Cancer Immunity, Vol. 4, p. 8 (25 August 2004) Submitted: 23 April 2004. Accepted: 14 July 2004.
Communicated by: G Riethmuller

Prevention and reversal of tumor cell-induced monocyte deactivation by cytokines, purified protein derivative (PPD), and anti-IL-10 antibody

Monika Baj-Krzyworzeka, Jaroslaw Baran, Rafal Szatanek, Danuta Stankiewicz, Maciej Siedlar, and Marek Zembala

Department of Clinical Immunology, Polish-American Institute of Pediatrics, Jagiellonian University Medical College, Cracow, Poland

Keywords: human, monocyte activation, cultured tumor cells, cytokines, purified protein derivative

Abstract

Upon contact with tumor cells when cocultured *in vitro*, human monocytes become unresponsive (deactivated) to restimulation and demonstrate decreased production of TNF-alpha and IL-12, and enhanced IL-10 secretion. The present study was undertaken to determine whether immunomodulatory agents (proinflammatory cytokines and PPD of tuberculin) could either prevent or reverse the deactivation of monocytes. Monocytes were treated with the agents either before or after being cocultured with tumor cells. Pretreatment of monocytes with IFN-gamma, either alone or in combination with TNF-alpha, GM-CSF, or PPD, significantly enhanced TNF-alpha and IL-12 production by deactivated monocytes. TNF-alpha, GM-CSF, and PPD alone were inactive. Treatment of monocytes following coculture with IFN-gamma, TNF-alpha, GM-CSF, PPD or IFN-gamma in combination with these agents reversed the depressed TNF-alpha release, whereas IL-12 production was enhanced by IFN-gamma alone. All the agents had no or only a limited effect on the enhanced IL-10 secretion by deactivated monocytes. However, treatment of cocultured monocytes with anti-IL-10 mAb significantly increased the production of TNF-alpha and IL-12 by deactivated monocytes. Moreover, coengraftment of deactivated monocytes with human pancreatic carcinoma cells into SCID mice caused an enhancement of the tumor growth that was alleviated by the treatment of monocytes *in vitro* with IFN-gamma alone or in combination with GM-CSF or PPD. These results suggest that activation of monocytes with certain proinflammatory cytokines and/or selective inhibition of IL-10 by a mAb may prevent or reverse monocyte deactivation caused by tumor cells.

Introduction

Despite progress in understanding the rationale for cancer immunotherapy and the identification of tumor antigens, the clinical application of this form of treatment for human cancer has had a limited rate of success (1). The antitumor response of the host is complex, and both innate and adaptive immunity are involved.

Monocytes/macrophages play an important role, and as tumor-infiltrating macrophages (TIMs), they may both inhibit and enhance tumor growth: this is the so-called macrophage balance hypothesis (2). Although they produce powerful effector molecules such as proinflammatory cytokines (for example, TNF-alpha, IL-12, and IL-18), reactive oxygen and nitrogen intermediates, and are involved in cytotoxic destruction of tumor cells (3, 4, 5, 6), TIMs are also able to inhibit the antitumor potential of macrophages, for example, by means of the IL-10 and TGF-beta produced by the TIMs themselves or induced by them in macrophages (7). This collectively may be called tumor-induced macrophage dysfunction (8) or tumor-driven macrophage polarization (7).

We have designed an *in vitro* model of TIMs: tumor interactions in which human monocytes are cocultured with cancer cells and from which monocytes are then isolated and their antitumor potential studied by determining the production of cytokines, reactive oxygen intermediates, and reactive nitrogen intermediates (3, 4, 5, 6, 9). Our recent studies provide evidence that monocytes preexposed to tumor cells show decreased antitumor activity, as defined by depressed TNF-alpha and IL-12 production, cytotoxicity, and enhanced IL-10 secretion (4). Hence, we suggest that contact of monocytes with tumor cells induces the polarization of mononuclear phagocytes toward the M2 (TNF-alpha- IL-12- IL-10+) phenotype; that is, similar or identical to that observed *in vivo* (7). Furthermore, we show that M1 to M2 polarization of monocytes is linked to selective unresponsiveness (deactivation), as it is limited to tumor cells although the response to lipopolysaccharide is unaffected. It is also associated with IL-1 receptor-associated kinase downregulation (4).

As these findings have obvious implications for immunotherapy targeted at enhancing TIM activity, we asked whether tumor-induced monocyte deactivation can be prevented or reversed by various monocyte immunomodulating agents, for example, IFN-gamma, TNF-alpha, GM-CSF, and PPD, either alone or in combination. Therefore, the basic question was to establish whether M1 (potent effector tumor-killing cells) to M2 (cells producing immunosuppressive cytokines and promoting angiogenesis) polarization of monocytes by tumor cells could be either prevented or reversed.

