Toll-like receptors: tools, assays, and implications for in vitro pyrogen tests
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T cell activation in whole blood induces IL-8, but no IL-6, and is dependent on IL-2, GM-CSF, and TNF production

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submitted
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Abstract

Stimulation of T cells in whole blood (WB) by anti-CD3 monoclonal antibodies (mAbs) results in T cell proliferation and concomitant production of cytokines such as IL-2, GM-CSF, TNF, IL-13, and IFNγ. Here we demonstrate that T cell activation in WB led to IL-8 production, but not IL-6 production. Neutralization of GM-CSF and TNF completely abrogated IL-8 production in anti-CD3-stimulated WB. Conversely, GM-CSF and TNF synergistically induced IL-8 production not only in WB, but also in mononuclear cells and isolated neutrophils. Again, no IL-6 was produced under these circumstances. Because IL-8 production in WB induced by GM-CSF and TNF was markedly lower than IL-8 production after T cell stimulation, we attempted to identify a putative additional factor involved, and found that IL-2 markedly enhanced GM-CSF/TNF-induced IL-8 production. At high concentrations IL-2 alone induced WB IL-8 production, which was abrogated by neutralization of GM-CSF and TNF. At lower IL-2 concentrations GM-CSF and TNF strongly synergized in IL-8 production. Our study demonstrates that IL-2, in a GM-CSF- and TNF-dependent fashion, induces WB IL-8 production. This finding may be of clinical importance as it may explain the activation of neutrophils which is frequently observed in patients undergoing IL-2 immunotherapy in advanced malignancy.

Introduction

In the late 1980s, the measurement of interleukin-6 (IL-6) production by mononuclear cells (MNC) was introduced as an alternative for the detection of pyrogens (fever causing agents) in pharmaceuticals with the rabbit pyrogen test or the Limulus amebocyte lysate (LAL) test.¹ To study the effects of coagulation on pro-inflammatory cytokine production, in addition to the effects of pyrogens such as endotoxins, our group developed a sensitive
assay based on cytokine production in diluted WB.\textsuperscript{2,3} The WB assay was first described by Kirchner et al. for the measurement of IFN production\textsuperscript{4} and later re-established.\textsuperscript{5} At present, the monocyte activation test (MAT), using mononuclear cells as well as diluted WB, has been internationally validated and generally established for the measurement of pyrogens.\textsuperscript{6} In contrast with the activation of innate immunity, studies on the activation of adaptive immunity have been limited mostly to MNC. For example, studies with monoclonal antibodies (mAbs) against CD3, which can initiate T cell activation in the absence of antigen and which have been commonly used to study polyclonal activation of T cells, have been predominantly conducted in MNC.

Previously, we observed that stimulation of WB with a combination of anti-CD3 and anti-CD28 mAbs leads, as expected, to production of T cell cytokines such as IL-2, TNF, GM-CSF, IL-13, and IFN\textsubscript{\gamma}. In those conditions hardly any IL-6 production was observed, but extremely high levels of IL-8 were found.\textsuperscript{7} This was particularly surprising because a) both IL-6 and IL-8 are predominantly produced by monocytes, but not by T cells, and b) the regulation of IL-6 and IL-8 production is very similar.\textsuperscript{8-10} Moreover, the previous and scarce studies by a different group on CD3/CD28-induced cytokine production also using WB did not investigate IL-6 and IL-8 production.\textsuperscript{11-13} Therefore, the aim of the present study was to investigate T cell proliferation and cytokine production in WB, after T cell activation by anti-CD3 mAbs, and to elucidate how T cell stimulation led to production of IL-8, but not IL-6. We found that WB IL-8 production, but not IL-6 production, paralleled T cell proliferation, and was dependent on IL-2, GM-CSF, and TNF production.

Materials & Methods

Cells & cell cultures

Blood was obtained from healthy volunteers after informed consent in line with the Sanquin Ethical Advisory Council. Blood samples were collected using endotoxin-free evacuated blood collection tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) containing sodium heparin.

