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Title: ***INHIBITORY EFFECT OF EXTRACELLULAR PURINE NUCLEOTIDE AND NUCLEOSIDE CONCENTRATIONS ON T CELL PROLIFERATION***

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ABSTRACT

The release of nucleic acids and derivatives after tissue-injury may affect cellular immune-response. We studied the impact of extracellular ribo-, desoxyribonucleotides and nucleosides on T-cell immunity. Peripheral-blood-mononuclear-cells (PBMCs) or isolated $CD3^+$T-cells obtained from 6 healthy donors were stimulated via CD3/CD28 Dynabeads or dendritic cells (DCs) in the presence or absence of pyrimidine-, purine-nucleotides and -nucleosides (range 2-200µM). Addition of deoxy-, guanosine-triphosphate (dGTP, GTP) and guanosine resulted in a partial inhibition of the induced T-cell-proliferation. Adenosine-triphosphate (ATP), adenosine and the pyrimidine-ribo- and -deoxyribonucleotides displayed no inhibitory capacity. Inhibitory effects of dGTP and GTP, but not of guanosine and ATP were culture-media-dependent and could be almost abrogated by use of lymphocyte-culture-media Xvivo15 instead of RPMI with standard-supplementation. In contrast to RPMI, Xvivo15 resulted in a significant down-regulation of the cell-surface-located ectonucleotidases CD39 (Ecto-Apyrase) and CD73 (Ecto-5´-Nucleotidase), critical for the extracellular nucleotides-hydrolysis to nucleosides, explaining the loss of inhibition mediated by dGTP and GTP, but not Guanosine. In line with previous findings ATP was found to exert immunosuppressive effects on T-cell-proliferation. Purine-nucleotides, dGTP and GTP displayed a higher inhibitory capacity, but seem to be strictly dependent on the microenvironmental conditions modulating the responsiveness of the respective T-lymphocytes. Further evaluation of experimental and respective clinical settings should anticipate these findings.

INTRODUCTION

1.Cellular decay leads to release of nucleic acids and their compounds (nucleotides, nucleosides, nucleobases) in the extracellular milieu.

Patients with leukaemia or solid tumours are confronted with a strong tumour cells´ decay during and after the chemotherapy treatment and irradiation, followed by the release of high amounts of nucleic acids into the extra-cellular milieu (Weber et al., Advan. Enzyme Regul. 2006, Cole et al., Radiation Research 1957). Moreover, an excessive protein rich (meat based) nutrition leads to a higher risk of hiperuricaemia and gout (H.K.Choi et al., N. Eng. J. Med. 2004,A. Corrado et al., 2006). Endonucleases, phosphodiesterases and phosphorylases degrade extra-cellular DNA and RNA at internal sites to oligonucleotides, free nucleosides, bases and ribose-1-phosphate. Only a part of the nucleosides and bases are re-utilised in the “salvage pathway” for purine and pyrimidine biosynthesis; the pyrimidine bases are degraded to β-aminoisobutyrate, NH_3 and CO_2. The decay of purine bases could produce an increasing of the concentration of purine decay products such as xanthine, hypoxanthine and uric acid in blood. As consequence the patients might develop secondary gout, due to highly nucleic acid turnover.

2. Nucleic acid-based therapies in the treatment of malignant and non-malignant diseases.

Formerly modified nucleobases as 5-Fluoruracyl, and Thiopurine such as 6-Thioguanine or 6-Mercaptopurine were used in cancer therapy. In the past years, especially purine nucleoside analogues such as Fludarabine, Cladribine, Pentostatine, Nelarabine, Immucilin H or 8-Chloroadenosine are used as cytotoxic drugs in the treatment of various leukaemias or autoimmune diseases – all of them inducing apoptosis after incorporation in DNA, blockade of intracellular

nucleoside cleavage or inhibition of RNA-synthesis (Robak T. et al, Curr. Med. Chem 2006). Other purine analogs are used for the treatment of viral diseases Varicella zoster virus (VZV), Eppstein-Barr virus (EBV), Cytomegalovirus (CMV) and Herpes simplex virus type (HSV) 1 or 2: Acyclovir or Gancyclovir are guanine analoga inhibiting the function of viral DNA-polymerase. New nucleic acid-based therapeutic strategies using antisense or immunostimulatory CpG, oligodeoxynucleotides, aptamers, ribozymes or Defibrotide have strongly gained in importance (Ku. et al, 2015)

3. Accumulation of purine nucleotides and nucleosides may affect the cellular response .

The effects of purine nucleotides and nucleosides have been studied in animal models: it could be shown that guanine nucleotide can inhibit rabbit neutrophile migration (Boonen et al, 1991); guanosine and inosine are able to preserve the viability of mouse neuronal and glial cells during chemical hypoxia (Litsky et al, Brain Research, 1998) and together with adenosine protect rat glia cells during mitochondrial inhibition and glucose deprivation (Jurkowitz et al, J. of Neurochem, 1998). It has been shown that certain concentrations of naturally occurring purine deoxy-rybonucleotides can be toxic for lymphoid cells (Henderson et al, Pharmacol. Ther.8, 1980); particularly the toxicity of deoxyguanosine (dGua) in purine nucleoside phosphorylase (PNP)- deficiency, a malignancy with severe impairment of the cellular immunity has been studied. The addition of dGua to B-cells isolated from healthy donors leads to intracellular accumulation of GTP and dGTP, resulting in inhibition of *in-vitro* B-cells´ proliferation (Scharenberg et al, Eur. J. Immunol, 1986). In cultured T-lymphoblasts, deoxyadenosine triphosphate (dATP) appears to be the toxicity mediator of 2´- dGua and 2´-deoxyadenosine (2´-dAde) (Mann, G.J. et al, J. Clin. Invest, 2006). The inhibition of PHA-stimulated T-cells´ proliferation induced with dGua could be prevented by hypoxanthine and deoxycytidine (dCyt) addition, which indicates the role of deoxycytidine kinase in the dGua phosphorylation to dGTP (Spaapen et al, Journal of Immunol, 1984). Human T- but not B-cells- are able to accumulate important amounts of dGTP –resulting in T-cell immunodeficiency in PNP-deficiency (Fairbanks et al, The Journal of Immunol, 1990). Studies on the cell line K562 reported inhibition and arrest of DNA-synthesis of the S-phase cell-cycle by GTP accumulation (Moosavi et al, J. of Biochem. and Molec. Biol, 2006); Jurkat cells' death could be achieved by appropriate high concentrations of dGua, due to intracellular accumulation of dGTP and dATP (Batiuk et al, J. Physiol. Cell, 2001).

4. Guanosine nucleotides and nucleosides may interfere with immunosuppressant drugs such as Mycophenolate Mofetil (MMF).

Based on the observation that lymphocytes´ proliferation is strictly dependent on the *de novo* synthesis of guanosine nucleotides, it was possible to develop immunosuppressive drugs targeting directly the *de novo* synthesis pathway, such as Mycophenolate Mofetil (MMF). MMF, the prodrug of the active metabolite mycophenolic acid, is used to prevent rejection after solid organ transplantation, e.g. kidney transplantation (Sanjay et. al., Transplant International, 2008). Its way of action involves the specifically, non-competitive inhibition of inosine-5´ monophosphate dehydrogenase (IMPDH), the key-enzyme of guanosine nucleotides *de novo* synthesis pathway. In allogeneic organ transplantation the immunosuppressive activity of MMF is frequently amplified through combination with calcineurin inhibitors as Cyclosporine A, which acts on the nuclear factor of activated T-cells (NFAT) and blocks the IL-2 production (Delalande et al., J. of Immunl. 2008). Important for the purine metabolism is the

action of two ecto-nucleotidases: CD39 (Ecto-Apyrase, NTPD-ase1) and CD73 (Ecto-5´-Nucleotidase) that are involved in the intracellular up-take of exogenous purine nucleotides; CD39 as ecto-phosphodiesterase hydrolyses adenine nucleotides triphosphates to di- and monophosphates and GTP to di-and monophosphates (J.Goding, J. of Leukocyte Biology 2000, D. Leal et al., Biochemica et Biophysica Acta 1721, 2005).

5. Aim of our work:

The aim of this work was to reveal the influence of exogenously added purine and pyrimidine nucleotides and nucleosides on healthy human T-cells´ proliferation (after stimulation with CD3/CD28 Dynabeads or dendritic cells (DCs) in mixed lymphocytes culture (MLC)) *in vitro*, thereby targeting the intracellular metabolism of nucleic acids and their possible interferences with immunosuppressive drugs such as MMF. T-cells´ proliferation was measured in various cell culture media, in the presence or absence of various growth factors or cell culture additives, with or without exogenously added purine nucleotides and nucleosides, using different proliferation assays such as CFDA-SE, XTT, or Trypan-blue viability test. We also investigated regulatory effects of exogenously added purine nucleotides and nucleosides in various cell culture media, on the cellular expression of CD39 (Ecto-Apyrase, NTPD-ase1) and CD73 (Ecto-5´-Nucleotidase) as ecto-enzymes involved in the degradation and intracellular up-take of purine nucleotides and nucleosides.

MATERIALS AND METHODS

1. Cell Sample Collection.

Cell culture media: Isolated peripheral blood mononuclear cells (PBMCs), T-cells, Raji- or Jurkat-cell lines used in our experiments were cultured in the following media: RPMI1640 cell culture medium (RPMI1640[1], Cell Concepts GmbH, Umkirch, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM/L L-Glutamine, 100U/ml Penicillin, 100µg/mL Streptomycin (Gibco, Germany); serum-free medium X-Vivo15 (X-Vivo15[2], Bio Whittaker Europe, Verviers, Belgium); Dulbecco's Modified Eagle's Medium (DMEM[3], Gibco, Germany) supplemented with 10% heat-inactivated FCS, 2mM/L L-Glutamine, 100U/ml Penicillin, 100µg/mL Streptomycin or Iscove´s Modified Dulbecco´s Medium (IMDM[4], Gibco, Germany) supplemented with 10% heat-inactivated FCS, 2mM/L L-Glutamine, 100U/ml Penicillin, 100µg/mL Streptomycin. Washes or re-suspensions of the cells were done in Phospate Buffer Saline, pH 7.2, without Ca^{2+} and Mg^{2+} (PBS, Gibco, Germany).

Peripheral blood mononuclear cells (PBMCs): PBMCs were isolated from heparinized blood of six healthy, voluntary donors, by density gradient centrifugation on a Biocoll separating solution (Biochrom AG, Berlin, Germany), washed and re-suspended in PBS. Viability of cells, measured by 0.4% trypan blue staining (Sigma-Aldrich Chemie GmbH, Munich, Germany), ranged between 98.0%-99.0%. PBMCs were adjusted to $1x10^6$ cells/mL in RPMI1640[1] cell culture medium and preserved in a humidified incubator (5% CO_2 at 37°C) until used for experiments or further T-cells isolation. The composition of PBMCs was: 33.0%-55.0% T-cells, 6.0%-7.2% B-cells, 3.9%-7.9% monocytes.

Cell lines: Possible inhibitory effects of purine nucleotides and nucleosides were also investigated on Raji (B-lymphocytes, human Burkitt lymphoma) and Jurkat (T-cells, ALL-Type) cell lines, provided from DSMZ Braunschweig, Germany. Both cell lines were cultured until use in $75cm^3$ sterile flasks (TPP, Trasadingen, Swiss) with 20mL RPMI1640[1] cell culture medium GmbH and preserved in a

humidified incubator (5% CO_2 at 37°C). The two cell lines were used in Tetrazolium salt (XTT)- based proliferation assays.

