Modulation of human dendritic cell function by therapeutic agents
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Chapter 2

Granulocyte-macrophage colony-stimulating factor has opposing effects on the capacity of monocytes versus monocyte-derived dendritic cells to stimulate the antigen-specific proliferation of a human T cell clone

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Summary
Granulocyte-macrophage colony-stimulating factor (GM-CSF) is widely used in combination with interleukin-4 (IL-4) to differentiate monocytes into potent T cell stimulatory cells, referred to as monocyte-derived dendritic cells (MoDC). These cytokines further increased the stimulatory function of MoDC when present during their incubation with antigen, as determined by the proliferative response of an allergen-specific T cell clone. Conversely, the incubation of freshly isolated monocytes with antigen in the presence of GM-CSF or GM-CSF and IL-4 strongly inhibited the specific stimulation of the T cells, as compared to monocytes pulsed in the absence of cytokines. This suppression was partly due to the secretion of prostaglandin E2 (PGE2) and IL-10 by GM-CSF treated monocytes, since the combined use of indomethacin and anti-IL-10 antibodies during GM-CSF incubation and antigen pulsing restored T cell growth to about 65% of control levels. As confirmed by culture supernatant transfer experiments, maximal inhibition of T cell stimulation was also dependent on the direct contact between the T cells and GM-CSF treated monocytes during antigen-presentation. Collectively, these results imply that GM-CSF can either inhibit or enhance the re-stimulation of primed T cells by antigen-presenting monocytes or MoDC, respectively.

Introduction
Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates growth and differentiation of granulocyte and monocyte/macrophage precursor cells (reviewed in [1]). It is also known to affect the function of mature myeloid cells by priming monocytes and neutrophils for enhanced adhesion [2], tumor cytotoxicity [3], or leukotriene production [4]. However, in addition to stimulatory effects on myeloid cells, previous reports showed that GM-CSF may also have suppressive effects via the induction of prostaglandin E2 (PGE2) synthesis. LPS and IFN-γ-induced TNF-α release by GM-CSF primed macrophages was inhibited due to GM-CSF-induced PGE2 production [5], and PGE2 has been demonstrated to inhibit the pro-inflammatory activity of monocytes/macrophages by decreasing the production of IL-12 [6] while augmenting IL-10 release [7]. Pronounced inhibitory effects by IL-10 on monocytes/macrophages have been reported (reviewed in [8]). In the presence of monocyte/macrophage APC, hIL-10 inhibited not only cytokine synthesis [9], but also proliferation of human T cells and T cell clones [10,11]. Moreover, PGE2 directly inhibits T cell proliferation by decreasing the expression of both IL-2 and IL-2Rα chain [12,13]. This may partly explain the observation that prostaglandins released by monocytes may mediate the suppression of T cell function in cancer patients [14,15] and that CD14+ cells present in GM-CSF-mobilized peripheral blood stem cell products can inhibit T cell function.

GM-CSF together with IL-4 promotes the differentiation of monocytes into dendritic cells in vitro [16,17]. Monocyte-derived dendritic cells (MoDC) are amongst the most potent stimulators of naive T cells but they are also superior as compared to other antigen-presenting cells (APC) at inducing antigen-specific recall responses of memory T cells. We wanted to investigate whether GM-CSF alone or in combination
Opposing effects of GM-CSF on monocytes versus MoDC

with IL-4 would also support a stronger APC function of monocytes as it does for MoDC. As a model system, we used a human T cell clone (TCC) specific for the major protein of house dust mites, Der p1, in association with the HLA-DPw4 class II molecule [18,19]. In this report, we show that GM-CSF alone or in combination with IL-4 drastically inhibits the capacity of allogeneic, HLA-DP-matched monocytes to induce antigen-specific proliferation of the TCC if the cytokines were present during the period of antigen-pulsing. The relevance of this finding for regulation of memory T cells by different types of APC in chronic inflammation will be discussed.

