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Alkylating drugs applied in non-cytotoxic doses as a novel compounds targeting inflammatory signal pathway

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ABSTRACT

Alkylating drugs (ADs) belonging to the nitrogen mustard family are commonly used as cytostatic and immunosuppressive agents. Our previous in vitro studies demonstrated that in the case of gradual dose decrease, the number of targets for alkylation in the cell is also reduced and the drug switches from brutal cytostatic to cell growth modifier. At doses of 0.3 μg/ml and lower, the effects of ADs are no longer associated with DNA damage or stress/MAPK pathways activation. Instead, the disruption of signal transduction by the IL-2β and/or TNFα cell surface receptors is observed. As a result, ADs in the doses 100-fold lower than cytostatic ones are capable to modify lymphocyte activity including the activity of regulatory T cells. We hypothesized that ADs may have a beneficial effect in the treatment of inflammatory diseases. Indeed, the application of non-cytotoxic doses of an AD melphalan reduces the severity of murine experimental colitis. Daily administration of melphalan (25 μg/kg body weight) markedly reduced the severity of DSS-colitis as determined by clinical and histological criteria. Moreover, the beneficial effect of melphalan was also shown in asthmatic patients. In 60% of these patients histological and ultrastructural signs of bronchial epithelium regeneration were also revealed. Thus, ADs at non-cytotoxic concentrations exert beneficial effect both in acute and chronic inflammatory diseases. Such anti-inflammatory activity is thought to be due to blocking of signal transduction through various cell surface receptor including IL-2R and TNFR. Consequently different steps of inflammatory cascade turn out to be inhibited.

1. Introduction

Alkylating drugs (ADs) are derived from sulfur mustards which were used as chemical warfare agents during World War I. The poisoned soldiers demonstrated leucopenia, bone marrow aplasia, dissolution of lymphoid tissue and ulceration of gastro-intestinal tract. The clinical course of bronchopneumonia in these subjects was characterized by the absence of leukocyte response [1]. Subsequent studies revealed that the susceptible tissues were those with rapid regenerative capacity. So bone marrow, lymphoid tissue and epithelium of gastro-intestinal tract turned out the principal targets for alkylating agents. These cytostatic effects prompted the creation of numerous antineoplastic drugs belonging to the nitrogen mustard family (cyclophosphamide, chlorambucil, melphalan). Subsequently, these drugs began to be used as immunosuppressive agents in the treatment of non-malignant diseases [2]. Thus, the efficacy of pulse cyclophosphamide treatment of severe connective tissue diseases, idiopatic pulmonary fibrosis, gastrointestinal vasculitis in

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systemic lupus erythematosus and acute steroid refractory bowel disease and nephrotic syndrome has been demonstrated [3–7]. The mechanism of such beneficial effect remains unclear, although the most of authors believe that it is associated with immunosuppressive activity of the drug. In the same time there are numerous works demonstrating that cyclophosphamide treatment is able to stimulate concomitant immunity due to regulatory T cell inactivation. In particular, a single dose of 150 mg/kg prevents a poorly immunogenic melanoma in mice [8]. It was also shown that a single injection of cyclophosphamide significantly accelerates the diabetes onset in non-obese diabetic mice [9,10]. Such diabetes acceleration is thought to occur through the selective depletion of regulatory T cells that otherwise inhibit the disease process in untreated mice [10]. These data are not in accordance with the common concept of mechanisms of cytostatic effects of alkylating agents, which are mainly associated with cross-linking of DNA double strands [11] and, at higher concentrations, with induction of DNA strand breaks [12]. Although DNA is not a unique target for alkylation in the cell the others (RNA and some proteins) do not play any role in the cytostatic effect realization if the drug is used at a DNA-altering dose. However, when the dose is gradually decreased, the number of targets for alkylating agents will also be reduced. Thus, after cell treatment with various concentrations of an AD, different scenarios will be realized. That may be demonstrated by the example of lymphocytes stimulated in vitro with a T cell mitogene (e.g. phytohaemagglutinin or concanavalin A; Fig. 1). If the concentration of ADs is high (more than 100 μg/ml or 300 μM), the cell dies within few hours due to irreversible DNA damage [13]. If the concentration of ADs is lower (30–100 μg/ml) numerous sites of DNA are also alkylated, but the damaged segments restored during DNA repair. However, the affected cells are anyway died due to apoptosis induction. It has been recently shown

