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Suppression of Dendritic Cell maturation: a novel mechanism of Azathioprine immune modulation.

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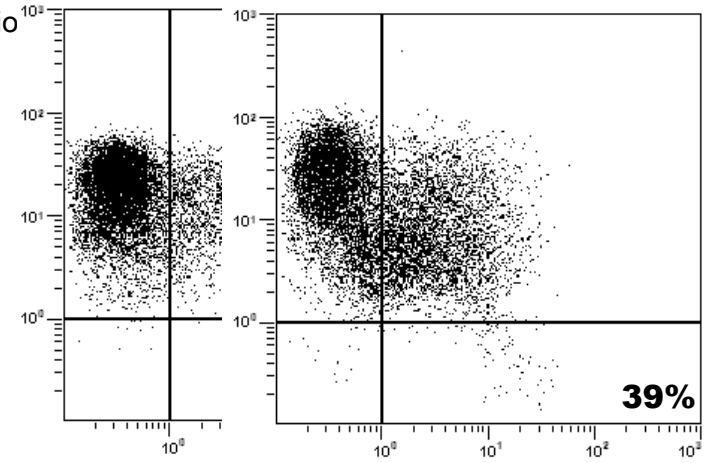


1. INTRODUCTION

1.1. Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) (Martin et al, 1992; Compston et al, 2002). MS has been considered to be an autoimmune disorder mediated by $CD4^+$ type 1 T helper cells, but recent studies have challenged this idea by indicating a role for other immune cells (Sospedra et al, 2005). During the course of MS, the humoral immune response becomes compartmentalized in the CNS generating intratecal production of oligoclonal IgG (Qin et al, 1998; Colombo et al, 2000). Moreover, studies performed on cloned T cells isolated from MS cerebrospinal fluid (CSF) demonstrated that this compartment is enriched of T cell clones using restricted T cell receptor (TCR) genes (Hafler et al, 1985; Lee et al, 1991). Even if other investigations failed to reproduce these findings (Rottevel et al, 1987), these data suggest that in MS also T cells may expand intrathecally as a result of antigen (Ag) specific stimulation. Recently, the possible contribution of $CD8^+$T cells as effectors of the pathological immune reactions damaging the CNS of MS patients and animals with experimental autoimmune encephalomyelitis (EAE), the animal model of MS, has been outlined (Sun et al, 2001; Huseby et al, 2001; Steinman 2001; Neumann et al, 2002; Liblau et al, 2002). T lymphocytes, $CD8^+$T cells could be demonstrated in MS brain lesions (Hayashi et al, 1986). In addition, analysis of microdissected $CD8^+$T cells in the brain lesions using single-cell PCR showed oligoclonal expansion of these cells (Babbe et al, 2000). Their role in MS pathogenesis has been strongly suggested by a study of the TCR repertoire in three separate compartments (brain, CSF and blood) of two MS patients (Jacobsen et al, 2002; Skulina et al, 2004). These data do not exclude that $CD4^+$T cells may also play an important role. It is likely indeed, that $CD8^+$ T cell function depends on “help” from CD4+ regulatory T cells and that $CD4^+$ effector cell function might directly contribute to inflammatory tissue injury. In a recent study, $CD4^+$ T cell activation has been

analysed in clinically isolated syndrome (CIS) suggesting an initial attack of MS. The authors showed that percentage of $CD25^+CD4^+$ T cells in CSF correlated negatively with myelin basic protein (MBP) CSF concentration and the presence of IgG oligoclonal bands (Jensen et al, 2004). Human $CD4^+$ regulatory T cells expressing high levels of CD25 are suppressive in vitro and have similar activity of mouse $CD4^+CD25^+$ regulatory T cells. It has been proposed that alteration in function of this cell sub-population may have implications in the breakdown of self-tolerance during MS (Viglietta et al, 2004). Indeed, in EAE $CD4^+CD25^+$ auto-Ag specific cells play an important role in genetic resistance to autoimmunity (Reddy et al, 2004).

As autoimmune pathogenetic mechanisms against CNS white matter underlie the development of the MS lesions, immune-suppressive medications have been successfully used in the therapy of this disease (Hommes et al, 2004): among them Azathioprine, a cytostatic agent, well tolerated, easy to administer and to monitor (Aarbake et al, 1997).

1.2. Thiopurine drugs

Azathioprine (Aza), 6-Mercaptopurine (6-MP) and 6-Thioguanine (6-TG) are thiopurine drugs (*Fig.1.2*) widely used as immunosuppressants/anti-inflammatory agents in organ transplantation and chemotherapy. Azathioprine is utilized to prevent rejection in kidney and heart transplantation (McGeown et al, 1988; Andreone et al, 1986; Chan et al, 1990) and to treat various autoimmune and chronic inflammatory diseases, such as inflammatory bowel diseases, rheumatoid arthritis, systemic lupus erythematosus, primary biliary cirrhosis, and multiple sclerosis (British and Dutch Multiple Sclerosis Azathioprine Trial Group 1988; De Silva et al, 1981; Ginzler et al, 1975; Christensen et al, 1985; Bouhnik et al, 1996; Fraser et al, 2002). On the other hand, 6-MP and 6-TG have been used particularly in the treatment of acute leukaemia (Erb et al, 1998).

6-MP and 6-TG were planned in 1950s by Gertrude Elion and George Hitchings as cytostatic agents primarily for suppressing neoplastic proliferation and initially were developed for therapy of childhood acute lymphoblastic leukaemia (Elion, 1967). Azathioprine (Aza) was designed later in order to prevent hydrolysis in the gut of the

unshielded mercapto-group of 6-MP. The immunosuppressive activity was described since 1958 (Elion, 1993) and in 1963 its activity on prolonging renal allograft survival was proved (Murray et al, 1963).

Along with the Azathioprine pharmacological and clinical development, other activities were observed (Elion 1993) and, for this discover, Elion and Hitchings in 1988 received the Nobel Prize in Medicine.

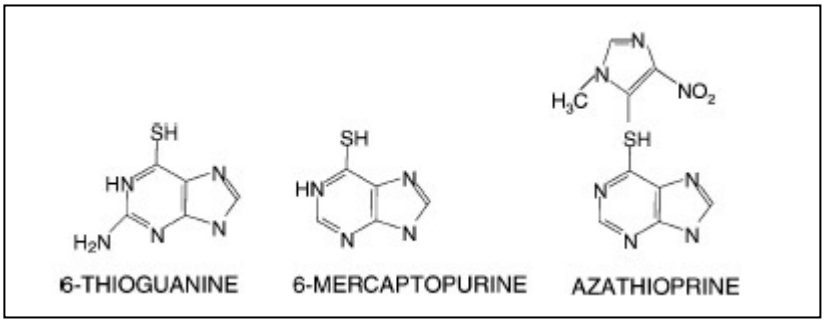


Fig. 1.2. Molecular structure of thiopurine drugs.

1.2.1. Metabolism of azathioprine

The Azathioprine molecule is composed of two moieties: mercaptopurine and an imidazole derivative. After oral administration and intestinal absorption, ranged from 50 to 72%, the pro-drug Azathioprine undergoes approximately 90% conversion to 6-MP and S-methyl-4-nitro-5-thioimidazole by non enzymatic attack by sulphydryl containing compounds such as glutathione or cysteine that are present in every mammalian cell. Whereas S-methyl-4-nitro-5-thioimidazole is excreted in urine, 6-MP enters cells where it is subject to three competing enzymes: xanthine oxidase (XO), thiopurine S-methyltransferase (TPMT) and hypoxanthine guanine phosphoribosyltransferase (HGPRT) (Van Os et al, 1996; Hoffmann et al, 2001; Lennard 1992).

Xanthine oxidase oxidizes 6-MP generating the inactive metabolite 6-thiouric acid (6-TA). Thiopurine S-methyl-transferase converts 6-MP to 6-methyl-mercaptopurine (6-MMP). Hypoxanthine-guanine-phosphoribosyl-transferase acts on 6-MP to produce 6-Thioguanine (6-TG); 6-TG is then metabolised further to form mono-di- and tri-phosphate Thioguanine nucleotides (TGNs). In particular, lymphocytes have been shown to

enzymatically convert 6-MP to 6-TG (Tiede et al, 2003). 6-TG and TGNs are thought to be the predominant active metabolites mediating the immunosuppressive properties of Aza, but they also seem to be associated with side effects of this drug as myelotoxicity (Coulthard et al, 2005). Most likely TGNs can exert cytotoxicity following incorporation into DNA as fraudulent bases causing DNA-protein cross-links, single strand breaks, interstrand cross-links and sister chromatid exchanges (Christie et al, 1984; Pan et al, 1990). The incorporation of TGNs into DNA has been shown to be recognised by the mismatch repair (MMR) system. Defects in the MMR pathway have been shown to be associated with resistance to 6-TG (Swann et al, 1996).

An intermediate in the HGPRT pathway, 6-thioinosine-monophosphate (TIMP), can be a substrate for TPMT, resulting in the production of S-methyl-thioinosine-monophosphate (MeTIMP), a strong inhibitor of de novo purine syntesis, which is believed to significantly contribute to the 6-MP cytotoxicity (Vogt et al, 1993). The level of TPMT activity would therefore be expected influence the production of MeTIMP and hence of *de novo* purine synthesis. The effects of TPMT activity levels on cytotoxicity was reported in 1987 by Van Loon (Van Loon et al, 1987). Moreover, the enzyme TPMT competing with HGPRT can prevent TGNs formation. The gene TPMT is subject to genetic polymorphism, leading to an almost 50-fold variation in enzyme activity among individuals. TPMT polymorphisms have been associated with mercaptopurine's therapeutic efficacy and also to its toxicity (Coulthard et al, 2005). Three single nucleotide polymorphisms (SNPs) account for over 90% of the clinically relevant TPMT mutations and bring to aminoacid substitutions that make the protein more susceptible to degradation through ubiquitylation. In Caucasian population about 0.3-0.6% of the individuals carry two mutant TPMT alleles and do not express functional TPMT. That results in greater conversion of 6-MP to 6-TGNs via the HGPRT pathway after mercaptopurine therapy and in myelosuppression, requiring doses to be reduced to as little as a tenth of the normal dose in order to tolerate therapy. Alternatively, high TPMT enzyme activity may result in larger 6-MMP production at the expense of 6-TGN, leading to decreased efficacy. A number of single nucleotide polymorphisms (SNP) for TPMT have been identified that cause diminished or absent

TPMT enzyme activity. About 5-10% of the Caucasians are heterozygous for this polymorphism, have intermediate levels of TPMT activity and require only modest dosage reductions. The remaining 90-95% of the population carry two wild-type alleles and have full TPMT activity (Coulthard et al, 2005).

The relative activities of XO, HGPRT and TPMT determine the net concentration of the active 6- TGN. For this reason attempt to prevent serious adverse events have been pursued looking for homozygous allelic variations. Nonetheless, the similar or higher frequency of toxicity in patients not carrying mutant alleles indicates that other factors, i.e. unpredictable allelic variation of other enzymes involved, may affect Aza metabolism and bioavailability. This finding and the very low frequency of homozygous subjects, suggest that genotyping for TPMT prior to Aza administration may not be efficient. In addition, in many pathological conditions, no correlation has been found between 6-TGNs blood concentrations and disease remission (Cara et al, 2004), indicating that TPMT genotype evaluation has low predictive value in the monitoring of thiopurine therapy as probably also other mechanisms are associated to the immunosuppressive activity of thiopurines (Cara et al, 2004; Lichtenstein 2004). On the other hand, combined net effects of the Aza metabolites may be easily evaluated by leuko/lymphocyte levels. Their reduction probably reflects mainly unspecific suppression of highly proliferating bone marrow precursors of white cell lineage, induced by both the 6-TGNs and the Me-TINPs and it may not necessarily be correlated to the immune-suppressive activity. However leuko/lymphocyte levels act as a marker of the active metabolites net bioavailability and correlates well with effective individual Aza/6-MP dosing (Colonna et al, 1994).

