Abstract. Background: Cancer is linked to defects in immunosurveillance. Vaccination studies using dendritic cells (DC) try to re-establish immune responses toward tumor cells. Tumor-derived products such as interleukin-10 (IL-10) have inhibitory effects on DC function, and tumor-bearing hosts exhibit a lower number of DCs, suggesting inhibitory effects of tumor-derived factors on the recruitment of precursor cells. Materials and Methods: We generated DCs in the presence and absence of IL-10. DCs were then characterized by flow cytometry and cDNA microarray analysis. Results: IL-10 interferes with differentiation of peripheral blood monocytes to DCs and induces cells with a distinct phenotype. Microarray analysis revealed that IL-10 exhibits inhibitory as well as stimulatory effects on the expression of several genes. Addition of IL-10 to the differentiation cocktail induces a sustained inhibitory effect on subsequent maturation stimuli. Conclusion: IL-10 inhibits DC function and redirects differentiation of DCs to cells with a different phenotype, thereby reducing the pool of potential DC precursors.

Dendritic cells (DCs) are used in many vaccination studies against tumors (1-4). Promising results were obtained in individuals with small tumor burden or vaccination before rechallenge with tumor cells. These studies highlight the prominent role of DCs in tumor surveillance, but also reveal a lack of DC function in established tumors. Cancer cells have developed numerous mechanisms to evade immunoprotection exhibited by DCs. By production of cytokines or growth factors, such as interleukin-10 (IL-10), IL-6, vascular endothelial growth factor (VEGF), or the increased expression of other short distance messengers, such as prostanoi ds or gangliosides, tumors inhibit a potent immune response (5-8). DCs within the microenvironment of tumors express a tolerogenic phenotype (9, 10). Thus, the number of functionally-active DCs in the tumor milieu is a prognostic marker (11). While many in vitro studies show the inhibitory effect of IL-10 on DC maturation, DC function and subsequent T-cell stimulatory ability (12-15), there is no evidence that IL-10 depletes the precursor pool. However, in addition to a disturbed DC function, total DC numbers in peripheral blood are found to be decreased in tumor-bearing hosts, indicating a defect in recruitment of precursor cells (16, 17). Since peripheral blood monocytes belong to the pool of DC precursors (18, 19), we investigated the effect of IL-10 on interleukin-4 (IL-4)/granulocyte-macrophage colony-stimulating factor (GM-CSF)-mediated monocyte differentiation to DC.

Materials and Methods

Isolation of monocytes. Heparinized peripheral blood was obtained from four different healthy individuals. Mononuclear cells were isolated by Biocoll density centrifugation (Biocoll Separation Solution; Biochrom AG, Berlin, Germany). Using cluster of differentiation 14 (CD14) microbeads and magnetic cell separation (MACS) medium size/positive selection (MS) columns (Miltenyi Biotech, Bergisch Gladbach, Germany) CD14-positive monocytes were obtained according to the manufacturer’s instructions (20). The mean purity was 87.7%±1.75% and was determined by fluorescence-activated cells scanning (FACS) analysis (data not shown).

Differentiation of monocytes into DCs. Monocytes were cultured in RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) containing 5% fetal calf serum and 1% penicillin/streptomycin under standard culture conditions (5% CO₂, 37°C). For differentiation into immature DCs, 700 U/ml recombinant human IL-4 (R&D Systems, Minneapolis, MN, USA) and 1,000 U/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; AL-Immunotools, Friesoythe, Germany) was added to the cultures. Cells
were cultured for seven days. Every other day half of the medium was exchanged and the respective cytokines were supplemented to the initial concentration given above. To some cultures, 10 ng/ml recombinant human IL-10 (R&D Systems) was added at the beginning of the culture period and exchanged similarly to the other cytokines. On day seven of culture, the medium was completely removed and the cells were washed extensively with phosphate-buffered saline (0.2 mol/l phosphate buffer pH 7.2 and 50 mmol/l NaCl). Maturation of DCs was induced by addition of 10 μg/ml hemocyanin from keyhole limpets (KLH; Sigma, Saint Louis, MO, USA), 50 ng/ml recombinant human tumor necrosis factor alpha (TNFα; R&D Systems) and 1.000 U/ml GM-CSF for three days.

