Activated Platelets Enhance IL-10 Secretion and Reduce TNF-α Secretion by Monocytes

Sif Gudbrandsdottir, Hans C. Hasselbalch and Claus H. Nielsen

*J Immunol* 2013; 191:4059-4067; Prepublished online 18 September 2013; doi: 10.4049/jimmunol.1201103

http://www.jimmunol.org/content/191/8/4059

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/09/18/jimmunol.1201103.DC1

**References**

This article cites 38 articles, 14 of which you can access for free at:

http://www.jimmunol.org/content/191/8/4059.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Activated Platelets Enhance IL-10 Secretion and Reduce TNF-α Secretion by Monocytes

Sif Gudbrandsdottir,*† Hans C. Hasselbalch,‡ and Claus H. Nielsen*

Activated platelets are known to modulate immune responses by secreting or shedding a range of immunomodulatory substances. We examined the influence of activated platelets on cytokine production by normal human mononuclear cells, induced by tetanus toxoid (TT), human thyroglobulin (TG), Escherichia coli LPS, or intact Porphyromonas gingivalis. Addition of platelets activated by thrombin-receptor–activating peptide enhanced IL-10 production induced by LPS (p < 0.001), TG (p < 0.05), and P. gingivalis (p < 0.01), and reduced the production of TNF-α induced by LPS (p < 0.001), TG (p < 0.05), and P. gingivalis (p < 0.001), and of IL-6 in LPS- and P. gingivalis–stimulated cultures (p < 0.001). Similar effects on IL-10 and TNF-α production were observed on addition of platelet supernatant to mononuclear cells, whereas addition of recombinant soluble CD40L mimicked the effects on IL-10 production. Moreover, Ab-mediated blockade of CD40L counteracted the effect of platelets and platelet supernatants on TNF-α production. Monocytes separated into two populations with respect to IL-10 production induced by TG; the high-secreting fraction increased from 0.8 to 2.1% (p < 0.001) on addition of activated platelets. Adherence of platelets increased TG- and TT-induced IL-10 secretion by monocytes (p < 0.05). In addition, activated platelets inhibited CD4+ T cell proliferation elicited by TT (p < 0.001) and P. gingivalis (p < 0.001). Our findings suggest that activated platelets have anti-inflammatory properties related to the interaction between CD40L and CD40, and exert a hitherto undescribed immunoregulatory action by enhancing IL-10 production and inhibiting TNF-α production by monocytes. The Journal of Immunology, 2013, 191: 4059–4067.

Moreover, fixed platelets inhibited Ag-induced proliferation of mouse splenocytes in vitro (14).

In this study, we investigated the capacity of platelets to modulate PBMC cytokine production and CD4+ T cell proliferation induced by physiologically relevant self- and non–self-antigenic stimuli. Our data suggest that activated platelets stimulate IL-10 release and inhibit TNF-α release from monocytes, in a CD40L-dependent manner, after stimulation of PBMCs with human thyroglobulin (TG), Escherichia coli LPS, or whole bacteria of the species Porphyromonas gingivalis.

Materials and Methods

Donors

Peripheral venous blood was drawn into tubes containing heparin, EDTA, or serum clot activator (BD, Plymouth, U.K.) from self-reported healthy volunteers recruited from laboratory staff and the Blood Bank at Copenhagen University Hospital Rigshospitalet. The Danish National Committee on Biomedical Research Ethics approved the study (protocol no. H-2-2011-040). Samples from 69 healthy donors were used (36 men and 33 women, age 36 ± 11 y [mean ± SD]).

Isolation of human PBMCs

PBMCs were isolated by gradient centrifugation of heparinized blood using LymphoPrep (Axis-Shield, Oslo, Norway), washed twice in PBS (Life Technologies, Invitrogen, Paisley, U.K.), and resuspended in RPMI 1640 buffer with HEPES (Biological Industries, Haemek, Israel), 1-glutamine (Life Technologies, Invitrogen), and gentamicin (Life Technologies, Invitrogen).