1.2.2. Toxicity

The most common adverse events observed during Aza treatment include gastrointestinal abnormalities (gastric or abdominal pain, seldom vomiting and diarrhoea), bone marrow suppression (defined as wbc<3000/mm^3 or lymphocytes<800/mm^3 or platelet<50.000) and abnormal liver function. Less frequently skin rash and myalgia can also be reported

(Yudkin et al, 1991; Massacesi et al, 1994; Craner et al, 2001; Bryant et al, 2001; Sudlow et al, 2003; Massacesi et al, 2005). However, simple leuKopenia or lymphopenia w/o myelosuppression are part of the action mechanism and should not be considered an adverse event. In prospective controlled clinical studies the adverse events frequency was between 45 and 55 % (Goodkin et al, 1991). Withdrawal rate due to unfavourable effects was lower, being reported about 30 %, but was 10-18% when compared to placebo (British and Dutch Multiple Sclerosis Azathioprine Trial Group 1988; Yudkin et al, 1991). However, any Aza toxicity can be prevented simply by careful monitoring of white blood cells and aminotransferases; in addition, serious adverse events due to hypersensitivity are usually ready reversible with dose reduction (Massacesi et al, 2005). Therefore most of the toxicity associated to Aza can be controlled by appropriate individual dose adjustment. Indeed very rarely, adverse effects and withdrawals were observed after the first year of treatment (Massacesi et al, 2005).

To date little is known about the metabolites that are responsible for the toxicity, although 6-MMP concentrations have been correlated to liver toxicity and 6-Me-TIMPs to bone marrow suppression, but no advantage is achieved by evaluation of serum metabolite concentrations (Reuther et al, 2003; Wright et al, 2004).

Carcinogenic activity of immune-suppression have been suggested, non–Hodgkin lymphomas being the malignancy more frequently associated (Kinlen et al, 1981; Opelz et al, 1993; Ciancio et al, 1997). However the data available are not conclusive, as recent studies indicate that the diseases requiring Aza therapy (autoimmune diseases, cancer or transplantation), may *per se* convey risk of cancer (Kwon et al, 2005; Fraser et al, 2002). Conflicting results have been reported in patients treated only with Aza, without any concomitant therapy: the main concern was raised by two studies by Kinlen et al. carried out many years ago, one in patients with renal graft transplantation, the other with reumathoid arthritis or other autoimmune diseases (Kinlen et al, 1981). These studies showed a substantial increase of cancer frequency rate for patients treated with Aza with respect to the general population. However, as risk of malignancies may be due to the disease itself, concurrent matched untreated patients carrying the same disease should be

the optimal control population (Fraser et al, 2002). Indeed, similar studies including the appropriate controls failed to confirm carcinogenic activity of Aza. For instance, no increase in cancer frequency was found in patients with lupus eritematosus sistemicus (LES) treated with Aza with respect to untreated patients, even after 22 years follow up (Nero et al, 2004). In addition, in patients with Inflammatory Bowel Diseases (IBD), augmented non–Hodgkin lymphoma frequency was observed if treated patients were compared to normal controls but not if compared to untreated patients (Kwon et al, 2005; Fraser et al, 2002). One of the largest study, by Fraser et al, retrospectively compared 627 IBD patients taking Aza with 1578 receiving no or other therapies for a mean of 7 years: high (4.5%) frequency of malignancies was observed, but identical in both the two groups (Fraser et al, 2002).

In MS, two studies with adequate statistical power to detect the cancer risk level, previously reported in other diseases, compared treated and untreated patients. In the first one, 300 patients treated for at least 3 years were followed for 17-20 years (Taylor et al, 2004), whereas in the second study, 442 patients treated for a mean of 4 years were followed for a median time of 7 years (Amato et al, 1993). Both failed to find significant cancer risk increase. However, the largest study was a case-control study, including 1191 patients followed for an average of 12$\pm$9 years (Confavreux et al, 1996). Among the 23 cases with malignancies and the 69 matched controls, respectively 14 (61%) and 34 (49%) had been treated with Aza. This difference, accounted for 1.7 odds ratio (OR), resulted not significant. However, a critical issue must be highlighted in this already classical study: the choice to include two cases receiving Aza for one month only and immediately before cancer diagnosis (respectively six and one month before). As for it is known of cancer biology, at the time of Aza administration the malignancy was probably already developed and it seems unlike that the minimal cumulative dose administered within one single month may have had any causal relation with the subsequent malignancies. If these two cases had not been included, even this marginal difference would disappear. Nonetheless comparing only patients with a treatment duration of more than 10 years the difference was significant (OR: 4.4), suggesting a cumulative dose effect. Nevertheless, Aza dose

related effect on carcinogenicity was not confirmed by a similar evaluation carried out in LES patients (Nero et al, 2004).

The data available overall support the safety of Aza at the usual immune-suppressive doses. This position is sustained in the Essential Medicine Formulary published on the web-site of the WHO, that does not include cancer risk among Aza adverse events (Mehta et al, 2004). If any carcinogenicity by this drug exists, it seems marginal and eventually appearing after more than 10 years treatment.

1.2.3. Azathioprine in MS treatment

Among immunosuppressive medications currently used in MS, Aza has been the most widely studied (Aarbake et al, 1997). The majority of these studies were carried out in the eighties and showed clinical efficacy (British and Dutch Multiple sclerosis Azathioprine Trial group, 1988; Ellison et al, 1989; Goodkin et al, 1991; Yudkin et al, 1991), but this efficacy was often considered marginal or inadequate to balance safety concerns (Yudkin et al, 1991). When, early in the nineties, Aza patent expired, clinical researchers neglected this treatment for MS, leaving a gap of studies that still needs to be filled. However, reviewing today the above mentioned works under the light of more recent achievements, Aza therapeutic effectiveness in MS may have been underestimated. This has probably been due to the limited knowledge, at the time those studies were planned, both on MS clinical course and pathogenesis and on Aza activity, safety and action mechanism. In addition, in the eighties the clinical results were not corroborated by more sensitive marker of disease as MRI evaluation of brain lesions (Cavazzuti et al, 1997; Miller et al, 1998). In the last years these considerations inspired new investigations to better evaluate the Aza usefulness in MS (Markovic-Plese et al, 2003; Fernandez et al, 2004; Palace et al, 1997). A recent work published by Massacesi et al (Massacesi et al, 2005) demonstrate for the first time that Azathioprine, administered at lymphocyte-suppressing doses, is effective in reducing MS new brain inflammatory lesions and is well tolerated.

1.2.4. New insights in the action mechanism of thiopurine drugs

Although Azathioprine has been in clinical use for about four decades, its precise mechanisms of action are still unknown. However, inhibition of *de novo* purine nucleotide biosynthesis with suppression of DNA and RNA synthesis and downregulation of B and T cell functions have been suggested as major therapeutic mechanisms of 6-MP (Röllinghoff et al, 1973; Abdou et al, 1973; Lennard, 1992).

The results obtained along several years indicate that Aza efficacy in various autoimmune diseases is good at well tolerated doses and the risk/benefit ratio is probably the most favourable among cytostatic immune-suppressive agents. This specificity allows many of those who have used and evaluated this medication, including the inventors, to believe that *the "action mechanism of thiopurines cannot be explained only by the cytostatic mechanism they were created for*" (Elion GB, cited by Maltzman et al, 2003).

Recent data produced by Tiede et al (2003) confirm that 6-MP and the metabolite 6 thioguanine triphosphate (6-Thio-GTP) are involved in more selective immune-regulatory mechanisms; they observed a unique and unexpected role for azathioprine and its metabolites in the control of T cell apoptosis by specific blockade of the small GTPase Rac1 activation upon CD28 costimulation, trough binding of azathioprine-generated 6-Thio-GTP to Rac1 instead of GTP. Consecutively, the activation of Rac1 target genes such as MEK, NF-kB and bcl-xL is suppressed, leading to a mitochondrial pathway of apoptosis. Azathioprine thus converts a costimulatory signal into an apoptotic one.

These findings may contribute to elucidate Aza action mechanism in prevention of organ transplantation rejection and in the treatment of autoimmune diseases. To pursue this goal, it seems reasonable to clarify the role of Azathioprine and its metabolites in modulation of immune responses, taking into consideration not only the lymphocyte populations, but also cells which promote and regulate their functions, as dendritic cells.

1.3. Dendritic cells

Dendritic cells represent a heterogeneous population of professional antigen presenting cells (APCs). DCs play a unique role in inducing selective immune responses against individual classes of pathogens and are involved in peripheral immune tolerance and in immune homeostasis maintenance (Gad et al, 2003). Immature dendritic cells capture, process and present antigens and consequently express high levels of costimulatory and major histocompatibility complex (MHC) molecules, in addition to secreting various cytokines and chemokines which initiate and/or enhance many T and B lymphocyte responses. These responses include induction of CD4+ T lymphocyte type 1 and type 2 subset differentiation, CD8+ T lymphocyte activation and enhancement of cytotoxic T lymphocyte activity and B lymphocyte maturation, Ig class-switching and antibody production (Gerloni et al, 1998; MacPherson et al, 1999).

Human DCs are all bone marrow-derived leukocytes (Katz et al, 1979) and comprise at least four types defined under cytokine-driven conditions in vitro. These include myeloid DCs (mDCs), dermal DCs or interstitial DCs (DDCs-IDCs), Langerhans cells (LCs) and plasmacytoid DCs (pDCs) (Dzionek et al, 2000; Rossi et al, 2005).

DCs have been found in most tissues, in lymphoid organs, where represent the major cell population, and in peripheral blood, where constitute the 0.2% of circulating leucocytes. Circulating DCs are distinct in two subsets: myeloid DCs and plasmacytoid DCs morphologically, phenotypically and functionally distinguished. mDCs have a monocytoid appearance, while pDCs are so named because of their morphological resemblance to plasma cells. Both subgroups have lacked lineage markers and express high levels of HLA-DR and CD4, but whereas mDCs are $CD11c^{+}$, $CD33^{+}$, $CD13^{+}$, $CD1c^{+}$ and $CD123^{low}$, pDCs are $CD11c^{-}$ and $CD123^{bright}$ (McKenna et al, 2005). DCs resident in peripheral tissues exhibit an immature phenotype characterized by low expression of costimulatory molecules such as CD40, CD80, CD86 and high ability to ingest by phagocytosis, endocytosis or pinocytosis a wide variety of antigens including microbial pathogens, dead or dying cells, immune complexes (Mahnke et al, 2002). DCs express pattern recognition receptors that bind pathogen-associated molecular patterns consisting of microbial or of damaged host tissues components, such as lipopolysaccharides, peptidoglycans, CpG

motifs, flagella and viral nucleic acids, these pattern recognition receptors include Toll-like receptors (TLRs). Dendritic cell subsets exhibit unique repertoires of TLRs, allowing specialized responses to each class of pathogen, (Proietto et al, 2004; Jarrossay et al, 2001) enhancing innate immune responses at the site of inflammation and driving adaptive immunity. For instance, pDCs exclusively express TLR9 and TLR7 by which are able to respond to viral CpG DNA and viral single stranded RNA, respectively. However, pDCs do not express TLR4 and therefore respond to lipopolysaccharide relatively weakly. In pDCs TLR9 stimulation determines an high production of type I interferons (IFN-α and IFN-β) promoting their own cell survival and increasing MHC expression by neighboring antigen-presenting cells, thus enhancing anti-viral immunity. Meanwhile, responses to bacterial infections may primarily be mediated by mDCs, that express TLR4. Engagement of the TLRs results in production of several proinflammatory cytokines, including type I interferons, tumor necrosis factor (TNF)-α, IFN-γ, IL-12, IL-6 and IL-1 (Siegal et al, 1999; Cella et al, 1999). Depending on the environment in which pathogens are encountered, both subtypes of DCs seem able to induce Th1 or Th2 T cell responses (Cella et al, 2000).
Another group of pattern recognition receptors expressed by DCs are the C-type lectin receptors, which bind the carbohydrate moieties of glycoprotein self-antigens and pathogens for processing and presentation on MHC molecules. Immature not activated DCs express these receptors, as Langherin (CD207), DC-SIGN (CD209), mannose receptor (CD206) and DEC205 (CD205), which are specialized for antigen capture and processing (Thery et al, 2001; Figdor et al, 2002). Activation and maturation down-regulate expression of C-type lectin receptors, as DC function changes from antigen uptake to antigen presentation. TLRs and C-type lectin receptors work in concert to balance immune tolerance with activation. DCs use C-type lectin receptors to present self-antigens and harmless environmental antigens in the steady state, thereby maintaining peripheral tolerance (Hawiger et al, 2001; Liu et al, 2002). Perturbation of the steady state by concomitant exposure to an activating stimulus like TLR-binding ligands or CD40L can reverse any tolerizing purpose of C-type lectin receptors and lead to immune activation (Gantner et al, 2003).