Materials and Methods

Reagents, antibodies and cytokines

The basal culture medium (CM) was RPMI 1640 that was further supplemented with NaHCO₃ (2 mg/ml), penicillin (50 μg/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), and 10% FCS (Gibco, Grand Island, NY). This culture was endotoxin free as determined by the Limulus amebocyte lysis assay (<10 pg/ml of endotoxin). Recombinant human (rh) GM-CSF (specific activity 6x10⁶ U/mg), rhIL-4 (specific activity 6x10⁶ U/mg), and rhIL-2 (specific activity 10⁷ U/mg) were produced by and obtained from Novartis Pharma (Basel, Switzerland). In some experiments, GM-CSF obtained from R& D Systems (Minneapolis, MN) was used to confirm the specific biological activity of the material produced by Novartis Pharma. The neutralizing anti-IL-10 antibody (JES3-9D7) was purchased from Pharmingen (San Diego, CA). Lyophilized protein extract of Dermatophagoides pteronyssinus (Dpt) was purchased from ARTU Biologicals (Lelystad, The Netherlands). The content of the major allergen Der p1 in the lot used was 18.5 μg/mg of Dpt protein extract. Indomethacin was obtained from Sigma (St. Louis, MO).

Isolation and culture of cells

The human T helper cell clone CFTS 4:3.1 (TCC) was isolated from a skin punch biopsy of a patient with atopic dermatitis as described previously [18,19]. It specifically recognizes the major allergen of house dust mite, Der p1, in association with the MHC class II restriction molecule HLA-DPw4 [20]. The TCC was propagated by stimulating the T cells every 14-16 days via immobilized anti-CD3 mAb (Leu4, Becton Dickinson) or by presentation of Dpt antigen on irradiated autologous EBV transformed B cells (EBV-B) in the presence of rhIL-2 and rhIL-4 (50 U/ml each) as described [18].

Primary human monocytes were obtained by countercurrent elutriation [21] of leukapheresis samples donated by healthy individuals. To obtain a higher purity of monocytes, residual T cells and B cells were negatively depleted using magnetic beads covalently coupled to anti-CD2 and anti-CD19 mAb (Dynal Inc., Oslo, Norway) using a magnetic bead-to-target cell ratio of 5/1. The resulting monocyte preparation contained >95% CD14⁺ cells, as judged by flow cytometry and was further cultured in CM in the absence or presence of different supplements as indicated in the text.

Monocyte-derived dendritic cells (MoDC) were obtained by culturing purified human monocytes at 8x10⁵ cells/ml in CM supplemented with rhGM-CSF (300 U/ml) and rhIL-4 (120 U/ml). Cultures were fed every other day (days 2, 4 and 6) by
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exchanging half of the medium for fresh CM that had been supplemented with cytokines. MoDC were harvested on day 7 of culture and frozen in liquid nitrogen for subsequent use as APC in T cell proliferation assays. Cell viability was higher than 90% after thawing, as determined by trypan blue exclusion.

**Antigen-specific lymphocyte proliferation assay**

For antigen-specific stimulation of the T cells, each type of APC was incubated in CM for 16 to 20 h with Dpt (250 μg/ml for EBV-B and 50 μg/ml for monocytes and MoDC) or without antigen as negative control. Where indicated, rhGM-CSF (300 U/ml), rhIL-4 (120 U/ml), indomethacin (100ng/ml), or anti-IL-10 antibody (10μg/ml) were added during the preincubation of APC with antigen. For the determination of soluble mediators secreted by treated or untreated monocytes, cell-free culture supernatants were harvested after 16-20 h. Subsequently, APC were irradiated (40 Gy), washed and added to the T cells that were used not earlier than 14-16 days after stimulation with Dpt or plate-bound anti-CD3 to ascertain a resting state of the cells. Cloned T cells (4x10^4 cells/well) were mixed with autologous EBV-B (4x10^4 cells/well), HLA-DPw4-matched human monocytes (4x10^4 cells/well), or HLA-DPw4-matched human MoDC (4x10^3 cells/well) and further cultured in a volume of 200μl CM per well of 96-well round-bottomed plates (Corning Costar, Badhoevedorp, The Netherlands). Indomethacin (100ng/ml), rhGM-CSF (300 U/ml) or rhIL-2 (50 U/ml) were also present during the lymphocyte proliferation assay, where indicated. At day 3, 1 μCi of ³H-thymidine was added per well during the last 16h and incorporated radioactivity was quantitated by liquid scintillation counting. Results are expressed as cpm ± SD and represent the mean of triplicate cultures.