Flow cytometry. Prior to staining, cells were incubated with 100 μg/ml of purified human immunoglobulin (Sigma) to avoid non-specific binding reactions. Cells were stained with fluorescently-labeled anti-CD14, anti-CD1a, anti-human leukocyte antigen DR (HLA-DR), anti-CD80, anti-CD83 (Immunootech, Marseille, France), anti-chemokine (C-C motif) receptor-1 (CCR1), anti-CCR2 and anti-CCR7 (R&D Systems), or the respective isotype control antibodies (fluorescein isothiocyanate-labeled IgG2b, phycoerythrin-cyanine 5.1-labeled IgG1, Immunotech; phycoerythrin-labeled IgG2a, Becton Dickinson, San Jose, CA, USA) suspended in phosphate-buffered saline. After 10 min of incubation (30 min for CCR1, CCR2 and CCR7), cells were washed in phosphate-buffered saline and the fluorescence intensity was measured using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). To exclude dead cells from the analysis, cells were stained with annexin V and propidium iodide (Immunotech) as described previously (21). Only cells which tested negatively for annexin V and propidium iodide were included in the analysis.

RNA isolation. After two days of culturing monocytes under the conditions described above, cells were harvested and washed extensively with phosphate-buffered saline. Total RNA was isolated using the Invitorn Spin Cell RNA Mini Kit (Invitorn, Berlin, Germany) according to the manufacturer’s instructions. To increase the purity, RNA was incubated with DNase (Quiagen, Caltag Laboratories, Burlingame, CA, USA). For quality and quantity control, an RNA sample was analyzed using the absorption ratio of 260/280 nm, as well as by agarose gel electrophoresis.

cDNA array analysis. To achieve appropriate concentrations for membrane labeling, the extracted mRNA samples were amplified using the SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA). In short, first-strand cDNA was synthesized and then used for long-distance polymerase chain reaction (PCR) with the Clontech Advantage PCR Kit. Optimal number of cycles was chosen by comparison with 1 kb DNA marker on 1.2% agarose gel electrophoresis. cDNA was then cleaned using Nucleospin columns (Clontech). cDNA was synthesized in the presence of (32P)-labeled dCTP (Amersham Pharmacia Biotech, Freiburg, Germany). After an additional cleaning step, the radioactively-labeled cDNA was hybridized overnight with the Clontech Atlas™ Human Cytokine and Receptor array (Clontech; Gene Expression Omnibus accession number GPL136). The nylon membrane was then washed and scanned by Phospho-Imager FLA-300 (Fujifilm, Tokyo, Japan). The resulting gene expression profiles were interpreted using the corresponding orientation grid and gene table from Clontech. For comparison of the different cell populations AIDA Array Metrix/AIDA Array Compare from Raytest (Straubenhardt, Germany) was used. The means of duplicate hybridizations were used for further evaluation. A total of nine housekeeping genes (ubiquitin C, tyrosine-3-monooxygenase, hypoxanthine phosphoribosyl transferase 1, glyceraldehyde-3-phosphate dehydrogenase, tubulin alpha, major histocompatibility complex class I, C (MHC IC), actin beta, ribosomal protein L13a, and ribosomal protein S9) were used for positive controls, and plasmid and bacteriophage DNAs as negative controls. The integral of the area of the spots was calculated using the pixel information depicted by AIDA Array Metrix. The integrated background intensity was subtracted from all spots in the evaluation. The mean integrated area of the housekeeping genes (references) was used for normalization of the spots. Only genes that exhibited similar expression in the different culture conditions in all samples were subjected to further investigation.

Statistical analysis. The paired t-test was used for statistical data evaluation. Differences were considered statistically significant with p<0.05.

