Monocytes activated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) rapidly secrete a number of monokines with different functional properties. Interleukin-4 (IL-4), a T-cell derived cytokine, has been shown to reduce the production of monokines with cytostatic activity for tumor cells, chemotactic activity for monocytes, and factors that stimulate thymocyte proliferation. This latter activity is mediated by a number of monokines like IL-1, tumor necrosis factor α (TNFα), and IL-6. To elucidate which cytokines produced by monocytes are controlled by IL-4, we tested the effect of IL-4 on the secretion of IL-1α, IL-1β, TNFα, and IL-6 induced by LPS or IFNγ. IL-4 was found to inhibit the secretion of IL-1β and TNFα by activated monocytes almost 100%. The secretion of IL-6 was found to be reduced 70% to 85% in the presence of IL-4, whereas there was no effect on the secretion of IL-1α (IL-1α is mainly cell-associated). Time-course experiments demonstrate that IL-4 reduces the secretion of monokines for a prolonged period of time (>40 hours). The reduced secretion of IL-1β and TNFα was specifically induced by IL-4 because anti-IL-4 antiserum completely restored normal monokine production. These data suggest that IL-4 plays a role in the regulation of immune responses by reducing the production of functionally important monokines.

**HUMAN RECOMBINANT interleukin-4 (IL-4) has pleiotropic effects.** IL-4 has been shown to act as a growth factor for activated T cells, thymocytes, natural killer cells, and B cells. IL-4 can enhance specific cytotoxic T lymphocyte (CTL) activity but suppresses IL-2-induced lymphokine activated killer (LAK) cell activity. IL-4 induces the expression of class II major histocompatibility complex (MHC) antigens on normal and malignant B cells as well as on peripheral blood monocytes.

Recently we demonstrated that monocytes cultured with IL-4 show a reduced production of factors with cytostatic activity against melanoma cells and with chemotactic activity for monocytes. Initial experiments showed that IL-4 inhibited the production of IL-1-like activity as judged by the reduced ability of culture supernatants of these cells to costimulate (with Con A) the proliferation of murine thymocytes. However, this thymocyte assay does not specifically detect monocyte products like IL-1β because tumor necrosis factor α (TNFα)β and IL-6β can also stimulate thymocyte proliferation.

This prompted us to study whether IL-4 reduces the production of one or more of these monokines. We used IL-1α, IL-1β, TNFα, and IL-6 immunosassays to directly study the effect of IL-4 on synthesis of these monokines by monocytes. Because monocytes generally need to be stimulated to produce significant levels of monokines in vitro culture,14–19 we studied monokine secretion after activation with lipopolysaccharide (LPS) or interferon γ (IFNγ). The data described here show that IL-4 inhibits the secretion of IL-1β, TNFα, and IL-6 by monocytes on activation, suggesting that IL-4 is involved in the regulation of the production of monokines by human monocytes.

**MATERIALS AND METHODS**

**Isolation and culture of human monocytes.** Human peripheral blood monocytes were isolated from 500 mL of blood of normal human donors, as described previously. Mononuclear cells were isolated by density centrifugation in a blood component separator, followed by fractionation into lymphocytes and monocytes by centrifugal elutriation. The monocyte preparation was over 95% pure, as judged by nonspecific esterase staining and contained more than 98% viable cells. Monocytes were cultured in Yssel’s medium containing human serum albumin (HSA) and supplemented with 1% autologous or pooled heat-inactivated human serum. This culture medium was endotoxin free as determined by the Limulus amebocyte lysate assay (<0.2 ng/mL of endotoxin). The monocytes were incubated at a concentration of 4 x 10⁶ cells/mL in Teflon bags (Jansen MNL, St Niklaas, Belgium).

After culture for 2 to 40 hours, monocyte culture supernatants were collected for determination of IL-1α, IL-1β, TNFα, and IL-6. The viability of the cells determined by trypan blue exclusion always exceeded 90%.