Preparation of platelets

Blood was collected in EDTA tubes (BD Bioscience, Plymouth, U.K.) and isolated by a one-step method previously described to yield functional platelets (19). In brief, 5 ml blood was layered over 5 ml Nycodenz density barrier (Axis-Shield) and centrifuged at 350 × g for 20 min at 20°C without brake. Platelet-rich plasma was harvested from the platelet-rich fraction, and centrifuged at 1200 × g for 10 min and supernatants were harvested for immediate use.

*Department of Infectious Diseases and Rheumatology, Institute for Inflammation Research, Copenhagen University Hospital Rigshospitalet, 2100 Copenhagen, Denmark; and †Department of Hematology, Copenhagen University Hospital Roskilde, 4000 Roskilde, Denmark

Received for publication April 16, 2012. Accepted for publication August 2, 2013.

This work was supported by Agen and GlaxoSmithKline (to S.G.).

Address correspondence and reprint requests to Sif Gudbrandsdottir, Department of Infectious Diseases and Rheumatology, Institute for Inflammation Research, Section 7541, Copenhagen University Hospital Rigshospitalet, Blegdamsvej 3, 2100 Copenhagen Ø, Denmark. E-mail address: sif.gudbrandsdottir@gmail.com

The online version of this article contains supplemental material.

Abbreviations used in this article: MFI, mean of fluorescence intensity; PF4, platelet factor 4; TG, thyroglobulin; TRAP, thrombin receptor agonist peptide; TT, tetanus toxoid.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201103
Cell cultures

Isolated PBMCs were labeled with CFSE at a final concentration of 1.5 μM for 10 min at 37°C before washing in RPMI 1640 (centrifuged at 400 × g, 5 min) and resuspension in RPMI 1640 buffer. PBMCs were then cultured in a Nunclon Delta microwell plate (Thermo Fischer Scientific, Roskilde, Denmark), 2.5 × 10⁸ cells/well, with 30 μl autologous serum and 80 μl RPMI 1640 buffer. The cells were incubated with 10 μg/ml TT (Statens Serum Institut, Copenhagen, Denmark), 0.05 μg/ml LPS (E. coli O55:B5 endotoxin; Lonza, Walkerville, MD), 2 × 10⁶/ml P. gingivalis strain ATCC 33277 (supplied by the Oral Microbiology Section, School of Dentistry, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark), or without Ag in the presence or absence of activated platelets (0.5–3.4 × 10¹⁰/l) at a total volume of 120 μl/well. Cells incubated without platelets or Ags served as negative controls. The cells were cultured for 7 d in a Heracell 150i CO₂ incubator (ThermoScientific, Waltham, MA). After 24 h of culture, 50 μl of the supernatants were harvested and assessed for cytokine content. Subsequently, 100 μl RPMI 1640 buffer was added to each well. On day 7, the PBMCs were harvested, pelleted (700 × g, 5 min), and resuspended in 100 μl buffer.

**FIGURE 1.** Influence of platelets on Ag-induced cytokine secretion by PBMCs. Purified PBMCs from healthy donors were stimulated with TT (n = 26), human TG (n = 15), E. coli–derived LPS (n = 9), or P. gingivalis (PG, n = 9) in the absence (open bars) or presence (closed bars) of TRAP-activated autologous platelets (plts). The content of (A) IL-2, (B) IFN-γ, (C) TNF-α, (D) IL-6, (E) IL-4, (F) IL-10, and (G) TGF-β in the culture supernatants was measured after 24 h. (H) The production of TNF-α in the presence of platelets was partly IL-10 dependent, as indicated by a significant inhibition upon addition of IL-10 Ab in cultures stimulated with LPS (n = 6) or P. gingivalis (n = 9). Means and SEM are shown. The detection limit of the analysis was the mean + 2 SD of supernatants from nonstimulated cultures (dotted line), and this value was inserted for cytokine levels below the detection limit. *p < 0.05, **p < 0.01, ***p < 0.001 using paired t tests on log-transformed data.
PBS before flow cytometric analysis. In additional experiments, platelet suspensions were replaced with 10 µl platelet supernatant, and after 24 h of incubation with PbMCs, culture supernatants were harvested and analyzed for cytokine content.