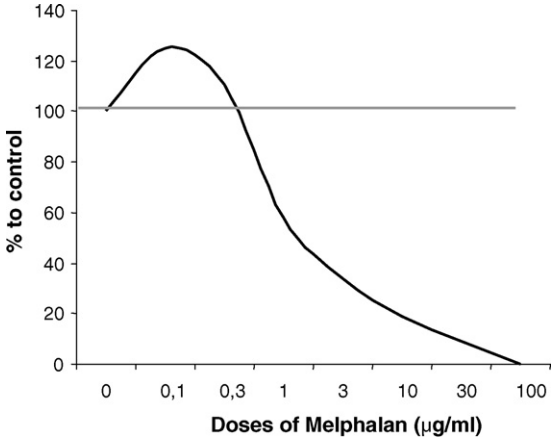

Fig. 1 – Effects of alkylating agents on proliferative response of murine spleen limphocytes stimulated with Con A. Freshly isolated spleen cells were exposed to mafosfamide or melphalan for 1 h at a concentration ranging from 0.01 to 100 μg/ml (or nearly 0.03–300 μM). Subsequently, the cells were washed, stimulated with optimal dose of Con A and cultured for 72 h. Cell proliferation was evaluated by [^{3}H]-thymidine incorporation.

that AD like other stress-induced agents, such as UV irradiation, heat shock, and protein synthesis inhibitors, activate both the JNK/SAPKs and another member of the MAPK family, the HOG1 homolog p38 MAPK [14]. Persistent activation of stress-induced kinases JNK/SAPK and p38 has been shown to trigger c-Jun-dependent CD95-L expression that is seemed to be rate-limiting step in the induction of apoptosis [15,16]. Moderate concentrations of ADs do not kill a cell but make it resistant to proliferative stimuli, possibly due to interference between mitogene signaling and stress/MAPK pathways that lead to the inhibition of IL-2 production in lymphocytes [17]. The activation of stress-induced kynases is believed to be independent of cytotoxic properties of ADs. Thus, JNK/SAPK activity is significantly induced even at relatively low concentrations (near 10 μM) that did not affect cell viability [16]. Nevertheless this dose range is much higher than those minimum concentrations, which can still modulate cultured lymphocyte proliferation [17,18]. Thus, ultra-low concentrations of ADs (0.3 μg/ml and lower) can augment the proliferative response of lymphocytes to phytohaemagglutinin (PHA) or concanavalin A (Con A) due to selective inhibition of suppressor cells [19] (see Fig. 2).

2. Immunomodulating effects of low concentrations of alkylating agents

Although skepticism developed regarding the existence of suppressor cells, studies in recent years have confirmed a central role of suppressor cell population in regulating immunity. Naturally occurring suppressor T cells (renamed regulatory T cells) constitutively express the transcription factor FoxP3 [20,21], CD25 [22], and glucocorticoid-induced TNF receptor (GITR) [23]. Regulatory T cells (Tregs) not only express IL-2Rα but also IL-2Rβ and the γc: that is, all of the subunits that are required to express a functional high-affinity IL-2R [24]. In the same time, Tregs do not secret IL-2 [25,26], so they depend on paracrine IL-2 for any responsiveness to this cytokine. In our previous experiments [19] it was shown that low concentrations of AD mafosfamide (a synthetic analogue of alkylating metabolite of cyclophosphamide) inhibit activity of suppressor cells induced by recombinant IL-2 (rIL-2). As seen in Fig. 2, addition of untreated suppressor lymphocytes to the culture of freshly isolated spleen cells significantly decreased their response to Con A. The pretreatment of suppressor cells with ultra-low doses of mafosfamide restored the level of lymphocyte response to mitogene. The effect of mafosfamide on suppressor lymphocyte activity can be mimicked by exposing of suppressor cells to anti-p75 mAb (antibody against β chain of IL-2R), but not to anti-p55 mAb (antibody against α chain of IL-2R). These data suggest that ADs are able to directly affect suppressor lymphocytes due to IL-2 signaling impairment. Similar results were obtained in our experiments with cytotoxic lymphocytes (CTL) [19]. As seen in Fig. 3, CTL were induced in a semi-allogeneic mixed lymphocyte culture. The cells were positive for IL-2Rβ but negative for IL-2Rα surface expression. Treatment with mafosfamide strongly suppressed the response of CTL to IL-2 stimulation. Thus, β chain of IL-2R and/or other components of IL-2 signal cascade seem to be critical molecular targets for