Upon internalization, exogenous antigens are subject of processing mechanisms and the resulting peptides are loaded onto class II MHC molecules for presentation to $CD4^+$T lymphocytes, whereas antigens synthesized in the cytosolic compartment are associated to class I MHC molecules and presented to $CD8^+$T lymphocytes. Remarkably, DCs can also cross-present antigens on the MHC class I and II molecules to autologous MHC-restricted T cells in spite of the MHC alleles expressed by the antigen source. Much has also been made of the distinction between apoptotic and necrotic cell death as a source of cross-presented antigen (Sauter et al, 2000). Whether antigen remains intact or denatured during apoptosis or necrosis, as well as any association with additional danger signals, are the greater determinants of effective cross-presentation and a tolerant or immune outcome (Albert et al, 1998 *J Exp Med*; Albert et al, 1998 *Nature*).

After phagocytosis and subsequent activation, DCs residing in peripheral tissues migrate to the draining lymph nodes, undergo further maturation, present to and stimulate T lymphocytes specific for the cognate peptide. Maturation of DCs is crucial for the induction of T lymphocyte immunity as demonstrated by Hugues et al (Hugues et al, 2004). This group determined that dendritic cell maturation due to inflammatory stimuli, such as lipopolysaccharide, results in prolonged contacts between DCs and T lymphocytes. The kinetics of this interaction differ from those observed for immature DCs in which only short-term contacts with T lymphocytes are established. Interestingly, the extended contact between T lymphocytes and mature dendritic cells resulted in efficient T lymphocyte activation and proliferation not observed with immature DCs. It is likely that the kinetics of dendritic cell/T lymphocyte interactions, along with dendritic cell phenotype (i.e., costimulatory molecule and cytokine expression) together determine whether immunity or tolerance is established. In addition to the kinetics of T lymphocyte interaction, dendritic cell maturation is crucial for appropriate migration to lymph nodes. DCs matured by inflammatory stimuli in the periphery become highly motile and readily traffic to local lymph nodes. Once in a lymph node, these mature cells interact with large numbers of T lymphocytes priming and activating them (Lindquist et al, 2004). Recent data have established an important role for DCs in innate immunity by enhancing reactivity of resting

NK and (Fernandez et al, 1999) and NKT cells (Fujii et al, 2003), which otherwise respond to the aggregate of activating and inhibitory signals on their targets. DC-stimulated NK and NKT cells become potent sources of IFNγ and other inflammatory cytokines, supporting the maturation of resident populations of DCs through a reciprocal interaction and eliciting an adaptive Th1 response mediated by cytotoxic effectors (Taniguchi et al, 2003; Gerosa et al, 2005). Many evidences suggest that DCs also control immunity by inducing T regulatory cells to promote antigen specific unresponsiveness of lymphocytes in primary and secondary lymphoid tissues (Yamazaki et al, 2003; Tarbell et al, 2004).

Progress in the study of DC biology exploded in the 1990s. Investigators developed cytokine-driven methods for expanding and differentiating DCs ex vivo in both mouse and human systems (Inaba et al, 1992; Sallusto et al, 1994) and further refinements continue to emerge (Thurner et al, 1999; Lutz et al, 1999). The DCs generated in vitro with cytokines should approximate resident populations that exist in vivo under steady-state conditions. The inflammatory cytokine combinations used in vitro for terminal DC maturation and activation mimic the physiologic activation via TLRs. The most accessible DC precursors are the CD14 monocyte in peripheral blood, which under the influence of GM-CSF and IL-4 differentiates into $CD14^{-}$, $CD11c^{high}$, $HLA\text{-}DR^{high}$, $CD80^{+}$, $CD86^{+}$, $CD40^{+}$, monocyte-derived DCs (moDCs) that, upon appropriate stimulation, express high levels of activation marker CD83, upregulate HLA-DR and the costimulatory receptors CD80, CD86, CD40.

Most current clinical studies use DCs for active immunotherapy trials in cancer. Dendritic cell-based “vaccines” consisting of re-infusion of autologous dendritic cells upon *ex vivo* manipulation have already been developed (Banchereau et al, 2001; Hsu et al, 1996; Thurner et al,1999; Timmerman et al, 2002). Most tumor antigens are poor immunogens because they are self-Ags or self-differentiation Ags, to which there is considerable tolerance. DCs provide a potential solution to this challenge by coupling tumor Ag with all of the requisite costimulatory ligands, cytokines, and chemokine-directed migration to secondary lymphoid organs. There they can stimulate incoming T cells to exit via efferent lymph into the periphery as cytolytic and helper T cell effectors (Rossi et al, 2005).

Actually the role of dendritic cells in preventing autoreactivity and/or tolerizing existing autoreactive T cells are becoming subject of extensive investigation (Dhodapkar et al, 2001; Yamazaki et al, 2003). Manipulation of immunity using DCs generated in vitro should therefore exploit the less mature and not-activated forms to promote tolerance and the activated and mature forms to break tolerance and advance immunity.

Therapies for autoimmune disorders and anti-graft rejection treatments have traditionally relied upon broadly immunosuppressive drugs. In view of their central role in eliciting and regulating immunity, the application of dendritic cells in immunotherapy is highly appealing.

1.4. Aim of the study

Considering the pivotal role of DCs in regulating immunity and the recent outcomes on Azathioprine action mechanism, in this study we proposed to investigated the possible immunosuppressive effects of 6-MP on in vitro matured and activated human monocyte-derived dendritic cells (moDCs).

According to autoimmune hypothesis of MS pathogenesis, the activation of autoreactive T cells is a central event in the development of autoimmune response in MS (Sospedra et al, 2005). At the time of MS diagnosis, an immunological process known as Ag/epitope spreading, has probably already occurred in most of the patients (Tuohy et al, 1999). This makes unfeasible, even at single patient level, antigen specific desensitization, a treatment strategy directed to a unique pathogenetic self-Ag (Bielekova et al, 2000). If the strategy of antigen specific desensitization will be confirmed not to be applicable (or even dangerous), immunosuppressive/modulating therapies will probably maintain a key role in the treatment of MS.

2. MATERIALS AND METHODS

2.1. PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were extracted from buffy coats or freshly collected blood samples of healthy donors by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences).

2.2. *Ex vivo* primary proliferation assay

Blood samples were collected from 12 patients with definite relapsing remitting MS (RR-MS) according to Poser criteria (7) treated with Azathioprine (per os 2.7 mg/Kg a day) before starting the therapy and 6 months after onset of treatment. PBMCs were frozen and later tested at the same time. When thawed, cell viability was assessed by propidium iodide incorporation. PBMCs were cultured at $1x10^5$ cells/well in 96-well U-bottom plates in presence or absence of leucoagglutinin (PHA-L, 2μ g/ml), Concanavaline A (ConA, 2μ g/ml) and Candida albicans (heat killed bodies, 10^6 bodies/ml). PHA and ConA were from Sigma-Aldrich, Candida albicans was a kindly gift of Marta Peruzzi, MD. Cultures were set up at least in quadruplicate. After 48-72 hours from stimulation, cultures were pulsed 8 hours with 1μ Ci [^{3}H]Thymidine (Amersham), then cells were harvested by a 96-well plate harvester (Harvester 96, Tomtec) and [^{3}H]Thymidine incorporation was measured on a scintillation counter (1450 Microbeta Plus, Wallac). Wells with ΔCPM>2000 (CPM= Counts Per Minute; ΔCPM= mean CPM of stimulated wells-mean CPM of not stimulated wells) and SI>2 (SI= Stimulation Index= mean CPM of stimulated wells/mean CPM of not stimulated wells) were considered positive. Differences were tested for statistical significance by X^2 test.

2.3. 6-MP preparation

6-Mercaptopurine Hydrate ($C_5H_4N_4S$, FW 152.2) was from Sigma. Powder was dissolved in DMSO (Sigma) at the concentration of $2.5x10^{-1}$M, then was diluted in culture medium to reach working concentration.

2.4. Dendritic cell cultures

Monocytes were isolated by positive selection with human anti-CD14 antibodies coupled to magnetic beads (MACS, Miltenyi Biotec) from PBMCs of healthy donors. Cells were incubated with anti-CD14 microbeads as indicated in data sheet and separated on a column placed in an appropriate magnetic field (mini or midi MACS system, Miltenyi Biotec); the magnetically labeled $CD14^+$cells were retained in the column, while the unlabeled cells run trough. After removal of the column from the magnetic field, the $CD14^+$cells can be eluted as positively selected cell fraction. Purity of separation was determined by flow cytometry analysis. $CD14^+$cells were cultured in 24-well plates (Grainer) at the density of $1x10^6$/well for 6 days in RPMI 1640 containing 10% FCS, 2mM L-glutamine, 1% sodium pyruvate, 1% penicilline-streptomycine, 1% Hepes buffer (Euroclone), GM-CSF 1000 u/ml (recombinant human purified protein, Chemicon) and IL-4 1000 u/ml (recombinant human purified protein, R&D Systems). Where indicated, 6MP was added at two different concentrations (10^{-6} and 10^{-5}M) from the onset of the culture, then the immature DCs were activated for 24 hours with 1μ g/ml of lipopolysaccharide (LPS, Sigma-Aldrich); in one experimental group 6MP was added only 24 hours with LPS. Maturation state was evaluated as surface markers expression HLA-DR, CD14, CD80, CD86 and CD83 by flow cytometry. Also viability and apoptosis were assessed by flow cytometry. The samples were analyzed on a 4-color flow cytometer (Epics-XL with Expo32 software acquisition and analysis, Beckman-Coulter).

2.5. Immune staining procedure

Cells were centrifuged and the pellet was resuspended in cold staining buffer, composed by PBS with 1% FCS, at the density of $1x10^5$-$1x10^6$ cells/100μ l, then cells were stained with appropriate antibody amount as recommended in data sheet, mixed very well and

incubated at 4°C in the dark for 20-25 minutes. After incubation period cells were washed 2 times in cold staining buffer, resuspended in 300-500μl of the same buffer and kept on ice until flow cytometry analysis. Where indicated propidium iodide (PI, Molecular Probes) was added to 100μl of immune stained cells at the final concentration of 5μg/ml for 5 minutes, then samples were diluted with 200-400μl of staining buffer and immediately examined.

In this work the following conjugated monoclonal anti-human antibodies were used: mouse IgG2a CD14 FITC (Miltenyi Biotec), mouse IgG1 CD80 FITC (Immunotech), mouse IgG2b k CD86 PE (Immunotech), mouse IgG1 HLA-DR ECD (Immunotech), mouse IgG2b CD83 PC5 (Immunotech), mouse IgG1 CD4 FITC (Immunotech), mouse IgG1 CD3 PE (Immunotech), mouse IgG1 CD8 PC5 (Immunotech), mouse IgG2b CD69 PE (Immunotech), mouse IgG1 k CD25 PE (BioLegend).

2.6. Apoptosis detection assay

Apoptosis was evaluated through flow cytometry technique using the Annexin V-FITC kit by Immunotech. Cell samples were washed in ice-cold PBS; after centrifugation the pellet was resupended in ice-cold binding buffer at the density of $5x10^5$-$5x10^6$ cells/ml and 1μl of annexin V-FITC and 5μl of propidium iodide were added to 100μl of cell suspension for 10 minutes on ice in the dark, then 200-400μl of binding buffer were added and samples were analyzed.

2.7. Dextran-FITC assay

Dendritic cell macropinocytosis capability was examined as described by Sallusto et al.1995 (ref). Briefly, $2x10^5$ not activated and LPS activated DCs were resuspended in 10% FCS RPMI 1640 medium and equilibrated at 37°C or 0°C for 10 min, then were pulsed with dextran-FITC (40000 m.w., Molecular Probes) at the concentration of 1mg/ml for 45 min at 37°C or 0°C. Cells were washed four times with cold PBS buffer containing

0.01% sodium azide (Sigma-Aldrich) and 1% FCS and analyzed by flow cytometry using PI to exclude dead cells. The background (cells pulsed at 0°C) was subtracted.

2.8. Mixed lymphocyte reaction (MLR)

Untouched $CD4^+$lymphocytes were magnetically isolated from PBMC of healthy donors by negative selection using the Miltenyi $CD4^+$ Isolation Kit II. Purity of separation was determined by flow cytometry analysis. $CD4^+$ T cells ($1x10^5$/well) were cultured in 96-well U-bottom plates (Nunc) with HLA-DR mismatched allogenic DCs, after 24 hours LPS activation, at three different concentrations ($1x10^2$,$1x10^3$ and $1x10^4$ DCs/well). DCs were obtained as described above. Cultures were set up in quadruplicate. T cell proliferation was determined by [^{3}H]Thymidine incorporation after 5 days of culture, as described in section 2.2.