Measurement of cell proliferation

PbMCs harvested on day 7 were stained with PE-labeled anti-CD4 and allophycocyanin-labeled anti-CD19, incubated in the dark for 30 min at room temperature, and washed once in 1 ml PBS (700 × g, 5 min). The samples were assayed on a FACSCalibur flow cytometer using the CellQuest program (Becton Dickinson Immunocytometry System, San Jose, CA). Th cells were gated as CD4hi/CD19lo events within a morphological lymphocyte gate, and proliferation (% divided cells) was measured as the percentage of cells with less than half the CFSE fluorescence of the prime peak containing undivided cells as described previously (20).

Measurement of HLA-DR and costimulatory molecule expression

Isolated PbMCs were incubated with or without Ag in the presence or absence of activated platelets as described earlier. After 24 h of culture, cells were harvested and stained with Per-CP-labeled anti-CD14, PE-labeled anti-HLA-DR, FITC-labeled anti-CD80, and allophycocyanin-labeled anti-CD86. Monocytes were identified as CD14+ cells within a morphological gate and surface marker expressions determined as geometric means of fluorescence intensities (MFIs) for each conjugate.

Cytokines in supernatants of cell cultures

The BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit II (BD Bioscience) was used to measure IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 protein concentrations in nonstimulated cultures + 2×10^6 PBMCs were washed (300 × g, 10 min, 4°C), labeled with anti-CD45/anti–IL-10 or anti-CD45/anti–TNF-α bispecific catch reagent, and incubated for 45 min at 37°C. During the secretion phase, PbMCs were suspended in 1 ml medium with 5% autologous serum under continuous rotation. The PbMCs were then washed and labeled with a combination of allophycocyanin-labeled IL-10 detection Ab, FITC-labeled anti-CD4 Ab, PE-labeled anti-CD14 Ab, and PerCP-labeled anti-CD61 Ab or a combination of PE-labeled TNF detection Ab.

FIGURE 2. Influence of activated platelets on IL-10 production by monocytes. PbMCs from nine healthy donors were incubated for 16 h with TT or human TG in the presence or absence of TRAP-activated autologous platelets (plts). The secretion of IL-10 by individual monocytes was measured by flow cytometry using a bispecific anti-CD45/anti–IL-10 capture reagent. (A) Representative histogram showing IL-10 secretion by monocytes stimulated with TG in the absence (gray line) or presence (black line) of platelets. M1 indicates monocytes secreting low amounts of IL-10 and M2 those secreting large amounts. (B) IL-10 secretion by the M1 fraction of monocytes after stimulation with TT or TG in the absence (open bars) or presence (closed bars) of activated platelets, after adjustment for baseline fluorescence (no Ag or platelets added), is shown as means and SD. **p < 0.01. (C) The percentage of monocytes within the M2 fraction is shown as individual observations. Horizontal lines represent mean values. ***p < 0.001.
Platelet-mediated regulation of IL-10 and TNF-α secretion by monocytes and CD4+ T cells

Because monocytes are known to be a major source of IL-10 (23, 24), we examined the secretion of IL-10 by monocytes after stimulation of PBMCs with TG or TT (Fig. 2). TG induced secretion of IL-10 and TNF-α in monocytes, whereas TT induced IL-2 and IFN-γ (Fig. 1).

Statistical analysis

Statistical analysis was performed using the two-tailed paired t test on log-transformed data (GraphPad Prism 4; GraphPad Software). The p values <0.05 were considered significant.