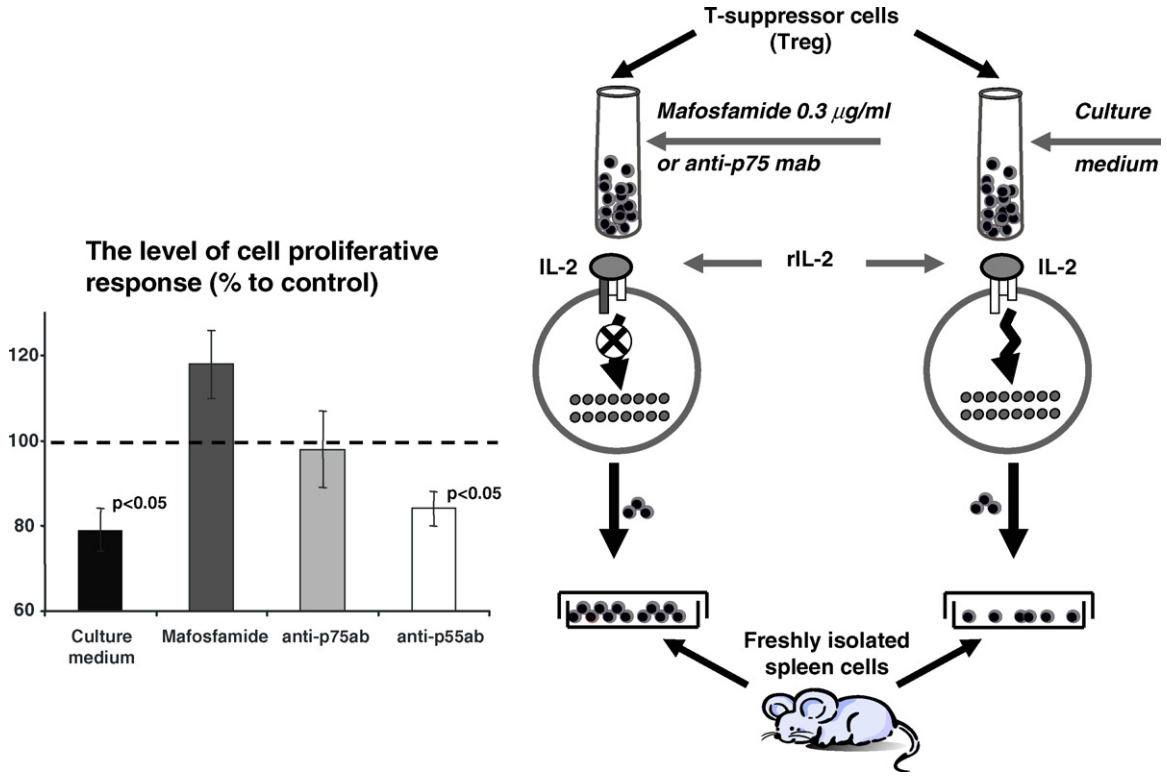

Fig. 2 – Low concentrations of alkylating agents impair IL-2 signaling pathway in regulatory T cells. To activate suppressor lymphocytes, feshly isolated murine spleen cells were incubated in the presence of rIL-2 (10 IU) for 24 h. For the evaluation of suppressor activity, rIL-2-treated cells were co-cultivated with the normal freshly isolated syngeneic spleen cells in ratio 1:1. Co-cultivation of normal spleen cells with the cells incubated without rIL-2 was used as a control. The cell mixtures were stimulated with optimal dose of Con A and incubated for 72 h. Cell proliferation was evaluated by [^{3}H]-thymidine incorporation. The suppressor cell sensitivity to mafosfamide was evaluated by pretreating the cells with different concentrations of the drug for 1 h. Subsequently, the cells were washed and cultivated in the presence of rIL-2 for 24 h as described above. It was demonstrated that the freshly isolated cells co-cultivated with the cells incubated without rIL-2 showed normal proliferative response to Con A. In contrast, the mixture of normal spleen cells and cells preincubated with rIL-2 demonstrated a significant decrease in response to mitogene.