2.9. CFSE labeling

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a dye that spontaneously and irreversibly binds to intracellular proteins. At each cellular division CFSE is equally distributed among the daughter cells, which, therefore, contain half the fluorescent dye compared with the parental cells resulting in multimodal flow cytometric histograms, with each cell generation clustering around half the fluorescence intensity of the previous one. On the basis of the CFSE fluorescence the number of cell divisions can be followed. Untouched $CD4^+$lymphocytes obtained as described previously were washed in PBS 1% FCS and resuspended at $20x10^6$cells/ml. Cell suspension was mixed in a ratio of 1:1 with a 5mM or 10mM CFSE solution (Molecular Probes) for 8 minutes at room temperature in the dark, then reaction was stopped by adding an equal volume of FCS and samples were centrifuged at 1250 rpm for 10 minutes. Next, cells were washed twice in culture medium at 1500 rpm for 10 minutes and were used in MLR assay.

2.10. Dendritic cell cytokine detection

DCs were cultured as described above. Supernatants were collected before adding LPS and after activation time, then stored at –80°C. IL-12 and IL-10 production was measured by double sandwich ELISA (Biosource), following the manufacturer's protocol.

2.11. T cell line generation

T cell lines (TCL) specific for FluHA peptide(306-318) were generated by modified split-well analysis from healthy donors as described by Vergelli et al. (ref). FluHA peptide(306-318) was kindly provided by AnnaMaria Papini, PeptLab, Dept Organic Chemistry, Florence University. Briefly, PBMCs were seeded in 200µl of T cell medium (TCM) (RPMI 1640 with 5% AB serum, 2mM L-glutamine, 1% sodium pyruvate, 1% penicilline-streptomycine, 1% Hepes buffer) at $2xl0^5$/well into 96-well U-bottom plates and stimulated with 5µg/ml of human FluHA peptide(306-318). Seven days later IL-2 20u/ml was added to each well. After another seven days, cells were washed once within the plate and resuspended in 200µl of TCM. Next, 50µl of the cell suspension was transferred into two adjacent wells of a separate 96-well U-bottom plate with 100µl of TCM containing $1xl0^5$ autologous irradiated (6000 Rad) PBMCs. One well was stimulated with 50µl of FluHA peptide solution at the final concentration of 5µg/ml, in the other well 50µl of TCM were added to reach the same volume of 200µl. T cell proliferation was evaluated by [^{3}H]Thymidine incorporation after 48-72 hours of culture. In parallel, the remaining wells in the original plates were restimulated with $1xl0^5$ autologous irradiated PBMCs and FluHA peptide (5µg/ml). One week later wells that showed a SI>2 in the proliferative assay were transferred into a 24-well plate and restimulated with $1xl0^6$ autologous irradiated PBL, 5µg/ml of FluHA peptide and IL-2 50u/ml. Cells were expanded by successive restimulation cycles as described above and periodically tested versus FluHA antigen.

2.12. Antigen specific proliferation test

$1x10^5$ TCL cells specific for FluHA peptide(306-318) were stimulated with $1x10^4$ autologous not activated DCs in 96-well U-bottom plates. Before test, DCs were incubated for 2 hours at 37°C in culture medium at the density of $1x10^6$/ml with or without 10μ g/ml of peptide. After that cells were washed twice and used in the antigen driven proliferation assay. Where indicated DCs were treated with 6MP at two different concentrations (10^{-6} and 10^{-5}M) for 6 days during the differentiation from monocytes (as described above) or during the 2 hours long antigen loading. Cultures were set up in quadruplicate. T cell proliferation was determined by [^{3}H]Thymidine incorporation after 48 hours of culture.

3. RESULTS

3.1. 6-MP partially suppresses recall antigen response in MS treated patients.

12 RR-MS outpatients diagnosed according to Poser criteria (Poser et al,1983) and taking a daily dose of 2,7 mg/Kg of Azathioprine were enrolled. Six months before and six months after therapy starting, PBMCs were isolated from patients, frozen and then tested at the same time for proliferation in response to Candida albicans (heat killed bodies, 10^6 bodies/ml); to verify their proliferation capability after thawing, response to not specific mitogen stimulation (PHA 2μ g/ml, ConA 2μ g/ml) was evaluated. While all the patients responded to PHA and ConA before and during therapy, we detected a statistically significant (*p value=0.04*, analysis performed by χ^2 test) reduction in number of positive responders (-42%) to Candida albicans after therapy (*Table 3.1*), suggesting an unspecific mechanism of antigen driven immunosuppression.

Table 3.1. The table reports the absolute number of responders (SI>2 and ΔCPM>2000) at the two time points.

N° of responders* to Candida albicans

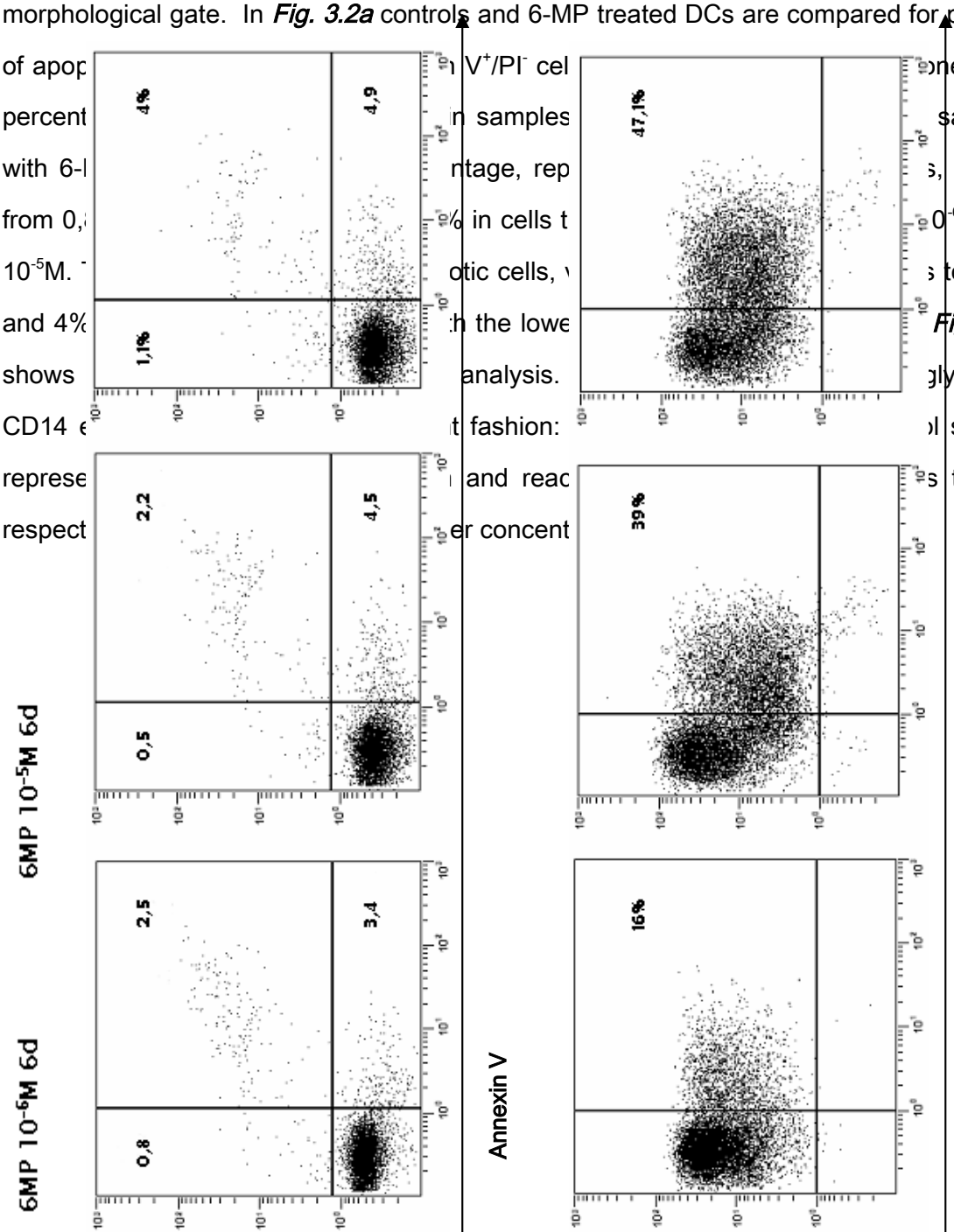
	+	-
before therapy	9	3
during therapy	4	8

N° of patients examined: 12 **p value=0.04*

3.2. 6-MP during DC maturation doesn't affect viability and maintains a CD14$^+$ phenotype in a higher percent of cells comparing to control.

Human CD14$^+$ monocytes were cultured 6 days with GM-CSF and IL-4 with or without 6-MP at two different doses (10^{-6}M, 10^{-5}M), then cells were analyzed for cell death, apoptosis

and CD14 membrane expression. As well known, during in vitro differentiation and maturation monocyte precursor cells lack CD14 surface expression, upregulate HLA-DR expression and display typical costimulation markers, such as CD80 and CD86. Interestingly , while cells treated with both doses of 6-MP didn't differ from control samples (without drug) concerning viability and apoptosis percentage (***Fig. 3.2a***), cells treated with drug significantly retained CD14 expression in a dose dependent manner (***Fig. 3.2b***), indicating that 6-MP impairs maturation process. ***Fig. 3.2a*** and ***Fig. 3.2b*** report dot plots from one experiment representative of three independent ones; analysis is referred to morphological gate. In ***Fig. 3.2a*** controls and 6-MP treated DCs are compared for percent of apop V$^+$/PI$^-$ cel ones: this percent n samples samples with 6-l ntage, rep s, ranges from 0,3 % in cells t 10^{-6}M and 10^{-5}M. T otic cells, v s to 2,2% and 4% h the lowe ***Fig. 3.2b*** shows analysis. gly retain CD14 e t fashion: ol sample represe and reac s treated respect er concent



ctr

PI

Fig. 3.2a

HLA-DR

Fig. 3.2b

A summary of CD14/HLA-DR phenotype examination relative to 3 independent experiments is reported in ***Fig. 3.2c***. The percentage of $CD14^{+}/HLA\text{-}DR^{+}$ cells, expressed as mean value±DEV.Q, ranges from 14,3%±1% in controls to 34,1%±4% in DCs treated with the lower concentration of 6-MP and 44,1%±3% in DCs with the higher dose of drug. Mean values and DEV.Q were compared by χ^2 test. 6-MP treated cells significantly maintain (1 at 10^{-6}M, *p value*<0,0(

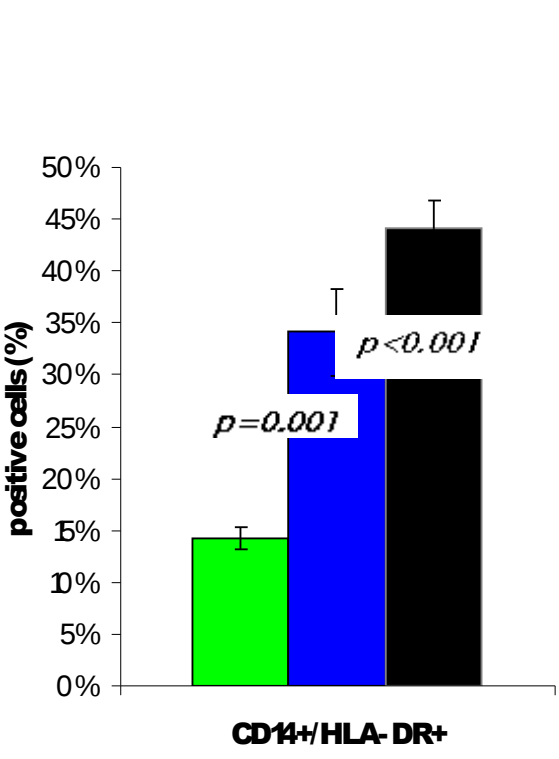
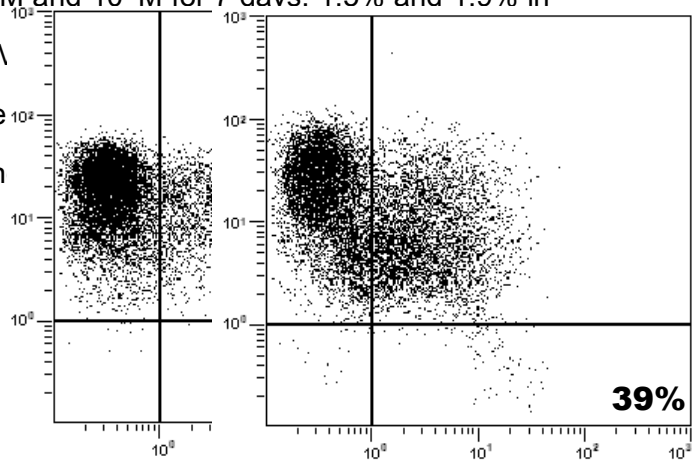


Fig. 3.2c. The histogram resumes three independent experiments and shows percentage of $CD14^+/HLA\text{-}DR^+$ cells, expressed as mean value±DEV.Q, in controls (green bar) and in samples treated for 6 days (6d) with 6-MP (added in culture at the beginning of culture) 10^{-6}M (blue bar) or 10^{-5}M (black bar). On blue and black bar top the *p values* (assessed by T-test) are reported.