Results

Effect of preactivation with different agents on the cytokine production by monocytes preexposed and restimulated with tumor cells

We reported previously that even brief contact with tumor cells leads to selective monocyte unresponsiveness to the same or different tumor cells, but not to lipopolysaccharide. We define unresponsiveness as decreased production of proinflammatory cytokines (TNF-alpha, IL-12) and enhanced release of IL-10 (4). In the present study, we used selected cytokines and PPD in order to determine their effect on monocyte deactivation. Monocytes were pretreated with these immunomodulatory agents before being cocultured with HPC-4 or DeTa tumor cells to establish whether tumor cell-induced deactivation of monocytes can be prevented (Figure 1). Following a short coculture with tumor cells or culture in the medium, monocytes (CD14+ cells) were isolated by FACS® sorting and restimulated with tumor cells for 18 h. Figure 2A shows that monocytes isolated from the coculture and restimulated with tumor cells produced approximately 40% of the amount of TNF-alpha produced by control monocytes. Pretreating monocytes with IFN-gamma or with IFN-gamma combined with TNF-alpha or GM-CSF before coculturing them with tumor cells enhanced TNF-alpha secretion 1.5- to 2.5-fold as compared to untreated monocytes. Neither TNF-alpha, GM-CSF, nor PPD used alone were able to increase TNF-alpha secretion. Adding TNF-alpha and GM-CSF to IFN-gamma had a synergistic effect as compared to each cytokine used alone. Paradoxically, PPD decreased the enhancing effect of IFN-gamma, although this combination also prevented the induction of monocyte deactivation.

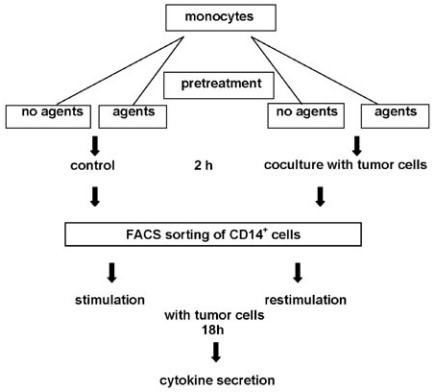

Figure 1. Flow chart of protocol 1, in which monocytes are pretreated with different agents before being exposed to tumor cells.

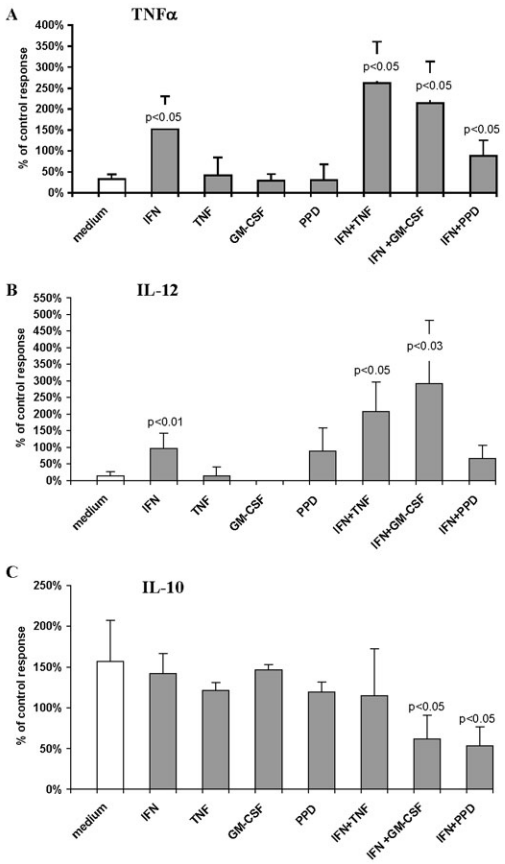

Figure 2. Secretion of (A) TNF-alpha, (B) IL-12, and (C) IL-10 by monocytes pretreated with different agents before coculture and restimulation with tumor cells (protocol 1). Cytokine release by tumor cell-stimulated monocytes (control response) was 4348 ± 860 pg/ml, 873 ± 346 pg/ml, and 2662 ± 963 pg/ml for TNF-alpha, IL-12 (p40), and IL-10, respectively. Cytokine release by monocytes preexposed and restimulated with tumor cells (medium) was 1507 ± 979 pg/ml, 110 ± 91 pg/ml, and 4166 ± 1104 pg/ml for TNF-alpha, IL-12 (p40), and IL-10, respectively. Mean cytokine release by control monocytes pretreated with different agents was 235 ± 372 pg/ml, 11 ± 39 pg/ml, and 9 ± 40 pg/ml for TNF-alpha, IL-12 (p40), and IL-10, respectively. The results are expressed as a percentage of the response obtained for control monocytes stimulated with tumor cells. The means from 8-10 independent experiments are shown, with error bars corresponding to 1 SD.

IL-12 release was significantly depressed in cocultured monocytes, but it was enhanced by IFN-gamma pretreatment and reached a level comparable to that of the control monocytes (Figure 2B). In this case, addition of TNF-alpha and GM-CSF further enhanced IL-12 release. It seems to be a synergistic effect, as TNF-alpha and GM-CSF alone had no influence. Although PPD alone also had no effect, it decreased the enhancing effect of IFN-gamma.

Cocultured monocytes (preexposed to tumor cells) showed an enhanced IL-10 release corresponding to approximately 150% of the control monocyte response (Figure 2C). Pretreatment of monocytes with different agents had no significant effect on IL-10 secretion, except that combining IFN-gamma with GM-CSF or PPD decreased its release. Tumor cells alone did not release any of the cytokines analyzed (data not shown).