low concentrations of ADs in lymphocytes. Such conclusion is able to explain enhanced proliferative response of lymphocyte pretreated with low doses of AD (see Fig. 1). It is well known that both effector cells and suppressor cells, so-called regulatory T cells, take part in lymphocyte response to mitogenes. Among the resting lymphocytes only regulatory T cells permanently express high affinity receptors for IL-2. The latter plays a very important role since it is a factor supporting the life of this cell subset [27]. So in the case of the resting lymphocyte treatment with low concentration of ADs the surface IL-2 receptors will be affected. As a result, due to growth factor signaling disturbance, regulatory T cells are eliminated. In the same time effector lymphocytes remain intact as they do not have an appropriate target. High affinity IL-2 receptors begin to express by this cell only after mitogen or antigen stimulation. That is why mitogene stimulation after AD removing results in unlimited proliferation out of regulatory T cell control.

IL-2R is not a unique receptor, which may be blocked with alkylating agents. Thus, low concentrations (0.1 μg/ml) of mafosfamide protect fibroblastoid cells (L929) against TNFα-induced apoptosis [28]. It is known that alkylating agents represent one of the most potent inducers of the cellular stress response, a specific program of gene expression, which includes the induction of JNK/SAPK activity and transcription activation of *c-fos* and *c-jun*, whose gene products have been proposed to be required for the cellular defense against cytotoxic agents [14,16,29,30]. To determine whether de novo protein syntheses are essential for protective activity of alkylating agents, we preincubated L929 cells with transcriptional inhibitor actinomycin D. Subsequently, the cells were treated with melphalan (L-phenyl-alanine mustard) and challenged by TNFα. As can be seen in Fig. 4, under these conditions we also observed a significant increase in cell viability. The influence of melphalan on TNFα-activated NFκB was tested by TransAM Kit (Active Motif). We did not observe an increase in NFκB activity in nuclear extracts of melphalan treated fibroblasts, possibly due to prolonged incubation with the drug (for 1 h). Thus, Donepudi et al. [31] have recently shown that exposure of P815 mastocytoma cells to ultra-low concentration of melphalan lead to rapid and transient NFκB activation, which can be found in nuclear extracts derived from the P815 cells treated with melphalan 30 min earlier, but not in nuclear extracts derived from the P815 cells treated with