For WB cultures, venous blood was diluted 1/10 with IMDM (Bio Whittaker, Verviers, Belgium) supplemented with 0.1\% FCS (Bodinco, Alkmaar, The Netherlands), 15 U/ml heparin (Leo Pharmaceutical products, Weesp, The Netherlands), penicillin 100 U/ml/streptomycin 100 \(\mu\)g/ml (Gibco, Merelbeke, Belgium), and 50 \(\mu\)M 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany). The supplementation of medium with 0.1\% FCS
was performed to ensure that all stimuli were homogeneously distributed and no stimuli (e.g. LPS) were lost due to adsorption to consumables.

Mononuclear cells (MNC) were isolated from freshly drawn blood by separation over a Percoll gradient (d = 1.078, Pharmacia Fine Chemicals, Uppsala, Sweden) and cultured in IMDM supplemented with 5% heat-inactivated FCS, penicillin 100 U/ml / streptomycin 100 μg/ml, 50 μM 2-mercaptoethanol, and 20 μg/ml human transferrin (Sigma-Aldrich) at a concentration of 40,000 cells/well.

Neutrophils were isolated from the pellet fraction of the Percoll gradient by lysis of the erythrocytes with ammonium chloride solution (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.2). The remaining neutrophils were washed 3 times in PBS containing 10% trisodiumcitrate (Sanquin, Amsterdam, The Netherlands) and 10% pasteurized plasma proteins (Sanquin Plasma Products) and cultured in IMDM supplemented with 5% heat-inactivated FCS, penicillin 100 U/ml / streptomycin 100 μg/ml, 50 μM 2-mercaptoethanol, and 20 μg/ml human transferrin at a concentration of 50,000 cells/well.

Donors expressing HR-FcγRIIa or LR- FcγRIIa were selected using MNC based on the functional FcγRIIa polymorphism, as described previously.16-18

All cells were cultured in 200 μl wells in flat-bottom microtitre plates (Nunc, Roskilde, Denmark) at 37°C in the presence of 5% CO2, in a humidified incubator.

Stimuli and monoclonal antibodies

Anti-CD3 murine IgE (CLB.T3/4E), anti-CD3 mIg1 (CLB.T3/4.1) and anti-CD28 (CLB.CD28/1) were from Sanquin, Amsterdam, The Netherlands. For T cell activation, the anti-CD3 murine IgE isotype was used, unless otherwise stated. AT-10, a FcRγIIa-blocking mAb produced by Dr. M Glennie, Tenovus Research Laboratory, Southampton, United Kingdom was a kind gift from Dr. J.G. van de Winkel, Immunotherapy Laboratory, Department of Immunology, University Medical Centre Utrecht, Utrecht, The Netherlands. Anti-GM-CSF was a kind gift from Dr. G. Trinchieri, then at the Wistar Institute, Philadelphia, PA, USA. Anti-TNF 5 and anti-TNF 7 were from Sanquin, and a stimulatory mAb to CD40 (clone MAB89) was from Abcam, Cambridge, UK. Etanercept (Enbrel®) was from Wyeth Pharmaceuticals, Hoofddorp, The Netherlands, Infliximab (Remicade®) from Centocor, Leiden, The Netherlands, and Adalimumab (Humira®) from Abbott, Hoofddorp, The Netherlands.

Human recombinant cytokines were used at a concentration of 5 ng/ml, unless otherwise indicated. Cytokines were purchased at the following companies: IL-1β: CellGenix, Freiburg, Germany; IL-3: Strathmann Biotec, Hamburg, Germany; IL-6: Sanquin Reagents; IL-7 and IL-15: R & D Systems, Abingdon, Ox, UK; IL-10 and IFNγ:
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Peprotech, London, UK; IL-12p70: Hoffman la Roche, Basel, Switzerland; GM-CSF: Sandoz, Basel, Switzerland. IL-4 was a kind gift from M. Schreier, Sandoz, Basel, Switzerland and IL-17 was kindly donated by Dr. R. Lutter, Amsterdam Medical Centre, The Netherlands. IL-2 and TNF were a kind gift from Dr. A Creasy, Cetus Corporation, Emeryville, CA, USA.

Lipooligosaccharide (LOS), derived from Neisseria meningitidis was a generous gift from Dr. J. Poolman, RIVM, Bilthoven, The Netherlands. E. coli LPS serotype O55:B5 and polymyxin-B were from Sigma-Aldrich.