Results

Role of IL-10 during early differentiation of monocytes to immature DCs. When CD14-positive monocytes were cultured with GM-CSF and IL-4 (herein called standard culture conditions) for a period of seven days, they exhibited the typical phenotype of immature DCs, with an almost complete loss of CD14 and high cell surface expression of HLA-DR and CD1a (Table I). Cells staining positively for HLA-DR were concomitantly negative for CD14. Expression of the chemokine receptors CCR1 and CCR2 was negligible. Compared to cells that were cultured in medium-alone for seven days, cell surface expression of CD14, CCR1 and CCR2 was significantly reduced in immature DCs, while the proportion of CD14-negative cells that stained positively for CD1a and HLA-DR was significantly increased. Addition of IL-10 to standard culture conditions at the beginning of the incubation period significantly inhibited IL-4/GM-CSF-induced down-regulation of CD14, CCR1 and CCR2 as well as up-regulation of CD1a. Moreover, IL-10 induced cells expressing a distinct phenotype (Table I). These cells expressed the highest percentage of HLA-DR while still being positive for CD14, CCR1 and CCR2 compared to cells cultured in medium-only. In addition, cells stained positively for CCR7 prior to exposition to maturation stimuli. Taken together, these changes in surface markers suggest the acquisition of a tolerogenic phenotype at day seven of culture.

Gene expression profile during early monocyte differentiation. Results obtained for cell surface expression indicated an early effect of IL-10 on differentiation from monocytes to immature DCs. Therefore, cDNA expression for various cytokine-related genes was evaluated 48 h after initiating the differentiation process using the Clontech Atlas™ Human Cytokine and Receptor Array. In addition to monocytes cultured under standard conditions, RNA was
isolated from monocytes cultured in medium-only, and in medium containing GM-CSF, IL-4 and IL-10. Many of the genes tested were found to be expressed at very low levels or were not expressed at all. Interestingly, cells cultured in medium-only exhibited the highest expression of many genes tested. Because large inter-individual differences were observed, gene expression profiles of DCs generated in the absence of IL-10 were set as 1 and fold changes for medium-only were compared to the control medium. This effect was reduced by IL-10 (Figure 1). Expression of IL-1 beta (IL1B), S100 calcium binding protein A9 (calgranulin A, S100A9), and CCR2 was reduced by IL-4/GM-CSF compared to the control medium. This effect was induced by addition of IL-10 (Figure 1). Expression of IL-1 beta (IL1B), S100 calcium binding protein A9 (calgranulin A, S100A9), colony stimulating factor 3 (CSF3), and diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor; HBEGF) was also reduced by IL-4/GM-CSF compared to the control medium. This effect was enhanced by the addition of IL-10, indicating divergent effects of IL-4 on IL-10-induced gene expression (Figure 2). IL-4/GM-CSF up-regulated the expression of interferon gamma receptor-2 (IFNGR2), golgi glycoprotein-1 (GLG1) and wingless type mouse mammary tumor integration site 8B (WTN8B) compared to the medium control. This effect was inhibited by the addition of IL-10 (Figure 3). On the other hand, the stimulating effect of IL-4/GM-CSF on platelet-activating factor receptor (PTAFR) was further increased by the addition of IL-10 (Figure 3).

Role of IL-10 during differentiation of immature DCs to mature DCs. In a next step, we investigated whether IL-10-treated monocytes were able to differentiate into fully mature DCs upon appropriate re-stimulation with KLH, TNFalpha and GM-CSF for three days after wash-out of IL-10. Immature DCs obtained by incubation under standard culture conditions during the first seven days exhibited a significant increase in cell surface expression of HLA-DR and CCR7 during stimulation for three additional days with KLH, TNFalpha and GM-CSF. In addition, these cells exhibited high expression of CD80 and CD83 (Table II), indicating a phenotype of mature DCs. Addition of IL-10 during maturation with KLH, TNFalpha and GM-CSF had only marginal, but significant effects of the maturation process. Significantly fewer of these cells expressed the co-stimulatory molecules CD80 and CD83. All other cell surface markers tested were expressed on comparable proportions of cells as those matured without IL-10; *p<0.05, after maturation (day 7) vs. before maturation (day 7).