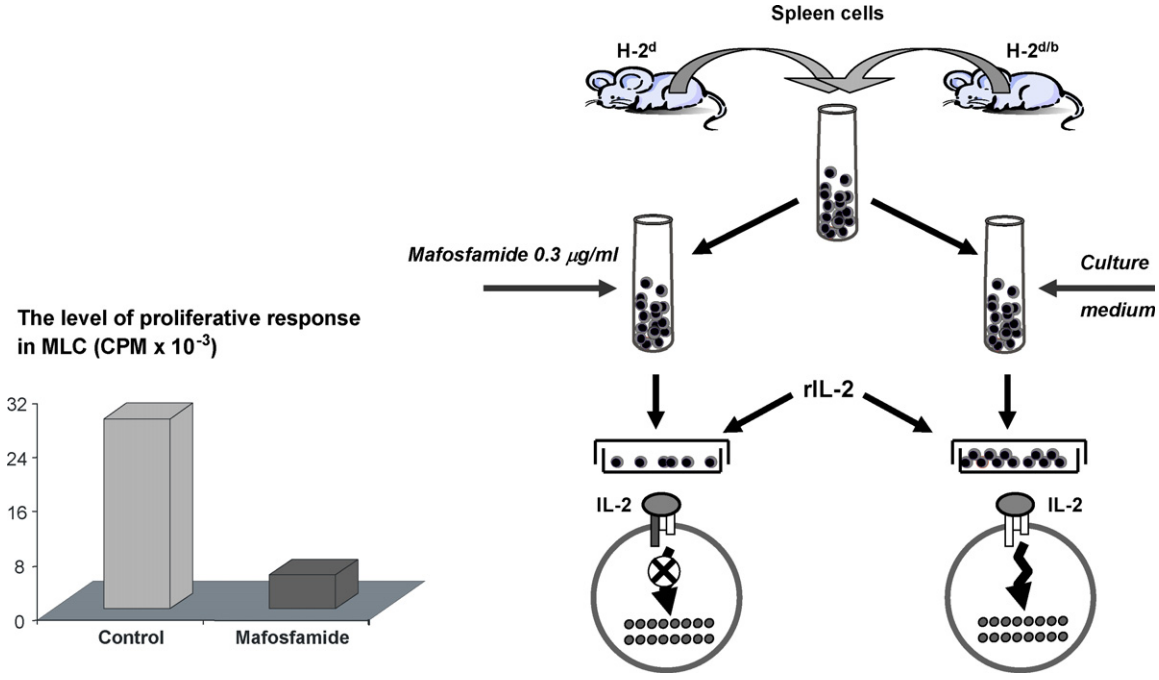

Fig. 3 – Low concentrations of alkylating agents impede proliferation of CTL simulated with rIL-2. CTL were induced in a semi-allogeneic mixed lymphocyte culture. Lymphocytes of C57BL/6 (H-2^b) and DBA/2 (H-2^d) mice were used as responding population and (C57BL/6 × DBA/2)F_1 (H-$2^{d/b}$) served as a source of stimulatory cells. Both the responder and stimulatory cells (10^6 cells of each population) were co-cultivated for 7 days. Subsequently, the cells were washed and incubated for 1 h with mafosfamide at different concentrations. Further, the cell were washed again and cultivated for 24 h in the presence of 10 IU rIL-2. Cell proliferation was evaluated by [^{3}H]-thymidine incorporation.

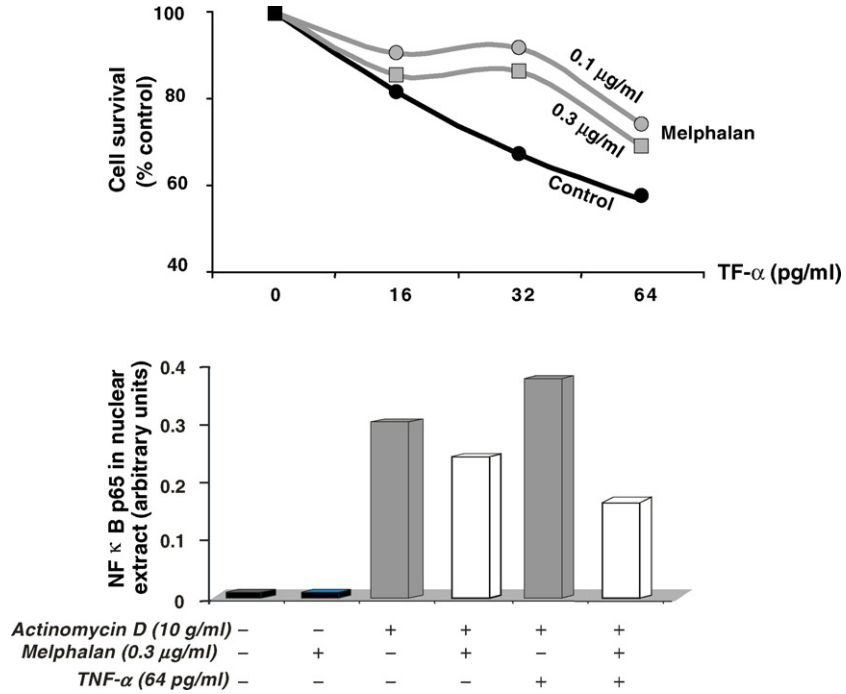

Fig. 4 – Protective effect of low doses of melphalan against TNF-α-induced cytotoxicity does not require de novo protein synthesis. A pool of L929 cells was preincubated for 1 h with actinomycin D. Subsequently, the cells were treated for another 1 h with melphalan and, then exposed to TNFα during 18 h (cell viability assessment) or during 15 min (monitoring of NFκB activation). Cells viability has been measured with crystal violet staining. Nuclear extract were prepared and tested for NFκB p65 using TransAM NFκB kits (Active Motif). The data were normalized to the absorbance of the standard nuclear extract provided as positive control for NFκB p65 activation.