**Recombinant IL-4, LPS, IFNγ, and anti-IL-4 serum.** Recombinant human IL-4 was obtained as a purified protein from supernatants of L cells transfected with the cDNA clone encoding IL-4 (specific activity 10⁷ U/mL), kindly provided by Dr S. Nagabhushan (Schering-Plough Corp, Bloomfield, NY). One unit of IL-4 was defined as the concentration of IL-4 resulting in half-maximal proliferation of phytohemagglutinin-activated T lymphoblasts. LPS (Escherichia coli 02-72:8) was obtained from Difco Laboratories (Detroit, MI). Human recombinant IFNγ (specific activity 10⁷ U/mg) was kindly provided by Drs P. Trotta and S. Nagabhushan (Schering-Plough Corp). The polyclonal rabbit anti-IL-4 antisera that blocks the biologic activity of IL-4 has been described previously. This antibody is specific for IL-4 and blocks IL-4–induced proliferation of T cells, B cells, and IL-4–induced immunoglobulin E synthesis.

**Detection of IL-1α, IL-1β, TNFα, and IL-6 in monocyte culture supernatant.** IL-1α in the culture supernatant of human monocytes was tested using the Endogen IL-1α enzyme-linked immunosorbent assay (ELISA) (Endogen Inc, Boston, MA). The sensitivity of this assay is 50 pg/mL of IL-1α and there is no cross-reactivity with IL-1β, IL-2, IFN, IL-6, TNFα, lymphotoxin, or other cytokines.

IL-1β in the culture supernatant of human monocytes was tested using the Cistron IL-1β ELISA (Cistron Biotechnology, Pine Brook, New Jersey).
The sensitivity of this assay is 20 pg of IL-1α and there is no cross-reactivity with IL-1β, TNFα or TNFβ.

TNFα in the culture supernatant of human monocytes was tested using the Biokine TNF Test Kit (T-Cell Sciences, Cambridge, MA). The sensitivity of this assay is 10 pg TNFα/mL and it shows no detectable cross-reactivity with any other cytokine.

IL-6 in the culture supernatant of human monocytes was tested using the Research & Diagnostics Systems Quantikine human IL-6 immunoassay (Minneapolis, MN). The sensitivity of this assay is 3.5 pg/mL and it shows no detectable cross-reactivity with other cytokines.

RESULTS

IL-4 inhibits production of IL-1β, TNFα, and IL-6 by human monocytes. IL-1α, IL-1β, TNFα, and IL-6 activity in the supernatant of IL-4–treated monocytes was tested using specific assays. Monocytes cultured for 4 hours in LPS secrete significant levels of IL-1β, TNFα, and IL-6 (Fig 1). IL-1α is not secreted but this correlates with the observation that IL-1α is predominantly membrane-associated, whereas IL-1β is mainly detected in the culture supernatant after cell activation. IL-4 downregulates the secretion of IL-1β, TNFα, and IL-6 by monocytes activated with LPS in a dose-dependent fashion (Fig 1). Addition of 10 U/mL of IL-4 already causes a 90% to 100% reduction of IL-1β and TNFα production by LPS-activated monocytes cultured for 4 hours (Fig 1). IL-4 also reduces the secretion of IL-6, but in contrast to blocking of the IL-1β and TNFα production no complete inhibition is found (70% to 85%). These data demonstrate that IL-4 reduces the secretion of IL-1β, TNFα, and IL-6 by LPS-activated monocytes in a dose-dependent manner.

Although the monocyte preparations we used in this study were over 95% pure we cannot exclude that IL-4 exerts its effect indirectly by acting through contaminating cells. Moreover, it has been described that IL-4 has lymphocyte-activating activity. Therefore, we mixed pure monocytes with increasing concentrations of fractionated lymphocytes and studied the effect of IL-4 on the production of TNFα. The effect of IL-4 on the TNFα production is most prominent if no lymphocytes are present (Fig 2) and the effect is measurable up to 50% contaminating lymphocytes. We have to take into account that in the presence of 50% lymphocytes the normal TNFα production of the monocytes is reduced with a factor 2 compared with 100% monocytes. Taken together these data demonstrate that IL-4 directly regulates this monocyte response and that lymphocytes have no effect.

The profound inhibition of IL-1β and TNFα production is specific for IL-4 because anti–IL-4 antiserum blocks the IL-4 effect. The inhibition of IL-1β and TNFα production by IL-4 is completely abrogated in the presence of an anti–IL-4 antiserum at dilutions of 1:500 and 1:5,000. Blocking of 20% to 30% was achieved at a 1:50,000 dilution (Fig 3A), whereas normal control rabbit antiserum was ineffective (Fig 3B). The anti–IL-4 antiserum used in this study also blocked IL-4–induced upregulation of MHC class II antigen expression and induction of CD23 expression on human monocytes.