Results

Influence of activated platelets on Ag-elicted cytokine production by PBMCs

PBMCs from 11 healthy donors were stimulated with the foreign recall Ag TT, the self-Ag TG, E. coli LPS, or the oral bacterium P. gingivalis in the presence or absence of TRAP-activated autologous platelets. Fig. 1 shows the cytokine content in supernatants harvested after 24 h of stimulation. The Ags elicited distinct cytokine profiles: TT induced production of IL-2 and IFN-γ (Fig. 1A, 1B), accompanied by small amounts of TNF-α, IL-6, and IL-4 (Fig. 1C–E), with no detectable IL-10 (Fig. 1F). TG, however, induced no production of IL-2, IFN-γ, or IL-4, moderate amounts of TNF-α and IL-6, and significant amounts of IL-10 in 5 of 11 experiments. LPS and P. gingivalis induced cytokine profiles similar to that of TG, but with more pronounced production of TNF-α and IL-6, and marked production of IL-10 in all experiments (Fig. 1F). Addition of activated platelets to the PBMC cultures had no significant effect on TT-elicted cytokine production, although tendencies toward decreased production of IFN-γ (p < 0.08; Fig. 1B) and TNF-α (p = 0.10; Fig. 1C) were observed. Activated platelets did, however, significantly reduce the TG-induced production of TNF-α (Fig. 1C) and the LPS- and P. gingivalis–induced production of TNF-α and IL-6 (Fig. 1C, 1D). Notably, activated platelets induced ~3-fold increases in the production of IL-10 after stimulation with TG, LPS, or P. gingivalis (Fig. 1F, Table I). Correspondingly, the presence of activated platelets reduced the TNF-α production induced by TG by 85% (p < 0.001), by LPS by 82% (p < 0.0001), and by P. gingivalis by 79% (p < 0.0001).

In a separate series of experiments, we found a significant increase in TGF-β concentrations after addition of activated platelets to PBMC cultures, irrespective of whether stimulating Ags were added (Fig. 1G). Additional measurements in supernatants from TRAP-activated platelets confirmed that these are a major source of TGF-β (data not shown). This led us to examine whether TGF-β might induce IL-10 and TNF-α production by PBMCs. Addition of rTGF-β had, however, no effect on the LPS-induced production of IL-10 or TNF-α, and Ab-mediated blockade of TGF-βRI on PBMCs did not affect the LPS-induced production of IL-10 or TNF-α (Supplemental Fig. 1).

To examine the interdependency between the LPS- or P. gingivalis–induced production of IL-10 and TNF-α, we blocked IL-10 in the PBMC-platelet cocultures with a monoclonal anti-IL-10 Ab (Fig. 1H). The inhibitory effect on TNF-α production, corresponding to that observed in Fig. 1C, was reversed by IL-10 blockade (p < 0.001 for both Ags). Conversely, when TNF-α was blocked by etanercept in cultures stimulated with P. gingivalis, we found a modest inhibition of IL-10 production (mean ± SEM: 264 ± 87 versus 196 ± 68 pg/ml; p < 0.05; n = 4; data not shown).

Platelet-mediated regulation of IL-10 and TNF-α secretion by monocytes and CD4+ T cells

PBMCs from nine healthy donors were stimulated with TT, human TG, or no Ag (no ag) and cultured for 16 h in the presence (closed symbols) or absence (open symbols) of TRAP-activated autologous platelets (plts). The proportions of CD4+ T cells secreting IL-10 or TNF-α as determined by flow cytometry are shown. Horizontal lines represent mean values. *p < 0.05, **p = 0.05, ***p < 0.01.
low amounts of IL-10 by the majority of monocytes (Fig. 2A, M1), whereas a minor subset produced high amounts of the cytokine (Fig. 2A, M2). Addition of activated platelets enhanced the TG-induced production of IL-10 by the low-secretting subset (p < 0.01; Fig. 2B) and caused a 2.5-fold increase in the proportion of high-secreting monocytes (mean ± SEM: 0.8 ± 0.2 versus 2.1 ± 0.6%; p < 0.001), as shown in Fig. 2C. In accordance with the data presented in Fig. 1F, TT did not cause IL-10 secretion (Fig. 2B, 2C).