Proliferation assays

Proliferation of WB or MNC was measured by thymidine incorporation. Briefly, 1/10 diluted WB or 40,000 MNC were seeded in 96-well flat bottom microtitre plates and incubated with the appropriate stimuli for the indicated time. \[^{3}H\]-thymidine (Amersham, Bucks, UK) was added at a final concentration of 1 µCi/ml (37 kBq/ml) during the last four hours of the incubation, after which the cells were harvested onto glass fibre filters (Wallac, Turku, Finland) and the radioactivity was measured by liquid scintillation counting.

Cytokine measurements

Following stimulation of WB, MNC, or neutrophil cultures, supernatants were harvested and stored at -20ºC until time of assay. IL-6, IL-8, and TNF were determined by ELISA kits (Peli-Kine-compact, Sanquin Reagents, Amsterdam, the Netherlands), according to the manufacturer’s instructions. The plates were read in an ELISA-reader (Labsystems Multiskan Multisoft, Helsinki, Finland) at 450 nm, with 540 nm as a reference.

Results

IL-8, but not IL-6 is produced in T cell-stimulated WB.

When WB cultures were stimulated with anti-CD3 alone (fig. 1A), or in combination with anti-CD28 (fig 1B), T cell proliferation and moderate TNF production were measured, similar as in T cell-stimulated MNC. Surprisingly, IL-8 was abundantly produced under these circumstances. Because regulation of IL-8 production is very similar to IL-6 production\(^8,9\) we were even more surprised by the finding that virtually no IL-6 was produced after T cell stimulation with anti-CD3 (fig. 1A). Even when WB was stimulated with both anti-CD3 and anti-CD28, IL-8 production was > 100-fold larger compared to IL-6 production, and IL-6 was only produced in minute quantities (fig. 1B). In contrast,
both IL-8 and IL-6 were produced in large quantities when LPS was used for stimulation (fig. 1C). Cytokine production in unstimulated WB was below 100 pg/ml for TNF, below 200 pg/ml for IL-8, and below 50 pg/ml for IL-6 (data not shown).

Correlation of T cell proliferation with TNF and IL-8 production in T cell-stimulated whole blood: the role of FcγRIIa crosslinking.

We next investigated the lack of IL-6 production in T cell-stimulated WB. Several reports demonstrated that Fcγ receptor crosslinking on monocytes and/or neutrophils by anti-CD3 mAbs induced cytokine production, including IL-6. Therefore, we stimulated WB with a murine IgG1 anti-CD3 mAb that selectively crosslinks the High Responder-form of the polymorphic FcγRIIa (HR-FcγRIIa). Experiments using MNC previously demonstrated that only T cells of donors expressing HR-FcγRIIa, and not the Low Responder-form of FcγRIIa (LR-FcγRIIa), proliferate in response to mIgG1 anti-CD3. As expected, in WB only T cells of donors expressing HR-FcγRIIa, and not LR-FcγRIIa, proliferated in response to mIgG1 anti-CD3 (fig. 2A). In addition, TNF (fig. 2B) and IL-8 (fig. 2C) were only produced in WB of donors expressing HR-FcγRIIa, but not LR-FcγRIIa, after mIgG1 anti-CD3 stimulation. Again, in these conditions where IL-8 was produced, no IL-6 was produced (data not shown). Stimulation of WB with mIgE anti-CD3, which activates T cells independent of accessory cells or FcγR allotype, led to equal T cell proliferation, TNF production, and IL-8 production in HR- and LR-FcγRIIa donors (data not shown).

Further support for the role of FcγRIIa crosslinking in the activation of cells is that both T cell proliferation (data not shown) and IL-8 production (fig. 2D) were almost completely abrogated by an FcγRIIa-blocking mAb after mIgG1 anti-CD3 stimulation. In contrast, when mIgE anti-CD3 was used for T cell stimulation, T cell proliferation (data not shown) and IL-8 production (fig. 2D) were unaffected by the FcγRIIa-blocking mAb. Thus, although FcγRIIa crosslinking led to T cell proliferation, TNF production, and IL-8 production, no IL-6 was produced under these circumstances.

Neutralization of GM-CSF and TNF in T cell-stimulated WB inhibits IL-8 production.

