The Journal of Immunology

RESEARCH ARTICLE | OCTOBER 15 2013

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

Sif Gudbrandsdottir; ... et. al

*J Immunol* (2013) 191 (8): 4059–4067.
https://doi.org/10.4049/jimmunol.1201103

# 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.**

Platelets are well-known as mediators of hemostasis and initiators of wound healing (1–3). Recent studies suggest that platelets also play a role in modulating adaptive immune responses (4–8). Upon activation, platelets are capable of secreting a range of immunomodulatory chemokines contained in their α-granula, including platelet factor 4 (PF4), RANTES, and CXCL7 (6, 9). After degranulation, transmembrane proteins of the α-granules emerge on the platelet surface, including the adhesion molecule P-selectin that facilitates interactions with the endothelium, monocytes, neutrophils, and lymphocytes (6, 10, 11). By shedding soluble and platelet microparticle-bound CD40L (10), platelets may also stimulate maturation of monocyte-derived dendritic cells (12, 13), as well as B cell proliferation, differentiation, and class switching (14–16).

Gerdes et al. (17) recently showed that platelets enhance production of the proinflammatory cytokine TNF-α and of the regulatory cytokine IL-10 in cultures of purified $CD4^+$ T cells, in a PF4-dependent manner. Accordingly, PF4 purified from supernatants of thrombin-stimulated platelets inhibited $CD4^+$ T cell proliferation induced by tetanus toxoid (TT) or a tuberculin derivative and augmented T cell production of IL-2 and IFN-γ (18). 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), L-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 band. Platelets were activated by incubation with 50 μM thrombin receptor agonist peptide (TRAP); KeraFAST, Winston-Salem, NC) for 5 min at 37°C before addition to the PBMC cultures. For experiments involving platelet supernatants, isolated activated platelets were 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 Amgen 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.



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), $2.5 \times 10^5$ 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), 10 μg/ml human TG (MyBioSource, San Diego, CA), 0.05 μg/ml LPS (*E. coli* O55:B5 endotoxin; Lonza, Walkersville, MD), $2 \times 10^6$/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 ($0.5–3.4 \times 10^{10}$/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 $CO_2$ 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