A significant increase in IL-10–secreting CD4+ T cells occurred after stimulation with TG (from 0.02 ± 0.003 [mean ± SEM] to 0.06 ± 0.014% of the CD4+ T cell population; p < 0.05), but the influence of activated platelets on this proportion was not significant (Fig. 3A). Stimulation with TT, however, induced a significant increase in the proportion of TNF-α–secreting CD4+ T cells (from 0.12 ± 0.032 to 2.0 ± 0.746% of the entire population; p < 0.05), and this proportion was halved (to 1.0 ± 0.492%) on addition of activated platelets (p = 0.05; Fig. 3B). We further assessed the possibility that platelets enhanced the amount of IL-10 produced by the individual CD4+ T cells without increasing the frequency of IL-10–producing cells. This was not the case (data not shown).

**Contribution of CD40L to the anti-inflammatory effect of platelets**

We next sought to identify the mechanism by which platelets affected PBMC cytokine production and speculated that CD40L, either surface-bound or shed from the platelets (10), might play a role in the process. Indeed, exogenously added CD40L did increase the LPS-elicited IL-10 production by PBMCs to a similar extent as activated platelets (Fig. 4A), but failed to mimic the effect of platelets on TNF-α production after stimulation with LPS or *P. gingivalis* (Fig. 4B). Somewhat contradictory, however, blockade of CD40L with an mAb did not affect IL-10 secretion, whereas it counteracted the inhibitory effect of platelets on TNF-α secretion (Fig. 4C, 4D).

**Figure 4.** CD40L–dependent immunoregulation by platelets. PBMCs were incubated with *E. coli* LPS, intact *P. gingivalis* (PG), or no Ag (no ag) in the presence (closed bars) or absence (open bars) of TRAP-activated autologous platelets (plts). (A and B) Recombinant sCD40L (shaded bars) was added instead of platelets (n = 8 for LPS; n = 4 for *P. gingivalis*). (C and D) Monoclonal anti-CD40L Ab was added at various concentrations (10, 40, and 100 μg/ml) to platelet-containing cultures (n = 9 for LPS; n = 4 for *P. gingivalis*). The content of IL-10 (A, C) and TNF-α (B, D) in the culture supernatants after 24 h is shown as means and SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

**Contribution of PF4-CXCR3 interaction to the anti-inflammatory effect of platelets**

An important role of PF4 in mediating immunoregulatory effects of platelets on T cells has been described (17, 18). We therefore examined whether blockade of CXCR3, the receptor for PF4, affected cytokine production in PBMCs cocultured with activated platelets. This was not the case (Supplemental Fig. 2).

**Influence of soluble platelet-derived factors on IL-10 and TNF-α production by PBMCs**

We next investigated whether the effects of platelets on LPS-stimulated PBMCs were associated with the presence of platelets or with soluble factors released from the platelets. To this end, we added supernatants from TRAP-activated platelets to LPS- or *P. gingivalis*–stimulated PBMCs. The supernatants proved almost as effective as platelet suspensions in enhancing LPS-induced IL-10 production (Fig. 5A) and inhibiting LPS- or *P. gingivalis*–induced TNF-α production (Fig. 5B). Incubation of the PMBCs with anti-IL-10 Abs counteracted, with borderline significance (p = 0.056, n = 4), the inhibitory effect of platelet supernatants on TNF-α production (data not shown).

Ab-mediated blockade of sCD40L in the supernatants did not counteract the supernatant-mediated enhancement of IL-10 production (Fig. 5C), although it did counteract the inhibitory effect on TNF-α production (Fig. 5D). Taken together, these findings point to CD40L secreted from platelets, or expressed on microparticles released from platelets, as a suppressor of TNF-α production.

**IL-10 production by monocyte-platelet aggregates**

We speculated that close physical contact between platelets and monocytes might promote IL-10 production; therefore, we examined aggregates containing both CD61+ platelets and CD14+ monocytes (Fig. 6). Even in the absence of exogenously added platelets, such aggregates were common (Fig. 6A–C), suggesting
that they had been formed in vivo. Their frequency increased on addition of exogenous platelets (Fig. 6D, Table II).