Next, we investigated which cells in WB produced IL-8 after T cell activation. Cells present in WB that are known for their capacity to synthesize IL-8 are neutrophils and monocytes. Since neutrophils are not capable of synthesizing IL-6, and isolated T cells stimulated with anti-CD3/CD28 do not produce IL-8 (data not shown), we hypothesized that following T cell stimulation with anti-CD3 mAbs only neutrophils would become activated, and not monocytes, which might explain why only IL-8 was produced. An
Figure 1: Correlation of T cell proliferation with production of TNF and IL-8 after T cell stimulation in whole blood.
Heparinized WB from 10 donors was diluted 1/10 and incubated with (A) anti-CD3 mAbs (1 µg/ml), (B) anti-CD3 and anti-CD28 mAbs (1 µg/ml and 5 µg/ml respectively) or (C) E. coli LPS (1 ng/ml). At day 4 (A and B), supernatants were harvested for determination of cytokine production, and proliferation of T cells was assessed in a 4 h [³H]-thymidine incorporation assay. Supernatant of WB cultures incubated with LPS (C) were harvested after 1 day stimulation. Data represent mean ± SEM of 10 donors. Cytokine production in unstimulated WB was below 100 pg/ml for TNF, below 200 pg/ml for IL-8, and below 50 pg/ml for IL-6 (data not shown).
alternative hypothesis is that T cell stimulation might lead to stimulation of monocytes to synthesize exclusively IL-8, and not IL-6. Incubation of isolated neutrophils with supernatant of anti-CD3/CD28-activated T cells induced IL-8 production, whereas incubation of neutrophils with aCD3/aCD28 mAbs or with supernatant of unstimulated T cells did not. Thus, activated T cells release soluble mediators that can induce IL-8 production, at least in neutrophils (data not shown).

Two cytokines produced by activated T cells that have been reported to increase neutrophil effector functions are GM-CSF and TNF. Therefore, we tested whether GM-CSF and TNF were involved in IL-8 production. Neutralization of GM-CSF or TNF markedly inhibited IL-8 release in T cell-stimulated WB with approximately 75% (fig. 3). The combined use of antibodies against GM-CSF and TNF even further inhibited IL-8 production with 95% (fig. 3). Also, anti-TNF therapeutics such as the anti-TNF mAbs Infliximab and Adalimumab, and the recombinant human soluble TNF-Receptor Etanercept inhibited anti-CD3/CD28 stimulated IL-8 production by approximately 75% (data not shown).

GM-CSF and TNF induce IL-8, but not IL-6 production in WB, MNC, and isolated neutrophils.

As we demonstrated that IL-8 production in T cell-stimulated WB is dependent on GM-CSF and TNF production, we investigated whether both cytokines, alone or in combination, were capable of inducing IL-8 production in WB, mononuclear cells, and isolated neutrophils (fig. 4). In WB, both GM-CSF and TNF stimulated IL-8 production. However, the combined use of GM-CSF and TNF synergistically potentiated IL-8 production 8-10 fold compared to the separate stimuli (fig. 4A). Also in MNC, GM-CSF and TNF cooperatively stimulated IL-8 production. To compare the sensitivity of WB and MNC for GM-CSF and TNF, both cytokines were titrated and the IL-8 production measured. At all concentrations between 0 and 5 ng/ml tested, GM-CSF and TNF dose-dependently induced WB IL-8 production (data not shown). A major difference between WB and MNC cultures is that MNC become activated during isolation, even when the materials or media used for isolation are free from endotoxins and other stimulatory contaminants. Also, we frequently observed that isolated MNC exhibited increased background IL-8 production. In our hands, background IL-8 production ranges from 1000-10,000 pg/ml in isolated MNC, whereas background IL-8 production in WB is always below 200 pg/ml (data not shown). Because of the background IL-8 production in MNC, we were unable to demonstrate reproducible dose-dependent effects of GM-CSF and TNF at low concentrations, on MNC IL-8 production.
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Figure 2: The effect of FcγRIIa-crosslinking by mIgG1 anti-CD3 on whole blood cytokine production. (A, B, C) WB of 5 HR and 5 LR donors was stimulated with mIgG1 anti-CD3 (1 µg/ml). After 4 days of stimulation, supernatants were harvested for determination of T cell proliferation (A), TNF production (B), IL-8 production (C), and IL-6 production (data not shown). For T cell proliferation assays (A), data represent median thymidine incorporation of triplicate samples. A representative experiment with 10 different donors is shown. *** p<0.001, ** p<0.01, Mann Whitney U-test.