Next we performed time-course experiments to characterize the kinetics of the effect of IL-4 on monokine secretion. The results in Fig 4 show that at all time points tested the secretion of IL-1β, TNFα, and IL-6 is reduced in the presence of IL-4. Up to 40 hours of culture reduced levels of IL-1β, TNFα, and IL-6 are found in the presence of IL-4 compared with control cultures in LPS only. From these data we can conclude that IL-4 inhibits the secretion of monokines for a prolonged period of time (up to 40 hours) and that the effect cannot be ascribed to a delayed secretion of monokines by IL-4.

In addition, we tested the effect of IL-4 on the secretion of
monokines induced by stimuli other than LPS, because it is described that not only LPS but also IFN$\gamma$ can activate monocytes to produce monokines. Production of enhanced levels of IL-1$\beta$ and TNF$\alpha$ by monocytes was found in the presence of IFN$\gamma$,$^{16,19}$ while IFN$\gamma$ has no effect on the production of IL-6.$^{17}$ We detected secretion of IL-1$\beta$ and of TNF$\alpha$ induced by IFN$\gamma$ (100 U/mL) in monocytes of three of six donors cultured for 20 hours. In all cases this secretion was reduced in the presence of IL-4 in a dose-dependent fashion (Fig 5). This result shows that IL-4 causes reduced IL-1$\beta$ and TNF$\alpha$ secretion in monocytes irrespective of whether the cells were activated with LPS or IFN$\gamma$.

**DISCUSSION**

We previously demonstrated that monocytes cultured in the presence of IL-4 show a reduced secretion of factors with chemotactic activity, cytotactistic activity, and factors that stimulate thymocyte proliferation.$^7$ In this study we show that IL-4 reduces the secretion of IL-1$\beta$ and TNF$\alpha$ by monocytes for almost 100%, whereas the secretion of IL-6 was reduced for 70% to 85% in the presence of comparable levels of IL-4 (Fig 1). These results are in accordance with data of Essner et al,$^{19}$ who demonstrate similar effects of IL-4 on the production of IL-1 and TNF$\alpha$ using bioassays to detect IL-1 (thymocyte assay) and TNF$\alpha$ (L929 cytotoxic assay). Because these assays possibly detect different factors, as already demonstrated for the thymocyte assay,$^6,13$ we used non-cross-reactive specific ELISAs, which confirm and extend their data. Although we did not find any effect on the secretion of IL-1$\alpha$, Weiss et al$^{19}$ demonstrated that monocyte cell-associated IL-1$\alpha$ and IL-1$\beta$ are reduced in monocytes cultured in the presence of IL-4. Yet their monocyte preparation was not pure and the cells were activated due to the isolation procedure.$^{11}$ In this study we show that a contamination of up to 50% lymphocytes does not influence the effect of IL-4 on monokine production.

Is the reduced secretion of IL-1$\beta$, TNF$\alpha$, and of IL-6 responsible for the observed reduction in cytotactic and chemotactic activity? The experiments performed in this study demonstrate that IL-1$\beta$ and TNF$\alpha$ secretion can be blocked for nearly 100% in the presence of IL-4. However, IL-4 does not completely block the production of cytotactic factors by monocytes as described previously.$^7$ These experiments showed that addition of 25% monocyte supernatant completely blocked the growth of A375 melanoma cells, and IL-4 displayed only a modest reducing effect on the produc-

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**Fig 2.** Effect of IL-4 on TNF$\alpha$ secretion by LPS (1 $\mu$g/mL) stimulated human monocytes in the presence of increasing concentrations of contaminating lymphocytes. TNF$\alpha$ was detected after 4 hours of cocultivation using a specific non-cross-reactive ELISA. Data of one representative experiment are shown.