Fig. 6E shows the production of IL-10 by monocytes with adherent platelets (black bars) and without (white bars). CD61+ monocytes with adherent platelets responded to both TT and TG with markedly increased production of IL-10 (p < 0.05 and p < 0.01, respectively), which was not the case for CD61− monocytes without adherent platelets. After addition of TRAP-activated platelets to PBMC cultures stimulated with TG or TT, the secretion of IL-10 by monocytes with adherent platelets was higher than the corresponding secretion by monocytes without adherent platelets (p < 0.05 for both stimuli). These findings suggest that activated platelets adhere to monocytes and facilitate IL-10 production. It should be noted that platelet-bearing monocytes from cultures without exogenously added TRAP-activated platelets (i.e., with adherent platelets bound in vivo or through handling procedures) produced significantly less IL-10 after stimulation with TG than platelet-bearing monocytes in cultures containing exogenously added TRAP-activated platelets (p < 0.01).

Influence of platelets on the expression of HLA-DR, CD80, and CD86 by monocytes

TT, but not TG or LPS, induced upregulation of monocyte HLA-DR expression and similar tendencies were observed for CD80 and CD86 expression (Supplemental Fig. 3). Addition of activated platelets to PBMCs increased expression of CD86 in cultures stimulated with TG (p < 0.009) and in unstimulated cultures (p < 0.02).

Platelet-mediated regulation of Ag-induced CD4+ T cell proliferation

Having thus established that activated platelets modulate cytokine production by monocytes, the precursor cells for Ag-presenting macrophages and dendritic cells, we examined the influence of activated platelets on Ag-induced proliferation of CD4+ T cells. Indeed, activated platelets markedly impaired the CD4+ T cell proliferation induced by TT (p < 0.001) and P. gingivalis (p < 0.001), as shown in Fig. 7A. The same effect was observed for cultures stimulated with TG (p < 0.05), whereas LPS did not elicit proliferation of CD4+ T cells (data not shown). In four ad-
Monocytes with adherent platelets (CD14+CD61+ events) are displayed in platelet marker CD61 and the corresponding quadrant statistics is shown. Cytos on the basis of forward/side scatter characteristics is illustrated. (B)

Although neither IL-10, TGF-β, nor CD40L, (data not shown) seemed to account for the inhibitory effect of platelets on CD4+ T cell proliferation almost as effectively as platelets (Fig. 7D).

**Discussion**

Over recent years, increasing attention has been paid to the possible immunomodulatory role of platelets (4–6, 8, 10, 12, 14, 15, 25). In this study, we showed that activated platelets modulate the function of both monocytes and CD4+ T cells in PBMC cultures stimulated with the foreign Ags *E. coli* LPS, TT, or *P. gingivalis*, or the self-Ag human TG, which is known to stimulate monocyte and CD4+ T cell responses in normal PBMCs (20, 21, 24).

Our key observation was that activated platelets, as well as supernatants from activated platelets, inhibited the Ag-elicited production of TNF-α and enhanced the corresponding production of IL-10, suggesting that platelets exert an anti-inflammatory effect via released soluble molecules or microparticles. These observations were made in six independent series of experiments (Figs. 1, 2B, 4A, 4B, 5C, 5D, 6E, Supplemental Fig. 2). The inhibitory effect of platelets on TNF-α production was reversed by addition of anti–IL-10 Ab, whereas blockade of TNF-α resulted in a modest inhibition of the IL-10 production. These data suggest that the platelet-mediated production of IL-10 is responsible for the corresponding decrease in TNF-α production. In addition to the effects on IL-10 and TNF-α, platelets increased the concentration of TGF-β in PBMC cultures in an Ag-independent manner. It has previously been shown that platelets are a major source of TGF-β (26, 27), and the platelet supernatants tested in this study did contain TGF-β.