(D) WB of a HR donor was stimulated with mIgE and mIgG1 anti-CD3 (0.1 µg/ml) together with anti-CD28 (1 µg/ml) in the absence or presence of total IgG or F(ab)_2-fragment of an FcγRIIa-neutralizing mAb (10 µg/ml). At day 4, supernatants were harvested for determination of T-cell proliferation (data not shown) and IL-8 production. Data represent mean IL-8 production ± SEM of duplicate samples. A representative of two experiments is shown.

In isolated neutrophils, IL-8 production induced by GM-CSF and TNF was somewhat different: GM-CSF dose-dependently increased IL-8 production, whereas TNF at low levels had little effect (fig. 4B). TNF at higher concentrations (>1 ng/ml) seemed to inhibit GM-CSF-induced IL-8 production (fig. 4B).

Interestingly, IL-8 production in WB and MNC induced by GM-CSF and/or TNF was not accompanied by IL-6 production (< 20 pg/ml, data not shown), whereas both IL-6 and
Figure 3: Neutralization of GM-CSF and TNF in T cell-stimulated whole blood inhibits IL-8 production.

WB was stimulated with anti-CD3 and anti-CD28 mAbs (1 µg/ml) for 3 days in the absence or presence of anti-TNF (anti-TNF 5 and anti-TNF 7, 5 µg/ml each) and anti-GM-CSF (10 µg/ml). After stimulation IL-8 in the supernatant was measured by ELISA. Data represent mean IL-8 production ± SEM of duplicate samples. One representative of at least 3 experiments is shown.

IL-8 were abundantly produced after stimulation with LOS, a ligand for TLR4 (fig. 4C). Similar results were found when LPS was used for stimulation (data not shown). Thus, similar as in anti-CD3/CD28-stimulated WB, GM-CSF and TNF induced IL-8, but not IL-6 production.

**IL-2 potentiates GM-CSF/TNF-induced IL-8 production in WB.**

We demonstrated that IL-8 production in T cell-stimulated WB is almost entirely dependent on GM-CSF and TNF production. However, IL-8 production in T cell-stimulated WB was always several fold higher compared with the IL-8 production induced by GM-CSF and TNF. Even after stimulation of WB with TNF (0.5 ng/ml) and GM-CSF at supraphysiological concentrations (50 ng/ml), IL-8 levels remained below those induced by T cell stimulation (data not shown). Therefore, we analysed whether other cytokines might be responsible for the observed difference between IL-8 production induced by T cell activation and by GM-CSF/TNF stimulation. To test this, WB was incubated with GM-CSF and TNF, and co-stimulated with several cytokines, some of which are known to be produced by activated T cells (fig. 5A). Strikingly, IL-2 powerfully enhanced GM-CSF/TNF-induced IL-8 production to comparable levels as those induced by anti-CD3/CD28 stimulation, whereas several other cytokines (IL-1β, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12p70, IL-15, IL-17, and IFNγ) were without effect (fig. 5A). IL-2, when acting alone, also potently induced IL-8 production, reaching levels approximately 50% of those induced by T cell stimulation (fig. 6A). However, maximum IL-8 levels, such as after T cell stimulation, were only reached when WB was stimulated with a combination of GM-
Figure 4: GM-CSF and TNF induce IL-8, but not IL-6 production in whole blood, mononuclear cells, and isolated neutrophils.

(A, C) WB and MNC of four donors were left untreated (control), stimulated with TNF, GM-CSF, or both cytokines (A). In parallel, WB and MNC of the same donors were stimulated with LOS (100 pg/ml, C) after 1 day of LOS stimulation (C) and 4 days of T cell stimulation (A), supernatants were harvested and cytokines (IL-6 and IL-8) measured by ELISA. Data represent mean cytokine production ± SEM of duplicate samples of four different donors.