Isolation of T-cells: Untouched T-cells were magnetically prepared from six different healthy donors` PMBCs using Pan-T-cells Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), after depletion of non-T-cells using a cocktail of biotin-conjugated antibodies (CD14, CD16, CD19, CD36, CD56, CD123 and Glycophorin A) and anti-Biotin MicroBeads. The percentage of $CD3^+$ T-cells in the isolated cell fraction was determined on FACScalibur (BD Biosciences, Heidelberg, Germany) and ranged between 98.0% and 99.6%. T-cells were isolated for XTT- and carboxy-fluorescein diacetate, succinimidyl ester (CFDA-SE) -based proliferation assays and for mixed lymphocytes culture (MLC).

2. Flow Cytometry.

In order to evaluate and quantify amounts and phenotypes of PBMCs, T-cells, monocytes and mature DC-subtypes we performed flow cytometric analyses using a panel of mouse monoclonal antibodies (moAbs) including isotype controls directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), cyanine-7 (Cy-7) or allophycocyanine (APC), as described (Kremser et al., J. Immunotherapy 2010). The antibodies were obtained from Caltag Laboratories[1] (Hamburg, Germany), BD Pharmingen[2] (Heidelberg, Germany), Beckman Coulter Immunotech[3] (Krefeld Germany) and Serotec[4] (Duesseldorf, Germany). FITC-conjugated moAbs were CD1b[2], CD3[2], CD4[1], CD39[4], CD83[1], CD86[1] as PE-conjugates moAbs were CD1a[1], CD3[2], CD56[3], CD73[4], CD80[1], CD206[3]. Cy-7-conjugated moAbs were CD4[1], CD14[3], CD19[1], 7AAD[3] and the APC-conjugated moAbs were: CD8[1], CD15[3], CD19[1], CD25[1], CD33[3], CD40[2], CD69[1], CD71[3].

PBMCs, T-cells, monocytes or mature DCs were re-suspended in RPMI1640[1] or in X-Vivo15[2] medium and stained with moAbs, according to manufacturers` instructions.

3. Proliferation Assays.

Influences of purine nucleotides and nucleosides on the cellular proliferation were evaluated on PBMCs or T-cells from different healthy donors, stimulated for 5-7 days with Dynabeads® Human T-activator CD3/CD28 for cell expansion and activation (Invitrogen, Germany) according to manufacturers´ instructions. These super- paramagnetic beads (4.5µm) combined with CD3 and CD28 antibodies provide primary and co-stimulatory signals, necessary for the activation and expansion of T-cells, without requiring antigens or antigen presenting cells.

CFDA-SE-based proliferation assay: carboxy-fluorescein diacetate, succinimidyl ester (CFDA-SE, CellTrace Proliferation Kit for Flow Cytometry, Invitrogen, Karslruhe Germany) is a colorless, nonfluorescent dye that passively diffuses into cells and turns to highly fluorescent as the intracellular esterases cleave its acetate groups to succinimidyl ester group and interacts with intracellular amines. The CFDA-SE dye formed in labelled cells is retained by the cells and is inherited by daughter cells after cell division. $10x10^6$ PBMCs or T-cells were stained with 1µL CFDA-SE in 10mL PBS (Gibco, Germany) at a final concentration of 1µM in the dark. Afterwards 10mL FCS were added in order to quench unspecific staining, followed by two washing steps in PBS. The efficiency of the staining was always at least 99.0%. CFDA-SE stained cells were re-suspended in RPMI1640[1] or in serum-free X-Vivo15[2] cell culture medium, adjusted to $1x10^6$ cells/mL, CD3/CD28 stimulated and plated ($2x10^6$ cells per well) in 96-wells round bottom plates (TPP, Trasadingen, Swiss) with or without exogenously

added nucleotides and nucleosides; the tested concentrations were 200-, 100-, 20-, 10-, 2µM. As negative control unstimulated cells were included in all the experiments. Proliferation during 5 days at 37° C in a humidified incubator with 5% CO_2, was measured by flow cytometry. A differentiation of viable, proliferating and non-proliferating cells from non-viable cells was made after addition of 0.2µM carbocyanine monomer TO-PRO3 iodide (Invitrogen, Karlsruhe, Germany) shortly before flow cytometric analyses. In order to quantify the cellular subtypes, 0.14x 10^5/mL APC Calibrate Beads (BD Bioscience, Heidelberg, Germany) were added shortly before analyses.

XTT- based proliferation assay: a.) We used the Tetrazolium Salt XTT Proliferation Kit II (Roche, Germany) as non-radioactive, alternative method for the quantification of cell proliferation and viability. Viable cells with a strong mitochondrial dehydrogenase activity are able to cleave the tetrazolium salt XTT in the presence of an electron-coupling reagent to soluble, orange coloured Formazan after an incubation time of four hours; the colour intensity of the Formazan, spectrophotometrically measured on the Sunrise™ Absorbance Reader (Tecan GmbH Germany) as optical density (OD) correlates with the intensity of cell proliferation. CD3/CD28 stimulated T-cells or PBMCs, as well as Raji or Jurkat cell lines, were plated at 1x10^5 cells/well in 96-well plates as triplicates and co-incubated for five days in RPMI1640[1] or in serum-free X-Vivo15[2], with or without various concentrations of exogenously added nucleotides and nucleosides (Larova GmbH, Teltow, Germany and Sigma-Aldrich Chemie GmbH, Munich, Germany). Concentrations of nucleotides and nucleosides tested in triplicates were 200-, 100-, 50-, 20-, 10-, 5-, 2-, 0.5µM. After five days the XTT proliferation assay was performed and the transformation of the tetrazolium salt XTT in Formazan developed by adding a mix of 50µl XTT reagent and 1µL electron-coupling reagent per 100µL cell suspension, after 4 hours co-incubation, at 37°C in a humidified incubator with 5% CO_2.

b.) Possible interfering effects of cell culture additives such as Insulin, Cortison, granulocyte macrophage colony-stimulating factor (GM-CSF), Biotine and high concentration of glucose on the T-cells proliferations` inhibition was tested using RPMI1640[1]as co-incubation medium, with or without purine nucleotides and nucleosides, enriched as follow: *1.)* with Insulin, 5µU/µL (physiological insulin concentration in blood), 5µg/µL (recommended as cell culture additive, e.g. from Roche, Germany, = 137.5µU/ml) and 10µg/µL (=275µU/ml), with or without 2g/L, 5g/L and 10g/L glucose (SigmaAldrich, Germany). *2.)* with GM-CSF: 1000U/ml, 10000U/ml). *3.)* with Cortison (2µM, 20µM, 200µM). Stimulated T-cells with/without purine nucleotides and nucleosides, co-incubated in RPMI1640[1] or X-Vivo15[2] were used as control. Possible effects of Biotine were studied in experiments using various media with a defined Biotine concentration, such as RPMI1640[1] (0.2mg/L Biotine), IMDM[4] (0.013 mg/L) or DMEM[3] (Biotine-free medium) and X-Vivo15[2] (unknown Biotine content) with or without guanine and adenine nucleotides and nucleosides.

Absorbance data (optical density, OD) obtained as described above were analysed with Magellan™ Standard Software; mean and standard deviations were calculated for each independent experiment using the Microsoft Office Excel 2004 Program. Moreover, we calculated the mean and standard deviation of mean values of triplicates obtained by a minimum of three independent experiments and the standard deviations. All results were represented as column diagrams, using Microsoft Office Excel 2004 Program.

4. Generation of Dendritic Cells (DCs) and Mixed Lymphocyte Culture (MLC).

DCs Generation: The effects of exogenously added nucleotides and nucleosides on the proliferation of DCs-stimulated T-cells were tested in mixed lymphocyte cultures (MLC). Dendritic cells (DCs), developed from isolated PBMCs from healthy donors, served as stimulator cells. DC were generated using the cytokine-based Monocytes Conditioning Medium (MCM-Mimic) method as described by us (Kremser et al, J. Immunotherapy 2010, Liepert et al, Cellular Immunity 2010,Grabrucker et al., 2010).

Quantification and Characterisation of DCs ***(Fig. 1)****:* After 7 days of culture DCs were harvested, counted with 0.4% Trypan blue and quantified. For analyses and quantification of DCs a refined gating strategy was applied as described by H. Schmetzer et al, Leukemia 2007. The DCs` generation was defined as successful if more than 10.0% DC (DCopt) could be generated in any given case (Kremser et al., J. Immunotherapy 2010, Dreyssig et al., J. Cancer molecules 2010, Grabrucker et al., J. Immunotherapy 2010, Liepert et al., Cellular Immunity 2010). Percentages of DCopt ranged between 19.5-23.5%, percentages of mature DCs between 50.4-60.4% and of viable DCs between 74.9-83.0%. Regularly conducted microscopical controls revealed the DC-typical morphology of large cells with irregular shapes and cytoplasmatic projections.

Mixed Lymphocytes Culture (MLC): T-cells ($CD3^+$) from PBMCs of six healthy donors isolated as described above, served as responder cells in co-cultures with DCs-stimulators, generated as described above. The generated DCs were irradiated (20Gy) and co-cultured (ratio1:2 and 1:10) in round bottom 96-well plates with allogeneic T-cells from another healthy donor in serum-free X-Vivo15[2] cell culture medium, in a final volume of 150µl per well, with or without 200µM exogenously added nucleotides and nucleosides, in a humidified incubator (37°C, 5%CO_2). After 24 hours, 150U/mL Interleukin-2 (IL-2, Roche, Germany) were added per well in order to sustain the proliferation of the T-cells. All micro-cultures were set up as triplicates. After 7 days, MLCs were co-incubated for 4 hours with 51µL XTT-mix labelling reagent and electron coupling solution per 100µL experimental volume in a humidified incubator (37°C, 5%CO_2). The degree of the T-cells´ proliferation under MLC conditions was determined as expression of colour absorbance (optical density, OD) measured with Sunrise™ Absorbance Reader (Tecan GmbH Germany) and analysed with Magellan™ Standard Software for each triplicate; mean and standard deviation of the three independent values of the triplicates, were calculated and represented as column diagrams, using Microsoft Excel 2004. The mean of (n=x) independent experiments were represented as stimulation index (SI) which was calculated as ($SI = OD_{\text{T-cells+DCs, with/ without exogenously added purine nucleotides and nucleosides}} / OD_{\text{T-cells}}$) (Buhmann et al, Blood, 1999) and represented as bar diagrams (Microsoft Office Excel 2004).

5. Statistical Methods. Means, standard deviations, ranges, variance and 2-tailed *t*-tests were conducted with a personal computer, using Microsoft Excel 2004. Differences were considered as statistically significant if the *p*-values were less than 0.05.

RESULTS

1. Guanine nucleotides, nucleosides and ATP inhibit (concentration dependent) proliferation of PBMCs and T-cells. (Fig .2 A,B,C,D).