3.3. 6MP inhibits membrane expression of CD83 during DC activation.

After 6 days of culture in GM-CSF+IL-4, DCs were activated with LPS for 24 hours. 6-MP (10^{-6}M, 10^{-5}M) was added in culture from day 1 (as above) or only the last 24 hours together with LPS (see Methods). After activation time, DCs were examined for cell death, apoptosis and immune phenotype. As previously mentioned, upon activating stimulation immature DCs acquire a fully mature phenotype, expressing at high levels the characteristic activation surface marker CD83 and upregulating HLA-DR, CD80 and CD86. Again, any significant variation in PI^+ and Annexin V^+ cell percentage comparing control and treated cells wasn't observed, as showed in ***Fig. 3.3a***, where one experiment representative of three independent ones is reported. The $AnnV^+/PI^-$ cell percentage (apoptotic cells) is 4,4% in control sample (cells without drug), 2% and 6,9% where 6-MP was present from the onset of culture (7d) respectively at 10^{-6}M and 10^{-5}M, 2,1% and 1,3% where 6-MP was administered for 24 hours (24h) together with LPS at the lower and the higher dose; the $PI^+/AnnV^-$ percentage (damaged viable cells) is 1,7% in control, 1,5% and 1,3% in cells treated respectively with 6-MP 10^{-6}M and 10^{-5}M for 7 days. 1.5% and 1.9% in samples treated respectively with 6-MP 10^{-6}M activation; the $PI^+/AnnV^+$ percentage (necrotic ce days 6-MP treated DCs respectively at the con

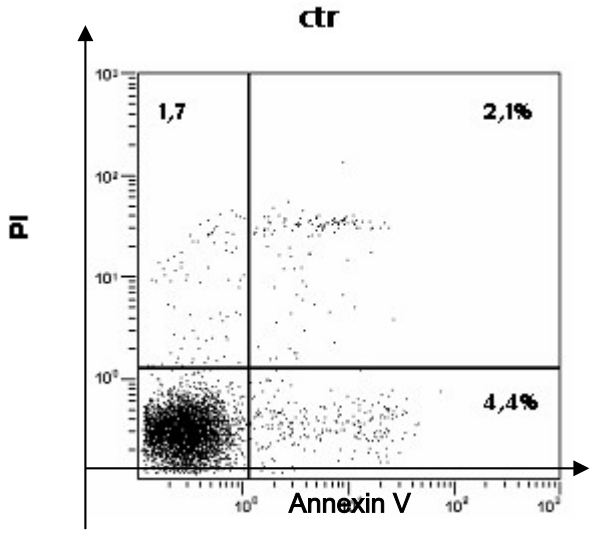


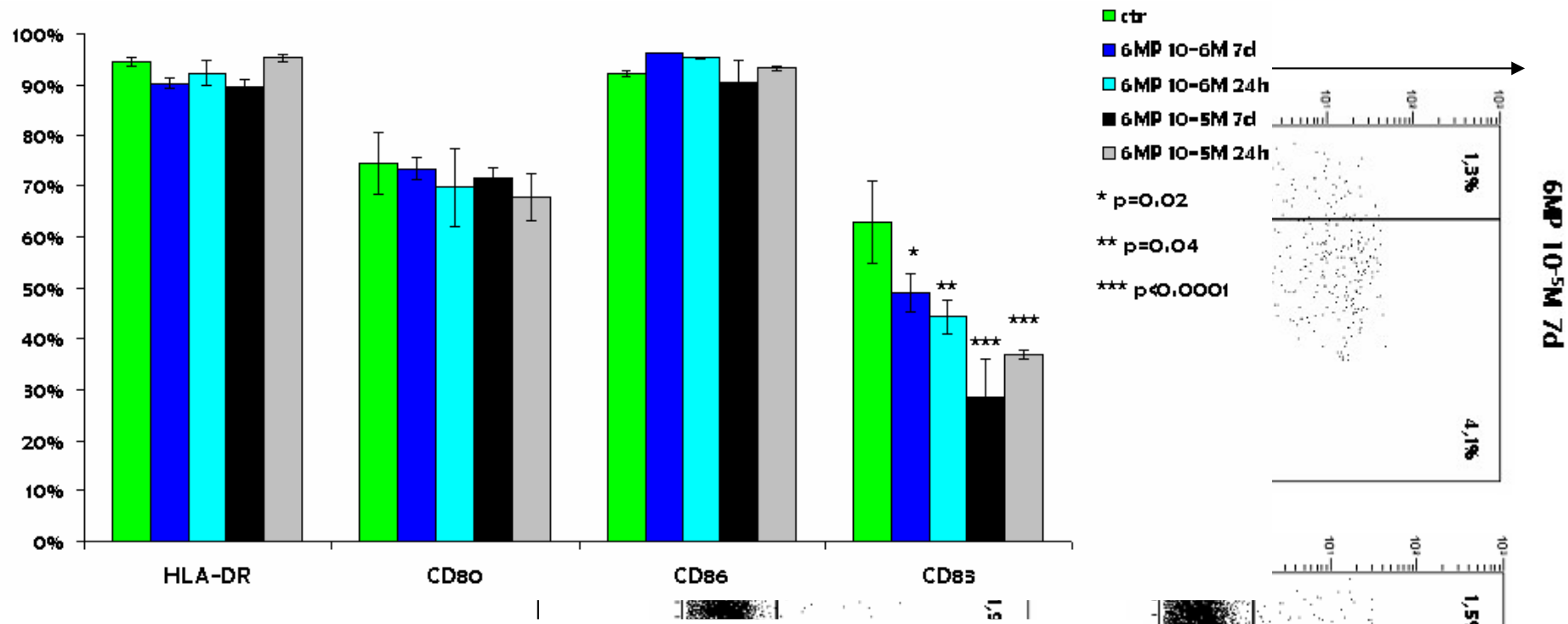
2,3% in samples treated for 24 hours during LPS activation respectively with the lower and the higher dose of drug.

From the phenotype point of view, 6-MP treatment, at both doses used, either if drug was added at the beginning of monocyte culture either if it was added for 24 hours during LPS activation, results in a significant (*p<0.05* at each dose tested, calculated by χ^2 test) reduction of $CD83^+$cell percentage in respect to controls (***Fig. 3.3b***). In particular, $CD83^+$ cell percentage decreases in a dose-dependent manner from 63%±8% (mean value of four independent experiments±DEV.Q) in controls to 49%±4% (*p=0,04*) and 29 %±8% (*p<0,0001*) in cells treated for 7 days respectively with 6-MP 10^{-6}M and 10^{-5}M, and to 44% ±3% (*p=0,02*) and 37%±7% (*p<0,0001*) in samples treated for 24 hours only during LPS activation respectively with the lower and higher dose of drug. Any alteration in HLA-DR, CD80 and CD86 expression was emerged.

These data suggest that 6MP has an inhibitory effect during activation processes.

Fig. 3.3a. After LPS activation, DCs were examined for cell death and apoptosis. Dot plots from one experiment representative of three independent ones are reported. Analysis is referred to morphological gate. 6-MP treatment doesn't affect cell viability.





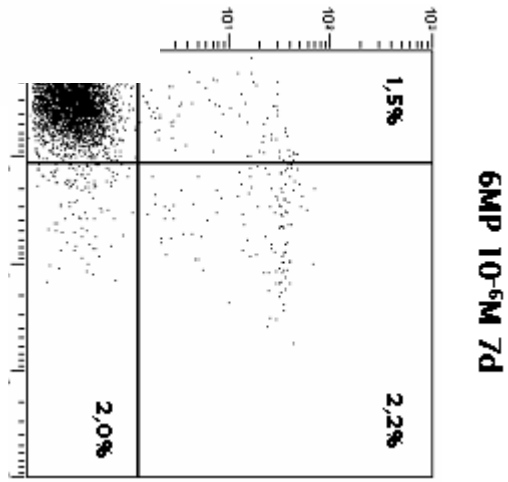
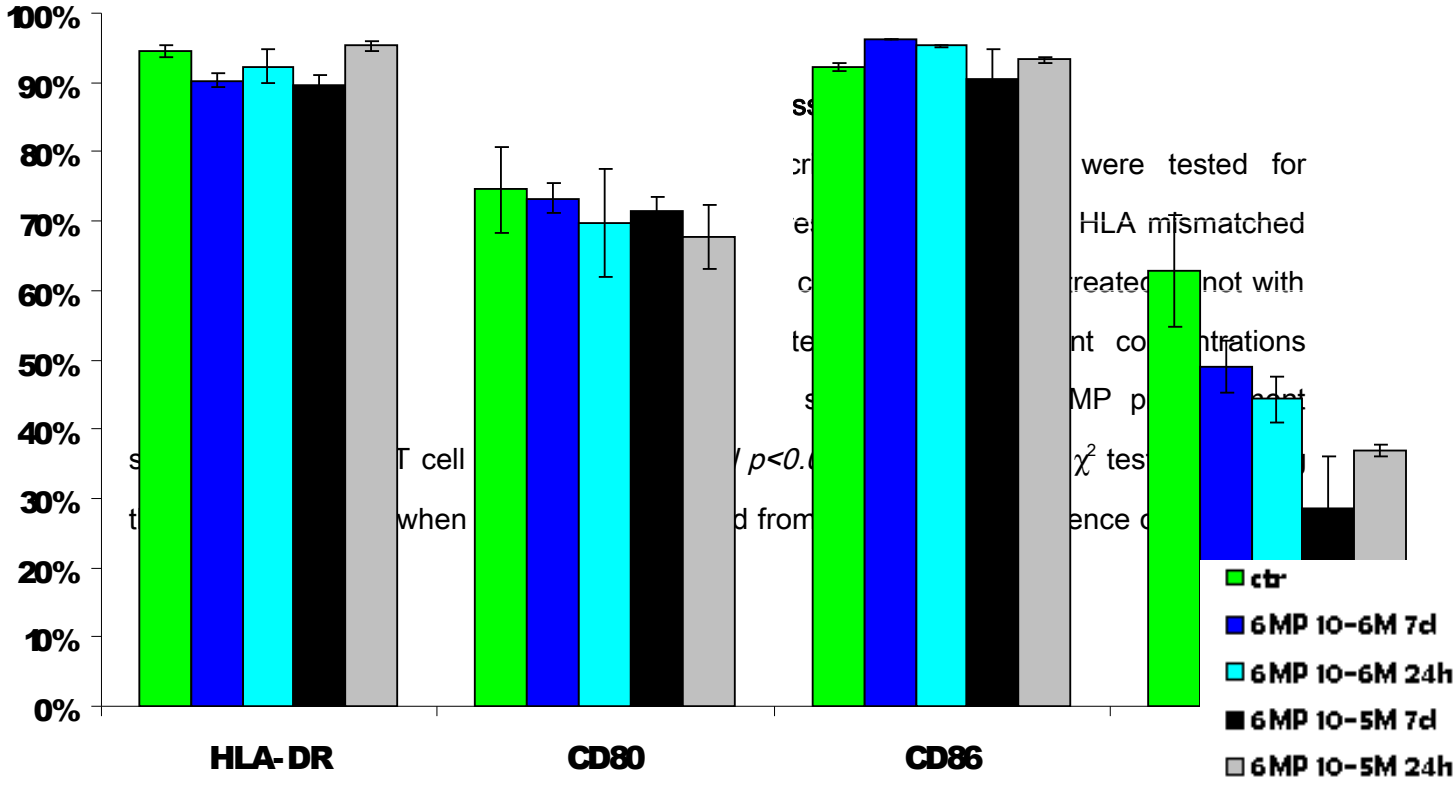


Fig. 3.3b. The figure summarizes four independent experiments and shows mean percentage±DEV.Q of cells expressing HLA-DR, CD80, CD86 and CD83 surface markers after LPS activation in the experimental conditions indicated. Analysis is referred to a morphological gate. 6-MP treatment doesn't impair HLA-DR, CD80, CD86 expression, but significantly reduces CD83^{+}cell percentage (*p values* are reported on each bar top).