Altogether, these results suggest that IFN-gamma alone or in combination with TNF-alpha, GM-CSF, or PPD can prevent tumor cell-induced monocyte deactivation, resulting in decreased proinflammatory cytokine production. However, none of these agents except for the combination of IFN-gamma and GM-CSF, as well as that of IFN-gamma and PPD, affected the enhanced IL-10 secretion.

Reversal of monocyte deactivation

In the second set of experiments, we asked whether tumor cell-induced monocyte deactivation could be reversed by treatment with immunomodulating agents. CD14+ monocytes isolated from the coculture with tumor cells (preexposure) or cultured in the medium (control) were treated with different cytokines or PPD. After washing, they were cultured with tumor cells for 18 h. The level of cytokines was then determined in the culture supernatants (Figure 3). Treatment with IFN-gamma or PPD alone, or with IFN-gamma in combination with TNF-alpha or PPD, significantly enhanced the depressed TNF-alpha secretion by cocultured monocytes until it reached or exceeded the level of secretion by control cells. TNF-alpha and GM-CSF alone, as well as the combination of IFN-gamma with GM-CSF, also increased TNF-alpha release, but to a lesser extent. PPD and TNF-alpha, but not GM-CSF, had an additive effect with IFN-gamma (Figure 4A). IFN-gamma, but not TNF-alpha or GM-CSF or PPD, significantly enhanced depressed IL-12 (p40) secretion by deactivated monocytes. However, TNF-alpha, GM-CSF, and PPD did not increase the effect of IFN-gamma (Figure 4B). IL-10 production by deactivated monocytes increased significantly (an approximately 2.3-fold increase as compared to control monocytes) and was neither inhibited by cytokines, by PPD given alone, nor by PPD given together with IFN-gamma (Figure 4C). These data suggest that IFN-gamma alone or in combination with other agents was able to reverse tumor cell-induced monocyte deactivation as measured by TNF-alpha secretion, whereas IL-12 release was affected by IFN-gamma only. None of the agents used affected the enhanced IL-10 secretion by deactivated monocytes.

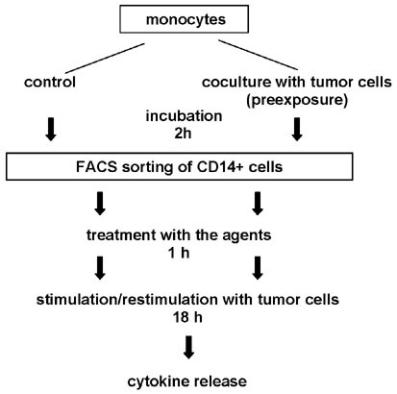

Figure 3. Flow chart of protocol 2, in which monocytes were treated with different agents following their exposure to tumor cells.

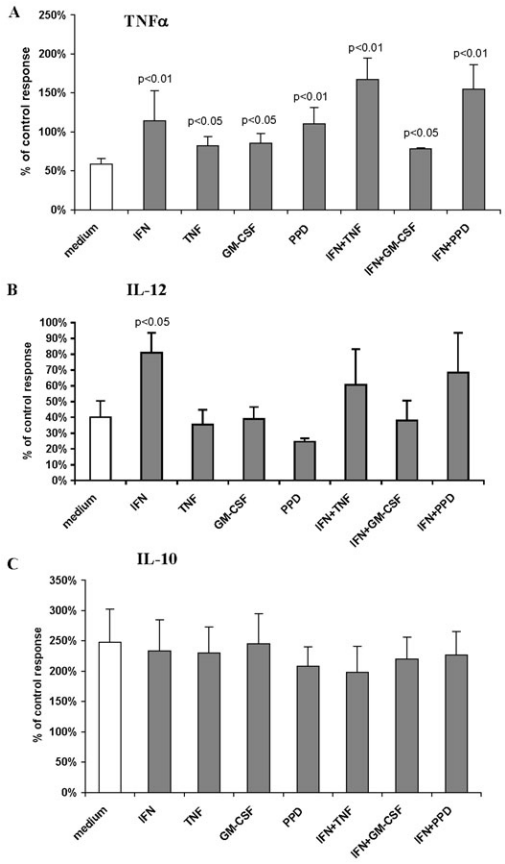

Figure 4. Secretion of (A) TNF-alpha, (B) IL-12, and (C) IL-10 by monocytes treated with different agents after coculture, before being restimulated with tumor cells (protocol 2). Cytokine release by tumor cell-stimulated control monocytes was 3225 ± 1200 pg/ml, 1442 ± 1140 pg/ml, and 1356 ± 908 pg/ml for TNF-alpha, IL-12 (p40), and IL-10, respectively. Cytokine release by monocytes that were cocultured and restimulated with tumor cells (medium) was 1943 ± 763 pg/ml, 595 ± 642 pg/ml, and 2875 ± 1621 pg/ml for TNF-alpha, IL-12 (p40), and IL-10, respectively. The results are expressed as the percentage of control monocytes stimulated with tumor cells. The means from 5-6 independent experiments are shown, with error bars corresponding to 1 SD.

