃, which are members of the G protein-coupled family of receptors [4]. It is well established that extracellular adenosine released from endothelium cells stops T cell proliferation, expansion and secretion of pro-inflammatory cytokines by stimulation of their A_{2A} surface receptors (A_{2A}R) that trigger a rise in intracellular 3',5'-cyclic adenosine monophosphate (cAMP) through activation of adenylyl cyclase [5]. This pathway has potential physiopathological and therapeutic consequences to downregulate the immune response due to spontaneous accumulation of adenosine at the T cell surface [6]. At inflamed site, recruited or resident T lymphocytes, as endothelium cells, are exposed to reduced oxygen pressure but little is known about the hypoxia effect on their own adenosinergic regulation. Because adenosine can be released from a single cell in an autocrine way [7] or be self surface produced by ectoenzymes [8], we have tested here whether in vitro exposure to reduced oxygen tension (mimicking hypoxia) can upregulate per se both the adenosine production and A_{2A}R expression of a cultured T cell line.

Materials and methods

Cell culture conditions

CEM, a CD4⁺ human T lymphoma cell line endogenously expressing A_{2A}R [9], were purchased from the American

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Tissue Culture Collection and grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM Hepes, 10% foetal calf serum and penicillin/streptomycin (100 U/ml, 100 µg/ml) in incubator at 37°C under 5% CO₂ and 95% air. When cells reached 70% confluence, they were either exposed to normal or hypoxia culture medium conditions. In normal conditions, 90 ml of culture medium containing 1×10^6 cells/ml were set into a 250-ml sterile infusion bottle under sterile laminar air flow at atmospheric pressure before closure of the cap. Hypoxia *in vitro* conditions was achieved by slowly flushing the 90-ml culture medium with 0.22 µm-filtered N₂ for 60 min into a 250-ml sterile infusion bottle with a needle inserted through a butyl rubber stopper transiently equipped with an air hole to expulse O₂. Flushed culture medium was then allowed to equilibrate at positive N₂ pressure in the capped bottle overnight at 37°C before cell exposure. Samples of cells cultured in normal and hypoxia conditions were harvested at the onset and after 3, 6, 9 and 12-h incubation at 37°C by the syringe puncture through the cap. The samples were immediately used for cell counting, pO₂, pH and lactate measurements. For cell parameters, the samples were distributed into tubes and centrifuged at 4°C (1,500×g, 15 min) to discard supernatant. Cell pellets were kept frozen at –80°C until use.

Viable cell counting

At 3-h time intervals, the CEM cells in culture bottle were suspended and withdrawn by a syringe for cell counting. Viable cells count was performed using the trypan blue dye exclusion method and a Malassez haemocytometer.

pO₂, pH and lactate measurements

Two millilitres of culture medium from bottles in normal and hypoxia conditions was taken into a dedicated syringe to monitor pH, pO₂ and lactate levels using a RAPIDSytem blood gas analyser (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA).

Adenosine assay

The technique used was previously described [10] with minor modifications. Intracellular adenosine concentration was measured using high-performance liquid chromatography equipped with a diode array detector (Chromsystems, Munich, Germany). Frozen cells (5×10^6) were mixed with 1 ml of cold stop solution (0.2 mM dipyridamole, 4.2 mM Na₂EDTA, 5 mM (9-erythro-3 nonyl) adenine, 79 mM α-β methylene adenosine 5'-diphosphates and 1 IU/ml heparin sulphate in NaCl 0.9%) to prevent adenosine degradation. Aliquot of 500 µl was injected into a 1-ml loop and eluted

with a methanol gradient (0–30% v/v) in ultrapure water containing 0.1% trifluoroacetic acid for 37 min on a LiChrospher C18 column (Merck, Darmstadt, Germany). Adenosine was identified by its elution time and spectrum. Measurement was made by comparison of peak areas with those given by standards. The intra-assay and inter-assay coefficients of variation ranged from 1% to 3%.