**Table I.** Cell surface expression of immature dendritic cells. Cluster of differentiation 14 (CD14)-positive monocytes were cultured in medium-alone, medium with 700 U/ml recombinant human interleukin-4 (IL-4) and 1000 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), or in medium with IL-4/GM-CSF in the absence of IL-10, respectively. Expression of the indicated surface antigens was assessed by flow cytometry after seven days of culture. Mean percentages of positive cells±standard error of four independent experiments are presented.

<table>
<thead>
<tr>
<th>Treatment (day 0-7)</th>
<th>Medium</th>
<th>IL-4/GM-CSF</th>
<th>IL-4/GM-CSF + IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>56.10±8.10</td>
<td>0.40±0.60*</td>
<td>35.58±19.6**</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>66.00±9.05</td>
<td>47.38±16.38</td>
<td>79.08±7.63**</td>
</tr>
<tr>
<td>CD14/HLA-DR</td>
<td>4.93±2.43</td>
<td>47.33±16.38*</td>
<td>49.33±19.33</td>
</tr>
<tr>
<td>CD1a</td>
<td>1.18±1.13</td>
<td>90.08±5.19*</td>
<td>6.40±5.3**</td>
</tr>
<tr>
<td>CCR1</td>
<td>23.53±5.94</td>
<td>2.30±1.7*</td>
<td>47.40±6.95**</td>
</tr>
<tr>
<td>CCR2</td>
<td>30.78±16.28</td>
<td>3.88±2.76*</td>
<td>21.60±8.7**</td>
</tr>
<tr>
<td>CCR7</td>
<td>11.7±2.3</td>
<td>0.50±3.5*</td>
<td>13.25±2.5**</td>
</tr>
</tbody>
</table>

**Table II.** Cell surface expression of mature dendritic cells. Cluster of differentiation 14 (CD14)-positive monocytes were cultured in medium-alone, medium with 700 U/ml recombinant human interleukin-4 (IL-4) and 1000 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), or in medium with IL-4/GM-CSF in the presence of 10 ng/ml recombinant human IL-10, respectively. After seven days, maturation was induced by addition of keyhole limpet hemocyanin, tumor necrosis factor-alpha, and GM-CSF. During maturation, IL-10 was present or absent. Expression of the indicated surface antigens was assessed by flow cytometry after 10 days of culture. Mean percentages of positive cells±standard error of four independent experiments are presented.

<table>
<thead>
<tr>
<th>Treatment (day 7-10)</th>
<th>IL-4/GM-CSF</th>
<th>IL-4/GM-CSF + IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maturation cocktail</td>
<td>No IL-10</td>
<td>IL-10</td>
</tr>
<tr>
<td>CD14</td>
<td>0.07±0.1*</td>
<td>0.60±0.6</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>96.10±2.5**</td>
<td>97.07±1.0**</td>
</tr>
<tr>
<td>CD1a</td>
<td>93.90±2.5*</td>
<td>92.93±1.8</td>
</tr>
<tr>
<td>CCR1</td>
<td>87.70±5.5**</td>
<td>82.13±6.8**</td>
</tr>
<tr>
<td>CCR2</td>
<td>92.10±2.5*</td>
<td>86.83±2.6**</td>
</tr>
<tr>
<td>CCR7</td>
<td>90.9±0.8*</td>
<td>86.83±2.6**</td>
</tr>
<tr>
<td>CD14</td>
<td>93.90±2.5*</td>
<td>92.93±1.8</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>93.90±2.5*</td>
<td>92.93±1.8</td>
</tr>
<tr>
<td>CCR1</td>
<td>0.63±0.5*</td>
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<tr>
<td>CCR2</td>
<td>1.40±0.8*</td>
<td>5.20±4</td>
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<tr>
<td>CCR7</td>
<td>17.40±5.9**</td>
<td>22.50±9.3</td>
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</table>

**HLA-DR:** Human leukocyte antigen DR; **CCR:** chemokine (C-C motif) receptor. *p<0.05, vs. Medium; **p<0.05, vs. IL-4/GM-CSF.