Effect of treating monocytes with IFN-gamma before or after coculture with tumor cells on TNF-alpha mRNA expression

In order to establish whether the immunomodulating agents used affected *de novo* cytokine production, IFN-gamma (as the most effective cytokine) was used to determine whether it had an effect on TNF-alpha mRNA expression in monocytes treated either before or after coculture with tumor cells. The cytokine mRNA level was determined by real time RT-PCR (4). Figure 5 shows that the accumulation of TNF-alpha mRNA in monocytes isolated from the coculture was decreased approximately 3.6-fold compared to that in control monocytes. Pretreatment of monocytes before, or treatment after, coculture with IFN-gamma increased TNF-alpha mRNA 2.5- and 3.4-fold, respectively. These data suggest that IFN-gamma, and presumably the other immunomodulatory agents used, increases *de novo* production of TNF-alpha, not just its secretion.

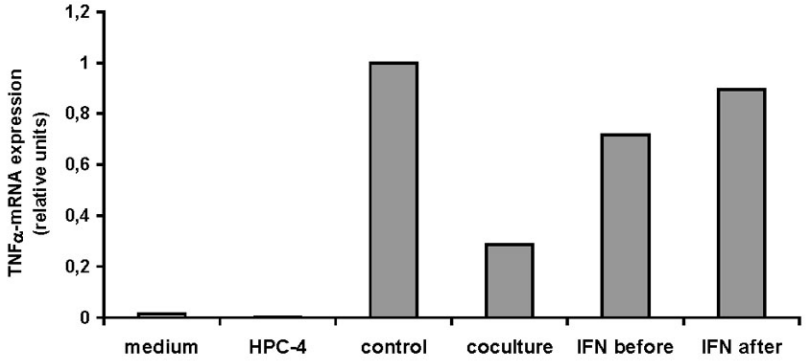

Figure 5. TNF-alpha mRNA expression in monocytes treated with IFN-gamma before or after coculture with tumor (HPC-4) cells. One representative experiment, out of the three performed, is shown.

Treatment with anti-IL-10 mAb reverses monocyte deactivation

Our previous data show that contact with tumor cells leads to the occurrence of the M2 (TNF-alpha- IL-12- IL-10+) phenotype of monocytes (4). IL-10 is a potent immunosuppressive cytokine that regulates the production of proinflammatory cytokines in an auto/paracrine fashion. Deactivated monocytes produce an increased amount of IL-10 following restimulation with tumor cells, so we have studied the effect of anti-IL-10 mAb on the production of TNF-alpha, IL-12, and IL-10 by cocultured monocytes (preexposed to tumor cells). Adding anti-IL-10 mAb to the culture of monocytes preexposed to and restimulated with tumor cells completely reversed the depressed monocyte activity, as it caused a significant increase in TNF-alpha and IL-12 production (Figure 6). Not surprisingly, no IL-10 was detected. Hence, the preexposure of monocytes to tumor cells primes the monocytes for enhanced IL-10 production during rechallenge. It in turn blocks TNF-alpha and IL-12 production. Therefore, the selective inhibition of IL-10 production reverses monocyte deactivation.

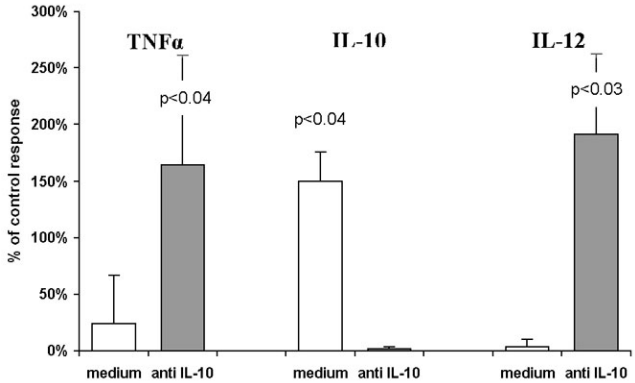

Figure 6. Effect of treatment with anti-IL-10 mAb on cytokine secretion by cocultured (deactivated) monocytes restimulated with tumor cells. Cytokine release by tumor cell-stimulated control monocytes was 3359 ± 4724 pg/ml, 1082 ± 449 pg/ml, and 547 ± 459 for TNF-alpha, IL-10, and IL-12, respectively. Cytokine release by monocytes that were cocultured and restimulated with tumor cells in the presence of isotype control for anti-IL-10 mAb (medium) was 1895 ± 3790 pg/ml, 1565 ± 396 pg/ml, and 39 ± 67 pg/ml for TNF-alpha, IL-10, and IL-12, respectively. The results are expressed as a percentage of the control monocyte response after stimulation with tumor cells. The means of three independent experiments are shown, with error bars corresponding to 1 SD.

Effect of treating monocytes preexposed to tumor cells with immunomodulatory agents on the growth of HPC-4 tumors in SCID mice

We also studied the effect of treatment of deactivated monocytes with IFN-gamma alone or in combination with GM-CSF or PPD on the growth of human HPC-4 tumors in SCID mice. Coengrafting the monocytes isolated from the coculture with tumor cells *in vitro* with HPC-4 cells enhanced tumor growth, as compared to control monocytes (Figure 7), indicating that deactivated monocytes facilitate tumor growth. Treatment of monocytes isolated from the coculture with IFN-gamma *in vitro* significantly suppressed this effect. A similar, though less pronounced, effect was seen when the combination of IFN-gamma with GM-CSF or PPD was used for monocyte activation. In keeping with the *in vitro* data, PPD slightly compromised the effect of IFN-gamma alone. These results indicate that deactivated monocytes show a growth-promoting effect *in vivo*, which can be partly reversed by treatment with IFN-gamma alone or in combination with GM-CSF or PPD.