ADA activity

Adenosine deaminase (ADA) activity measurement was carried out as previously described [11]. Briefly, 1×10^6 frozen cells were lysed in 500 µl of ultrapure water. One hundred twenty-five microlitres of lysate was mixed with 750 µl of 28 mM adenosine and 125 µl of 7% BSA in 0.9% NaCl and incubated for 36 min at 37°C. The reaction was stopped by cold immersion at 4°C, and the quantity of ammonium formed was determined with a Synchron LX 20 analyser (Beckman Coulter Inc, Villepinte, France). The intra-assay and inter-assay coefficients of variation ranged from 3% to 5%.

cAMP assay

Intracellular cAMP level was measured on 1×10^6 frozen cells with the Amersham Biotrak kit (GE Healthcare Life Sciences, Buckinghamshire, UK) by a competitive enzyme immunoassay according to the manufacturer's instructions.

HIF-1α and A_{2A}R measurements

Hypoxia-inducing factor-1α (HIF-1α) production and A_{2A}R expression were assessed by quantitative Western blotting procedure as previously described [9]. Briefly, frozen cell pellets were solubilised with 4% SDS aqueous solution by 30-min sonication at 47 kHz. After protein quantification by microBCA (Pierce Biotechnology, Rockford, IL, USA), 20 µg of cell solubilise were diluted in 65.2 mM Tris–HCl buffer, pH 8.3, containing 10% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol and subjected to standard electrophoresis procedure in Mini Protean II system (Bio-Rad, Hercules, CA, USA). Separated proteins in 12% acrylamide minigel were transferred onto a PVDF membrane. Blotted membrane was placed into the blot holder of the SNAP i.d. protein detection system (Millipore, Billerica, MA, USA), saturated with non-fat dried milk and incubated 20 min with the appropriately diluted mouse monoclonal antibody, anti-A_{2A}R (Adonis) [12] and anti-HIF-1α (clone 241809, R&D Systems, Minneapolis, MN, USA). Blots were visualised by horseradish peroxidase labelled anti-mouse IgG Fab-specific antibodies and enhanced chemiluminescent substrate (SuperSignal West Femto, Pierce Biotechnology, Rockford, IL, USA) using a Kodak Image Station 440CF

(Eastman Kodak Company, Rochester, NY, USA). The staining intensities of the bands were densitometrically measured with the public domain NIH Image software developed at the US National Institutes of Health.

Results and discussion

In vitro hypoxia conditions

The non-physiological relevance of classic in vitro culture conditions was particularly emphasised for hypoxia studies that obviously can only be done in vivo like most of physiopathological experiments [13, 14]. However, in vitro cell culture is required for testing comportment of unique types of cells in special conditions deprived of influence of their natural environment. To mimic at best the in vivo process of hypoxia by using in vitro cultured T cells under 5% CO₂ and 95% air (i.e. hyperoxia conditions), the culture medium was flushed with N₂ to obtain a 92-mmHg fall in oxygen tension that shift from hyperoxia (~145 mmHg) to moderate hypoxia (~53 mmHg) in vitro conditions, and we postulated that this

change was representative of a similar shift which can occur in vivo from normal oxygen tension in blood vessels with circulating lymphocytes (40–100 mmHg) to anoxia or severe hypoxia conditions for tissue lymphocytes at inflamed site (4–20 mmHg). So, in this study, we used the terms of normal and hypoxia conditions for comparing our in vitro conditions. Figure 1a shows that pO₂ remained stable throughout the 12-h experiment both in normal (145.5±5.6 mmHg) and hypoxia (53.4±9.2 mmHg) in vitro conditions. Figure 1b shows that pH of culture medium gradually diminished over the time in both conditions, but hypoxia conditions systematically induced a 0.2±0.04 pH increase of culture medium. This rise of pH was due to the flush of N₂ that lowered the CO₂ as the O₂ in the culture medium (not shown). However, pH values always remained compatible with cell culture conditions along the 12-h incubation. Effectively, as shown in Fig. 1c, CEM cells continuously grow to reach a plateau value at 9 h, and the number of viable cells remained unchanged throughout the experiment both in normal and hypoxia in vitro conditions. Furthermore, blue dead cells were never observed during the 12-h experiment in both conditions, and longer timings were excluded to avoid cell death in hypoxia