(B) Neutrophils were stimulated with different concentrations of GM-CSF and TNF for 3 days, after which supernatants were harvested and the IL-8 production measured by ELISA. Data represent mean IL-8 production ± SEM of duplicate samples of 1 donor. A representative experiment is shown.
Figure 5: IL-2 potentiates GM-CSF/TNF-induced IL-8 production in WB.

WB, in the presence of polymyxin-B (10 µg/ml), was stimulated with both GM-CSF and TNF together with a third cytokine as indicated. In parallel, WB was stimulated with anti-CD3 and anti-CD28 (0.1 µg/ml and 1 µg/ml, respectively). After 4 days supernatants were harvested for determination of IL-8 production. Data represent mean ± SEM of duplicate samples of two donors. Cytokine-induced IL-8 production is expressed as % of IL-8 production induced by anti-CD3/CD28 stimulation which was defined as 100%.

CSF/TNF and IL-2 (fig. 6A). When WB was stimulated with IL-2 in the presence of neutralizing mAbs to GM-CSF and TNF, IL-8 production was completely abrogated, indicating that the effects of IL-2 on IL-8 production were mediated by GM-CSF and TNF (fig. 6B). Finally, titration experiments revealed that IL-2 alone induced WB IL-8 production at high concentrations (>2.5 ng/ml) only, whereas in the presence of GM-CSF/TNF, IL-2 already at low concentrations dose-dependently induced WB IL-8 production (fig. 6C). IL-2 alone or in combination with GM-CSF and TNF also induced IL-6 production, albeit to a much lower extent (approximately 100-fold less) than IL-8 production (fig. 6D). Remarkably, IL-6 production induced by IL-2 combined with GM-CSF/TNF was higher (approximately 4-fold) than IL-6 production induced by anti-CD3/CD28 (fig. 6D).
Discussion

In this study, we unexpectedly found that IL-8, a potent activator and chemoattractant for neutrophils, is produced in T cell-stimulated WB. Moreover, this IL-8 production was entirely correlated with T cell proliferation and TNF production. Since the regulation of IL-6 and IL-8 production is very similar, we were also surprised by the finding that IL-8 production in T cell-activated WB was not accompanied by IL-6 production.

In general, activation of WB with LPS or other ligands for Toll-like receptors (TLRs) expressed on monocytes and/or neutrophils leads to approximately 10-15 fold higher levels of IL-8 compared to IL-6. However, in T cell-activated WB, 100-1000 fold higher levels of IL-8, compared to IL-6, are produced. Also crosslinking of HR-FcγRIIa by a murine IgG1 switch variant of the anti-CD3 mAb induced proliferation, TNF and IL-8 production, but not IL-6 production in WB. Therefore, we next attempted to determine which factor(s) in T cell-stimulated WB selectively induced the production of IL-8, and not IL-6. Subsequent experiments demonstrated that neutralization of GM-CSF and TNF markedly inhibited IL-8 production in T cell-stimulated WB. Conversely, GM-CSF and TNF co-operatively induced IL-8, but not IL-6 production in WB, MNC, and neutrophils. Several studies demonstrated co-operative stimulatory effects of GM-CSF and TNF on neutrophil functions.

Therefore, it is tempting to speculate that the stimulatory effects on neutrophils observed were caused by IL-8 induced by GM-CSF and TNF. Our experiments showed that the effects of GM-CSF and TNF on IL-8 production were not confined to neutrophils only, but that GM-CSF and TNF also co-operatively induce the production of IL-8 in WB and MNC. Thus, similar as after T cell stimulation, GM-CSF and TNF induced production of IL-8, but not IL-6, both in WB and MNC.

Although some research groups have reported that T cells produce minute amounts of IL-8 (< 1 ng/ml) after T cell activation, it is generally accepted that isolated T cells do not produce IL-8 after anti-CD3 stimulation. In addition, we (data not shown) and others have observed that contamination of isolated T cell cultures with even less than 1% monocytes may cause a marked induction of IL-8 production after anti-CD3 stimulation (data not shown). Because T cells do not produce IL-8, we next sought to determine the contribution of monocytes and neutrophils to the total IL-8 production in WB after GM-CSF/TNF stimulation.