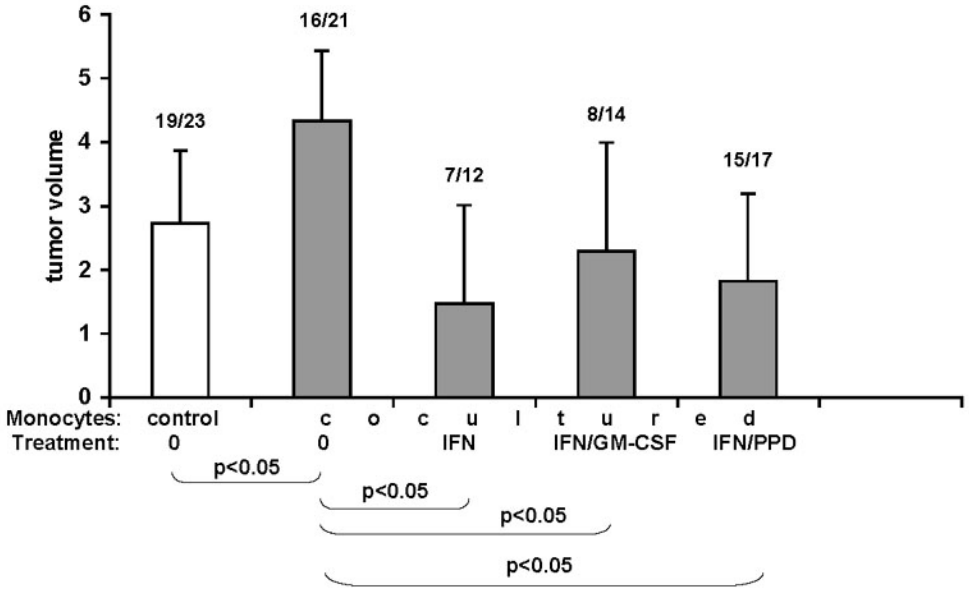

Figure 7. Growth of HPC-4 tumors in SCID mice coengrafted with deactivated monocytes from the coculture treated with the indicated immunomodulatory agents. Tumor volume and the number of tumor-bearing mice per group at 63 d after coengraftment are shown.

Discussion

Tumor cell-induced deactivation of monocytes is characterized by decreased production of TNF-alpha and IL-12 and by enhanced secretion of IL-10 upon *in vitro* challenge with tumor cells (4). In this study, several immunomodulatory cytokines and PPD were used to determine whether this tumor cell-induced unresponsiveness could either be prevented (by pretreatment of monocytes before contact with tumor cells) or reversed (by stimulation of deactivated monocytes). The data presented show that pretreating monocytes with IFN-gamma before they were in contact with tumor cells resulted in preventing the depressed TNF-alpha and IL-12 secretion, as well as the depressed TNF-alpha mRNA levels. Furthermore, TNF-alpha and GM-CSF acted synergistically with IFN-gamma, as the former cytokines alone had no effect. This appears to indicate that the

deactivating effect of tumor cells on monocytes can be prevented by these cytokines. This may be analogous to endotoxin tolerance, whereby IFN-gamma, and to a lesser extent GM-CSF, prevented the inhibition of TNF-alpha production by lipopolysaccharide-tolerized human monocytes by promoting IL-1 receptor-associated kinase expression (10). It was demonstrated earlier that in tumor cell-induced monocyte deactivation, IL-1 receptor-associated kinase expression is depressed (4). This was observed in lipopolysaccharide-tolerized monocytes (10). It is likely that a similar mechanism may be operative in the present system for the effects seen with IFN-gamma alone or in combination with GM-CSF or TNF-alpha.

Reversal of deactivation, as defined by the derepression of TNF-alpha release, was seen following treatment of monocytes from the coculture with IFN-gamma alone, or in combination with TNF-alpha, GM-CSF, or PPD. The depressed IL-12 release was only affected by IFN-gamma. However, posttreatment of monocytes was less effective than pretreatment. This is likely to be associated with an insufficient ability of these agents to significantly alter enhanced IL-10 production by deactivated monocytes (4). Whereas the combination of IFN-gamma and GM-CSF, as well as that of IFN-gamma and PPD, prevented an enhanced IL-10 release by tumor-preexposed monocytes, none of the agents used reversed it. It may be associated with different signaling pathways for proinflammatory cytokines, which are mostly IL-1 receptor-associated kinase/NF-kappaB-dependent, and for IL-10 production, which is regulated by STAT3 and is NF-kappaB-independent (4, 11). The enhanced IL-10 production seems to be an important mechanism in causing monocyte unresponsiveness, as shown by the effect of anti-IL-10 mAb, which caused a significant enhancement of TNF-alpha and IL-12 production by the cocultured monocytes. This is in keeping with other data showing that anti-IL-10 mAb reverses the inhibition of IL-12 production by TIMs isolated from ovarian cancer (12). However, none of the agents used reversed an enhanced IL-10 release by tumor-preexposed monocytes.

There was a notable difference between the prevention and the reversal of monocyte deactivation, as evidenced by the effect of PPD, which was inactive when used for pretreatment but almost as active as IFN-gamma when used for treatment of unresponsive monocytes. At the moment, it is unclear what the mechanism underlying this differential activity of PPD is, but it should be noted that this poorly characterized preparation contains many different immunoregulatory moieties (13).