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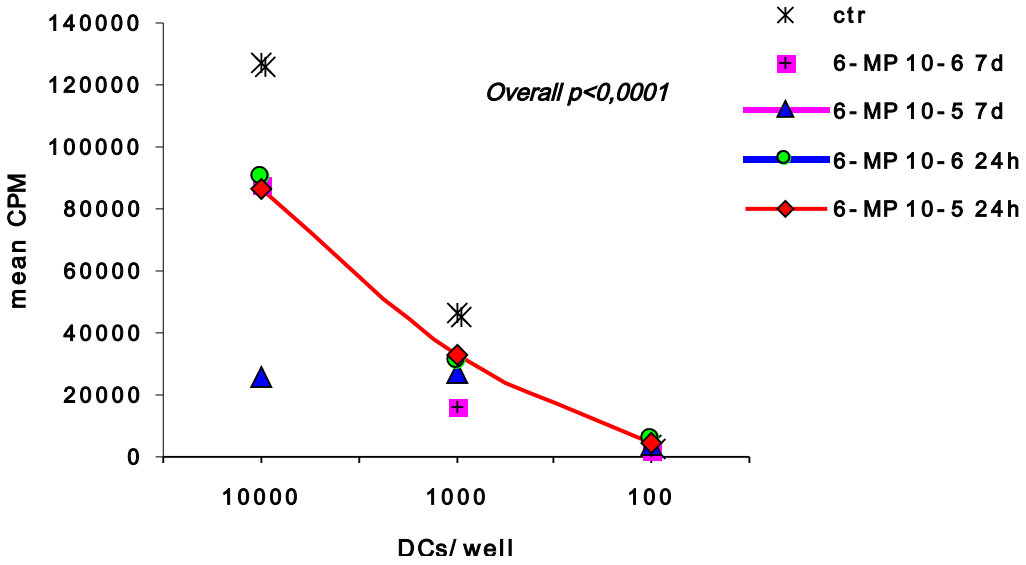
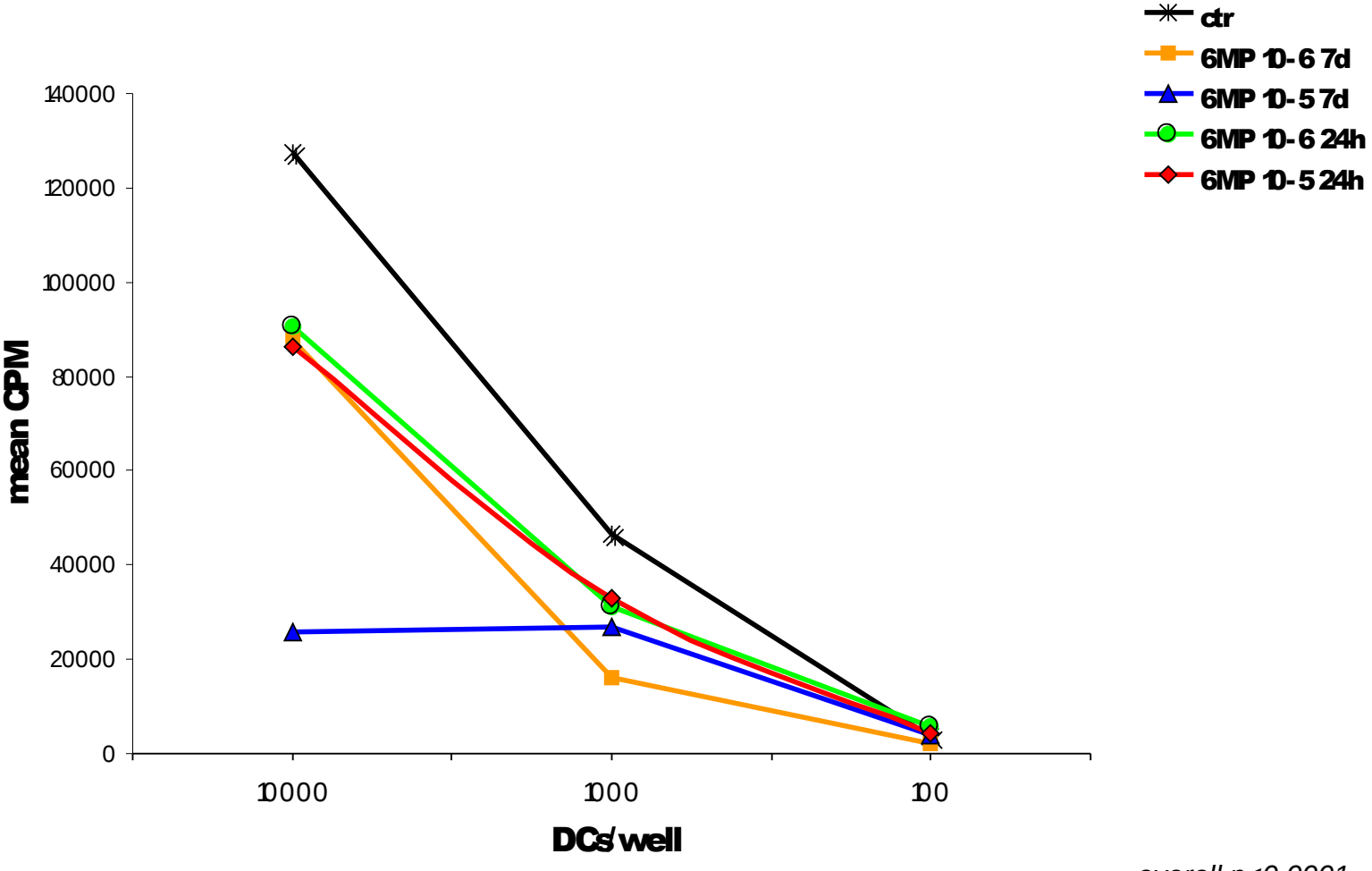
dose of 6-MP from the onset of culture (***Fig. 3.4***). According to phenotype data, 6-MP treated DCs result less efficient in allostimulation assay compared to control cells.





overall p<0.0001

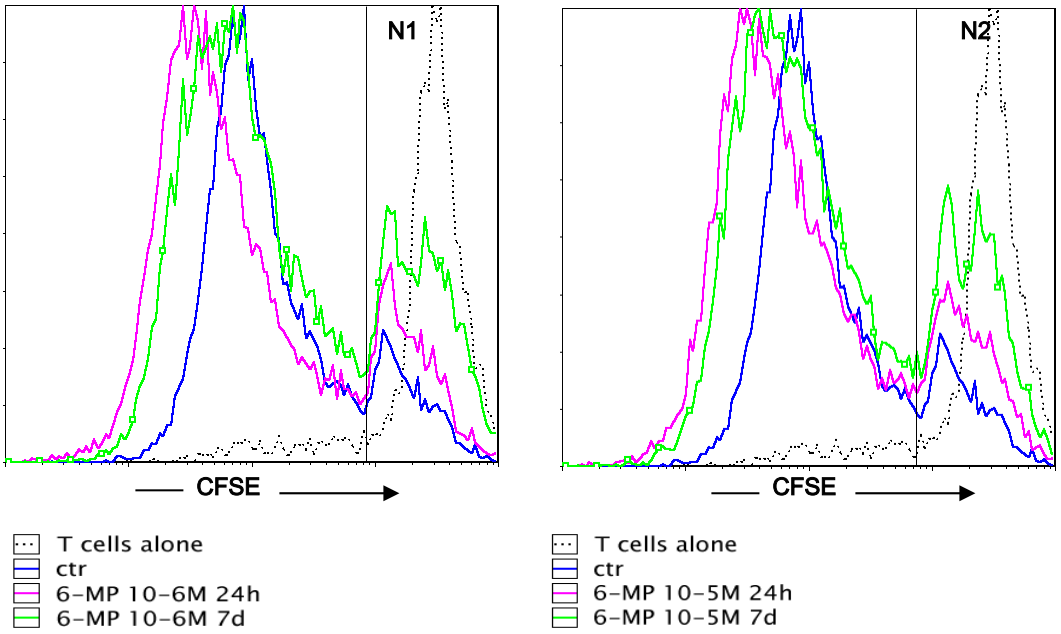


Fig. 3.4. MLR performed with CFSE stained T lymphocytes. $1x10^5$ $CD4^+$T cells/well were cultured with three different doses of DCs ($1x10^4$,$1x10^3$,$1x10^2$/well). CFSE fluorescence distributions after 5 days of co-culture are compared by overlapping histograms each other (CFSE fluorescence intensity on *x* axis, event counts on *y* axis). Plots show proliferative response corresponding to T cells stimulated with $1x10^3$ DCs/w treated with 6-MP at two different concentrations (10^{-6}M histogram on the left, 10^{-5}M histogram on the right) and for different time (7 days, distribution delimited in green, or 24 hours, in pink) or not treated (controls, in blue). Hatching delimited distributions indicate basal proliferation of T cells without DC stimulation. CFSE positive cells included in regions N1 and N2 (high mean fluorescence intensity) represent not divided cell population. We can observe a larger percentage of not divided cells in treated samples in respect to the controls. CFSE positive cells are gated on PI negative events.

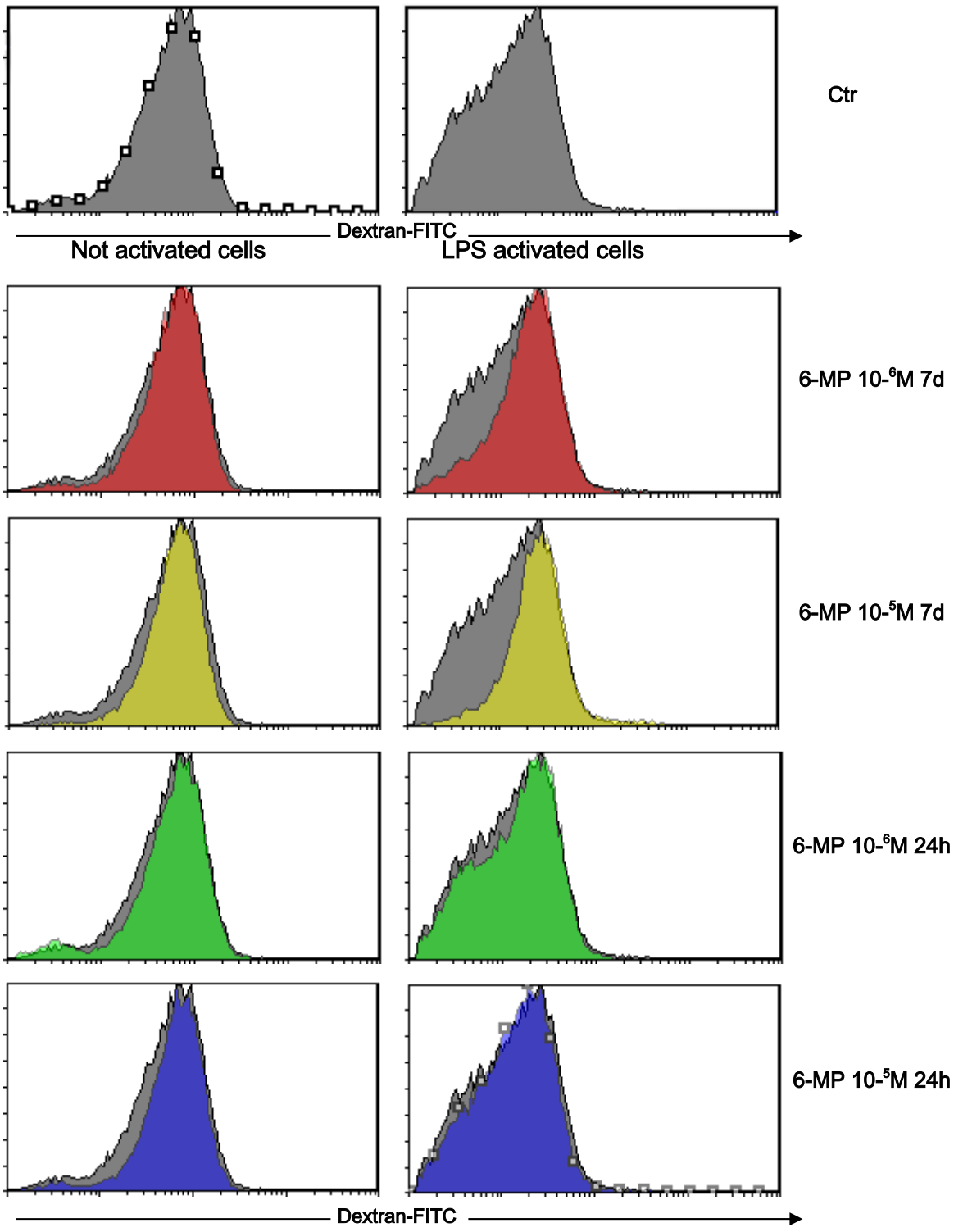
3.6. 6-MP treated DCs maintain high Dextran-FITC uptake capability after LPS activation.