A problem that occurs in these experiments is that isolated neutrophils rapidly undergo spontaneous apoptosis during cell culture, which may lead to underestimation of IL-8 production by neutrophils in WB. Although GM-CSF treatment of isolated neutrophils has been shown to prevent apoptosis, a number of studies indicated that TNF induces or
Figure 6. IL-2-induced IL-8 production in whole blood is dependent on GM-CSF and TNF production

(A) WB was stimulated with anti-CD3/CD28 (as in fig. 5), GM-CSF/TNF, IL-2, or with the combination GM-CSF/TNF and IL-2. (B) WB was stimulated with anti-CD3/CD28 (data not shown) or IL-2 in the absence and presence of neutralizing mAbs to GM-CSF (10 µg/ml) and TNF (anti-TNF 5 and anti-TNF 7, 5 µg/ml each). Cytokine-induced IL-8 production is expressed as % of IL-8 production induced by anti-CD3/CD28 stimulation (A and B) (C, D) WB was stimulated with different concentrations of IL-2 in the absence (open circles) or presence (filled circles) of GM-CSF and TNF. At day 4, supernatants were harvested for determination of IL-6 and IL-8 production. Dotted lines (C, D) indicate cytokine production induced by anti-CD3/CD28 stimulation. Data represent mean ± SEM of duplicate samples of 3 (A) or 2 (B, C, D) donors.
accelerates neutrophil apoptosis, even in the presence of GM-CSF. Studies by Van den Berg et al demonstrated that low concentrations of TNF exerted anti-apoptotic effects, whereas TNF concentrations above 1 ng/ml rapidly induced apoptosis in isolated neutrophils, which might explain why in our experiments TNF did not stimulate GM-CSF-induced IL-8 production in isolated neutrophils (fig. 4B). Although the presence of monocytes in neutrophil cultures delays apoptosis of neutrophils, and little is known about the life-span of neutrophils in more physiological circumstances such as in diluted WB, it remains to be elucidated to what extent neutrophils in WB become apoptotic under the influence of TNF. Thus, the fact that isolated neutrophils have a short life-span renders answering the question as to their relative contribution to total IL-8 production in T cell-stimulated WB difficult. The determination of the relative contribution of MNC to total IL-8 production in T cell-stimulated WB encounters other difficulties. We (data not shown) and others consistently observed increased background IL-8 production in isolated MNC, which greatly influences the sensitivity of the system, and which impedes the measurement of specific IL-8 induction by low concentrations of GM-CSF and/or TNF. The increased background IL-8 production was not caused by minor contamination with endotoxins, because polymyxin-B, a polycationic compound that specifically inhibits endotoxins, failed to inhibit background IL-8 production (data not shown). The activation of MNC during isolation might also lead to background TNF production, which might explain why GM-CSF alone strongly stimulated IL-8 production in MNC (fig. 4A). Thus, although it is clear that MNC present in WB constitute an important source of IL-8 production after GM-CSF/TNF stimulation, high background IL-8 production of MNC prevented a detailed and accurate determination of the MNC contribution to WB IL-8 production. Because of the background cytokine production in MNC we continued our experiments in WB, a system which has very low background IL-8 production and which is very sensitive to low concentrations of cytokines and/or stimuli.