We could already show that nucleic acid-based drugs like Defibrotide antagonise Fludarabine, Acyclovire or Mycophenolate mofetyl (MMF), the pro-drug of immunosuppressant mycophenolic acid

(MPA) (R. Buhmann, T.Yang, M. Schifferer**,** *Blood* -ASH Annual Meeting Abstracts-2008 112: Abstract 1614). Furthermore, exogenously added polydeoxyribonucleotides (dNTP) and polyribonucleotides (NTP) *in vitro* interfere with MMF. This could mean that an increased extracellular nucleic acid concentration (e.g. after solid tumor decay, chemotherapy, radiation) could interfere with the intracellular metabolism of nucleic acids and influence it. Based on these observations we co-incubated isolated, CD3/CD28 stimulated PBMCs from three different healthy donors, with 200µM purine and pyrimidine ribo- and deoxyribonucleotides; we used 2µM MMF as control for the inhibition of proliferation **(Fig.2, A)**. No inhibition of PBMCs´ proliferation for the tested purine and pyrimidine ribo- and deoxyribonucleotides could be shown, with the exception of the stimulated PBMCs co-incubated with 200µM dGTP or GTP. For these two nucleotides, the inhibitory effects were comparable with those obtained in the presence of 2µM MMF. Co-incubation of isolated, stimulated T-cells or PBMCs with pyrimidine ribo- and deoxyribonucleotides did not show an inhibition of T-cells´ proliferation nor any interactions with 2µM MMF or CsA (data not shown). Therefore we focused on the analyses of possible inhibitory effects of purine nucleotides and nucleosides on the proliferation of stimulated T-cells.

We compared the *in vitro* inhibitory capacity of purine nucleotides and nucleosides on stimulated T-cells from four healthy donors using CFDA-SE labelled, CD3/CD28 stimulated T-cells, co-incubated for 5 days with various concentrations (200-, 100-, 50-, 20µM) of GTP, dGTP, Guanosine (Gua), ATP, dATP, Adenosine (Ade) **(Fig.2, B)**; Guanine nucleotides and nucleosides demonstrated strong inhibitory effects of the T-cells´ proliferation at 200µM and weak inhibition at 100µM and 50µM. Using adenosine nucleotides and nucleosides, inhibitory effects were observed only with 200µM ATP. Moreover, we also tested other concentrations of purine nucleotides and nucleosides such as 20µM and 2µM and compared the effects on the stimulated T-cells´ proliferation, using an alternative XTT-proliferation assay, addressing mitochondrial dehydrogenase activity **(Fig.2, C)**. Based on these results, we decided to use 200µM of exogenous purine nucleotides and nucleosides added to culture media as an optimised concentration with maximal anti-proliferative effect on stimulated T-cells for further experiments. This concentration was tested in six independent experiments with CD3/CD28 stimulated T-cells from different healthy donors, co-incubated for 5 days with 200µM guanine and adenine nucleotides and nucleosides, using XTT-proliferation assays (**Fig.2, D):** comparing the proliferation of stimulated T-cells with/without the influence of 200µM guanine nucleotides or ATP it can be seen that the maximal inhibition of proliferation was achieved by addition of GTP (p_{GTP} <0.001), dGTP p_{dGTP} <0.001), Gua (p_{Gua} <0.001) and ATP (p_{ATP} =<0.001); co-incubation of stimulated T-cells with dATP or Ade, 200µM each, did not significantly inhibit T-cells´ proliferation.

We conclude that exogenously added guanine nucleotides, nucleosides and ATP inhibit concentration dependent the proliferation of T-cells´ *in vitro,* as confirmed by two different assays (CFDA-SE- and XTT- based proliferation assays). The maximal anti-proliferative effect on the CD3/CD28 stimulated T-cells was observed with 200µM guanine nucleotides, nucleosides or ATP; no comparable, inhibitory effects could be measured when stimulated T-cells were co-incubated with exogenously added dATP, Ade or pyrimidine ribo- and deoxyribonucleotides.

2. Exogenously added guanine nucleotides, nucleosides and ATP- but not dATP or Ade- inhibit T-cell proliferation in mixed lymphocyte cultures (MLC, Fig. 3). In order to provide evidence that exogenously added purine nucleotides and nucleosides are able to inhibit the *in-vitro* T-cell proliferation, we investigated possible effects in a more complex T-cell proliferation system, using dendritic cells (DCs) generated from healthy donors as T-cell stimulators in mixed lymphocyte cultures. The intensity of the T-cells´ proliferative response (T-cells obtained from other (allogenic) healthy donors) was measured using the XTT-proliferation assay. The proliferative response of DC-stimulated T-cells in MLCs and the possible inhibitory effects of guanine and adenine nucleotides and nucleosides are presented as mean of stimulation index (SI) for 4 independent experiments with cells from healthy donors, as bar diagrams (**Fig.3)**. Compared to the stimulation index for the MLC-control (defined as 100% proliferation), a significant reduction of the proliferation to 60.9% (p=0.042) was measured after addition of GTP, to 52.0% (p=0.002) after addition of dGTP, to 54.5% (p=0.02) after addition of Gua and to 56.0% (p=0.037) after addition of ATP (200µM each), whereas the addition of dATP (96.4%) or Ade (94.2%) could not inhibit T-cells' proliferation **(Fig.3).** Comparable results were found co-incubating CD3/CD28 stimulated T-cells with GTP, dGTP, Gua and ATP (200µM each) as well as for dATP and Ade (200µM each, **Fig.1E**). Compared to MLC-control, the proliferation of T-cells in MLC was significantly inhibited, after addition of GTP (p_{GTP}=0.036), dGTP (p_{dGTP}=0.005), Gua (p_{Gua}=0.009) or ATP (p_{ATP}=0.015), whereas the addition of dATP and Ade did not significantly inhibit T-cell proliferation in MLC. That means that the addition of dGTP, GTP, Gua and ATP induces an inhibition of stimulated T-cells´ proliferation *in vitro,* not only after CD3/CD28 Dynabeads T-cell stimulation, but also in a more complex stimulator-responder system (MLC), with DCs as T-cells' stimulators.

Therefore we investigated potential inhibitory effect of exogenously added purine nucleotides and -nucleosides on Jurkat- and Raji- cell lines, cultured in RPMI1640[1] *in vitro*. Cumulative data of three independent experiments revealed that only guanine nucleotides and nucleosides at 200µM tested concentration were able to inhibit both Jurkat- and Raji-cells (data not shown). Comparing the proliferation values of the two cell lines after three or six days co-incubation with each 200µM exogenously added purine nucleotides and nucleosides, we could demonstrate inhibitory effects of GTP, dGTP or Gua on Jurkat-cells' proliferation after three days and on Raji-cells´proliferation after six days of co-incubation, whereas by the Raji-cells we measured an anti-proliferative effect by co-incubation with 200µM GTP, dGTP or Gua after six days; the start of inhibition for the Raji cells´ proliferation was already measured after co-incubation with 200µM Gua, but not with 200µM GTP or dGTP.

3. Guanine nucleotides, nucleosides and ATP have different culture medium dependent inhibitory effects on CD3/CD28 Dynabeads stimulated T-cells´ proliferation (Fig. 4). Different cell culture media are recommended to be used for T-cell proliferation assays (e.g. RPMI1640[1], X-Vivo-15, IMDM or D-MEM). Since the composition of these media differ, we determined their influence on the T-cells´ proliferation, with or without adding guanine and adenine nucleotides or nucleosides. Therefore, isolated, CD3/CD28 stimulated, $CD3^+$ T-cells were co-incubated for 5 days with or without 200µM guanine and adenine nucleotides and nucleosides in different cell culture media, as described

below; the intensity of the proliferation was detected and compared in XTT- or CFDA-SE proliferation assays. Interestingly, the inhibitory effects of guanine nucleotides, nucleosides and ATP were different if stimulated T-cells were co-incubated in RPMI1640[1]compared to X-Vivo15[2]. With addition of 200µM GTP (or dGTP), we demonstrated a down-regulation to 40.2% (or 48.7%) of stimulated T-cells´ proliferation co-cultured in RPMI1640[1] but only to 97.3% (or 89.2%) when co-cultured with 200µM GTP (or dGTP) in X-Vivo15[2] medium. No differences in the inhibition of stimulated T-cells` proliferation could be measured for 200µM Gua, in RPMI1640[1] or in X-Vivo15[2], as shown in five independent experiments with different healthy donors cells **(Fig.4, A)**. The effects of adenosine nucleotides and nucleosides were comparable for the two cell culture media. Similar effects were confirmed in five independent experiments. We performed Trypan-blue viability tests on stimulated T-cells under the influence of exogenously added guanine and adenine nucleotides and nucleosides given above, and we could demonstrate that X-Vivo15[2] medium sustained the cell viability by co-incubation with 200µM GTP or dGTP, compared with co-incubation in RPMI1640[1] **(Fig. 4, B).** Co-incubation of stimulated T-cells with guanine and adenine nucleotides and nucleosides in DMEM[3] or IMDM[4] cell culture media, yielded inhibitory effects comparable with those obtained in RPMI1640[1] as co-incubation medium (data not shown).

In order to clear the role of various additives in serum–free medium X-Vivo15[2] on T-cells´ proliferation with or without exogenously added guanine and adenine nucleotides or nucleosides we performed XTT- proliferation assays, using RPMI1640[1] medium enriched with Insulin, Glucose, GM-CSF or Cortison, as described in “Materials, Methods”. As a control we used stimulated T-cells co-incubated in RPMI1640[1] or X-Vivo15[2] medium, with/without purine nucleotides and nucleosides.

In addition, we tested the influence of Biotine on the inhibition of stimulated, proliferating T-cells co-incubated with or without exogenously added dGTP, GTP or ATP. As co-incubation media we used RPMI1640[1] (0.2mg/L Biotine), IMDM[4] (0.013 mg/L), DMEM[3] (Biotine-free medium) and X-Vivo15[2] (unknown Biotine content). In all those cell culture media, supplemented with additives or directly used as described above, using XTT-proliferation assays, we could measure and quantify values of T-cells´ proliferation corresponding to a “RPMI1640[1]-pattern-like” way of action of purine nucleotides and nucleosides and not to a serum–free “X-Vivo15[2] pattern”.

We conclude that none of the analysed substances, tested in the mentioned concentrations and added in RPMI1640[1] were able to influence the inhibitory effect of purine nucleotides and nucleosides in the same manner as the serum-free medium X-Vivo15[2].

4. Exogenously added guanine nucleotides and nucleosides interfere concentration dependent with the immunosuppressant drug Mycophenolate mofetil (MMF) (Fig.5, Fig.6)

We investigated possible interferences of T-cells´ proliferation with exogenously added purine and pyrimidine ribo- and deoxyribonucleotides with the anti-proliferating, immunosuppressive effect of MMF, a potent inosine-monophosphate dehydrogenase (IMPDH) -inhibitor and blocker of *de novo* biosynthesis of guanine nucleosides. CFDA-SE labelled, CD3/CD28 stimulated PBMCs or T-cells were co-incubated for 5 days with exogenously added purine and pyrimidine ribo- and deoxyribonucleotides (200µM), with or without 2µM MMF, and with or without the calcineurine inhibitor Cyclosporine A (CsA) 2µM as control. We observed that the proliferation of T-cells or PBMCs was

inhibited not only by 2µM MMF or 2µM CsA, but also with a mixture of 2µM MMF and 200µM dGTP or GTP **(Fig. 5)**. The strong inhibitory effects of the proliferation induced by co-incubation of the stimulated PBMCs with 2µM MMF were comparable with those measured by co-incubation of the stimulated PBMCs with 200µM dGTP or GTP **(Fig.2).** In further experiments isolated, CD3/CD28 stimulated T-cells from healthy donors were co-incubated for 5 days with various dGTP concentrations, with or without 2µM MMF and 2µM CsA as a control **(Fig. 6)**. Cumulative data of three independent experiments showed that the immunosuppressive effect of 2µM MMF but not of 2µM CsA, could be partially abrogated by co-incubation with 200µM and 10µM dGTP and strongly abrogated by co-incubation with 50-, 20- and 100µM dGTP. Lower dGTP concentrations (e.g. 5, 2, 1, 0.5µM) could not abrogate the immunosuppressant of 2µM MMF or 2µM CsA **(Fig. 6)**. **Table 1** gives an overview of our proliferation data, obtained when stimulated T-cells were co-incubated with or without 2µM MMF and various concentrations of dGTP, GTP or Gua. For a better comprehension, the OD values were adjusted to 100% stimulated, proliferating T-cells. We could demonstrate that the immunosuppressive effect of 2µM MMF could be abrogated with GTP, dGTP or Gua, with an optimum of the inhibitory concentrations between 100µM and 20µM; no interferences were measured by co-incubation of 2µM CsA with various concentrations of GTP, dGTP or Gua **(Table 1).**

5. CD39 and CD73-expression is medium-dependent and may influence the inhibitory capacities of guanine nucleotides and nucleosides.