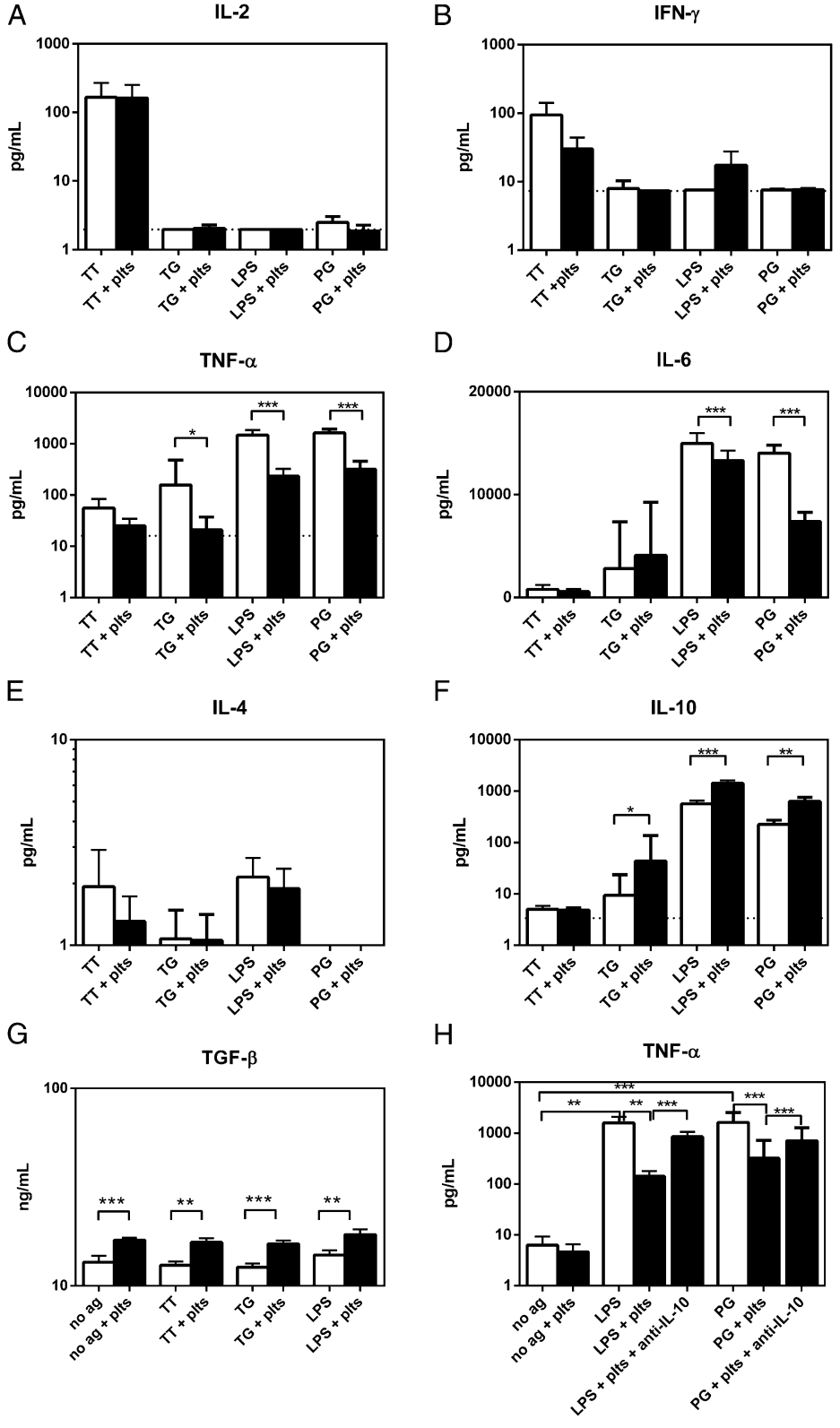


**FIGURE 1.** Influence of platelets on Ag-induced cytokine secretion by PBMCs. Purified PBMCs from healthy donors were stimulated with TT ($n$ = 26), human TG ($n$ = 15), *E. coli*–derived LPS ($n$ = 9), or *P. gingivalis* (PG, $n$ = 9) in the absence (open bars) or presence (closed bars) of TRAP-activated 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.

Table I. IL-10 and TNF-α production in the presence and absence of TRAP-activated platelets

| Stimulating Ag | Mean Ratios ± SEM | | | |
|---|---|---|---|---|
| | TT | TG | LPS | PG |
| IL-10 ratio with or without platelets | $n = 8$<br>0.93 ± 0.28 (NS) | $n = 8$<br>3.09 ± 0.83 ($p < 0.05^a$) | $n = 8$<br>2.67 ± 0.21 ($p < 0.0001^a$) | $n = 9$<br>3.40 ± 0.66 ($p < 0.01^a$) |
| TNF-α ratio with or without platelets | $n = 6$<br>0.41 ± 0.32 (NS) | $n = 7$<br>0.15 ± 0.11 ($p < 0.001^a$) | $n = 9$<br>0.18 ± 0.06 ($p < 0.0001^a$) | $n = 9$<br>0.21 ± 0.06 ($p < 0.0001^a$) |

The enhancing effect of activated platelets on IL-10 production was quantified only in experiments where the IL-10 concentration in cultures without platelets was above the detection limit.

[a]The $p$ values indicate the probability for the ratio being equal to 1.

PBS before flow cytometric analysis. In additional experiments, platelet suspensions were replaced with 10 μl platelet supernatant, and after 24 h of incubation with PMBCs, 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 $CD4^{high}CD19^{-}$ 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 in culture supernatants as described previously (21), according to the manufacturer's instructions. A FACSCalibur flow cytometer (BD Bioscience) was used for data acquisition, and the data were subsequently analyzed using the FCAPArray Software (SoftFlow, Burnsville, MN). The detection limit for each cytokine was defined as the mean value of concentrations in nonstimulated cultures + 2 × SD.

TGF-β was measured in culture supernatants and supernatants from TRAP-activated platelets using the TGF-β1 Multispecies Singleplex Bead Kit (Invitrogen, Camarillo, CA) and the Luminex$^{100}$ detection system (Bio-Rad) according to the manufacturer's instructions.

### *Cytokine secretion assay*

After 16 h of culture, IL-10 and TNF-α secretion by $CD4^{+}$ T cells and $CD14^{+}$ monocytes was assessed using MACS Cytokine Secretion Assays (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In brief, $1 \times 10^6$ 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,