Fig. 1 Effect of acute hypoxia on CEM cell culture. CEM cells were seeded in sterile bottles under normal (N) and hypoxia (H) conditions for 12 h. Medium with cultured cells were sampled at the onset (0) and 3, 6, 9 and 12-h incubation. Data shown are the temporal profile in the

culture medium of oxygen pressure (a), pH (b), cell number (c) and lactate production (d). Results are mean ± SD of triplicates. *p < 0.05 for hypoxia value compared to normal value by *t* test

conditions. Under aerobic conditions, the source of cellular energy is glycolysis coupled to oxidative phosphorylation. During glycolysis, glucose is converted to pyruvate with the production of ATP. Then, pyruvate enters the tricarboxylic acid cycle to produce electrons kept by oxygen through the mitochondrial respiratory chain. This chain of electron transport reactions produces ATP through the action of ATP synthase and this is the oxidative phosphorylation [15]. Under hypoxia conditions, glucose is converted to pyruvate which does not reach mitochondria and accumulates in cytoplasm. As a result, pyruvate is reduced in lactate and released from the cell. Figure 1d shows that CEM cells used glycolysis as their main source of energy irrespective of the pO_2 in culture medium because they continuously produced lactate in normal and hypoxia conditions. However, we noticed that hypoxia increased the anaerobic glycolysis in CEM cells by significantly increasing the lactate production in the culture medium at 9 and 12 h. This is in agreement with previous observations that, after stimulation, the glucose catabolism in immune cells increased [16] and that inhibitors of glycolysis impaired the function of immune cells whereas inhibitors of oxidative phosphorylation did not [17–19]. Our results also fit nicely with a recent report that concludes to a role of hypoxia-induced adenosine via $A_{2A}R$ in high glucose and lactate plasma levels [20].

Hypoxia-adenosinergic mechanism

During hypoxia, ATP is dephosphorylated to adenosine via endo-apyrase and endo-5'-nucleotidase, and adenosine kinase salvage pathway is suppressed leading to increased intracellular adenosine levels. Extracellular ATP is directly catabolized into adenosine by ecto-apyrase (CD39) and ectonucleotidase (CD73) cell membrane enzymes. Adenosine can be degraded into inosine by ADA localised inside the cell or linked to CD26 at the cell surface. Adenosine level in and out of the cell is also dependant of equilibrative nucleoside transporters. Under hypoxia conditions, extracellular adenosine accumulates due to decreased cell uptake by transcriptional repression of transporters, and thus, adenosine stimulates the $A_{2A}R$ to produce cAMP. HIF-1 α is the oxygen-regulated subunit of the transcription factor heterodimer HIF-1. Under hypoxia, HIF-1 α is stabilised and can dimerize with the constitutively expressed HIF-1 β to coordinate the transcription of genes involved in adenosine metabolism and transport (for a review, see [21]). Figure 2 shows the temporal profile of the production or expression of five hypoxia-related parameters: HIF-1 α , adenosine, ADA, $A_{2A}R$ and cAMP levels in CEM cells. Levels of all these products changed with time in hypoxia conditions showing various temporal profiles. Figure 2a shows that HIF-1 α is immediately produced upon hypoxia conditions and raised a plateau value at 9 h. Based on recent literature