Although we clearly established that WB IL-8 production after T cell activation was entirely dependent on GM-CSF and TNF, we presumed that a third factor was involved. This presumption was based on the fact that IL-8 production in T cell-stimulated WB was always several-fold higher than WB IL-8 production induced by GM-CSF and TNF. Experiments with a "third" cytokine, in addition to GM-CSF and TNF, demonstrated that IL-2 induced WB IL-8 levels comparable to those as observed after T cell stimulation. Since IL-2 is produced by proliferating T cells, the IL-2-induced production of IL-8 in GM-CSF/TNF-stimulated WB is consistent with our finding that IL-8 production completely paralleled T cell proliferation.
Actions of IL-2 are mediated through binding to a specific surface IL-2 receptor consisting of the IL-2Rα-, β-, and γ-chain. Apart from activated T cells, also monocytes and neutrophils express the β-chain of the IL-2R. Therefore, we hypothesize that IL-2 induced by T cell activation, via IL-2 receptors expressed on monocytes and neutrophils, stimulates IL-8 production. Indeed, other research groups demonstrated that IL-2 was capable of stimulating IL-8 gene expression in isolated monocytes and isolated neutrophils. Our data demonstrate that the stimulatory effect of IL-2 on IL-8 production is entirely dependent on GM-CSF and TNF, because neutralization of GM-CSF/TNF completely abolished IL-2-induced IL-8 production. In the presence of GM-CSF/TNF, low concentrations of IL-2 (< 1.25 ng/ml) already markedly stimulated IL-8 (fig. 6C), whereas IL-6 was hardly produced under these circumstances (fig. 6D). Only at higher IL-2 concentrations (> 1.25 ng/ml) IL-6 was produced (fig. 6D). Other research groups reported small or no effects of IL-2 on monocyte IL-6 production. However, in our experiments IL-6 production induced by IL-2, alone or in combination with GM-CSF/TNF, was always 100-fold lower than IL-8 production (fig. 6C and D). Also, IL-6 production induced by anti-CD3/CD28 remained below the IL-6 levels induced by IL-2 and GM-CSF/TNF, whereas IL-8 production induced by IL-2/GM-CSF/TNF and by anti-CD3/CD28 was comparable. Therefore, it can be concluded that IL-2 generated after T cell activation, together with GM-CSF and TNF, predominantly stimulates IL-8 production, and not IL-6 production. Further studies are underway in our laboratory to elucidate the interplay between GM-CSF, TNF, and IL-2, on IL-8 production. The finding that the effects of IL-2 on WB IL-8 production are completely dependent on GM-CSF and TNF, whereas supraphysiological levels of TNF and GM-CSF were unable to induce IL-8 production to comparable levels as after T cell activation, may suggest that additional (T cell) factors are involved. In this regard the role of the T cell cytokine IFNγ in WB IL-8 production merits further studies. Previous studies using isolated monocytes demonstrated that IFNγ upregulates monocyte expression of the IL-2 receptor β subunit, but suppresses monocyte IL-8 production.

In contrast to previous studies, we were unable to demonstrate co-stimulation of IL-8 production by other "third" cytokines such as IL-3, IL-7, IL-12, and IL-15. IL-15 has been reported to increase both neutrophil and monocyte IL-8 production. Also we found that IL-15 moderately stimulated IL-8 production in isolated neutrophils (data not shown). However, we were unable to demonstrate this effect in WB. This is particularly surprising because IL-15 shares a number of biologic effects with IL-2 and was able to activate both the β- and γ-chain of the IL-2 receptor. To date, we cannot explain the lack of WB IL-8 induction by IL-15. However, it should be noted that in our co-stimulation experiments
polymyxin-B was included to prevent stimulation of cells by endotoxin contamination. This is of particular interest as we observed that GM-CSF and TNF increase the sensitivity of WB for endotoxin and other TLR ligands (data not shown).

Our study demonstrates that T cell proliferation is accompanied by IL-8 production, and that this IL-8 production is entirely dependent on the interplay between IL-2, GM-CSF and TNF. This may be of importance from a mechanistic point of view and may illustrate how adaptive immunity (activation of T cells) may lead to enhanced activity of innate immunity (neutrophil chemotaxis). In addition, our study may provide an explanation for the activation of neutrophils observed in patients who receive IL-2 immunotherapy for advanced malignancy.\textsuperscript{49,50} Also other groups hypothesized that IL-8 generation induced by IL-2 plays a major role in the chemotaxis and activation of neutrophils.\textsuperscript{51} Consequently, activated neutrophils may cause indirect endothelial injury through the release of vasoactive mediators or direct damage by elastase, lysosomal enzymes, and the generation of oxygen free radicals\textsuperscript{52} possibly leading to the sepsis-like vascular leak syndrome (VLS) associated with this therapy.\textsuperscript{53,54} Finally, our study illustrates that the effects of T cell activation on monocyte and/or neutrophil cytokine production can be accurately studied in WB without concomitant problems such as the increased background cytokine production frequently observed in MNC. The WB system is a highly suitable system for the sensitive measurement of cytokine production not only after TLR triggering such as in the monocyte activation test, but also after T cell stimulation.

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**References**


Induction of IL-8 in T cell-stimulated whole blood