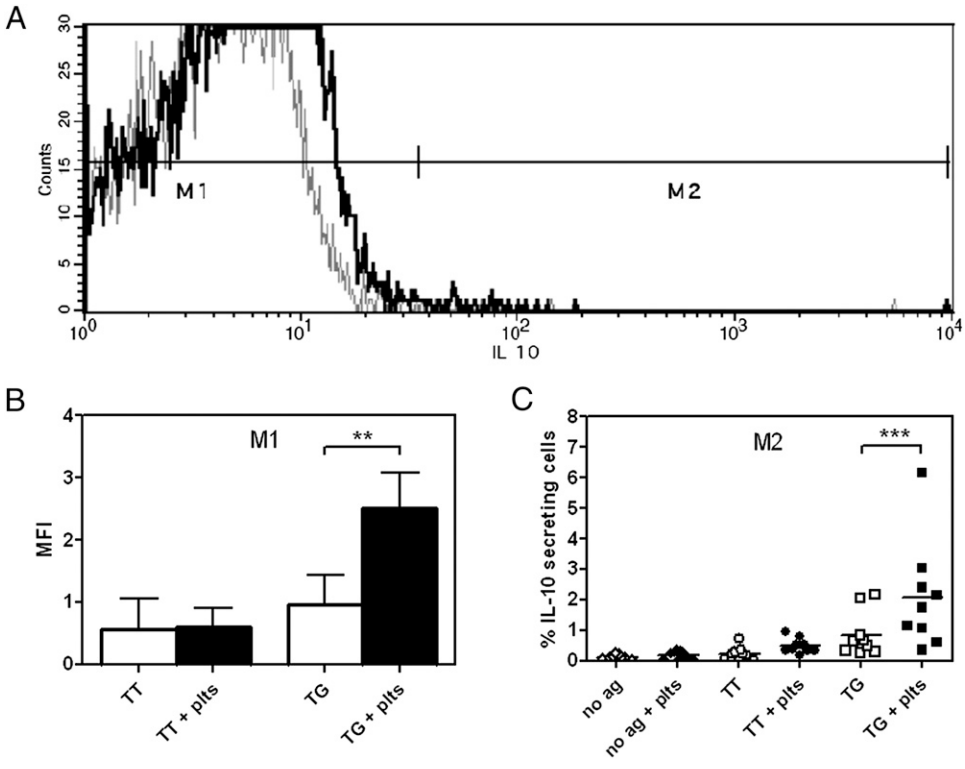


**FIGURE 2.** Influence of activated platelets on IL-10 production by monocytes. PBMCs from nine healthy donors were incubated for 16 h with TT or human TG in the presence or absence of TRAP-activated autologous platelets (plts). The secretion of IL-10 by individual monocytes was measured by flow cytometry 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 SEM, $^{**}p < 0.01$. (**C**) The percentage of monocytes within the M2 fraction is shown as individual observations. Horizontal lines represent mean values. $^{***}p < 0.001$.

allophycocyanin-labeled anti-CD14 Ab, FITC-labeled anti-CD4 Ab, and PerCP-labeled anti-CD61 Ab for 10 min on ice and in the dark. After a final wash, PBMCs were assayed using a FACSCalibur flow cytometer (BD Bioscience) and CellQuest software (Becton Dickinson Immunocytometry System). Th cells were gated as $CD4^{high}CD14^{-}$ cells within a morphological lymphocyte gate. Monocytes were gated as $CD14^{high}CD4^{int}$ cells within a morphological monocyte gate. To exclude the possibility of IL-10 overflow from IL-10–secreting cells to neighbor cells, PBMCs were suspended in 1 and 10 ml buffer during the secretion phase. No detectable differences between subsequent binding of anti–IL-10 detection Ab were observed (data not shown), suggesting that overflow was not a problem at the cell concentration used, as also found by others (22).

### *Assessment of the effects of TGF-β and soluble CD40L on PBMC cytokine production*

Isolated PBMCs, stimulated with LPS, PG, or not stimulated, were cultured in the presence of TRAP-activated autologous platelets, 2 ng/ml recombinant human TGF-β (R&D Systems Europe, Oxon, U.K.) or 10 ng/ml recombinant soluble CD40L (Invitrogen). Supernatants were harvested at day 1, and the cytokine content was measured using the BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit II (BD Bioscience) as described previously (21).

### *Blockade of CXCR3, CD40L, TGF-βR1, IL-10, and TNF-α*

For blockade of CXCR3, PBMCs were preincubated with 2 μg/ml monoclonal anti-CXCR3 Ab (R&D Systems, Minneapolis, MN) for 30 min at 37°C before cultivation as described earlier. For blockade of CD40L, platelets were preincubated for 10 min at 37°C with 10, 40, or 100 μg/ml monoclonal anti-CD40L (R&D Systems). For blockade of TGF-βR1, PBMCs were preincubated with anti–TGF-βR1 (SB525334; Tocris Bioscience, R&D Systems) for 10 min at 37°C. For blockade of IL-10, PBMCs with or without platelets were cultured with 10 μg/ml anti–IL-10 (eBioscience, San Diego, CA). For blockade of TNF-α, PBMCs stimulated with TT or *P. gingivalis* were cultured with 2.5 μg/ml etanercept (Wyeth Europa, Berkshire, U.K.) in the absence of platelets.