In order to evaluate the maturation state of 6-MP treated and control DCs, their endocytic activity was measured by Dextran-FITC assay. The Dextran-FITC uptake is mediated by mannose receptor, a surface C-type lectin (Sallusto et al, 1995). The capacity to interiorize and process antigen is a constitutive property of immature DCs. Normally, fully activation of DCs results in a downregulation of endocytic ability compared to not activated ones.

The uptake ability was evaluated as percentage of Dextran-FITC positive events, excluding dead cells by PI co-staining. We found that immature DCs treated with both doses of 6-MP (10^{-6}M and 10^{-5}M), either when drug was added in medium at the first day of culture either when it was administered at day 6 for 24 hours only, were perfectly able to engulf Dextran-FITC like immature control cells (***Fig. 3.6***), as percentage of Dextran-FITC positive DCs was between 92% and 97% of vital cells (data relative to one experiment representative of three independent ones). From the other hand, after LPS activation, while controls downregulated endocytic capability (reduction of 35% respect to not activated state), DCs treated with both doses of 6-MP for 7 days almost preserved this capability in a dose dependent fashion (reduction of 18% in DCs treated with 6-MP 10^{-6}M and of 9% in DCs treated with 6-MP10^{-5}M respect to corresponding not activated condition). Interestingly, DCs treated with 6-MP just during LPS activation reduced their endocytic ability at the same manner of activated control cells. According to phenotype data, DCs treated with 6-MP from the onset of culture are less activated by LPS stimulation compared to control cells, but if 6-MP is administered at day 6 for 24 hours together with LPS we do not detect any variation in respect to the controls.

Fig. 3.6. Dextran-FITC uptake. Figure reports in the left column the assay on not activated DCs and in the right column the assay on LPS activated ones (one experiment representative of three independent ones); the different conditions tested are represented in plots with different colors: controls (ctr) in gray, samples treated with 6-MP from the onset of culture (7d) in red (10^{-6}M) and yellow (10^{-5}M), samples treated with 6-MP only during LPS stimulation (24h) in green (10^{-6}M) and blue (10^{-5}M). Each plot relative to 6-MP treated samples is overlapped to corresponding control histogram (in gray) for both conditions examined (before and after LPS activation). Histograms show Dextran-FITC fluorescence intensity on *x* axis and event counts on *y* axis.

Not activated cells LPS activated cells


Ctr
Dextran-FITC
Not activated cells
LPS activated cells
6-MP 10-6M 7d
6-MP 10-5M 7d
6-MP 10-6M 24h
6-MP 10-5M 24h
Dextran-FITC

3.7. 6-MP doesn't affect cytokine production.

Supernatans of LPS activated and not activated DCs were checked for IL-12 and IL-10 production by ELISA assay. 6-MP treatment doesn't affect the DC ability to secrete IL-10 (*Fig. 3.7*) and IL-12 (data not shown).

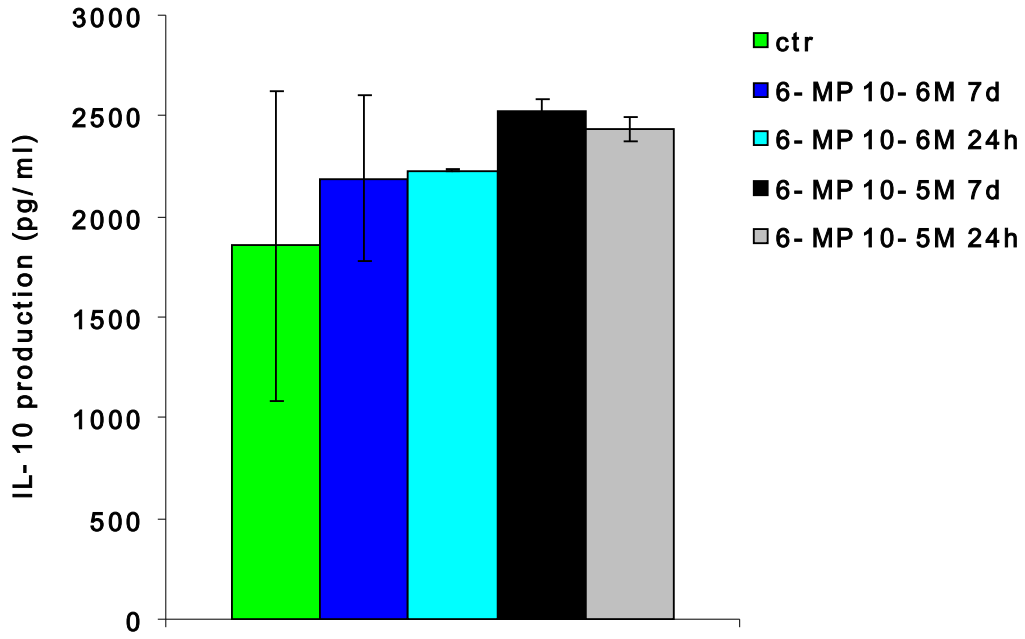


Fig. 3.7. Graph shows IL-10 production (on *y* axis, expressed as pg/ml) after LPS activation (one experiment representative of three independent ones). Supernatants was dosed in duplicate for each experimental condition and corresponding mean values (pg/ml) ±SD are reported. Any significant difference is detectable between control and treated samples.

3.8. 6-MP reduces DC stimulatory functions in antigen driven T cell proliferation.

Finally, the DC characteristic antigen presenting ability was investigated in antigen specific proliferation test. TCL cells specific for FluHA(306-318) peptide were stimulated with DCs loaded for 2 hours with or without peptide. 6-MP was administered to DCs at two different concentrations (10^{-6}M and10^{-5}M) or at the onset of culture (thus present in culture for 6 days) or during antigen loading (2 hours), as described in section 2.12; when DCs were pre-treated with both doses of 6-MP T cell proliferative response was weaker than in control sample. The maximum of inhibition (34%) was observed when drug was added during antigen loading at the higher dose (one preliminary experiment) as showed in ***Fig. 3.8***.

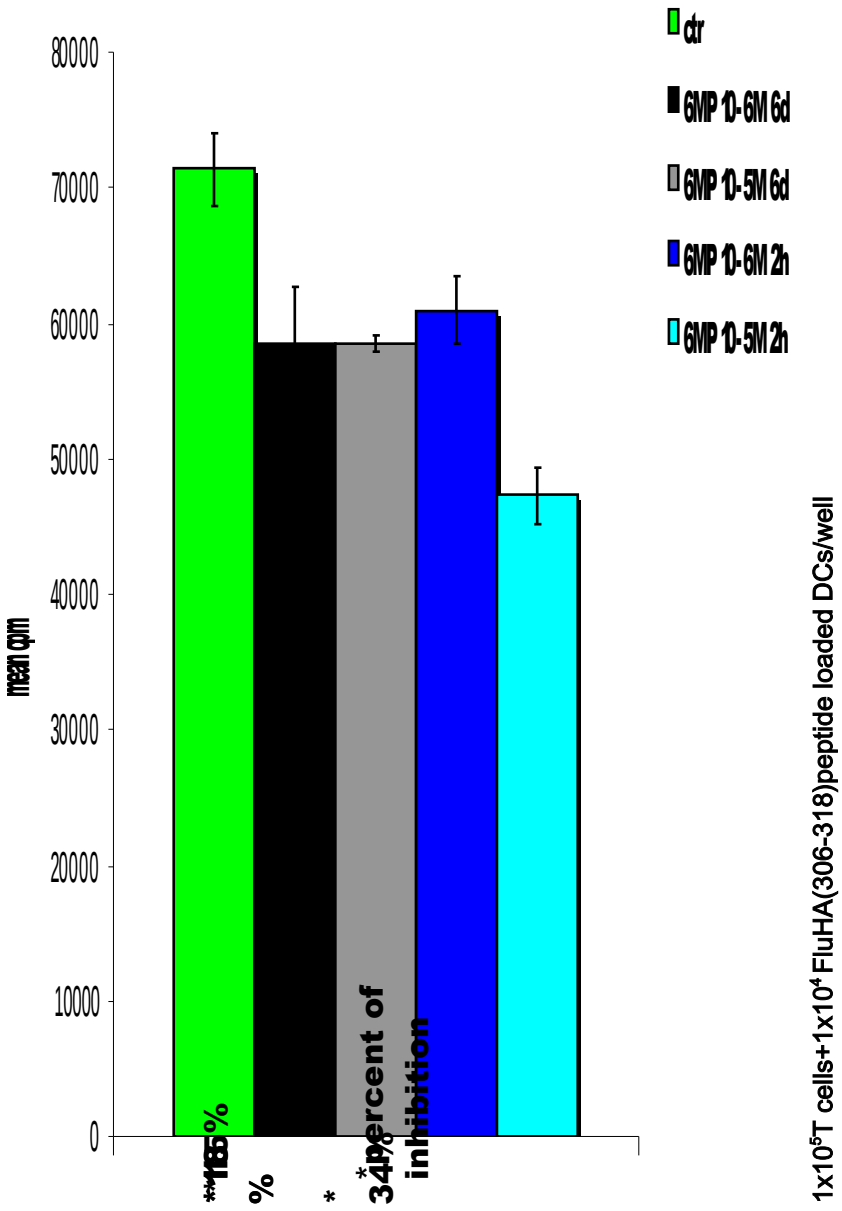


Fig. 3.8. Antigen specific proliferation assay (one preliminary experiment). All experimental conditions were tested in quadruplicate. T cell proliferation is expressed as mean cpm ±SD (*y* axis). For every condition we used 1x10^5 T lymphocytes+1x10^4 DCs/well. On each bar top percent of inhibition of proliferative response in respect to control sample is reported.

4. DISCUSSION

Azathioprine is an immune-suppressive agent largely used either in the prevention of transplant rejection or in the therapy of autoimmune diseases, such as multiple sclerosis. Although inhibition of purine nucleotide biosynthesis based on random incorporation of 6-thioguanine nucleotides (6-TGNs) into DNA has been suggested as the action mechanism, a general inhibition of nucleic acid synthesis is not sufficient to explain the relatively low bone marrow toxicity of the thiopurines associated to therapeutic efficacy on immune mediated disorders (Massacesi et al, 2005). Recent works by Neurath group (Tiede et al, 2003; Poppe et al, 2006) demonstrated that Rac1, a small guanosine triphosphatase (GTPase) of the Rho family, is a molecular target of azathioprine and 6-MP in naïve $CD4^{+}$T lymphocytes upon CD28 costimulation. Specifically, they found that azathioprine metabolite 6-Thio-GTP bind to Rac1 instead of GTP suppressing Rac1 activation. If T cells were stimulated via anti-CD3/CD28 Abs for at least 5 days, Rac1 inhibition led to a mitochondrial pathway of apoptosis. If T cell stimulation was shorter (3 days), 6-Thio-GTP binding to Rac1 blocked Vav guanosine exchange activity, causing an accumulation of 6-Thio-GDP-loaded, inactive Rac proteins, and these events resulted finally in the suppression of T cell-APC conjugation.

These findings may help explain our preliminary results about *ex vivo* proliferative response to recall antigens by PBMCs from 12 RR-MS patients before and during Azathioprine therapy. We found a statistically significant reduction in number of positive responders to Candida albicans after Aza therapy starting, although PBMCs from all patients were perfectly able to proliferate if stimulated with non-specific mitogens. In a recent work Massacesi et al (2005) demonstrated that in RR-MS patients treatment with Aza at doses bringing to lymphopenia determines a remarkable decrease of the new brain lesion accumulation, without increasing infections, such as candidosis; according to these data, our *ex vivo* results may signify that Aza therapy, without affecting resistance to

Candida infection, suppresses *in vivo* Candida specific memory T cell frequency or function, probably by inducing apoptosis in antigen activated T lymphocytes trough the mechanism described by Tiede et al (2003).