The results presented suggest that two main mechanisms of monocyte deactivation by tumor cells may be operative: (1) an IL-10-dependent mechanism, as demonstrated by the effect of anti-IL-10 mAb, and (2) an IL-10-independent mechanism, as shown by a lack of consistent effect of IFN-gamma alone, or in combination with cytokines or PPD, on IL-10 production by deactivated monocytes. Several factors may be involved, such as the expression of surface molecules that are important for monocyte-tumor cell interactions. We previously reported the role of CD44, HLA-DR, and CD29 molecules in signaling for TNF-alpha, reactive oxygen intermediates, and reactive nitrogen intermediates generation (3, 9). Therefore, we have checked the expression of these molecules following treatment with IFN-gamma, both with and without TNF-alpha, GM-CSF, or PPD. The only noticeable difference was the upregulation of HLA-DR; the effect on CD44 and CD29 expression was variable (data not shown). Monocytes isolated from the coculture show a higher expression of HLA-DR (14). Further upregulation of this molecule was observed following IFN-gamma treatment. Hence, at least one of the mechanisms that may be linked to an IL-10-independent pathway of prevention/reversal of monocyte deactivation may be the upregulation of HLA-DR, which is known to be involved in signaling for TNF-alpha production by monocytes stimulated with tumor cells (3).

In vivo data showed that the growth of HPC-4 tumors in SCID mice was enhanced by deactivated monocytes as compared to control monocytes. This effect was partly reversed by IFN-gamma, either alone or in combination with GM-CSF or PPD. This is an important point, as it indicates that downregulation of TNF-alpha and IL-12 production *in vitro* by deactivated monocytes is associated with their growth-promoting effect *in vivo*. Our unpublished data indicate that the latter is associated with an increased angiogenesis. Furthermore, the significantly compromised function of monocytes preexposed to tumor cells can be reversed, at least partly, by IFN-gamma with or without GM-CSF or PPD. These data are in keeping with observations that IFN-gamma can

reverse mouse macrophage deactivation induced by implanted biomaterials (15) and monocyte deactivation in septic patients (16). This may have some practical implications for the role of immunomodulatory agents targeting TIMs in the prevention or reversal of their M2 polarization in the tumor bed (7).

Abbreviations

PPD, purified protein derivative; TIM, tumor-infiltrating macrophage

Acknowledgements

This study was supported by the National Committee for Scientific Research (Grants no. 6 PO5A 096 20 and 6 PO5A 095 21). We wish to thank Ms. Barbara Hajto for her skillful technical assistance.

References

1. Davis ID, Jefford M, Parente P, Cebon J. Rational approaches to human cancer immunotherapy. *J Leukoc Biol* 2003; **73**: 3-29. (PMID: 12525559)

2. Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumor-associated macrophages. *Immunol Today* 1992; **13**: 265-70. (PMID: 1388654)

3. Zembala M, Siedlar M, Ruggiero I, Wieckiewicz J, Mytar B, Mattei M, Colizzi V. The MHC class-II and CD44 molecules are involved in the induction of tumour necrosis factor (TNF) gene expression by human monocytes stimulated with tumor cells. *Int J Cancer* 1994; **56**: 269-74. (PMID: 7508890)

4. Mytar B, Woloszyn M, Szatanek R, Baj-Krzyworzeka M, Siedlar M, Ruggiero I, Wieckiewicz J, Zembala M. Tumor cell-induced deactivation of human monocytes. *J Leukoc Biol* 2003; **74**: 1094-101. (PMID: 12960282)

5. Zembala M, Siedlar M, Marcinkiewicz J, Pryjma J. Human monocytes are stimulated for nitric oxide release *in vitro* by some tumor cells but not by cytokines and lipopolysaccharide. *Eur J Immunol* 1994; **24**: 435-9. (PMID: 8299693)

6. Mytar B, Siedlar M, Woloszyn M, Ruggiero I, Pryjma J, Zembala M. Induction of reactive oxygen intermediates in human monocytes by tumor cells and their role in spontaneous monocyte cytotoxicity. *Br J Cancer* 1999; **79**: 737-43. (PMID: 10070862)

7. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes [review]. *Trends Immunol* 2002; **23**: 549-55. (PMID: 12401408)

8. Elgert KD, Alleva DG, Mullins DW. Tumor-induced immune dysfunction: the macrophage connection. *J Leukoc Biol* 1998; **64**: 275-90. (PMID: 9738653)

9. Mytar B, Siedlar M, Woloszyn M, Colizzi V, Zembala M. Cross-talk between human monocytes and cancer cells during reactive oxygen intermediates generation: the essential role of hyaluronan. *Int J Cancer* 2001; **94**: 727-32. (PMID: 11745469)

10. Adib-Conquy M, Cavaillon JM. Gamma interferon and granulocyte/monocyte colony-stimulating factor prevent endotoxin tolerance in human monocytes by promoting interleukin-1 receptor-associated kinase expression and its association to MyD88 and not by modulating TLR4 expression. *J Biol Chem* 2002; **277**: 27927-34. (PMID: 12032143)