**Polyclonal stimulation of CD4^+ T cells**

96-well round bottom plates were seeded with 2x10^5 monocytes for incubation in CM with increasing amounts of GM-CSF in the presence or absence of indomethacin (100ng/ml). After 16 h, monocytes were washed two times with CM in the microtiter plates. The monocytes were not irradiated. T cells were collected from high density fractions of elutriations of the same PBMC donors as used for the preparation of monocytes. 1x10^8 T cells/ml were incubated with paramagnetic beads covalently coupled to anti-CD4 monoclonal antibodies (CD4^+ T cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany) as described by the manufacturer. Subsequently, 1x10^5 purified CD4^+ T cells were seeded into the wells that contained cytokine-treated or untreated monocytes or no APC as a control. T cell activation was induced by adding soluble anti-CD3 mAb (200 ng/ml, Pharmingen, San Diego, CA). After 48 h of co-culture, the proliferative response of the T cells was assessed by adding 1 μCi/well of ³H-thymidine for another 16 h. Results are expressed as cpm ± SD and represent the mean of quadruplicate cultures.

**Immunoassays**

Cell-free supernatants were harvested at 20 h from cytokine treated or untreated monocyte cultures or at 24 h from the co-culture of the T cells and monocytes for the determination of prostaglandins and cytokines. PGE₂ was determined with the
Immunooassay kit from R&D (Abingdon, UK). The sensitivity of this assay was 36.2 pg/ml. Production of IL-10 was measured by cytokine specific ELISA from Predicta (Genzyme, MA). The lower detection limit of this ELISA was 5 pg/ml. IL-1β, TNF-α, IL-6, TGF-β1, TGF-β2, and heterodimer IL-12 specific ELISA were obtained from R&D Systems (Minneapolis, MN). The sensitivity of these assay kits was 1 pg/ml, 4.4 pg/ml, 0.7 pg/ml, 7 pg/ml, 7 pg/ml, 5 pg/ml respectively.

Results

GM-CSF profoundly decreases the capacity of monocytes to induce antigen-specific growth of TCC

The human house dust-mite specific T cell clone CFTS4:3.1 was used to assess the stimulatory capacity of different APC. Autologous, EBV-transformed B cells, HLA-DP matched, allogeneic MoDC or monocytes were pulsed with specific antigen and subsequently added in varying numbers to cultures of resting 4:3.1 TCC. Consistent with published data, MoDC were superior to monocytes or EBV-B cells in their capacity to stimulate T cell proliferation over a wide range of APC/T cell ratios. As shown in Fig. 1 and also indicated by results obtained in a series of similar experiments (n=8), the proliferative response induced by monocytes when used at a 1/1 ratio (4x10^4 APC/well), was comparable with the degree of T cell proliferation stimulated by MoDC at a 1/10 ratio (4x10^3 APC/well). Therefore, these numbers of APC/well were used in subsequent experiments to allow for a comparison between the relative stimulatory capacity of MoDC and monocytes.

![Figure 1. MoDC are more potent stimulators of TCC than monocytes or autologous EBV-B cells. Different numbers of antigen-pulsed APC, autologous EBV-transformed B cells (B) or allogeneic MoDC and monocytes (Mo) obtained from HLA-Dpw4 matched donors, were co-cultured with TCC (4x10^3 cells/well). The proliferative response was determined at day 3. The results represent the mean cpm (± SEM) of three different experiments. Proliferation values of non-Dpt stimulated TCC have been subtracted.](image-url)
MoDC were generated in accordance with published protocols by culture of monocytes in media containing GM-CSF and IL-4 for seven days after which they are referred to as immature dendritic cells based on their phenotype [16]. MoDC were subsequently washed and pulsed with antigen in the presence or absence of freshly added GM-CSF or GM-CSF and IL-4 to address the influence of both cytokines on antigen presentation and stimulatory capacity. As shown in Fig. 2, the proliferative response of the specific T cell clone was slightly increased by adding fresh cytokines, particularly GM-CSF, to MoDC when pulsing with antigen.