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only expressed on one third of these cells. There was no significant increase in cells with HLA-DR or CD1a expression upon maturation. Addition of IL-10 for the first and second incubation period did not significantly change these inhibitory effects. Thus, even when IL-10 is not present during maturation, its presence during early differentiation from monocytes to immature DCs significantly influences the maturation process, indicating a sustainable effect on DC differentiation.

Discussion

Several tumor-derived molecules, such as IL-10, are known to have an inhibitory effect on DC maturation or T-cell stimulatory ability of DCs (22-24). Many studies investigated the role of IL-10 during maturation of immature to mature DCs, showing an inhibitory effect on the expression of co-stimulatory and antigen-presenting molecules (25-28), which might lead to an impairment of DC function. However, in addition to inhibition of DC function, tumor-bearing hosts exhibit a reduction in DC numbers, indicating an early inhibitory event during differentiation of precursors to DCs. To explore the potential mechanisms for this, we analyzed the effect of IL-10 on early differentiation of IL-4- and GM-CSF-stimulated monocytes. We found that IL-10 led to a cell population lacking common markers of immature DCs (29, 30). Cells remained positive for CD14, CCR1 and CCR2, expressed HLA-DR, but stained only weakly positively for CD1a, suggesting that IL-10 redirects DC differentiation to a distinct cell population. To further test this hypothesis, we investigated whether the inhibitory effects of IL-10 were reversible by washing-out IL-10 and re-stimulating the cells with a maturation-inducing cocktail containing TNFα, KLH and GM-CSF. These analyses showed that the inhibitory effects of IL-10 were only partially reversible. The aberrant cell phenotypes (CD14−, CD1a−, HLA-DR+ and CD14−, HLA-DR+, CCR1+) remained almost unchanged, indicating that IL-10 induced a stable phenotype making cells resistant to further maturation. Our data is in line with that of Fortsch...
et al. (31) and Buelens et al. (13) who showed that the effect of IL-10 on the expression of CD1 on IL-4/GM-CSF-treated monocytes was dose-dependent and led to a cell population with high CD14 and almost no CD1a expression. Allavena et al. found similar results for IL-13/GM-CSF-treated monocytes (26). While the former used day-seven immature DCs for their phenotypic characterization, Thommsen et al. were able to show that the inhibition of CD1a induction by IL-4/GM-CSF is already present at day three of the culture period (15). In our culture system, at day seven, cells expressing CCR1 and CCR2 were not found at relevant percentages, indicating a rather mature phenotype. On the other hand, cells did not exhibit significant staining for CCR7 and exhibit a relatively low frequency of CD14-negative and HLA-DR-positive cells compared with other studies (32, 33). We, therefore, questioned whether the effects of IL-10 really do take place during differentiation of monocytes to immature DCs, or whether they are part of the maturation process. Gene expression analyses revealed that IL-10 starts to block IL-4/GM-CSF-driven monocyte differentiation to immature DCs as early as 48 h after culture initiation. The induction of IFNGR2, GLG1 and WNT8B was inhibited by the addition of IL-10 to levels comparable to cells cultured in medium-alone. In addition, down-regulation by IL-4/GM-CSF of CTGF, S100A8 and CCR2 was reverted by the addition of IL-10. Induction of CCR2 mRNA and its ligand monocyte chemoattractant protein 2 (MCP2) by IL-10 was reported by Sun et al. (34). In the present study, we showed that CCR2 mRNA and cell surface expression is increased on immature and mature DCs by the addition of IL-10. In contrast, results for CCR1 in the array analysis did not parallel those obtained for cell surface expression (data not shown). While Williams et al. reported that CCR1 mRNA can be induced by IL-10 (27), Li et al. showed that IL-10 prolongs CCR1 mRNA half-life rather than increasing mRNA levels (35). Post-transcriptional or post-translational mechanisms might explain the discrepancy between increased cell surface