Using a capture-assay fixing IL-10 on the surface of the cells producing it, we identified the primary source of IL-10 as monocytes, the majority of which released small amounts, whereas ~1% secreted large amounts upon stimulation with TG. In contrast, <0.1% of the CD4+ T cells contributed to the TG-elicited production. These observations fully agree with our previous findings under similar conditions but with intracellular staining, where CD4+ T cells nonetheless controlled the TG-induced IL-10 production by monocytes (24). In this study, IL-10 release by the low-secreting majority of monocytes was approximately doubled and the proportion of high-secreting monocytes was more than doubled by addition of TRAP-activated platelets (Fig. 2).

Activated platelets adhere to various leukocyte populations, including monocytes (28, 29), partly because of interactions between P-selectin and P-selectin glycoprotein ligand (28–32). This allows direct interaction between surface-bound molecules on platelets and monocytes, such as that of CD40L with CD40. We observed that the IL-10 secretion by monocytes with adherent platelets was significantly higher than the secretion from monocytes bearing no platelets, regardless of whether the stimulating Ag was TT or TG. It is likely that very high local concentrations of immunomodulatory mediators can be achieved upon cell–cell contact, and that secretory clefts sealed off from the external environment are formed, as described for cytotoxic T cells and target cells (33). This would give the Abs used in this study poor access to their respective Ags, resulting in underestimation of the effect of the respective receptor–ligand interactions on the cytokine production examined.

We considered that three platelet products TGF-β, PF4, and CD40L, all known to exert immunomodulatory effects (4–6, 17, 18), were responsible for the effects observed in this study. TGF-β was likely not the mediator, because addition of exogenous human rTGF-β had no effect on the LPS-induced production of IL-10 and TNF-α, nor did Ab-mediated blockade of TGF-βRI on PBMCs. Likewise, blockade of CXCR3, the receptor for PF4, had no effect. By contrast, CD40L apparently played an important role: first, recombinant human sCD40L mimicked the effect of activated platelets on IL-10 production, but not on TNF-α production.
(Fig. 4A, 4B). Second, Ab-mediated blockade of CD40L counteracted the inhibition of LPS-induced TNF-α production induced by intact platelets or platelet supernatants, but had no effect on the corresponding IL-10 production (Figs. 4C, 4D, 5C, 5D). These findings suggest that CD40L is involved in the induction of IL-10 release and inhibition of TNF-α release from PBMCs. It can be speculated that the threshold for induction of IL-10 production by CD40L is relatively low and, therefore, can be overcome by addition of recombinant sCD40L. Inhibition of TNF-α production, in contrast, may require a high signal strength that cannot be provided by sCD40L. Intact platelets or microparticles contained in platelet supernatants may bear CD40L at a sufficient density to cross-bind multiple CD40 molecules on target cells, however. A high threshold for TNF-α induction and a low threshold for IL-10 induction would also explain why anti-CD40L Abs canceled out the platelet-mediated inhibition of TNF-α production, but failed to inhibit the corresponding IL-10 production.

A difference between the effects of membrane-bound CD40L and sCD40L has been demonstrated in other situations. Thus, binding of cell-bound CD40L leads to internalization of CD40 and recruitment of TNFR-associated factors, whereas binding of sCD40L leads to endocytosis of CD40 and activation of TNFR-associated factor-independent signaling pathways (34).

Our main finding regarding T cells was that CD4+ T cell proliferation induced by P. gingivalis or TT was reduced by the presence of activated platelets. Accordingly, Gerdes et al. (17) found that platelets inhibited anti-CD3/anti-CD28–driven CD4+ T cell proliferation. We investigated whether the platelet-mediated reduction of CD4+ T cell proliferation could be explained by an inhibitory effect on the monocyte expression of HLA-DR, CD80, or CD86. We found no such inhibitory effect, but rather a slight upregulation of CD86 on TG-stimulated monocytes, in accordance with a previous study showing that PF4 increases the expression of CD86 on monocyte-derived dendritic cells (35). Others have reported that blockade of TNF-α reduces TT-induced CD4+ T cell proliferation (36), and we confirmed these findings in P. gingivalis–stimulated cultures and observed a similar trend in TT-stimulated cultures. We therefore propose that the inhibitory effect