In order to further elucidate inhibitory effects of dGTP, GTP and Gua on stimulated T-cells´ proliferation we analysed the expression profiles of two important ecto-enzymes -CD39 and CD73-, involved in the degradation, transport and up-take of purine nucleotides from extra-cellular milieu into the lymphocytes. The expression of CD39 and CD73 was analysed on CD3/CD28 stimulated T-cells, co- incubated with or without 200µM GTP, dGTP or Gua, by flow-cytometric analyses on day 0, 3 and 5 in two different cell culture media: RPMI1640[1] and X-Vivo15[2]. The differences between the CD39- and CD73- expression profiles for the stimulated T-cells after culture in the two media are presented in **Table 2**. X-Vivo15[2] caused a two to six-fold up-regulation of CD39-expression on T-cells co-incubated with dGTP, GTP or Gua, compared with the CD39-expression on stimulated T-cells, day five, whereas RPMI1640[1] produced only a weak up-regulation. Similarly, for the CD73 expression, we could measure a three- to ten-fold up-regulation in X-Vivo15[2] when stimulated T-cells were co-incubated with dGTP, GTP or Gua, compared with the CD73 expression on stimulated T-cells-control on day five. Using RPMI1640[1] as co-incubation medium for stimulated T-cells, with or without guanine nucleotides and nucleosides we measured only a minimal up-regulation of CD73-expression compared with the marker expressions on stimulated T-cells on day five.

In all, these observations might provide evidence for the different inhibitory capacities of GTP and dGTP in RPMI1640[1] compared with X-Vivo15[2].

DISCUSSION

1. Physiological degradation of extra-cellular nucleic acids

Human lymphocytes´ proliferation is mediated in great part by the *de novo* pathway of purine synthesis and less by the *salvage* pathway. Nevertheless high extra-cellular GTP and dGTP concentrations activate the key-enzyme of the *de novo* pathway, inosine monophosphate

dehydrogenase (IMPDH), and leads to a higher guanine nucleotides´ and nucleosides´ turn-over with a possible block of DNA-synthesis through the inhibition of ribonucleoside reductase. The inability of lymphocytes to synthesise new DNA directly affects their proliferating capacity. It is known that a high dATP concentration also inhibits the activity of ribonucleoside reductase by binding on the active sites of the enzyme, resulting in a lack of new DNA-synthesis. That means not only that exogenously added guanosine nucleotides and nucleosides have an inhibitory capacity on stimulated T-cells´ proliferation *in vitro*, but also that similar effects have to be anticipated regarding the adenosine nucleotides and nucleosides. This report investigates the potential of exogenously added purine nucleotides and nucleosides to inhibit *in vitro* stimulated T-cells´ proliferation and moreover, possible interferences with immunosuppressive drugs such as Mycophenolate mofetil, the pro-drug of mycophenolic acid, used to prevent acute or chronic allograft rejection after organ transplantation. The essential finding of this report is, that exogenously added guanine nucleotides, nucleosides and ATP can inhibit - concentration dependent- the *in vitro* T-cells´ proliferation, achieved either with CD3/CD28 Dynabeads or with dendritic cells (DCs) used as stimulators in mixed lymphocyte cultures (MLC).

2. Pathogenic role of extra-cellular accumulated nucleic acids as a consequence of cell decay after chemotherapy, irradiation or protein-rich based nutrition

Nucleic acids accumulate into the extra-cellular space not only as a consequence of cellular degradation after tumour chemotherapy or irradiation, but also as a consequence of excessively protein rich, meat-based nutrition. Due to the enzymatic degradation to nucleotides, nucleosides and nucleo-bases, nucleic acids are partially re-utilized into the cell for the purine and pyrimidine biosynthesis. A high nucleic acid turnover can lead to high concentration of purine decay products in blood (e.g. xanthine, hypoxanthine, uric acid) and therefore to various forms of gout. Moreover, nucleosides or nucleotides can influence immunological reactions. Therefore many nucleic acid-based drugs are developed and successfully used in the treatment of malignancies or viral diseases.

3. Pathogenic role of extra-cellular purine nucleotides´ accumulation, as a consequence of hereditary deficiency of adenosine deaminase (ADA) or purine nucleoside phosphorylase (PNP)

*E*xtracellular accumulation of purine nucleotides occurs in case of hereditary deficiency of two key-enzymes of the purine catabolism: purine nucleoside phosphorylase (PNP) and adenosine deaminase (ADA), both resulting in severe combined immunodeficiency SCID (Mann et al., 2006). Whereas ADA-deficiency causes dATP-accumulation in erythrocytes and lymphocytes, ADP-accumulation in lymphocytes and adenosine accumulation in plasma through block of adenosine deamination to inosin, the PNP-deficiency is responsible for a high dGTP level and accumulation in lymphocytes as a result of an impaired degradation of inosine and guanosine to hypoxanthine. Both enzymes` deficiencies trigger the inhibition of other key-enzymes of the DNA-synthesis, e.g. ribonucleotide-reductase or deoxynucleotidyl-transferase: ADA-deficiency affects T-, B- and natural killer- cells (NK), while a PNP-deficiency is rather responsible for T-cells´ deficiency (Speckmann et. al. 2010). Experiments on cultured T-cells show that co-culture with dGua has a cyto-toxic effect in vitro (Fairbanks et al., 1990). Based on those observations, we could measure and quantify inhibitory effects of (r)Gua, GTP, dGTP and ATP on the *in vitro* stimulated T-cells´ proliferation. Moreover we

could show that the co-incubation of CD3/CD28 stimulated PBMCs isolated from healthy donors, addressing directly T-cells, with adequate 200µM exogenously added guanine nucleotides, nucleosides and ATP, inhibited cell proliferation. No comparable effects were observed by co-incubation with pyrimidines, dATP or Ade. Among the tested adenine nucleotides and nucleosides, only ATP was able to generate inhibitory effects on stimulated T-cells´ proliferation, comparable with the effects obtained with guanine nucleotides and -nucleoside added to stimulated T-cells. This could be interpretated by a high concentration of ATP leading rather to GMP-synthesis than to AMP-synthesis, by using ATP as energy source for the conversion of IMP to GTP.

4. Exogenously added guanine nucleotides, nucleosides and ATP have concentration-dependent inhibitory effects on the in vitro proliferation of stimulated T-cells and may inhibit various cell lines´ proliferation .

Purine nucleosides/nucleotides added to the K562 cell line are known to induce a cell cycle arrest in the early S-phase, resulting in down-regulated DNA-synthesis (Moosavi et al., 2006) or an inhibited proliferation of Jurkat-cell line (Batiuk et al., 2001). We confirm these findings: both Jurkat- and Raji-cells´ proliferation *in vitro* could be inhibited when co-incubated with 200µM GTP, dGTP or Gua for six days but not by 200µM ATP. The T-cell line Jurkat responded more sensitive to exogenously added purine nucleotides or nucleosides compared to the B-cells like Raji, what could be explained by a more rapid metabolism. The increase of GTP followed by a decrease of ATP could be explained by ATPs' consume as energy source for the conversion GMP→GDP→GTP, targeting nucleoside diphosphate kinase, the equilibrating enzyme for the guanine-adenine nucleotide pool (Batiuk et al., 2001).

In order to further elucidate the influence of purine nucleotides/nucleosides on T-cells and to deduce possible haematopoietic consequences, we performed refined experiments, applying exogenously nucleotides or nucleosides to CD3/CD28 stimulated T-cells, thereby addressing a more complex, cellular and specific T-cell stimulatory pathway. Our results demonstrated a concentration-dependent inhibition of T-cells` proliferation by using highly specialised antigen-presenting cells such as dendritic cells (DCs) as T-cell stimulators. Moreover, the percentage of inhibited T-cells` proliferation, obtained after T-cells stimulation by CD3/CD28 Beads, were comparable with those from T-cells` stimulation index, obtained with DCs in MLCs.

Modifying the cellular microenvironment in our experimental settings, we could further demonstrate that the proliferation inhibitory effect of purine nucleotides and nucleosides in X-Vivo15[2] cell culture medium (presumed to contain additives like Glucose, Biotin, GM-CSF or Cortison) was different compared to RPMI1640[1]. Based on these results, we presumed differenced expressions, medium-dependent, of ecto- apyrase (CD39) or ecto-5´- nucleotidase (CD73). We studied the cell surface expression of the two enzymes in our inhibitory model, either in RPMI1640[1] or in X-Vivo15[2]. The two enzymes are directly involved in the cellular metabolism of exogenously purine: CD39 (E-NTPDase, EC 3.6.1.5) is an ecto-phosphodiesterase which hydrolyses not only ATP to ADP and AMP but also GTP, to GDT and GMP (J. Goding, J. of Leukocyte Biology 2000, D. Leal et al., Biochemica et Biophysica Acta 1721, 2005). Recently the role of CD39 has been elucidated in more detail: meanwhile it is regarded as an important checkpoint molecule with a central role in the mediation of

tumormediated immunosuppression – that can be addressed by CD39-blocking antibodies (Bonnefoy et al., 2015; Bastid et al., 2015). The role of CD73 (ecto-5´- nucleotidase, EC 3.1.3.5) is the extracellular dephosphorylation of AMP and GMP to the corresponding adenosine or guanosine, enabling the intracellular up-take and re-conversion to ATP or GTP, targeting the purine salvage pathway (Chakraborty et al., 2009). Moreover it has been shown, that in case of an expression of CD73 on γδ Tcells downregulates their Tcell activity and that a modification of CD73 expression on (Th17) Tcells manipulates the pro- or anti-inflammatory effects of these Tcells (Liang et al., 2016). Here we could demonstrate, that the CD39 expression on stimulated T-cells in RPMI1640[1] was much stronger up-regulated compared to X-Vivo15[2], demonstrating a stronger degradation of purine nucleotide triphosphates to purine di- and monophosphtes; In contrast, the CD73-expression on stimulated T-cells was gently down-regulated in RPMI1640[1], compared to X-Vivo15[2]. That could be an evidence for a very low dephosphorylation ratio of nucleotide monophosphates to corresponding nucleosides. CD39 expression on stimulated T-cells was strongly up-regulated by co-incubation with or without guanine nucleotides or nucleosides in X-Vivo15[2] (ranking: [GTP> dGTP> Gua> stimulated T-cells without guanine nucleotides or nucleosides]), compared with the up-regulation of stimulated T-cells, co-incubated under the same conditions, but in RPMI1640[1]. CD39-expression was also up-regulated by co-incubating stimulated T-cells with or without adenine nucleotides or nucleosides in X-Vivo15[2] (ranking: [ATP> Ade> dATP> stimulated T-cells without adenine nucleotides or nucleosides]), whereas in RPMI1640[1], a down-regulation was measured (ranking: [stimulated T-cells without adenine nucleotides or nucleosides > ATP> dATP> Ade]). The expression of CD73 on stimulated T-cells, co-incubated in the two different media, under the same conditions as CD39, showed a similar pattern as the CD39-expression.The impact of culture media (RPMI, X-vivo (with or without serum components added)) on morphological, antigen-expressions, genetic repertoires, cellular expansions as well as functional characteristics of (regulatory) T-cells or celllines has already been shown (Golab et al., 2013; Pandolfino et al., 2010; Block et al., 2008). Especially with respect to the use of (serum-free or serum-supplemented) media in the use of a generation and preparation of cells for adoptive immunotherapeutic cell transfer of cells these findings have to be considered.