11. Benkhart EM, Siedlar M, Wedel A, Werner T, Ziegler-Heitbrock HW. Role of Stat3 in lipopolysaccharide-induced IL-10 gene expression. *J Immunol* 2000; **165**: 1612-17. (PMID: 10903771)

12. Sica A, Saccani A, Botazzi B, Polentarutti N, Vecchi A, van Damme J, Mantovani A. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. *J Immunol* 2000; **164**: 762-7. (PMID: 10623821)

13. Rowland SS, Ruckert JL, Cummings PJ. Low molecular mass protein patterns in mycobacterial culture filtrates and purified protein derivatives. *FEMS Immunol Med Microbiol* 1998; **23**: 21-5. (PMID: 10030543)

14. Mytar B, Baran J, Gawlicka M, Ruggiero I, Zembala M. Immunophenotypic changes and induction of apoptosis of monocytes and tumour cells during their interactions *in vitro*. *Anticancer Res* 2002; **22**: 2789-96. (PMID: 12529998)

15. Boelens JJ, van der Poll T, Dankert J, Zaat SA. Interferon-gamma protects against biomaterial-associated Staphylococcus epidermidis infection in mice. *J Infect Dis* 2000; **181**: 1167-71. (PMID: 10720548)

16. Docke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, Volk HD, Kox W. Monocyte deactivation in septic patients: restoration by IFN-gamma treatment. *Nat Med* 1997; **3**: 678-81. (PMID: 9176497)

17. Iwanuma Y, Chen FA, Egilmez NK, Takita H, Bankert RB. Antitumor immune response of human peripheral blood lymphocytes coengrafted with tumor into severe combined immunodeficient mice. *Cancer Res* 1997; **57**: 2937-42. (PMID: 9230205)

Materials and methods

Isolation of cell populations

PBMCs were isolated from EDTA-blood of healthy donors by the standard Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were separated from mononuclear cells by counterflow centrifugal elutriation with a JE-6B elutriation system equipped with a 5 ml Sanderson separation chamber (Beckman-Coulter, Palo Alto, CA, USA), as previously described (5). The cells were suspended in RPMI 1640 medium (Biochrom, Berlin, Germany) with 25 µg/ml gentamycin (Biochrom, Berlin, Germany), 2 mM glutamine (Gibco, Paisley, UK) and 10% FBS (Biochrom, Berlin, Germany). Monocytes were 90-96% pure, as judged by flow cytometry analysis (FACS® Calibur, BD Biosciences Immunocytometry Systems, San Jose, CA, USA) using anti-CD14 mAb (BD Biosciences Pharmingen, San Diego, CA, USA).

Cell lines

The following human cell lines were used: HPC-4 (pancreatic adenocarcinoma) and DeTa (colorectal adenocarcinoma) (9). Cells were cultured by biweekly passages in RPMI 1640 with 5% FBS. Cell lines were tested regularly for mycoplasma contamination using a PCR-ELISA kit according to the manufacturer's procedure (Roche, Mannheim, Germany).

Immunomodulating agents

The recombinant cytokines IFN-gamma and GM-CSF were purchased from Sigma (St. Louis, MO, USA), TNF-alpha from R & D System (Minneapolis, MN, USA), and PPD from Statens Serum Institute (Copenhagen, Denmark).

Cell culture and monocyte treatment protocols

In these experiments, monocytes deactivated by a short exposure (2 h) to tumor cells (coculture) were used. For details, refer to Mytar *et al*. (4). Two protocols were used for monocyte treatment: monocytes were either preincubated with immunomodulating agents before coculture (protocol 1, prevention of deactivation) or were preexposed to tumor cells (HPC-4 or DeTa), isolated from the coculture and then incubated with the agents indicated (protocol 2, reversal of deactivation). In the first protocol (Figure 1), isolated monocytes (1×10^6/ml) were incubated for 1 h either in the medium (control) or in the presence of IFN-gamma (400 U/ml), TNF-alpha (10 pg/ml), GM-CSF (500 µg/ml), PPD (50 µg/ml), or in combinations of IFN-gamma with TNF-alpha, GM-CSF, and PPD in the doses indicated. Afterward, the cells were washed and their viability was checked by flow

cytometry after staining with PI (Sigma, St. Louis, MO, USA). Then control and pretreated monocytes were cultured with tumor cells in a 1:0.3 ratio for 2 h (preexposure) in 5 ml polypropylene Falcon 2063 tubes to avoid cell attachment (Falcon/BD Biosciences, San Diego, CA, USA). After staining with PE-labeled anti-CD14 monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA, USA), CD14+ cells were sorted out by FACS® Vantage SE cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) equipped with a Turbo Sort module using Cell Quest v.3.1 software. The ion laser Innova Enterprise II (Coherent, Santa Clara, CA, USA), operating at 488 nm, was used as a light source. After setting CD14+ (monocytes) and CD14- (tumor cells) gating, sorting was performed using a 70-µm nozzle tip and a drop drive frequency of 65 kHz, 1.5 drop envelopes, in the so-called normal-R sort mode. Sorted cells were collected into water-cooled (constant temperature circulator, Neslab Instruments, Inc., Portsmouth, NH, USA) polystyrene Falcon 2057 tubes (Falcon/BD Biosciences, San Diego, CA, USA) precoated with FBS to avoid plastic charging and cell attachment. The purity of sorted cells was checked by flow cytometry and exceeded 98%. As a control, CD14+ cells were sorted out from monocytes cultured in the medium alone (so-called dummy sorting). CD14+ monocytes isolated by sorting were stimulated/restimulated with tumor cells (cocultured at the same ratio). Supernatants were then collected and tested for cytokine content. In the second protocol (Figure 3), monocytes were cocultured with tumor cells (preexposure) for 2 h, immunostained with PE-labeled anti-CD14 mAb, and sorted. Isolated CD14+ monocytes which had been preexposed to tumor cells or control CD14+ monocytes were stimulated with different agents for 1 h at 37°C, washed, and then cultured for 18 h with tumor cells (stimulation/restimulation) before collecting the supernatants for cytokine determination.