![Bar chart](image)

**Figure 2.** Treatment of monocytes, but not EBV-B cells or MoDC with GM-CSF during antigen-pulsing strongly inhibits antigen-specific stimulation of T cells. The TCC was stimulated by autologous EBV-B cells (B), allogeneic monocytes (Mo) or MoDC that were matched for the expression of HLA-DpW4 as restriction element. APC were incubated with antigen (Dpt) for 20h in presence or absence of GM-CSF (300 U/ml) or with GM-CSF and IL-4 (300 U/ml and 120 U/ml, respectively). Data represent the mean cpm (± SEM) of at least 4 independent experiments, except for GM-CSF treated MoDC 2 experiments were performed. Proliferation values of non-Dpt stimulated TCC have been subtracted.

This result led us to investigate the influence of these cytokines on the stimulatory capacity of monocytes or autologous EBV-B cells. Unexpectedly, the proliferative response of the TCC was greatly diminished when antigen was presented by monocytes pulsed in the presence of GM-CSF and IL-4 as compared to monocytes pulsed in the absence of either cytokine (Fig. 2). T cell growth stimulation was further decreased (90%) if GM-CSF alone was present during the incubation of monocytes with antigen. As determined in GM-CSF titration experiments, the inhibition of TCC proliferation reached a plateau when using 300 U/ml of GM-CSF, which therefore was the concentration used in all subsequent experiments. This was also confirmed by using a commercial sample of GM-CSF (R&D Systems) in some of the titration
Opposing effects of GM-CSF on monocytes versus MoDC

experiments (data not shown). In contrast, the APC function of autologous EBV-B cells was unaffected by GM-CSF ± IL-4.

**GM-CSF treated monocytes secrete PGE\textsubscript{2} and a variety of immunomodulatory cytokines**

GM-CSF has been reported to induce the secretion of prostaglandins by monocytes [5,22,23]. Therefore, supernatants harvested after 20 h from monocyte cultures containing GM-CSF or the combination of GM-CSF and IL-4 were assayed for the presence of PGE\textsubscript{2} by specific ELISA. As shown in Table 1, high levels of PGE\textsubscript{2} were found in the supernatants of GM-CSF treated monocytes. The production of PGE\textsubscript{2} was almost completely suppressed when indomethacin was present during the incubation of monocytes with GM-CSF. Interleukin-4 also inhibited GM-CSF induced PGE\textsubscript{2} production to about 90%.

Since GM-CSF may also modulate or directly induce the secretion of other mediators, the same supernatants were assayed for the presence of various cytokines. As can be seen in Table 1, IL-10, IL-1\textbeta, TNF-\textalpha, and IL-6 were produced by monocytes treated with GM-CSF regardless of whether indomethacin was present or not. The addition of IL-4 reduced the production of IL-10 by about 40%, but it also inhibited the secretion of the potentially T cell stimulatory cytokines IL-1\textbeta, TNF-\textalpha, and IL-6. After subtraction of values found in FCS containing medium (ca. 0.75 ng/ml) the levels detected for TGF-\textbeta1 were always below 0.1 ng/ml and not significantly affected by GM-CSF. TGF-\textbeta2 or IL-12 could not be detected in supernatants of GM-CSF treated monocytes.