Taken together our data show that guanine nucleotides, nucleosides and ATP inhibit concentration dependent the proliferation of T-cells or even influence their vitality, accompanied by a changed cellular expression profile of the nucleotide-processing surface markers (CD39, CD73), what might go along with a modified functionality of Tcells . Influences of additives or other factors such as Glucose, Biotin, GM-CSF or Cortison, on T-cells´ proliferation might also be assumed.

6. Possible interferences of exogenously added guanine nucleotides or nucleosides with immunosuppressive therapy

Results of our previous experimental data showed inhibitory effects of exogenously added guanine nucleotides or nucleosides on in vitro stimulated T-cells. Therefore we studied possible interactions between exogenously added guanine nucleotides, nucleoside and the immunosuppressant MMF. MMF as the pro-drug of mycophenolic acid (MPA) targets directly and specifically IMPDH, the key-enzyme of the *de novo* purine synthesis, responsible for the conversion of inosine monophosphate to xanthosine monophosphate, an important precursor of guanosine nucleotides necessary for DNA-

synthesis (D. Golshayan et. al., 2009). Our data showed that the anti-proliferative effect of 2 µM MMF on CD3/CD28 stimulated T-cells could be partially abrogated with 50-, 20- or 100 µM of each guanine nucleotides or nucleosides. Other tested concentrations of exogenously added guanine nucleotides or nucleosides showed non-significant interactions with anti-proliferating effect of 2µM MMF. These results lead us to the conclusion that the interference dGTP, GTP, Gua -MMF requires certain guanine nucleotide or nucleoside concentrations. These observations are in line with the different pathways of the two immunosuppressive drugs with respect to targeting the intracellular metabolism of nucleic acids: a direct action of MMF on the *de novo* pathway of purine synthesis is shown, that points to the important interacting role of exogenously added guanine nucleotides, nucleosides as participants to the *salvage* pathway and the *de novo* pathway of purine synthesis.

7. Conclusions

We conclude that a) proliferation of stimulated T-cells *in vitro* can be inhibited using adequate concentrations of guanine nucleotides, nucleosides and ATP, but not by dATP or adenosine. *b.)* The inhibitory effects occurred by both CD3/CD28 stimulation and by DCs stimulation of T-cells in MLC. *c.)* Our data point to concentration dependent interferences ex-vivo between the immunosuppressant MMF, a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH) and therefore of the *de novo* pathway of guanine nucleotides and guanine nucleosides or nucleotides, but not with Cyclosporine-A (CsA), known as calcineurine- inhibitor. *d.)* Serum-free media such as Vivo15[2] removed the inhibitory effect of GTP and dGTP, but not of Gua and ATP, on the stimulated T-cells´ proliferation. Despite many analysed cell culture media additives, we could not find the compound or compounds that generate such effects. *e.)* A possible explanation of these different effects could be the different pattern of CD39- or CD73- expression on stimulated T-cells, co-incubated with or without guanine nucleotides and nucleoside, in the two different cell culture media – what might result in functional differences of the resulting cells. We hypothesise that *a.)* High extra-cellular concentration of nucleic acids might have a negative influence on proliferating T-cells by inhibiting the cellular proliferation and therefore T-cell-based immunity. *b.)* Exogenously added purine nucleotides or nucleosides might interfere with the nucleic-acid-based treatment via intracellular nucleic acid metabolism, thus decreasing the efficacy of these drugs. It might be necessary to adjust MMF-based immunosuppressive therapy with the extra-cellular, serum concentration of guanine nucleotides or nucleosides in transplanted patients during immunosuppressive therapy. *c.)* A purine-poor diet might be recommended for patients receiving MMF after organ transplantation in order to avoid an attenuation of the immunosuppressants´ effect and to favour a better transplant recovery. Prospective clinical trials are required to study and probably confirm *in vitro* findings also as *in vivo d.)* Nevertheless, it could be interesting to use the inhibitory capacity of guanine nucleotides, nucleosides or ATP as platform for experimental models concerning ADA-or PNP-deficiency, and also for developing of future purine nucleotides or nucleosides- based drugs. Further experiments will be necessary, in order to elucidate the cellular mechanisms that lead to such inhibitory effects for the guanine nucleotides, nucleoside and ATP and to interferences with nucleic acid-based therapy.

Figure 1: Flow-cytometric quantification strategy for DCs and mononuclear cell populations, before and after culture (MCM-Mimic).

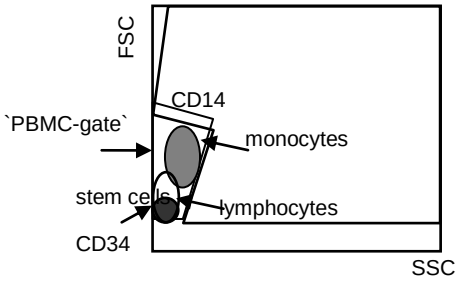
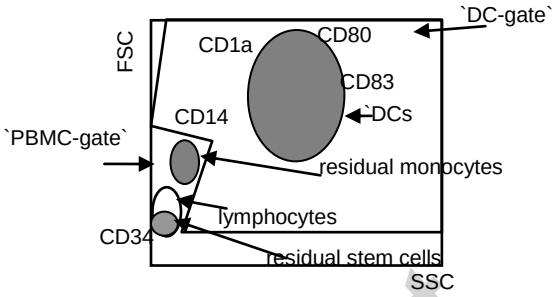
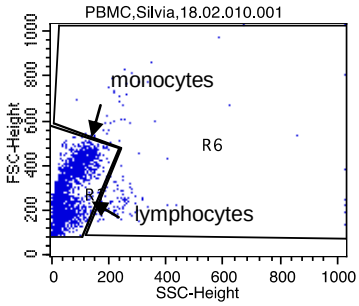
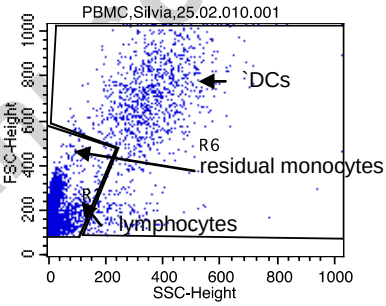
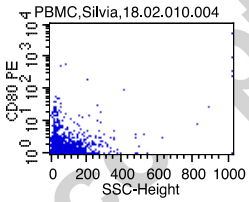
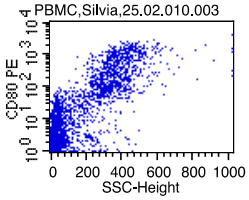
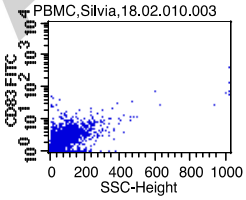
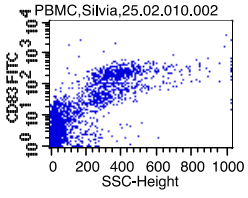
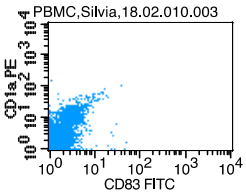
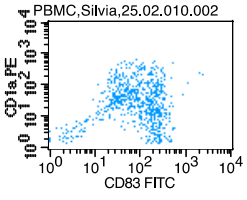
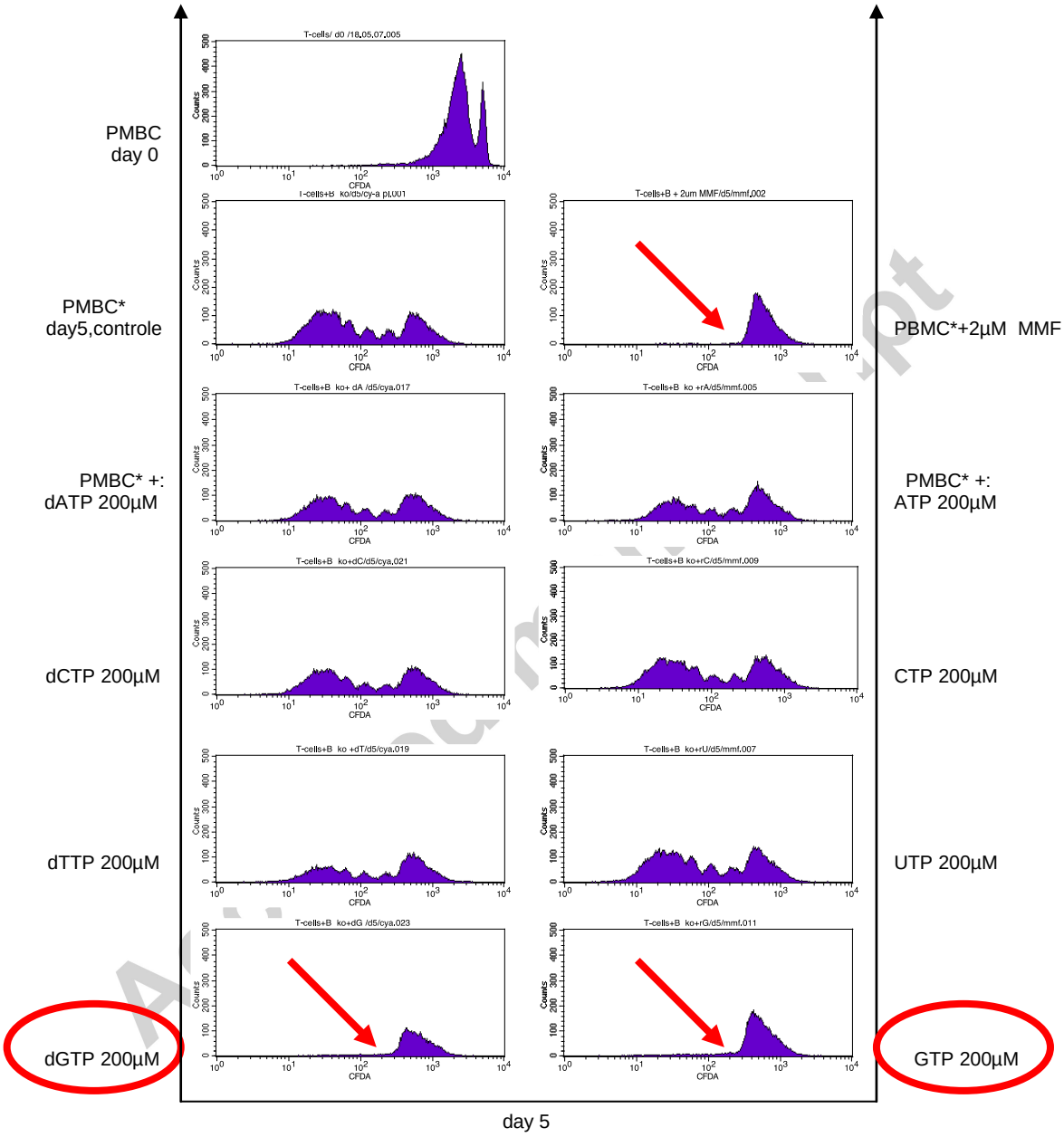
PBMCs before culture	PBMCs after culture
(A): Schematic gating of cell populations to quantify DCs.	
	