![FIGURE 7. Influence of platelets on TT- or TG-induced CD4+ T cell proliferation. PBMCs from healthy donors were labeled with CFSE and stimulated with TT or P. gingivalis (PG) for 7 d in the presence (+ plts, closed symbols) or absence (open symbols) of TRAP-activated autologous platelets. The consequent proliferation of CD4+ T cells was assessed by flow cytometry. The proportion of CD4+ T cells having undergone more than one cell division at day 7 is shown, adjusted for baseline proliferation. (A) Proliferation after stimulation with TT (n = 24) or P. gingivalis (n = 13) with or without platelets. (B) Addition of anti–IL-10 to P. gingivalis–stimulated cultures with platelets (n = 9). (C) Addition of anti-CD40L to cultures stimulated with TT or P. gingivalis with platelets (n = 9). (D) Addition of anti–TNF-α to cultures stimulated with TT (n = 5) or P. gingivalis (n = 4) without platelets. The p values were calculated by paired t test on log-transformed data. *p < 0.05, **p < 0.01, ***p < 0.001.]

Table II. Frequencies of monocyte-platelet aggregates defined as the percentage of CD4intCD14+CD61+ cells in the PBMC population

<table>
<thead>
<tr>
<th>Stimulating Ag</th>
<th>No Ag</th>
<th>No Ag + Platelets</th>
<th>TT</th>
<th>TT + Platelets</th>
<th>TG</th>
<th>TG + Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4intCD14+CD61+ % of total PBMCs</td>
<td>1.84 ± 0.73</td>
<td>2.82 ± 1.05*</td>
<td>2.45 ± 1.09</td>
<td>4.58 ± 4.5*</td>
<td>3.91 ± 2.71</td>
<td>7.95 ± 4.24b</td>
</tr>
</tbody>
</table>

*p < 0.05 (compared with no Ag).

bp < 0.01 (compared with TG alone or no Ag).
of activated platelets on CD4+ T cell proliferation is directly related to their inhibition of TNF-α, in accordance with findings of Brown et al. (37). Unlike other investigators (8, 17), we found no effect of platelets on the T cell production of TNF-α, IFN-γ, IL-2, or IL-4. In particular, platelets influenced neither the frequency of IL-10–secreting CD4+ T cells nor the IL-10 production per individual cell, which disagrees with Gerdes and colleagues (17), who found that platelets enhanced IL-10 production by CD4+ T cells. This discrepancy might be because of their use of monoclonal anti-CD3/anti-CD28 as a stimulus, which is likely to be much stronger than the more physiological antigenic stimuli used in this study. Using thrombin-generated platelet gels, Naldini et al. showed that platelets reduced the release of IFN-γ by LPS-stimulated PBMCs (8, 38), and supporting their data, we did find a tendency toward platelet-mediated inhibition of TT-induced IFN-γ production (Fig. 1B).

In summary, our data indicate that platelets and soluble factors released from platelets are capable of enhancing Ag-elicted IL-10 production and of inhibiting the corresponding TNF-α production by monocytes. The physiological consequences may be counteraction of exaggerated proinflammatory immune responses in vivo. Supporting this view, platelet-deficient mice suffer exaggerated systemic inflammatory responses to thermal injury, culminating in increased nonhemorrhagic mortality (39), CD40/CD40L interactions seemed to account, at least in part, for the effects of platelets on cytokine secretion. This is a hitherto undescribed mechanism by which platelets regulate the immune system.

Acknowledgments
We thank Ole Christensen, Pia Meincke and Winnie Hansen (Institute for Inflammation Research, Copenhagen University Hospital Righospital) for excellent technical assistance in the laboratory, Morten Bagge Hansen (Department of Clinical Immunology, Copenhagen University Hospital Righospital) for providing the TRAP, and Alistair Reeves (Ascribe Medical Writing and Translation, Wiesbaden, Germany) for editing the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References