[22], we suggest that hypoxia allowed HIF-1 α to repress the transcriptional activation of adenosine kinase. Consequently, adenosine rapidly increased, peaking as soon as 3 h (Fig. 2b), and conversely, ADA continuously decreased until 6 h and remained almost stable up to 12 h (Fig. 2c). In turn, $A_{2A}R$ expression was upregulated peaking at 6 h and rapidly returned to normal values (Fig. 2d). Finally, cAMP accumulated with a peak at 9 h (Fig. 2e). Adenosine has immunomodulatory properties on $A_{2A}R$ [23], and as expected in hypoxia conditions, we observed a rise in adenosine intracellular T cell level which was probably representative of the adenosine level released by the cells in the culture medium (not tested). The observed decrease of total (cytoplasmic and cell surface CD26 associated) ADA activity is in agreement with the enhancement of adenosine metabolism and fits very well with previous studies which revealed inhibition of ADA and adenosine kinase as a metabolic adaptation to elevated adenosine levels in rat pheochromocytoma (PC12) cells chronically exposed to hypoxia [7]. High adenosine levels with decreased ADA activity were also observed in mononuclear cells of hemodialysed patients with immune defect [11]. In contrast, hypoxia was reported to increase total and cell surface ADA protein level and enzymatic activity in cultured endothelial cells [24]. However, the ADA rise was evidenced in a 12–72-h range of hypoxia exposure but not for a lesser timing (3–12 h) as reported here. We have not extended hypoxia exposure of T cells in our method because cells started to die after 12 h in hypoxia conditions. We can only speculate that ADA first decreased and then increased in a second time (after 12 h) by feedback regulation to counteract excessive adenosine production as suggested by the virtually V-shaped curve of ADA (Fig. 2c) and the weak level of adenosine at 12-h hypoxia exposure (Fig. 2b). Alternatively, because ADA was only tested for its enzymatic activity, it was possible that ADA protein expression immediately increased (as soon as 3 h) but its enzymatic activity was transiently inhibited under hypoxia conditions by unknown regulatory mechanism. However, the observed rise both in adenosine production and $A_{2A}R$ expression may explain the increased level of intracellular cAMP was able to inhibit the effector functions of T cells [25]. These results support the view that the increase in extracellular adenosine in inflamed tissue microenvironment is due to local hypoxia [26]. They further indicate that hypoxia upregulates adenosine production and $A_{2A}R$ expression in T cells which are key partners of the immune system.

Clinical relevance and therapeutic implications

On T cells, $A_{2A}R$ are the predominant adenosine receptor subtype [25, 27]. The consensus emerging from numerous recent studies using adenosine receptor knockout mice is

Fig. 2 Effects of acute hypoxia on five adenosinergic parameters in CEM cells. Experimental conditions were as in Fig. 1. Data shown are the temporal production/expression in CEM cells of HIF-1 α (a), adenosine (b), ADA (c), A_{2A}R (d) and cAMP (e). Results (mean $\pm$ SD of triplicates) are expressed as percentage of value in hypoxia conditions relative to value in normal conditions. Representative Western blots of

triplicates for HIF-1 α production and A_{2A}R expression are inserted in a and d, respectively. The mean basal values $\pm$ SD at the onset (time 0) for adenosine production, ADA activity and cAMP level are $1.01 \pm 0.03 \mu\text{M}/5 \times 10^6$ cells, $34.7 \pm 1.2 \text{ IU}/10^6$ cells and $103.6 \pm 4.6 \text{ fmol}/10^6$ cells, respectively

that A_{2A}R is the major receptor subtype in dictating lymphocyte responses [28]. A_{2A}R agonists inhibit T cell activation by inducing intracellular cAMP production, and genetic deficiency or pharmacological antagonism of A_{2A}R increase inflammatory tissue damage. In agreement with this reasoning, it was previously reported in experimental mouse model of induced T cell-dependent acute hepatitis that A_{2A}R-deficient mice are much more sensible to liver damage and exhibit high pro-inflammatory cytokines levels compared to wild-type mice [29]. In the same model, circulating adenosine increases in mice exposed to hypoxia which become resistant to liver damage [30]. Considering these data, the immunosuppressive effect of hypoxia would be of

potential relevance in vivo to reduce tissue damages and loss of vital functions during inflammatory diseases. In contrast, hyperoxia appears deleterious especially for patients with pulmonary inflammation by weakening of A_{2A}R signalling. Supporting this point is the feature that oxygenation of mice severely exacerbates inflammatory lung damage and even causes death in the majority of mice whereas hypoxia suppresses lung damage by the A_{2A}R-dependent mechanism [31]. When oxygen supplementation is required as for respiratory distressed patients, our results provide additional arguments for administration of A_{2A}R agonists to restore protection by upregulating the adenosinergic immunosuppressive pathway as recently proposed

[6]. In this regard, Adonis, the newly developed agonist-like monoclonal antibody [12] that inhibits the cell growth and upregulates the A_{2A}R expression of CEM T cells [9] would be candidate as a therapeutic drug.