Figure 2. Interleukin-10 (IL-10) enhances IL-4/granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced down-regulation of IL-1 beta (IL1B), colony stimulating factor 3 (CSF3), S100 calcium-binding protein A9 (S100A9), and heparin-binding epidermal growth factor-like growth factor (HBEGF). Cluster of differentiation 14 (CD14)-positive monocytes were cultured in medium-alone, medium with 700 U/ml recombinant human IL-4 and 1000 U/ml recombinant human GM-CSF, or in IL-4/GM-CSF in the presence of 10 ng/ml recombinant human IL-10, respectively. After 48 h of culture, cells were harvested and RNA was isolated, reverse-transcribed and amplified using radioactively-labeled dCTP. After incubation with the Clontech Human Cytokine/Receptor Array, gene expression was evaluated by Phospho-Imager scanning. Results are shown as relative expression. Expression of cells cultured under standard conditions in the presence of IL-4/GM-CSF was set as 1. Triangles, diamonds and squares indicate data of three different individuals.
expression and the lack of increased mRNA in our experiments. D’Amico et al. showed that CCR1 and CCR2 expressed by IL-10-treated cells work as decoy receptors and therefore taper the innate immune response (36). Expression of IL1B, S100A9, CSF3, and HBEGF was further reduced by the addition of IL-10. The signal for PAFR was highly increased by IL-10. IL-1β is a known inducer of DC maturation and an enhancer of DC IL-12 production, therefore promoting DC/T-cell interaction (37, 38). By further reducing IL-1β, as early as 48 h after initiation of the differentiation process, IL-10 starts to exert its inhibitory effects on DC maturation. Taken together, these data show that IL-10 not only inhibits the maturation of immature to mature DCs, but also exhibits sustained effects during differentiation of precursors to immature and mature DCs. It displays not only an inhibitory effect on IL-4/GM-CSF-driven differentiation induction, but also favors the development of a distinct cell population. IL-10-induced impairment of DC maturation in patients with elevated IL-10 levels can result in immunosuppression. For instance, serum from patients with systemic lupus erythematosus with high IL-10 levels was shown to inhibit DC maturation in vitro (39). Polymorphisms in the IL10 locus are associated with clinical outcome in patients with hematological malignancies (40, 41). Immune escape by hematopoietic tumor cells, e.g., Hodgkin’s lymphoma cells (42), is a common phenomenon and the serum levels of IL-10 are of prognostic significance for these patients (43). In addition to direct effects on T-cells, IL-10 might interfere with DC maturation in these patients, resulting in long-lasting immunosuppression. In addition to IL-10-producing tumor cells, IL-10-producing mesenchymal stem cells (MSCs) can inhibit DC maturation (44). MSCs are immunosuppressive cells which have been used clinically with variable success for the treatment of patients with graft-versus-host disease (45-47). It might be interesting to analyze the function of

Figure 3. Interleukin-10 (IL-10) inhibits IL-4/granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced up-regulation of interferon gamma receptor 2 (IFNGR2), golgi glycoprotein 1 (GLG1) and wingless type mouse mammary tumor integration site 8B (WNT8B), and increases IL-4/GM-CSF-induced up-regulation of platelet-activating factor receptor (PTAFR). Cluster of differentiation 14 (CD14)-positive monocytes were cultured in medium alone, medium with 700 U/ml recombinant human IL-4 and 1000 U/ml recombinant human GM-CSF, or in IL-4/GM-CSF in the presence of 10 ng/ml recombinant human IL-10, respectively. After 48 h of culture, cells were harvested and RNA was isolated, reverse-transcribed and amplified using radioactively-labeled dCTP. After incubation with the Clontech Human Cytokine/Receptor Array, gene expression was evaluated by Phospho-Imager scanning. Results are shown as relative expression. Expression of cells cultured under standard conditions in the presence of IL-4/GM-CSF was set as 1. Triangles, diamonds and squares indicate data of three different individuals.
DC in patients after MSC transplantation. In conclusion, our data reveal that IL-10 redirects differentiation of DCs to cells with a different phenotype, thereby reducing the pool of potential DC precursors. This may constitute a potential mechanism of tumor immune escape.

References


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