melphalan 60 min earlier. Nevertheless, 1 h treatment with melphalan moderately decreased actinomycin D-induced NFκB activation and markedly reduced the transcription factor activity in nuclear extracts of actinomycin D pretreated cells challenged with TNFα (Fig. 4). Taken together, these data favor the concept that specific alkylation of components in the cytoplasm or cell membrane by nitrogen mustards interferes with cytokine signaling pathways and implicates in the mechanisms of cell activation and cell death. Accordingly, previous in vivo experiments had shown that cyclophosphamide and sarcolysin (*D*-isomer of melphalan) inhibit the tail muscle resorption in *Rana temporaria* and *Pelobates fuscus* tadpoles during amphibian metamorphosis [32], which is known to be associated with larval cell apoptosis [33].

3. ADs in the treatment of inflammation

The results indicate that ADs applied in low non-cytotoxic doses disturb inflammatory signal pathways, including the block of cell surface receptor for IL-2 and TNFα. On the basis of these data we postulated that application of ultra-low doses of ADs may result in a beneficial effect in the treatment of some inflammatory diseases [28]. Indeed, we succeed in showing that daily administration of melphalan (25 μg/kg body weight) markedly reduced the severity of experimental colitis in mice as determined by survival rate and histological criteria (Fig. 5). Murine experimental colitis was induced by the replacement of drinking water with 5% solution of dextran sulfate sodium (DSS). Both systemic and local anti-inflammatory effects had been observed. We believe that beneficial effect of melpahalan in DSS-colitis is particularly associated with inhibition of apoptotic cell death pathway in colon epithelium exposed to TNFα, which has been detected in colon as early as 1 day after the start of DSS treatment, with peak production occurring between days 5 and 7 [34,35]. Other possible mechanism(s) of melphalan beneficial effect in DSS-induced colitis seems to be related with facilitation of epithelial repair. This proposal is indirectly confirmed by the results of our recent investigation in asthmatic patients treated with inhalation of ultra-low doses of melphalan (five daily inhalations of 0.1 mg of the drug). In this study 60% of melphalan-treated patients had demonstrated the histological signs of bronchial epithelium regeneration. Moreover, in these patients a systemic anti-inflammatory effect of the drug had been found. For patients of placebo group neither signs of regeneration, nor systemic anti-inflammatory effect had been revealed [36,37]. In our opinion, anti-inflammatory effect of melphalan observed both in human patients and in DSS murine model may be also associated with direct action of the drug on activated lymphocytes expressing IL-2R.

4. Concluding remarks

It is well recognized that many chemotherapeutic drugs actively suppress cell-mediated immunity. Cyclophosphamide and melphalan, ADs commonly used in chemotherapy, are paradoxical in this regard. Whereas these drugs do have