**Table I.** Effect of GM-CSF, IL-4, and indomethacin on cytokine production by monocytes

<table>
<thead>
<tr>
<th></th>
<th>TGF-\textbeta1</th>
<th>PGE\textsubscript{2}</th>
<th>IL-10</th>
<th>IL-1\textbeta</th>
<th>TNF-\textalpha</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/ml</td>
<td>ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>25.4</td>
<td>0.3 ± 0.1</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>40.5</td>
<td>41.0 ± 7.9</td>
<td>4.7 ± 0.7</td>
<td>14.1 ± 2.5</td>
<td>7.5 ± 3.8</td>
<td>218.6 ± 22.3</td>
</tr>
<tr>
<td>GM-CSF/indo</td>
<td>n.t.</td>
<td>0.3 ± 0.1</td>
<td>5.0 ± 1.1</td>
<td>15.6 ± 2.0</td>
<td>5.7 ± 3.2</td>
<td>277.3 ± 10.9</td>
</tr>
<tr>
<td>GM-CSF/IL-4</td>
<td>n.t.</td>
<td>3.5 ± 0.6</td>
<td>2.9 ± 0.3</td>
<td>3.8 ± 0.5</td>
<td>0.6 ± 0.2</td>
<td>80.1 ± 12.2</td>
</tr>
</tbody>
</table>

1) Freshly isolated monocytes (1x10\textsuperscript{6}/ml) were stimulated with GM-CSF in the absence or presence of indomethacin (indo) or with GM-CSF and IL-4. Data represent mean cytokine production (± SEM) of at least 4 independent experiments. 2) Values for TGF-\textbeta1 found in serum containing medium were subtracted; n.t.: not tested.
PGE$_2$ but not IL-10 is the major monocyte-derived soluble inhibitor of antigen-specific T cell proliferation

The issue of whether PGE$_2$ and/or IL-10 were directly responsible for T cell inhibition was addressed in culture supernatant transfer experiments. Supernatants were harvested from cytokine treated or untreated monocytes and added to the co-culture of the TCC with either antigen-pulsed monocytes, autologous B cells or MoDC. As shown in Fig. 3, supernatants harvested from GM-CSF-treated monocytes were found to inhibit T cell clone proliferation regardless of the type of APC used for antigen-presentation. In contrast, supernatants harvested from untreated or GM-CSF and indomethacin treated monocytes did not significantly affect T cell growth. This result suggests that PGE$_2$ is the major soluble factor secreted by GM-CSF-treated monocytes that negatively and directly affects allergen-specific T cell proliferation.

Figure 3. Indomethacin relieves inhibition of T cell growth mediated by supernatant of GM-CSF treated monocytes. Human T cell clones (4x10$^4$/well) were stimulated by antigen-pulsed EBV-B cells (4x10$^5$/well), monocytes (4x10$^5$/well), or MoDC (4x10$^5$/well) in a 1:1 mixture of culture medium and SN of monocytes that were incubated for 20h in the absence (gray bars) or presence of either GM-CSF (black bars) or GM-CSF and indomethacin (hatched bars). The degree of T cell proliferation induced by APC in the presence of SN of monocytes not treated with GM-CSF was set at 100% response and compared to the antigen-specific T cell proliferation induced by the relevant type of APC in the presence of SN(GM-CSF) or SN(GM-CSF/indo). Data represent the mean (± SEM) of 3 independent experiments. Proliferation values of non-Dpt stimulated TCC have been subtracted.

In the next series of experiments we asked which of the mediators produced during the co-culture of antigen-pulsed monocytes and T cell clone were actually responsible for the inhibition of T cell proliferation. Therefore, culture supernatants were harvested 24 hours after addition of APC to the T cells and the content of IL-10 and PGE$_2$ was determined by specific ELISA. As shown in Table 2, high PGE$_2$ levels correlated with strong TCC suppression (13% growth response). On the other hand,
the highest level of IL-10 was found in the supernatant of the medium control, e.g. T cells stimulated by monocytes pulsed with antigen in the absence of cytokines. It is of note that we still observed an inhibition of T cell growth as compared to the medium control although PGE₂ synthesis was decreased by 90% or even 99% through inclusion of indomethacin or IL-4, respectively, in combination with GM-CSF during antigen pulsing of monocytes (Table 2).