(B): Cell shapes before and after DC-culture in MCM-Mimic.	
(B1): DCs are characterised by high FSC and SSC.	
	
(B2): Cells gain CD80 after conversion to DCs.	
	
(B3): Mature DCs co-express CD83.	
	
	

Figure 2: In vitro proliferation of PBMCs and T-cells can be inhibited with purine nucleotides and nucleosides.

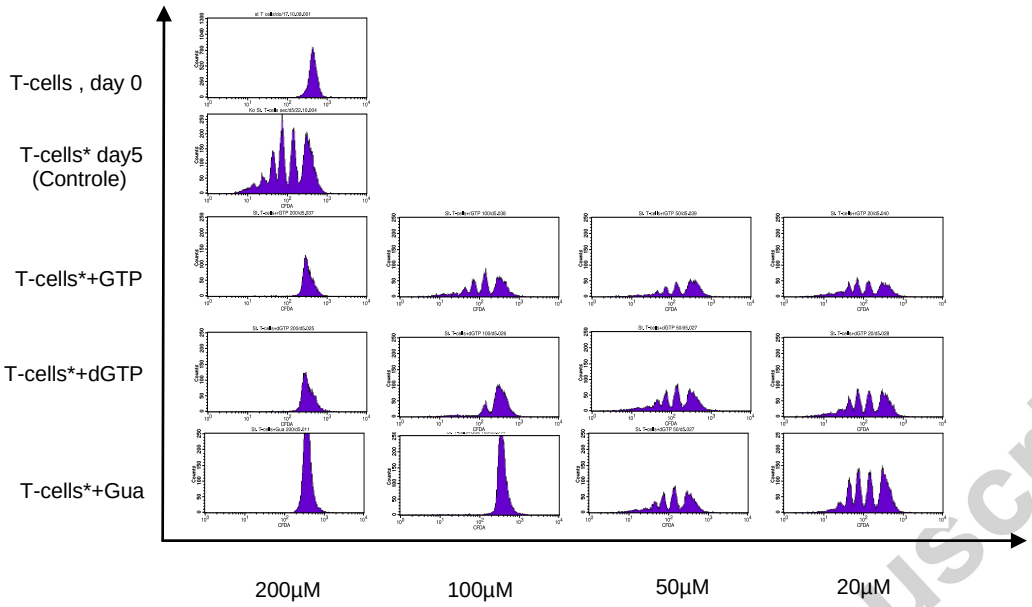
(A): **d**GTP and GTP (200µM) inhibit proliferation of CD3/CD28 stimulated PBMCs (PBMCs*), demonstrated by CFDA-SE assays. One representative histogram out of three independent experiments is given.



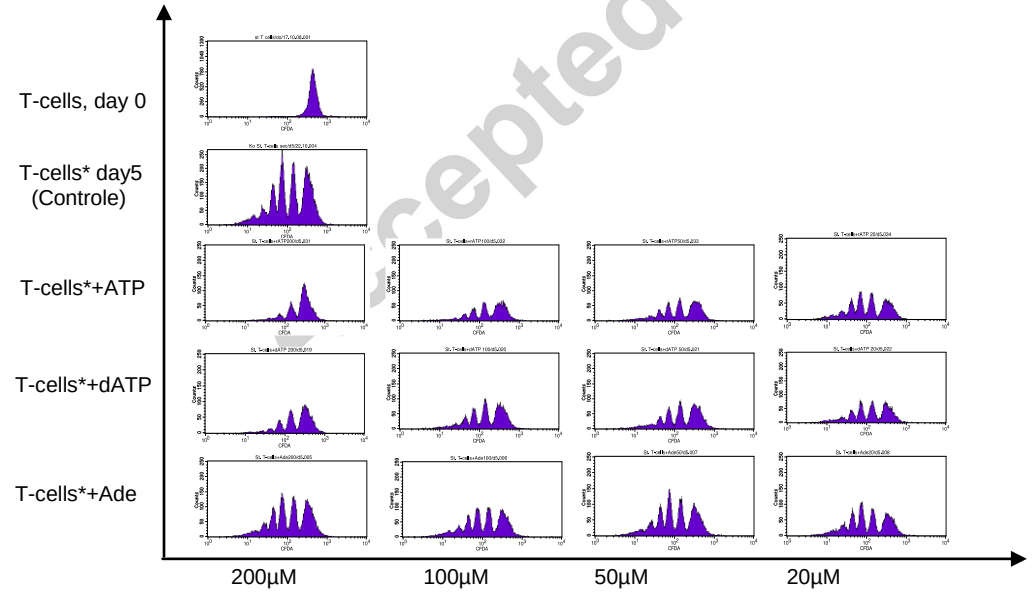
PBMC day0 = PBMC + CD3/CD28 Dynabeads, measured 2 hours after stimulation.

PBMC* = CD3/CD28 Dynabeads stimulated PBMCs.

(B): Purine nucleotides and nucleosides inhibit concentration dependent CD3/CD28 stimulated T-cells´ proliferation (T-cells*), as demonstrated by CFDA-SE assays. One representative histogram out of four independent experiments is given.



(I)

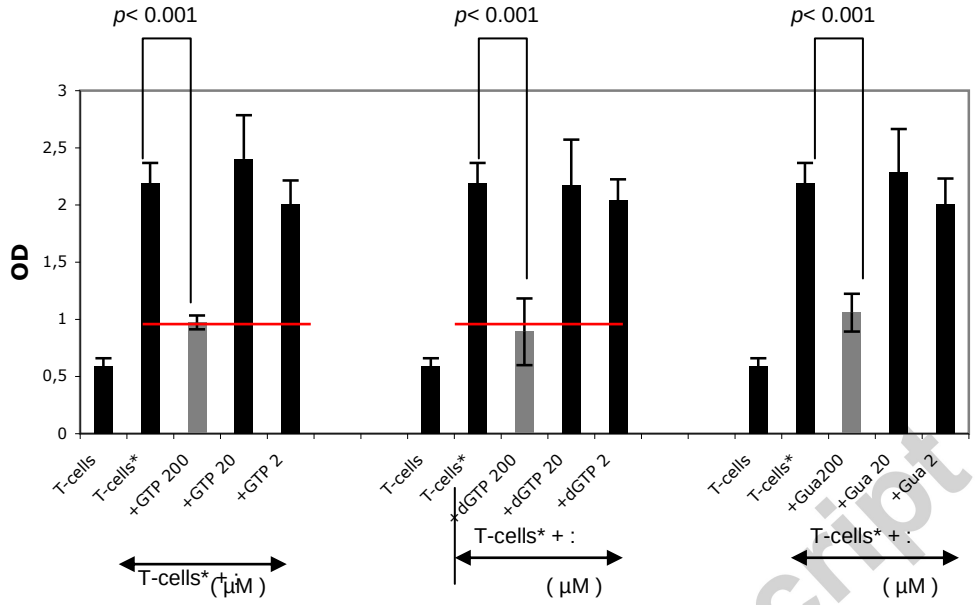


(II)

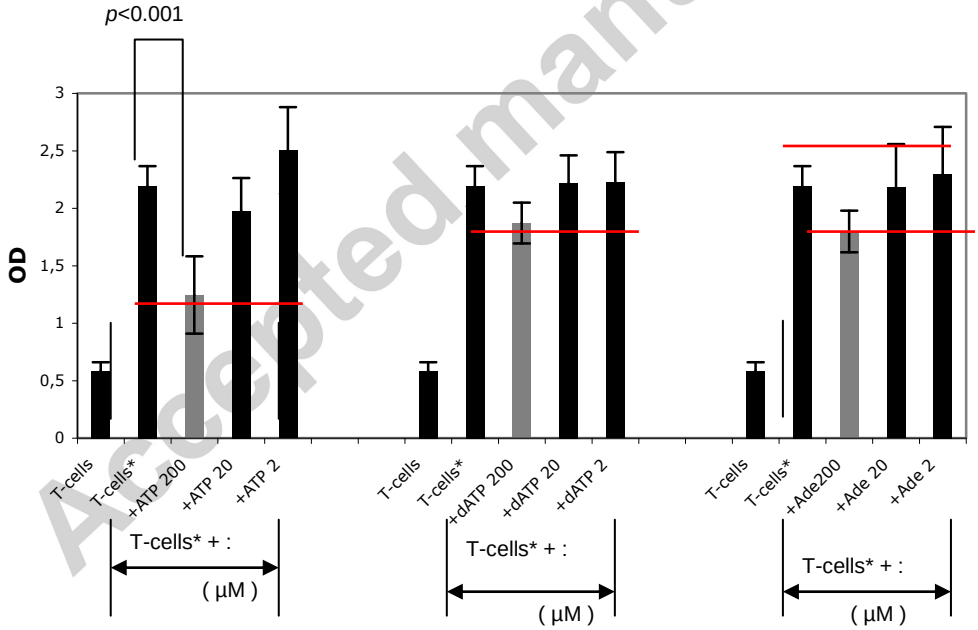
T-cells do= T-cells + CD3/CD28 Dynabeads, measuread 2 hours after stimulation.

T-cells*= CD3/CD28 Dynabeads stimulated T-cells.

(C): XTT-proliferation assays confirm concentration dependent inhibitory effects of purine nucleotides and nucleosides on CD3/CD28 stimulated T-cells´ (T-cells*) proliferation. Cumulative data of four independent experiments are given.



(I)



(II)

T-cells= unstimulated T-cells.

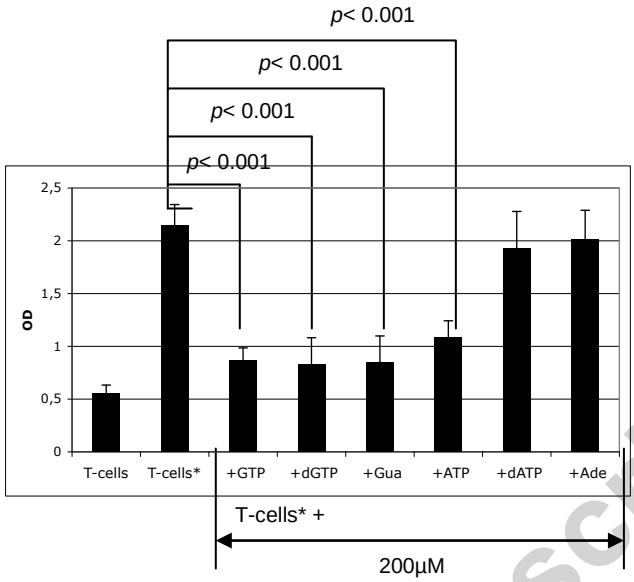
T-cells*= CD3/CD28 Dynabeads stimulated T-cells.

columns = T-cells*+ inhibitory, 200µM purine nucleotides or nucleosides.