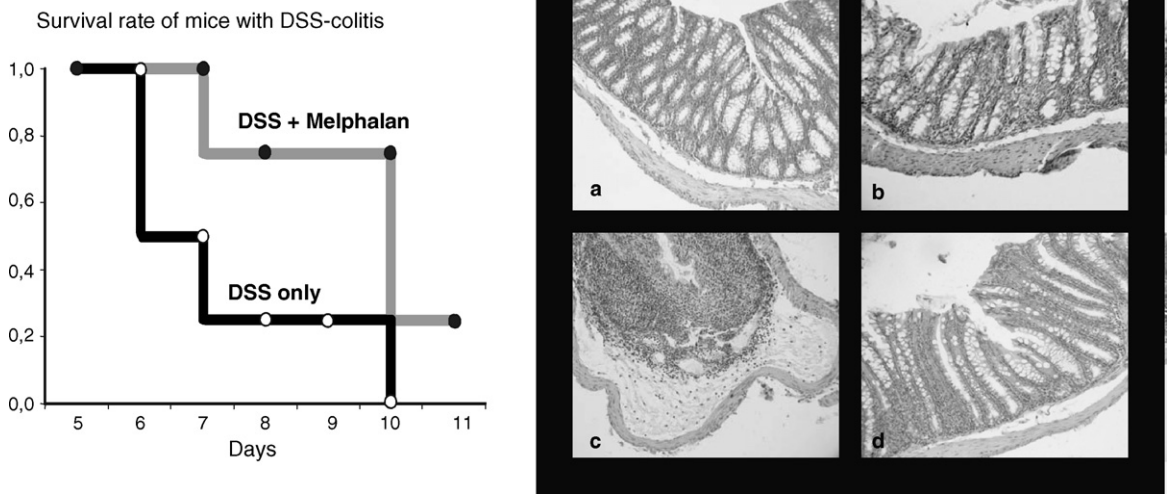

Fig. 5 – Survival rate and histological changes in mice with DSS-colitis treated with melphalan. Male BALB/cJLac mice weighing 18–20 g were used. For colitis induction, normal drinking water was replaced with a DSS solution (W.M. 36,000–50,000) for 10 days. Melphalan was administered daily intraperitoneally beginning of the day of DSS exposure. Control group was daily injected with phosphate buffered saline. (a) The colon from a normal BALB/c mouse. (b) The colon from a normal mouse treated daily with melphalan at a dose of 25 μg/kg body weight during 5 days; the histological examination reveals a moderate inflammatory cell infiltration without any crypt damage. (c) The colon from a mouse received a 5% aqueous solution of DSS ad libidum during 5 days; it can be seen a complete crypt destruction, massive cell infiltration and oedema. (d) The colon from a mouse received a 5% aqueous solution of DSS ad libidum during 5 days and treated daily with melphalan at a dose of 25 μg/kg body weight; in spite of the signs of slight oedema and cell infiltration, the crypt architecture is not disturbed. Shown here are 6-μm sections stained with hematoxylin and eosin; original magnification ×200.

immunosuppressive qualities and indeed are commonly used as a suppressant in autoimmune conditions such as arthritis and lupus nephritis, they have also been shown to have immunepotentiating activity in some setting [38–42]. Generally, these potentiating effects have been observed when the drugs are administered before antigen exposure or, importantly, in low doses [43–45]. There is no contradiction here. We speculate that alkylating agents exert simultaneously both suppressive and stimulatory effects. Since maintenance of peripheral tolerance is active and steady state process, Treg are seemed to be steadily activated. These cells constitutively express high-affinity receptor for IL-2, the cytokine, which is seemed to be the factor of their growth and survival [20–24]. Activated effectors including both $CD4^+$ and $CD8^+$ lymphocyte subsets are also strongly dependent on cell surface receptor signaling. Once activated the lymphocytes need IL-2 and other cytokines for their expansion and differentiation. Blocking cytokine signaling ADs switch off simultaneously both Treg and activated effectors. As a result, a binary effect may be achieved in the case of autoimmune or aggressive inflammatory processes: on the one hand, the expansion of dangerous T cell clones is stopped and the effector cells become inactive, on the other hand, the elimination of defective Tregs is occurred. During tumor immunotherapy, ADs can eliminate tumor specific Tregs [46,47] and simultaneously enhance the engraftment of adoptively transferred tumor-reactive effector T cells [48] by creating space [49–51]. Thus, pulse therapy with moderate or high dose of cyclphosphamide not only erases unfavorable immune response but allows to redirect newly minted one in desirable route. In patients with autoimmune disorders the combination of ADs and steroids results in switch to beneficial Th2 type response [52], whereas in subjects with tumors the combined treatment with ADs and adjuvants augments the activity of Th1 type lymphocytes [44–46]. We believe that such ''eraser effect'' may become more safe and precise if ADs are used in low and ultra-low doses.

Thus, bimodal activity of ADs is believed to be associated with their ability to disturb signal pathways of cell surface receptors including ones for IL-2 and TNF. Using these signal pathways as a therapeutic target for low doses of ADs is a good chance for numerous patients with various diseases associated with disturbance of immune response.

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