These data indicated that (i) IL-10 did not mediate T cell growth inhibition directly and (ii) GM-CSF plus IL-4 treated monocytes were able to inhibit T cell proliferation during co-culture although PGE₂ synthesis was reduced to background levels.

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**Table II.** Cytokine production during antigen-specific TCC stimulation by monocytes

<table>
<thead>
<tr>
<th>Mo treatment:</th>
<th>TCC response (%)</th>
<th>PGE₂ (ng/ml)</th>
<th>IL-10 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>100</td>
<td>0.5 ± 0.2</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>13.0 ± 1.9</td>
<td>24.2 ± 8.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>GM-CSF/indo</td>
<td>36.1 ± 3.8</td>
<td>2.3 ± 1.4</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>GM-CSF/IL-4</td>
<td>32.6 ± 2.4</td>
<td>0.2 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

Prior to co-culture with TCC (5x10⁵ cells), monocytes (5x10⁵ cells) were incubated with Dpt in the absence or presence of either GM-CSF or GM-CSF and indomethacin (indo) or IL-4. Cell-free supernatant was collected 24h after specific T cell stimulation. The percent of TCC response (± SEM) is shown relative to that induced by antigen-pulsed non-treated monocytes. The levels of IL-10 and PGE₂ are expressed as the mean (± SEM) of at least 3 independent experiments.

**TCC proliferation is partially restored by indomethacin, anti-IL-10 antibody or exogenous IL-2**

Since previous reports demonstrated IL-10 to inhibit antigen presentation by mononuclear cells, we tested whether the addition of neutralizing anti-IL-10 antibody alone or in combination with indomethacin would restore the ability of monocytes to fully activate the TCC. As shown in Fig. 4, antigen-pulsing of monocytes in the presence of GM-CSF and anti-IL-10 antibody slightly relieved inhibition of TCC proliferation. This effect was comparable to that achieved by blocking PGE₂ through the addition of indomethacin. However, by combining anti-IL-10 antibody with indomethacin at the time of monocyte incubation with antigen and GM-CSF, T cell proliferation was restored to about two-thirds of the level that was stimulated by monocytes pulsed in the absence of GM-CSF. Moreover, if indomethacin was supplemented to the co-culture of monocytes and T cells in order to block ongoing PGE₂ synthesis, T cell growth was still inhibited by about 35% when compared to
control level of TCC proliferation induced by non-treated antigen-pulsed monocytes in the presence of exogenous indomethacin.

One of the most obvious reasons for reduced growth response would be the failure to synthesize sufficient IL-2, perhaps due to small quantities of PGE$_2$ that were not detected in the ELISA. To exclude this possibility, excess amounts of IL-2 (50 U/ml) were added at the start of the T cells stimulation assay. As shown in Fig. 4, GM-CSF or GM-CSF and indomethacin treated, antigen-pulsed monocytes induced not more than about half-maximal proliferation of the TCC when compared to the control level of TCC proliferation induced by non treated antigen-pulsed monocytes in the presence of exogenous IL-2.

<table>
<thead>
<tr>
<th>present during co-culture:</th>
<th>Mo treatment:</th>
<th>Ag-specific TCC growth response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>medium</td>
<td>20 40 60 80 100 120 140</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GM-CSF/indo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GM-CSF/anti-IL-10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GM-CSF/indo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>GM-CSF</td>
<td></td>
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<td></td>
<td>GM-CSF</td>
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</table>

Figure 4. TCC proliferation is partially restored by indomethacin, anti-IL-10 or exogenous IL-2. Prior to co-culture with TCC, monocytes were incubated with Dpt in culture medium that was further supplemented with GM-CSF, indomethacin (indo), or anti-IL-10 antibody as indicated. Indomethacin or IL-2 was also added to the co-culture of T cells and cytokine treated or non treated monocytes. Shown is the percent of growth response of TCC relative to that induced by antigen-pulsed non treated monocytes. Data represent mean (± SEM) of at least 4 experiments. Proliferation values of non-Dpt stimulated TCC have been subtracted.