= inhibition level of proliferating, CD3/CD28 Dynabeads stimulated T-cells, using 200µM guanine **(I)** or 200µM adenine **(II)** nucleosides or nucleotides.

OD= optical density

D): 200µM is the optimal concentration of exogenously added purine nucleotides and nucleosides, with maximal inhibition of CD3/CD28 stimulated T-cells` proliferation (T-cells*), demonstrated by XTT-proliferations assays. Cumulative data of six independent experiments are given.

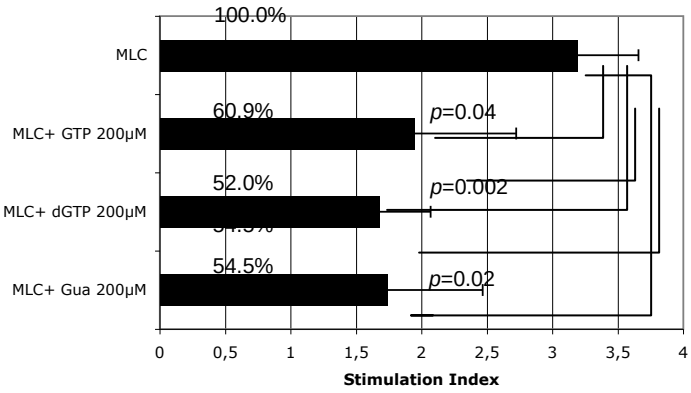


T-cells= unstimulated T-cells.

T-cells*= CD3/CD28 Dynabeads stimulated T-cells.

OD= optical density

Figure 3: Guanine nucleotides, nucleosides and ATP- but not dATP or Ade inhibit T-cells` proliferation in mixed lymphocytes culture (MLC). Csomparison of inhibitory effects of guanine and adenine nucleotides and nucleosides on mixed lymphocyte cultures (MLC), measured as XTT-proliferation assays. Mean values of stimulation indices and p-values of four independent experiments are given.



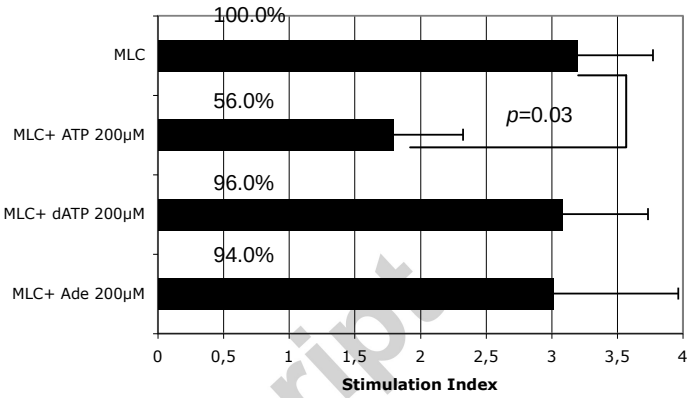
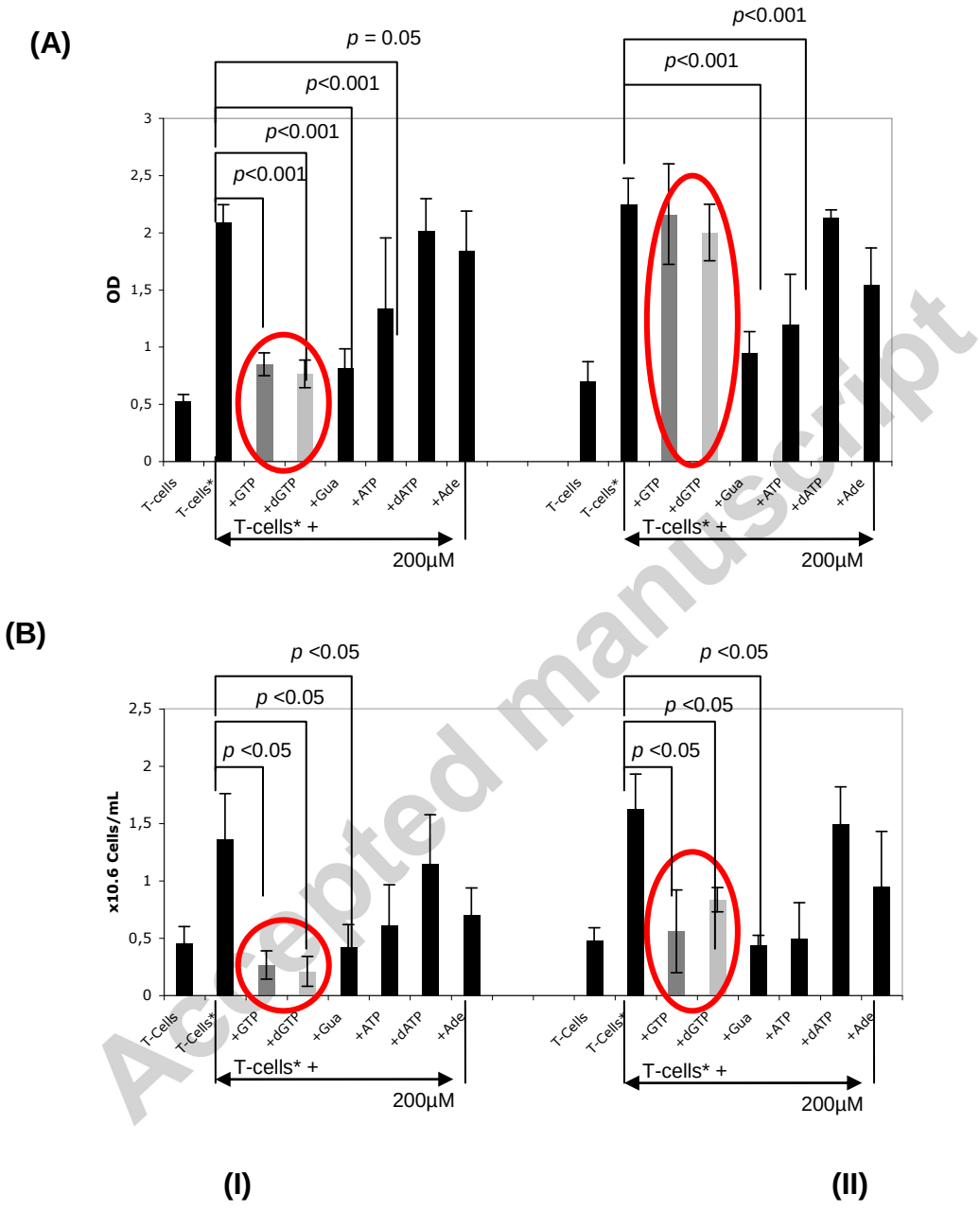


Figure 4: Different inhibitory effects of exogenously added GTP and dGTP on proliferating, stimulated T-cells (T-cells*) by co-incubation in RPMI1640[1] (I) or in serum-free X-Vivo15[2] medium (II), demonstrated as XTT-proliferation assays (A) or Trypan-blue viability-tests (B). Cumulative data of four independent experiments are given.



T-cells= unstimulated T-cells.

T-cells*= CD3/CD28 Dynabeads stimulated T-cells.

(I) = T-cells* incubated in RPMI1640.

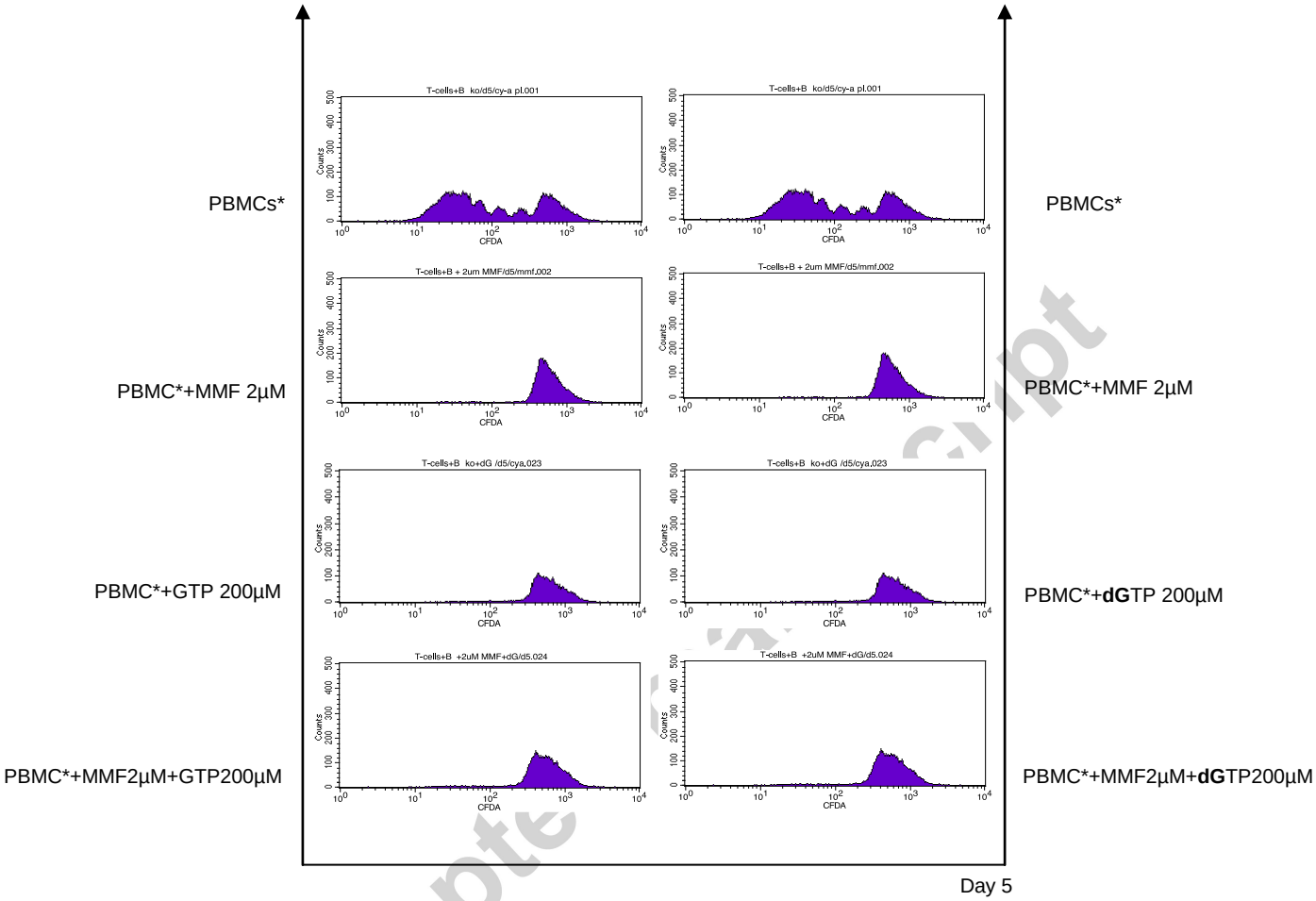
(II) = T-cells* incubated in X-Vivo15.

columns = T-cells*+ 200µM GTP

columns = T-cells* +200µM **dG**TP.