Determination of cytokines

Appropriate ELISA kits (BD Biosciences Pharmingen, San Diego, CA, USA) were used to measure the concentrations of TNF-alpha, IL-10, and IL-12 (p40) in culture supernatants, according to the manufacturer's instructions. The detection level was 20 pg/ml for TNF-alpha and 10 pg/ml for both IL-10 and IL-12.

Determination of cytokine mRNA expression by Light Cycler PCR

The RNA was extracted from monocytes by the single-step isolation method using TRIZOL reagent (Gibco-BRL, Grand Island, NY, USA) according to the manufacturer's protocol. The first strand cDNA was obtained from the total RNA samples (2 µg) with Moloney murine leukemia virus reverse transcriptase (Sigma, St. Louis, MO, USA) and oligo-dT (Gibco, Paisley, UK) primer as specified by the manufacturer's protocol. The PCRs for TNF-alpha, IL-10, and IL-12 (p40) were performed using the Light Cycler system (Roche Diagnostics, Mannheim, Germany). The following primer pairs were used:

TNF-alpha: 5'-CAG-TCA-GAT-CAT-CTT-CTC-GA-3' (sense) and 5'-TCA-CAG-GGC-AAT-GAT-CCC-AAA-3' (antisense)
IL-10: 5'-GGA-CTT-TAA-GGG-TTA-CCT-GG-3' (sense) and 5'-GAA-CTC-CTG-ACC-TCA-AGT-GA-3' (antisense)
IL-12 (p40): 5'-AAC-TGG-ACC-TTG-CAC-CAG-AG-3' (sense) and 5'-AGA-CTC-TCC-TCA-GCA-GCT-GG-3' (antisense)

In brief, 3 µl of the cDNA were used for each quantitative Light Cycler PCR run, using the Light Cycler-DNA Master SYBR Green I kit from Roche (Mannheim, Germany). Amplifications were carried out in a total volume of 20 µl with a final $MgCl_2$ concentration of 3 mM, and with 0.5 µM of each primer. Each Light Cycler PCR run consisted of 45 cycles with an initial denaturation time of 5 min at 95°C. For TNF-alpha, the cycling profile was set at 95°C for 0 seconds, 62°C for 10 seconds, and 72°C for 19 seconds; for IL-10, 95°C for 0 seconds, 62°C for 10 seconds, and 72°C for 40 seconds; and for IL-12 (p40), 95°C for 0 seconds, 60°C for 25 seconds, and 72°C for 40 seconds. The fluorescent signals generated during the informative log-linear phase were used to calculate the relative amount of mRNA. Melting curve analysis was performed to verify the specificity of the amplified products. The mRNA expression is indicated as the fold difference compared to the unstimulated monocytes (4).

Treatment with anti-IL-10 monoclonal antibody

CD14+ cells sorted out from the coculture with tumor cells were restimulated with tumor cells for 18 h in the presence of anti-IL-10 mAb (clone MAB217, R & D Systems, Inc., Minneapolis, MN, USA). The irrelevant mouse IgG2b (R & D Systems) was used in parallel. Supernatants were then collected and tested for cytokine content.

Coengraftment of monocytes and tumor cells into SCID mice

Freshly harvested HPC-4 cancer cells (5×10^5) were mixed with monocytes in a 1:1 ratio in a total volume of 0.2 ml saline and injected subcutaneously into the dorsal middle line area of 6- to 8-week-old SCID mice. Mice of the same sex were used in any given experiment. We used control monocytes (cultured in the medium) and monocytes that were isolated from the coculture with tumor cells (preexposed) for 2 h and then were either left untreated or were treated with IFN-gamma alone or with IFN-gamma and GM-CSF or PPD for 1 h. Tumor growth was checked every 7 d for 63 d. The longest dimension, *a*, and the perpendicular width, *b*, were measured, and the tumor volume was calculated according to the formula: $v = (ab)^2/2$ (17). The number of mice per group that developed tumors was also recorded.

Statistical analysis

Statistical analysis was performed by paired Student's *t*-test using Excel software for all experiments except those involving SCID mice, for which the Mann-Whitney nonparametric test was applied. Differences were considered significant at $P < 0.05$.

Contact

Address correspondence to:

Marek Zembala
Department of Clinical Immunology
Polish-American Institute of Pediatrics
Jagiellonian University Medical College
Wielicka str. 265, 30-663
Cracow, Poland
Tel.: + 48 12 658 24 86
Fax: + 48 12 658 17 56
E-mail: mizembal@cyf-kr.edu.pl