**GM-CSF mediated inhibition of monocyte APC function is transient**

Generally, GM-CSF is believed to convert monocytes into APC with enhanced T cell stimulatory function. Therefore we reasoned that the suppression of T cell growth by monocytes presenting antigen after their culture in the presence of GM-CSF should be transient. To address this question, monocytes were treated for 1, 2, or 5 days, respectively, with GM-CSF and were subsequently co-cultured with the TCC. The TCC response induced by monocytes treated for 1 day with GM-CSF was inhibited, as shown before (Fig. 5). However, monocytes treated for two days with GM-CSF...
induced a TCC response comparable to the control level of TCC proliferation induced by monocytes pulsed 1 day with antigen in the absence of cytokines. Monocytes treated for five days with GM-CSF were superior to non treated monocytes in their capacity to stimulate T cell proliferation.

**Figure 5.** Inhibitory effect by GM-CSF treated monocytes on TCC proliferation is transient. Monocytes were either pulsed 1 day with antigen in the absence of cytokines, washed, and used as APC or were incubated with GM-CSF for 1 day, 2 days, or 5 days respectively. During the last day of cytokine treatment the medium was supplemented with Dpt. Different numbers of antigen-pulsed monocytes were co-cultured with TCC (4x10^4 cells/well). The proliferative response was determined at day 3. Data represent the mean cpm (± SEM) of 4 independent experiments.

**Figure 6.** GM-CSF dose-dependently decreases monocyte accessory function for polyclonally stimulated autologous CD4+ T cells. 2x10^5 monocytes were seeded per 96-well and treated with increasing concentrations of GM-CSF in the absence (▼) or in the presence of indomethacin (■). After 16 h, they were washed in CM and 1x10^5 autologous CD4+ T cells were added to each well followed by the addition of soluble anti-CD3 mAb (200 ng/ml final concentration). The proliferation was determined at day 3. No proliferation was detected in wells that contained only monocytes or T cells (cpm <250). Results obtained in one of three similar experiments are shown (means of cpm ± SD).

**GM-CSF dose-dependently decreases the accessory function of monocytes for anti-CD3 activated autologous polyclonal CD4+ T cells**

Having demonstrated the reduced capacity of GM-CSF treated monocytes to stimulate the antigen-specific proliferation of an HLA-DP matched T cell clone we asked whether the same phenomenon can be seen in a non-cognate model of T cell activation. To address this question we have used highly purified CD4+ T cells that were activated by soluble monoclonal anti-CD3 antibody in the presence of autologous
monocytes treated before with increasing doses of GM-CSF. Since PGE$_2$ induced by GM-CSF is likely to be one of the mediators responsible for inhibition of T cell proliferation, we also included indomethacin during the incubation of the monocytes and their co-culture with the T cells. The results of a representative experiment are shown in Fig. 6. It is obvious that GM-CSF incubation of the monocytes prior to the addition of T cells and anti-CD3 antibody dose-dependently decreases their accessory function. The use of indomethacin both during the incubation of monocytes with GM-CSF and during the co-culture period partially restored the growth response of T cells cultured in the presence of monocytes treated with higher doses of GM-CSF.

Discussion
In this study, we have shown that GM-CSF mediated a strong inhibition (ca. 90%) of specific memory T cell proliferation when present during the incubation of monocytes with antigen. The strong suppressive effect of GM-CSF was slightly reversed by IL-4 since the combination of both cytokines with antigen during APC pulsing still resulted in about 70% inhibition of TCC proliferation. In contrast, a small stimulatory effect on T cell growth was obtained if MoDC were pulsed with antigen in the presence of GM-CSF or GM-CSF and IL-4. Because both cytokines are widely used to promote the differentiation of monocytes into potent antigen-presenting cells in vitro [16,17], a profound suppression of specific recall T cell responses as a result of treating monocytes with GM-CSF alone or in combination with IL-4 was not anticipated. Therefore, we also investigated the stimulatory capacity of monocytes that were incubated with GM-CSF for two or five days. This experiment revealed that the inhibition of T cell growth by GM-CSF treated monocytes was transient.