Conclusion

Accumulating evidences suggest that adenosine–A_{2A}R pathway is involved in immune suppression via T regulatory cells [26]. T cells with regulatory phenotype (CD4⁺ CD25⁺) are controlled by hypoxia via HIF-1 α [32] and produce themselves extracellular adenosine that contributes to A_{2A}R-mediated immunosuppression [8]. These assertions were hampered by the lack of reports on the complete sequence of hypoxia-related adenosine events on a same cell at a same time. The in vitro study reported here shows that a T cell line cultured alone without any other cells can detect a significant (92 mmHg) decrease in pO₂ of culture medium by producing HIF-1 α to upregulate the expression of its own A_{2A}R which are self-activated by auto-production of adenosine. We reasonably suggest that this finely tuned T cell mechanism occurs in vivo as part of immune regulation. Such a regulatory mechanism may auto-stop T cells hyperproducing adenosine under hypoxia environment in absence or deficit of A_{2A}R on other surrounding cells. In contrast, in conditions of low adenosine levels relative to the A_{2A}R cell surface expressed, administration of A_{2A}R agonists may be required to help deficient T cells to weaken inflammatory damages.

Acknowledgements Y. By is a recipient of a grant from the Assistance Publique, Hôpitaux de Marseille, France.

References

- Harris AL (2002) Hypoxia—a key regulatory factor in tumour growth. *Nat Rev Cancer* 2:38–47
- Sitkovsky MV, Ohta A (2005) The ‘danger’ sensors that STOP the immune response: the A₂ adenosine receptors? *Trends Immunol* 26:299–304
- Gorlach A (2005) Control of adenosine transport by hypoxia. *Circ Res* 97:1–3
- Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 53:527–552
- Sitkovsky M, Lukashev D (2005) Regulation of immune cells by local-tissue oxygen tension: HIF1 α and adenosine receptors. *Nat Rev Immunol* 5:712–721
- Ohta A, Sitkovsky M (2009) The adenosinergic immunomodulatory drugs. *Curr Opin Pharmacol* 9:501–506
- Kobayashi S, Zimmermann H, Millhorn DE (2000) Chronic hypoxia enhances adenosine release in rat PC12 cells by altering adenosine metabolism and membrane transport. *J Neurochem* 74:621–632
- Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, Kuchroo VK, Strom TB, Robson SC (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204:1257–1265
- By Y, Durand-Gorde JM, Condo J, Lejeune PJ, Fenouillet E, Guieu R, Ruf J (2010) Monoclonal antibody-assisted stimulation of adenosine A_{2A} receptors induces simultaneous downregulation of CXCR4 and CCR5 on CD4⁺ T-cells. *Hum Immunol* 71:1073–1076
- Guieu R, Sampieri F, Bechis G, Halimi G, Dussol B, Berland Y, Sampol J, Rochat H (1999) Development of an HPLC/diode array detector method for the determination of human plasma adenosine concentrations. *J Liq Chromatogr* 22:1829–1841
- Dussol B, Fenouillet E, Brunet P, Purgus R, Sauze N, Carrega L, Mercier L, Zouher I, Bechis G, Berland Y, Guieu R (2004) Kinetic study of adenosine concentrations and the expression of adenosine deaminase in mononuclear cells during hemodialysis. *Kidney Int* 66:1640–1646
- By Y, Durand-Gorde JM, Condo J, Lejeune PJ, Mallet B, Carayon P, Guieu R, Ruf J (2009) Production of an agonist-like monoclonal antibody to the human A_{2A} receptor of adenosine for clinical use. *Mol Immunol* 46:400–405