The second part of this study was focused on investigating the possible immunosuppressive effects of 6-MP on *in vitro* matured and activated monocyte derived human DCs (moDCs). We analyzed phenotype and function of DCs treated with 6-MP or during all the differentiating/maturating culture time, or only during the activation phase with LPS or Flu antigen. We found that monocytes cultured in presence of 6-MP during differentiation to DCs significantly retain CD14 expression. As well known, throughout *in vitro* differentiation and maturation monocyte precursor cells lack CD14 surface expression, so, our data indicate that 6-MP affects DC maturation process. In addition, we observed that 6-MP inhibits surface expression of DC activation marker CD83: inhibition was more evident if 6-MP was present for all culturing time, but was still significant if 6-MP was added just together with LPS. These findings suggest that 6-MP may have effects both during maturation and activation processes, without impairing cell viability, neither HLA-DR and costimulatory marker expression.

In order to analyze their functions, 6-MP treated DCs were tested for endocytic capability, allostimulatory ability, antigen presenting competence and cytokine production. Immature DCs have the constitutive ability to interiorize and process antigens and, normally, upon fully maturation they downregulate this activity. Trough Dextran-FITC uptake test, we showed that after LPS stimulation 6-MP treated DCs save their endocytic capability, but only when 6-MP was present from the onset of monocyte culture; indeed, when 6-MP was added just during LPS activation, DCs reduced their endocytic ability at the same manner of activated control cells. These results confirm the phenotype data indicating that 6-MP interferes with DC maturation processes; from the other hand they suggest that 6-MP administration for 24 hours to moDCs at the last step of culture maturation/activation is not sufficient to affect the molecular mechanisms modulating the endocytic capability. Moreover, 6-MP treated DCs resulted significantly less efficient in mixed lymphocyte reaction and in antigen specific proliferation assay compared to control cells. To clarify

how T cell proliferation in MLR was reduced when DCs were treated with 6-MP, the allo-reaction was performed with CFSE stained $CD4^+$T lymphocytes and proliferative response was evaluated together with CD69 and CD25 T cell early activation marker expression, respectively 12 hours and 24 hours after MLR onset. As we found 6-MP treated DCs do not affect CD69 and CD25 expression on T cells, we can conclude that mechanism by which 6-MP suppresses DC allostimulatory function, considering also that we didn't detect any alteration in IL-12 and IL-10 production, is not cytokine mediated. According to phenotype data, since among surface maturation markers analyzed only CD83 expression (reported as % of positive cells) was significantly diminished by 6-MP treatment, we can assume that 6-MP induced inhibition of DC allostimulatory ability may be partially due to reduction of CD83 expression. CD83 is a cell surface molecule involved in $CD4^+$T lymphocyte development in the thymus and in cell–cell interactions (Fujimoto et al, 2002; Lechmann et al, 2002), therefore it is reasonable to believe that 6-MP may in part suppress DC stimulatory functions by affecting the DC-T cell contact. This assumption may also explain our findings about antigen driven proliferation experiments on T cell lines specific for Flu-HA(306-318) peptide stimulated with immature DCs pre-pulsed for 2 hours with or without peptide: we observed that 6-MP significantly impaired DC antigen presenting ability, both when added in medium at the beginning of DC differentiation culture and when present during antigen loading. In these experimental conditions, maximum inhibition was achieved when 6MP was added during antigen loading at the higher dose. As the capacity to uptake antigens, at least the one mediated by mannose-receptor (Sallusto et al, 1995), seems not affected by 6-MP treatment, we can hypothesize again an involvement of CD83 molecule. Actually, a reduced CD83 surface expression may alter the DC-T cell contact during antigen presentation, resulting in a not-efficient T cell stimulation.

Taken together, our data show that 6-MP has an inhibitory effect on DC functions at several levels: during maturation, activation and antigen presentation without affecting survival and cytokine production.

Based on recent outcomes produced by Poppe et al (2005), we can speculate that 6-MP effect on DCs may be mediated by Rac1/Rac2 Rho-GTPase inhibition. The importance of small Rho family GTPases in T cells during T cell priming has been widely documented (Li et al, 2000; Gomez et al, 2000), their role in DCs is object of increasing interest (Kobayashi et al, 2001; Benvenuti et al, 2004). DCs exist in two functionally and phenotypically distinct states, immature and mature (Mellman et al, 2001). Immature DCs are widely distributed throughout the body and occupy sentinel positions in many non-lymphoid tissues. They constantly sample their environment for antigens by phagocytosis, macropinocytosis and pinocytosis. After antigen engulfing and activation by proinflammatory cytokines, immature DCs differentiate into mature DCs, which have a reduced potential for Ag uptake but have a high capacity for Ag presentation and T cell stimulation (Mellman and Steinman, 2001). This transition is accompanied by dramatic cytoplasmic reorganization characterized by a redistribution of MHC class II from intracellular compartments to the plasma membrane, up-regulation of surface costimulatory molecules and T cell adhesion molecules. DCs also remodel their profile of chemokine receptors that facilitate migration and homing to lymphoid organs (Mellman et al, 2001). Finally, DCs project long dendritic processes that further increase opportunities for T cell capture and interaction (Mempel et al, 2004). All of these changes depend on rearrangement of actin cytoskeleton, which in turn is mediated by small GTPases of the Rho family, (Nobes et al, 2000; Burns et al, 2001; West et al, 2000; Swetman et al, 2002). Many evidences indicate that Rho GTPases play a crucial role in several events, such as membrane trafficking, transcriptional regulation, cell growth control, chemotaxis, endocytosis (Yanagawa et al, 2003), differentiation, antigen presentation (Shurin et al, 2005), apoptosis (Boettner et al, 2002), intracellular transport of secretory vesicles and exocytose (Nassar et al, 2000). Disposable data together show that each Rho GTPase might mediate different effector pathways in DCs. For example, recently Benvenuti et al. (2004) found that Rac1 and Rac2 but not Rho itself control the formation of dendrites in mature DCs, their polarized short-range migration toward T cells and T cell priming. Other investigators have been suggested for Rac1 a special involvement in modulation of DC

capacity to endocytose apoptotic cells and prime T lymphocytes via cross-presentation (Kerksiek et al, 2005). It has been reported that Cdc42 plays a major role among Rho GTPases in the regulation of DC adhesion and endocytosis (Garrett et al, 2000). Moreover, it has been showed that Rho inactivation in DCs was associated with inhibited interaction between DCs and $CD4^+$T cells and 80% reduction of DC allo-stimulatory property in MLR, although the surface expression of MHC, costimulatory and adhesion molecules were unaffected (Kobayashi et al, 2001).

DCs represent a heterogeneous population of professional antigen presenting cells that initiate primary immune responses. Besides linking innate and adaptative immunity, DCs also control immunity based on their ability to induce antigen specific unresponsiveness of lymphocytes, phenomenon known as immunologic tolerance, in primary and secondary lymphoid tissues. Immunologic tolerance normally prevents reactions against self-antigens. Lack or loss of self-tolerance is likely to result in autoimmune responses. Interactions between lymphocytes and antigen presenting cells are critical for self-tolerance assessment, either in thymus (central tolerance) either in peripheral lymphoid tissues (peripheral tolerance). Central tolerance occurs during immune system maturation in the thymus, where developing lymphocytes with marked reactivity against self-antigens are eliminated by clonal deletion or inactivated by anergy induction. However, great number of self-reactive lymphocytes escape these central negative-selection processes and form a peripheral pool of potentially autoimmune-disease-mediating lymphocytes (Abbas et al, 2002). In addition, some self-antigens do not access the thymus, as environmental proteins located in the airway and intestine or sequestered into immunologically privileged sites like CNS, while others may be expressed later in life, when T and B lymphocyte repertoire has already been formed. Therefore, central tolerance needs to be supported by peripheral mechanisms. It has been proposed that under non-pathologic steady state conditions DCs continuously sample self-Ags from normal peripheral tissues (Banchereau et al,1998) and present self-Ags to $CD4^+$ and $CD8^+$T cells, including complete or partial T cell tolerization through deletion or induction of unresponsiveness (Banchereau et al, 1998; Hawiger et al, 2001). Other mechanisms of

tolerance occur extra-thymically and include activation of antigen specific T suppressor cells and clonal deletion (Abbas, 2002). The failure of any of the mechanisms involved in self-recognition and elimination or down regulation of self-reactive clones may result in autoimmunity.

Given their pivotal role in controlling immunity, DCs are logical targets for treating autoimmune diseases. Disruption of the costimulatory pathways has been shown to be effective in blocking the pathogenic process in several models of autoimmune diseases (Salomon et al, 2001; Van Parijs et al, 1997**)**. Insufficient IL-12 production and or decreased expression of costimulatory molecules by APCs with a concomitant increased secretion of IL-10, that in turn blocks DC maturation and inhibits IL-12 production during Ag presentation, have been implicated in T cell anergy and tolerance induction. At the moment, two different strategies to selectively enhance DC tolerogenic properties are under extensive investigation: one based on pharmacological impairment of DC maturation; the second trough the use of genetically modified DCs, engineered to express anti-inflammatory cytokines like IL-10 and TGF-b (Takayama et al, 1998), or to express costimulation-blocking agents such CTLA-4 (Takayama et al, 2000)**,** or to express FasL provoking lymphocyte apoptosis (Matsue et al, 1999) or, finally, transfected to block directly NF-kB (Bonham et al, 2002).

Altough DCs seem to be absent in the CNS parenchyma under physiological non-pathological conditions, the presence of DCs at the blood-brain barrier and meninges in rodents (Mc Menamin PG 1999; Serafini et al, 2000; Kivisakk et al, 2004) and in non-inflamed human brain tissue (Matyszak et al, 1996; Greter et al, 2005) has been showed. Recently, Greter et al (2005) demonstrated that DCs associated with CNS vessels could process and present myelin antigens in the context of MHC class II molecules and then restimulate adoptively transferred Ag-experienced T cells. So, they proposed that DCs, strategically located at the brain barrier level, act as sentinels for CNS, scanning for physiologic self and foreign antigens, presenting these to cognate T cells migrating trough CNS vessels and promoting the CNS invasion by T encephalitogenic cells. Under pathological conditions, it is possible that DCs co-migrate with T cells into CNS, where

inflammatory foci arrange in a fashion similar to secondary lymphoid organs (Prineas JW 1979; Raine et al,1984).

Furthermore, a recent study on animal models of MS (McMahon et al, 2005) provided evidences that epitope spreading occurs within the inflamed CNS. At the time of MS diagnosis, Ag-epitope spreading has probably already occurred in most of the patients (Tuohy et al, 1999), making unfeasible, even at single patient level, a treatment based on antigen specific desensitization (Bielekova et al, 2000). If the strategy of antigen specific desensitization will be confirmed not to be applicable (or even dangerous), immunosuppressive/modulating therapies will probably maintain a key role in MS treatment. In addition, it is now clear that, late in the MS course, chronic inflammatory infiltrates persist in the CNS beyond an intact blood-brain barrier (BBB) (Massacesi, 2002; Uccelli et al, 2005; Ambrosini et al, 2005); consequently, immune-therapies not trespassing BBB will not be able to suppress the autoimmune responses ongoing intratecally (Massacesi, 2002). Therefore, DCs sited at the interface of CNS and immune system, pivotal players in development of CNS inflammation as discussed above, could represent a novel therapeutic target for the treatment of multiple sclerosis and other CNS inflammatory diseases. As Azathioprine does not easily cross the BBB, it has been well accepted that it acts mainly on the lymphoid organs, resulting thus more effective early in the course of MS, during the RR phase. We can now believe that Aza may acts also on DCs located at the brain barrier level.

Our data show that 6-MP in culturing medium, without affecting DC survival and cytokine production, strongly inhibits cell maturation, costimulation marker expression and allostimulatory ability. Based on these results, we suggest a double action for 6-MP mediated immunosuppression on both sides of immune-synapses: one on T lymphocytes, as recently demonstrated (Tiede et al, 2003; Poppe et al, 2005), another on dendritic cells. These data provide new insight into the mechanisms of action of Azathioprine, indicating for the first time that its immunosuppressive effect may be partially due to the inhibition of DC maturation.

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