As demonstrated by others and in this study, GM-CSF can induce monocytes to secrete mediators that negatively affect T cell functions, particularly PGE$_2$ and IL-10. In fact, PGE$_2$ appeared to be the major soluble inhibitor, as shown by the transfer of supernatants obtained from GM-CSF or GM-CSF and indomethacin treated monocytes to co-cultures of T cells and antigen-pulsed APC (EBV-B cells, MoDC or untreated monocytes). Conversely, IL-10 did not seem to inhibit T cell growth directly because high IL-10 levels were found in the supernatant of T cells co-cultured with untreated antigen-pulsed monocytes (Table 2). The results shown here would be more consistent with an indirect inhibition of antigen presentation via the release of IL-10 from GM-CSF treated monocytes. This is in line with a previous report showing IL-10 mediated inhibition of antigen-specific T cell proliferation only if monocytes but not if EBV transformed B cells were used as APC [10]. Later it was shown that IL-10 decreased the level of antigen/MHC class II complexes by reducing ability of monocytes to efficiently transport newly synthesized, peptide loaded MHC class II molecules to the plasma membrane [24]. Although the level of expression of HLA class II antigens or, more specifically, HLA-DP molecules on monocytes was not decreased after 20 h of incubation with GM-CSF (FACS data not shown), it still remains possible that IL-10 secreted during antigen pulsing caused a decrease of the fraction of HLA-DP molecules that became successfully loaded with specific peptides. Consistent with this assumption is the partial restoration of T cell growth induced by monocytes treated with GM-CSF in the presence of anti-IL-10 antibody.
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It is noteworthy, that IL-2 added to the co-culture of the T cell clone and antigen-pulsed, GM-CSF treated monocytes failed to relieve growth inhibition to more than about 50%. Even the combination of indomethacin and anti-IL-10 did not completely restore the T cell response. Taken together, these data indicate a cell contact dependent inhibition by GM-CSF treated monocytes as opposed to insufficient co-stimulation. The latter was addressed by FACS analysis of the relative expression level of CD40, CD80, and CD86 on monocytes treated with GM-CSF as compared to untreated monocytes. We could not detect a significant modulation of the expression of these surface receptors (data not shown).

Several reports describe GM-CSF induced immune suppressor cells that resemble immature cells of the monocyte lineage [14,15,25]. Recently, CD14+ monocytes present in mobilized stem cell products have been reported to inhibit T cell function only via cell-cell contact [26]. At least in part, this is consistent with the results obtained in this study since the inhibition seen in co-culture experiments was always far more pronounced than the suppression obtained in supernatant transfer experiments. Soluble factors like PGE₂ and IL-10 are known as general inhibitors of naïve T cell proliferation. This led us to investigate the GM-CSF mediated reduction of monocyte accessory function in a non-cognate stimulation system. As shown in Fig.6, GM-CSF dose-dependently decreased the capacity of purified monocytes to support the polyclonal proliferation of autologous T cells activated by soluble anti-CD3 mAb. As seen in the cognate situation, the accessory function was partially restored by addition of indomethacin during monocyte incubation and T cell coculture.

Recently Kalinski et al. [27] showed that PGE₂ facilitates the cytokine-induced final maturation of MoDC and that these PGE₂-matured MoDC could bias the differentiation of naïve Th cells toward Th2 due to their greatly diminished capacity to secrete bioactive IL-12. These data together with the results shown in the present study would imply that monocytes infiltrating an inflammatory environment, for example the synovial joints in rheumatoid arthritis, where GM-CSF is abundantly expressed [28,29], are activated to secrete high levels of PGE₂ and thereby influence not only the final maturation process of MoDC but also contribute to the direct inhibition of Th1 cell proliferation and an anti-inflammatory effect via immune deviation from a Th1 into a Th2 dominated response.

References

Chapter 2


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