**Fig 3.** (A) Addition of increasing concentrations of anti-IL-4 antiserum to human monocytes cultured in the presence of 100 U/mL of IL-4 completely restores IL-1$\beta$ and TNF$\alpha$ levels secreted in the culture medium after 4 hours of culture with 1 $\mu$g/mL of LPS. (B) Normal rabbit control serum was ineffective. Monokine levels were determined using specific non-cross-reactive ELISAs. Data of one representative experiment are shown.
IL-4 inhibits IL-1β, TNFα, and IL-6 secretion

When we used neutralizing antibodies against IL-1β, TNFα, and IL-6 to neutralize chemotactic activity of the monocyte culture supernatants, we did not observe any significant neutralizing activity (not shown). From this experiment we can conclude that although IL-1 and TNFα have chemotactic activity for monocytes,34,35 the chemotactic factor(s) secreted in reduced amounts probably are not IL-1β, TNFα, or IL-6. A more likely candidate for this activity is the recently cloned monocyte chemotactic and activating factor (MCAF), secreted by monocytes.36,37

How IL-4 influences the production of IL-1β, TNFα, and IL-6 is not yet clear. Does IL-4 act directly on the production of these monokines or through intermediate factors? Because IL-1 can induce the production of IL-6 in fibroblasts and thymocytes,13,38 and monocytes,39 it is possible that the partially reduced IL-6 production is a consequence of reduced IL-1 production. In addition, IL-1 and TNF can induce each other's release by monocytes.40,41 Although our experiments indicate that IL-1β and TNFα secretion are regulated similarly by IL-4, the regulation of TNF and IL-1 production by monocytes is not identical and depends reportedly on their differentiation state, exposure to lymphokines, microbial products, or other inflammatory inhibitors.42 Preliminary studies indicate that IL-1α, IL-1β, TNFα, and IL-6 steady-state messenger RNA (mRNA) levels are reduced in monocytes cultured in the presence of IL-4 (not shown), but more detailed studies are required to assess the relative contributions of changing rates of transcription and/or mRNA stability in determining the influence of IL-4 on monocyte steady-state mRNA levels.

IL-1 has been described as a costimulatory cytokine secreted by Ag presenting monocytes involved in T-lymphocyte activation.43,44 Recently, TNFα and IL-6 were also treated monocytes is mediated by IL-6 or can be ascribed to other yet unknown monocyte factors with cytostatic activity against melanoma cells.

Fig 4. Time course of the production of IL-1β (A), TNFα (B), and IL-6 (C) by LPS (1 μg/mL) stimulated monocytes with or without 100 U/mL of IL-4. Monokine levels were determined using specific non-cross-reactive ELISAs. Data of one representative experiment are shown.

Fig 5. IL-4 reduces IFNγ induced secretion of IL-1β and TNFα by human monocytes. Monocytes were cultured for 20 hours in 100 U/mL of IFNγ and increasing concentrations of IL-4. Monokine levels in the culture supernatant were determined using specific non-cross-reactive ELISAs. Data of one representative experiment are shown.
found to mediate enhancement of T-cell responses.\textsuperscript{45-47} In addition, the quantitative variation in expression of class II MHC Ag plays a major role in T-cell activation by Ag-presenting cells (APC).\textsuperscript{48} A number of studies (reviewed in reference 43) show that many of the modulators of IL-1 production also affect the expression of MHC class II Ag in a coordinated fashion. This is of interest because we showed that IL-4 is able to upregulate the MHC class II Ag expression of human monocytes in a dose-dependent manner.\textsuperscript{7} Thus, IL-4 downregulates IL-1\(\beta\) (and TNF\(\alpha\) and IL-6) production by human monocytes, but at the same time it enhances the expression of class II MHC antigens on these cells. Preliminary experiments investigating the APC function of human monocytes cultured in the presence of IL-4 indicate that the APC function of human monocytes can be enhanced by IL-4 despite a reduced IL-1\(\beta\) secretion (Te Velde AA: manuscript in preparation). Similar results were reported by Zlotnik et al.,\textsuperscript{49} who showed that IL-4 enhanced the antigen-presenting capacity of murine bone marrow-derived macrophages.

A number of groups have initiated studies to investigate the possibility of combining IL-2 and IL-4 in immunotherapy.\textsuperscript{\textsuperscript{45,50}} Major drawbacks of high-dose IL-2 therapy are toxic side effects, which in part may be caused by the production of IL-1 and TNF.\textsuperscript{18,51} Preliminary in vitro data indicate that pre-exposure or simultaneous exposure to IL-4 reduces production of monokines by human monocytes activated by IL-2. Based on these in vitro results, it is tempting to speculate that IL-4 in combination with IL-2 may be used to generate cell populations from mononuclear cell suspensions with specific CTL activity\textsuperscript{5} and, potentially, with less toxic side effects.

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