- Atkuri KR, Herzenberg LA, Niemi AK, Cowan T, Herzenberg LA (2007) Importance of culturing primary lymphocytes at physiological oxygen levels. *Proc Natl Acad Sci U S A* 104:4547–4552
- Caldwell CC, Kojima H, Lukashev D, Armstrong J, Farber M, Apasov SG, Sitkovsky MV (2001) Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. *J Immunol* 167:6140–6149
- Saraste M (1999) Oxidative phosphorylation at the fin de siècle. *Science* 283:1488–1493
- Greiner EF, Guppy M, Brand K (1994) Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. *J Biol Chem* 269:31484–31490
- O’Flaherty JT, Kreutzer DL, Showell HJ, Ward PA (1977) Influence of inhibitors of cellular function on chemotactic factor-induced neutrophil aggregation. *J Immunol* 119:1751–1756
- Simchowicz L, Mehta J, Spilberg I (1979) Chemotactic factor-induced generation of superoxide radicals by human neutrophils: effect of metabolic inhibitors and antiinflammatory drugs. *Arthritis Rheum* 22:755–763
- MacDonald HR, Koch CJ (1977) Energy metabolism and T-cell-mediated cytotoxicity. I. Synergism between inhibitors of respiration and glycolysis. *J Exp Med* 146:698–709
- Maeda T, Koos BJ (2009) Adenosine A₁ and A_{2A} receptors modulate insulinemia, glycemia, and lactatemia in fetal sheep. *Am J Physiol Regul Integr Comp Physiol* 296:R693–R701
- Eltzschig HK (2009) Adenosine: an old drug newly discovered. *Anesthesiology* 111:904–915
- Morote-Garcia JC, Rosenberger P, Kuhlicke J, Eltzschig HK (2008) HIF-1-dependent repression of adenosine kinase attenuates hypoxia-induced vascular leak. *Blood* 111:5571–5580
- Gomez G, Sitkovsky MV (2003) Differential requirement for A_{2A} and A₃ adenosine receptors for the protective effect of inosine in vivo. *Blood* 102:4472–4478
- Eltzschig HK, Faigle M, Knapp S, Karhausen J, Ibla J, Rosenberger P, Odegard KC, Laussen PC, Thompson LF, Colgan SP (2006) Endothelial catabolism of extracellular adenosine during hypoxia: the role of surface adenosine deaminase and CD26. *Blood* 108:1602–1610
- Huang S, Apasov S, Koshiba M, Sitkovsky M (1997) Role of A_{2A} extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood* 90:1600–1610
- Sitkovsky MV (2009) T regulatory cells: hypoxia-adenosinergic suppression and re-direction of the immune response. *Trends Immunol* 30:102–108

27. Koshiba M, Rosin DL, Hayashi N, Linden J, Sitkovsky MV (1999) Patterns of A2A extracellular adenosine receptor expression in different functional subsets of human peripheral T cells. Flow cytometry studies with anti-A2A receptor monoclonal antibodies. *Mol Pharmacol* 55:614–624
28. Hasko G, Linden J, Cronstein B, Pacher P (2008) Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* 7:759–770
29. Ohta A, Sitkovsky M (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414:916–920
30. Chouker A, Thiel M, Lukashev D, Ward JM, Kaufmann I, Apasov S, Sitkovsky MV, Ohta A (2008) Critical role of hypoxia and A2A adenosine receptors in liver tissue-protecting physiological anti-inflammatory pathway. *Mol Med* 14:116–123
31. Thiel M, Chouker A, Ohta A, Jackson E, Caldwell C, Smith P, Lukashev D, Bittmann I, Sitkovsky MV (2005) Oxygenation inhibits the physiological tissue-protecting mechanism and thereby exacerbates acute inflammatory lung injury. *PLoS Biol* 3:e174
32. Ben-Shoshan J, Maysel-Auslender S, Mor A, Keren G, George J (2008) Hypoxia controls CD4 + CD25+ regulatory T-cell homeostasis via hypoxia-inducible factor-1alpha. *Eur J Immunol* 38:2412–2418