OD= optical density

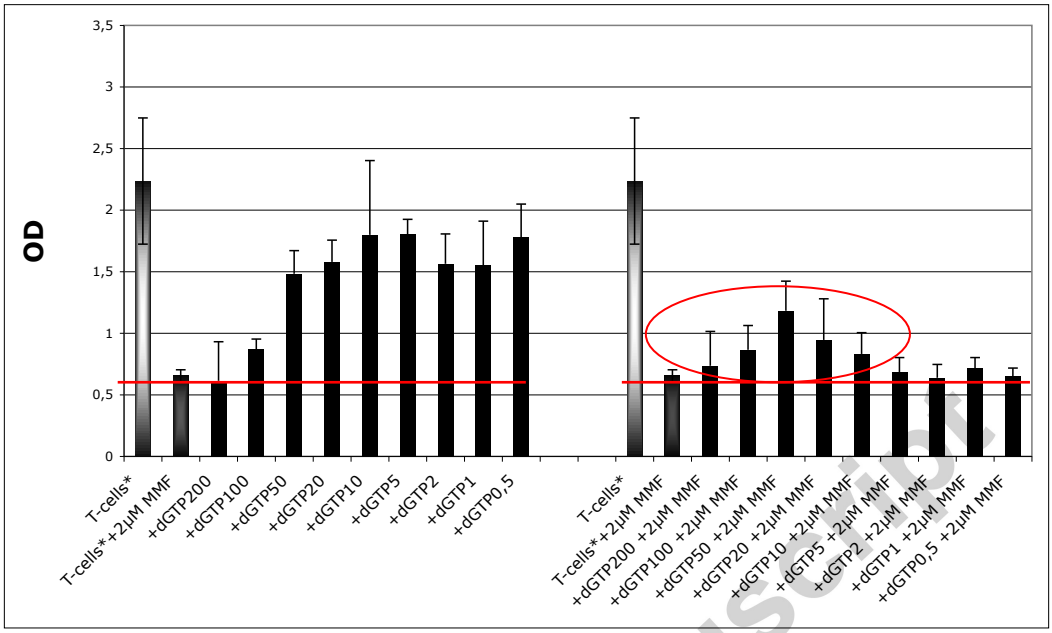
Figure 5: In vitro proliferation of CD3/CD28 stimulated PBMCs can be inhibited with guanosine nucleotides, with or without MMF, demonstrated as CFDA-SE assays. One representative histogram out of four independent experiments is given.

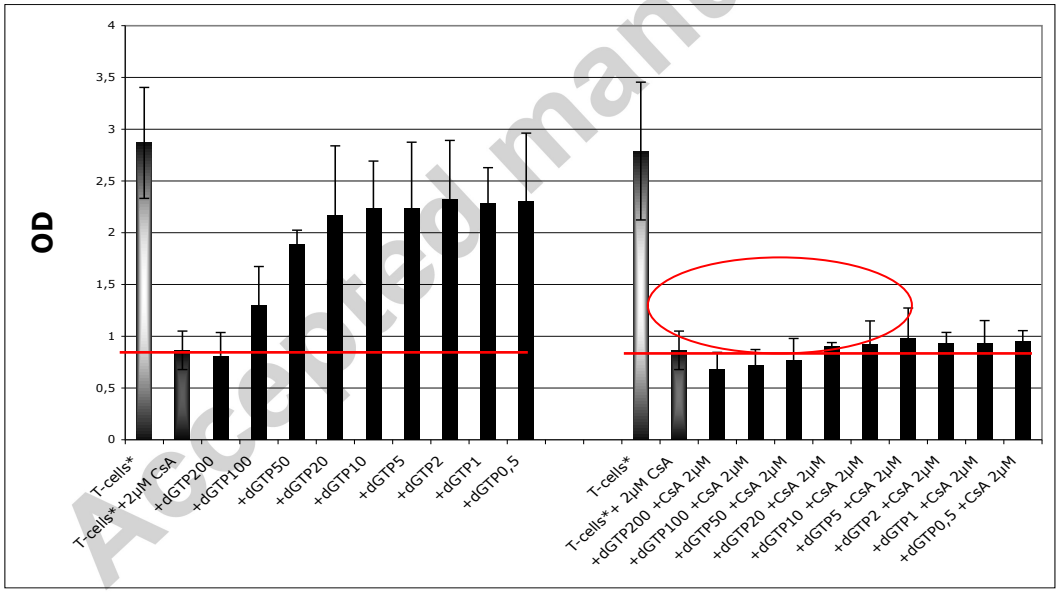


PBMCs* = CD3/CD28 Dynabeads stimulated PBMCs.
MMF= Mycophenolate mofetile.

Figure 6: dGTP inhibits (concentration dependent) CD3/CD28 stimulated T-cells` (T-cells*) proliferation and interfere with the immunosuppressant MMF (I) but not with CsA (II).
Cumulative data of three independent experiments are given.

(I)





(II)

T-cells*= CD3/CD28 Dynabeads stimulated T-cells.

columns = T-cells*.

columns = T-cells*+2µM MMF (I) or +2µM CsA (II).

columns = T-cells* with dGTP various concentrations, with or without MMF or CsA (2µM).

= inhibition level of proliferating,CD3/CD28 Dynabeads stimulated T-cells, using 2µM MMF(I) or 2µM CsA (II).

= interference of various dGTP concentrations with 2µM MMF(I) ; no interferance of dGTP 2µM CsA (II).

Table 1: Percentage of proliferating T-cells (T-cells*), co-incubated with or without 2µM MMF and various concentrations (µM) of dGTP, GTP or Gua.

T-cells* [%]		100	100	100
T-cells* + 2µM MMF [%]		31	31	31
		+dGTP	+GTP	+Gua
T-cells* [%]+ 2µM MMF +:	200µM	37	42	23
	100µM	42	57	52
	50µM	57	55	53
	20µM	47	52	48
	10µM	40	41	35
	5µM-0.5µM	31-34	34-38	31-35

***Table 2**: **Percentage of CD39- or CD73-expression on stimulated T-cells (T-cells*) co-incubated with or without 200µM dGTP, GTP or Gua, in RPMI1640[1] or X-Vivo15[2]**.*

		RPMI1640[1]	X-Vivo15[2]
CD39-expression [%] on : T-cells*	day0	16	16
	day5	23	8
T-cells*+200µM GTP	day5	30	49
T-cells*+200µM dGTP	day5	26	28
T-cells*+200µM Gua	day5	26	19
CD73-expression [%] on: T-cells*	day0	25	27
	day5	16	3
T-cells*+200µM GTP	day5	22	23
T-cells*+200µM dGTP	day5	18	7
T-cells*+200µM Gua	day5	19	9

LEGENDS TO FIGURES

Figure 1: Flow cytometric quantification strategy for DCs and mononuclear cells populations, before and after culture (MCM-Mimic).

(A): Special gating strategy used to detect and quantify DCs: schematic gating of the PBMC cell population before (left side) and after (right side) culture, in MCM-Mimic method for DC generation; low degrees of DC antigens (DCA, e.g.CD80, CD83, CD1a) can be detected by FACS-analyses on untreated PBMCs, high degrees- after culture with MCM-Mimic method; DCs co-expressing CD83 and CD1a were defined as "mature DCs".

(B1): DCs are characterized by high FCS and SSC: FCS/SSC- projections demonstrate the gain of high SSC/FSC DCs profiles (right side) for PBMCs after culture in MCM-Mimic method compared to uncultured PBMCs (left side). **(B2, B3):** Gain of CD80 (B2) and CD83 (B3) positivity on DCs (right side), compared with uncultured PBMCs (left side). **(B3):** gain of positivity for co-expressed CD83 and CD1a, for mature DCs after MCM-Mimic method culture (right side) compared with uncultured PBMCs (left side).

Figure 2: In vitro proliferation of stimulated PBMCs and T-cells can be inhibited with purine nucleotides .

(A): Proliferation of CD3/CD28 stimulated PBMCs (PBMCs*) can be inhibited by co- incubation with 2µM MMF but also only with GTP or dGTP (200µM). One representative of three independent histograms, CFDA-SE proliferation assay are given. **(B): (I)** Guanine nucleotides and nucleosides strongly inhibit T-cells´ proliferation, at concentrations >100µM; **(II)** among adenosine nucleotides and nucleosides, only 200µM ATP is able to inhibit the T-cells proliferation. One representative of four independent histograms, CFDA-SE proliferation assay are given. **(C):** a concentration dependent inhibition of CD3-CD28 stimulated T-cells´ proliferation using GTP, dGTP, Gua**(I)** and ATP**(II)** can be re-confirmed by XTT- proliferation assays. Cumulative data of four independent experiments, obtained with XTT- proliferation assays. **(D):** Cumulative data of six independent experiments, obtained with XTT- proliferation assays.

Figure 3: Guanine nucleotides, nucleosides and ATP- but not dATP or Ade can inhibit T-cells proliferation in mixed lymphocytes culture (MLC); Proliferative responses of purified CD3+ T-cells (responders) to allogeneic DCs (stimulators), added in a ratio of 1:2, can be inhibited after co-culture with 200µM exogenously added guanine nucleotides, nucleosides and ATP, but not dATP or Ade. Mean of stimulation index and *p*- values of four independent experiments, measured by XTT-proliferation assays are given; stimulation index was calculated as ($OD_{\text{T-cells+DCs, with/ without exogenously added purine nucleotides and nucleosides}}$ / $OD_{\text{T- cells}}$).

Figure 4: Different inhibitory effects of exogenously added GTP and dGTP on proliferating, stimulated T-cells (T-cells*) by co-incubation in RPMI1640[1] (I) or in serum-free X-Vivo15[2] medium (II), demonstrated as XTT-proliferation assays (A) or Trypan-blue viability-tests (B); Co-culture of CD3/CD28 stimulated T-cells with 200µM guanine nucleotides and nucleosides, parallel in RPMI1640[1] **(I)** and in serum–free X-Vivo15[2] **(II)** shows an abolished inhibition in X-Vivo15[2] for 200µM GTP and 200µM dGTP compared with the strongly inhibition of both 200µM GTP and 200µM dGTP in RPMI1640[1]; inhibition with 200µM Gua remain unaffected in X-Vivo15[2] and is slightly

favoured by 200µM ATP and 200µM Ade as in RPMI1640[1]; Cumulative data of five independent experiments, obtained with XTT- proliferation assays are given ***(A)***.Trypan-blue viability tests of stimulated T-cells, co-incubated as described above, demonstrate that the cell viability is sustained by X-Vivo15[2] compared with RPMI1640[1] used as co-incubation medium. Cumulative data of four independent experiments, obtained with Trypan-blue viability test are given ***(B)***.

Figure 5: In vitro proliferation of CD3/CD28 stimulated PBMCs can be inhibited with guanosine nucleotides, with or without MMF: Inhibition of the stimulated PBMCs´ proliferation can be achieved not only by co-incubation with 2µM MMF, 200µM- GTP or –dGTP, but also with a mixture of MMF and GTP or dGTP; One representative of four independent histograms, CFDA-SE proliferation assay are given.

Figure 6: dGTP inhibits (concentration dependent) CD3/CD28 stimulated T-cells´ (T-cells*) proliferation and interferes with the immunosuppressant MMF (I) but not with CsA (II): Proliferation of CD3/CD28 stimulated T-cells can be inhibited in a concentration dependent manner, by co-incubation with dGTP in various concentrations; the immunosuppressive effect of 2µM MMF (I) but not of 2µM CsA (II) could be slightly abolished by co-incubation with 200µM, 100µM or 2µM dGTP and strongly interfered by co-incubation with 50µM and 20µM dGTP; a decrease of the interference effect was observed for dGTP concentrations < 20µM. Cumulative data of three independent experiments, obtained with XTT- proliferation assays are given.

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