### *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-elicited 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-elicited 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*

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

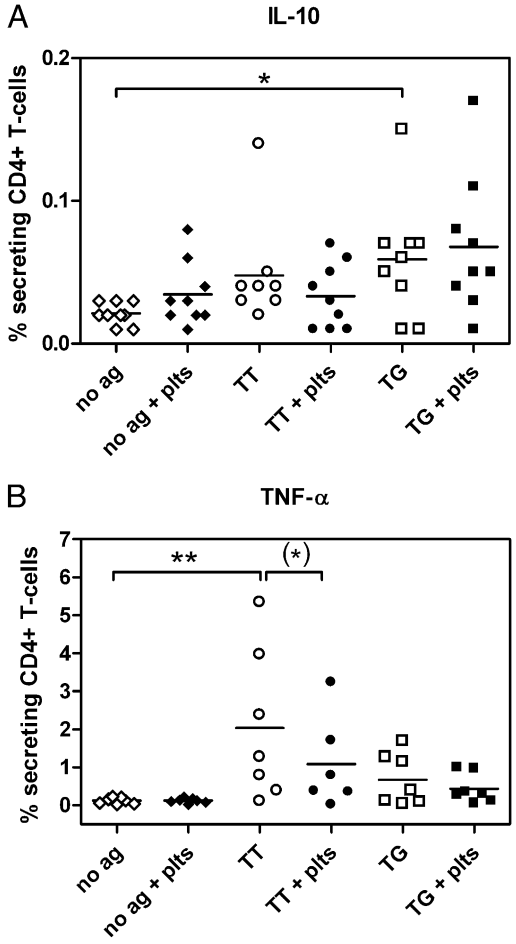


**FIGURE 3.** Proportion of IL-10– or TNF-α–secreting $CD4^+$ T cells in Ag-stimulated PBMC cultures. PBMCs from nine healthy donors were stimulated with TT, human TG, or no Ag (no ag) and cultured for 16 h in the presence (closed symbols) or absence (open symbols) of TRAP-activated autologous platelets (plts). The proportions of $CD4^+$ T cells secreting (**A**) IL-10 or (**B**) 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-secreting 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).

### *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

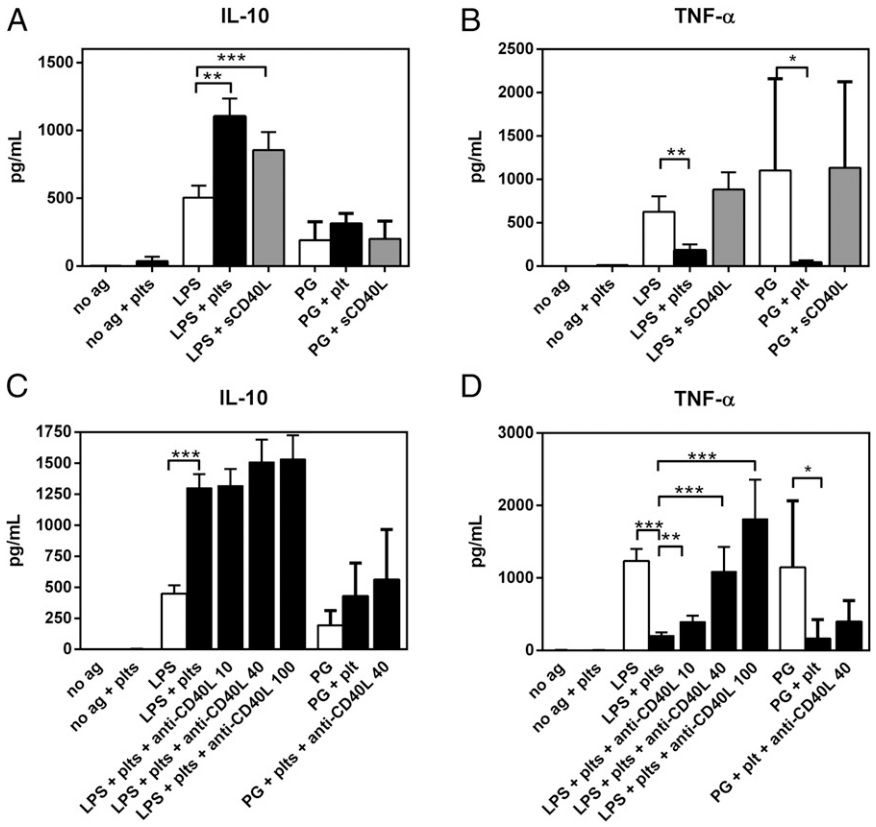


**FIGURE 4.** CD40L-dependent immunoregulation by platelets. PBMCs were incubated with *E. coli.* LPS, intact *P. gingivalis* (PG), or no Ag (no ag) in the presence (closed bars) or absence (open bars) of TRAP-activated autologous platelets (plts). (**A** and **B**) Recombinant sCD40L (shaded bars) was 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. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

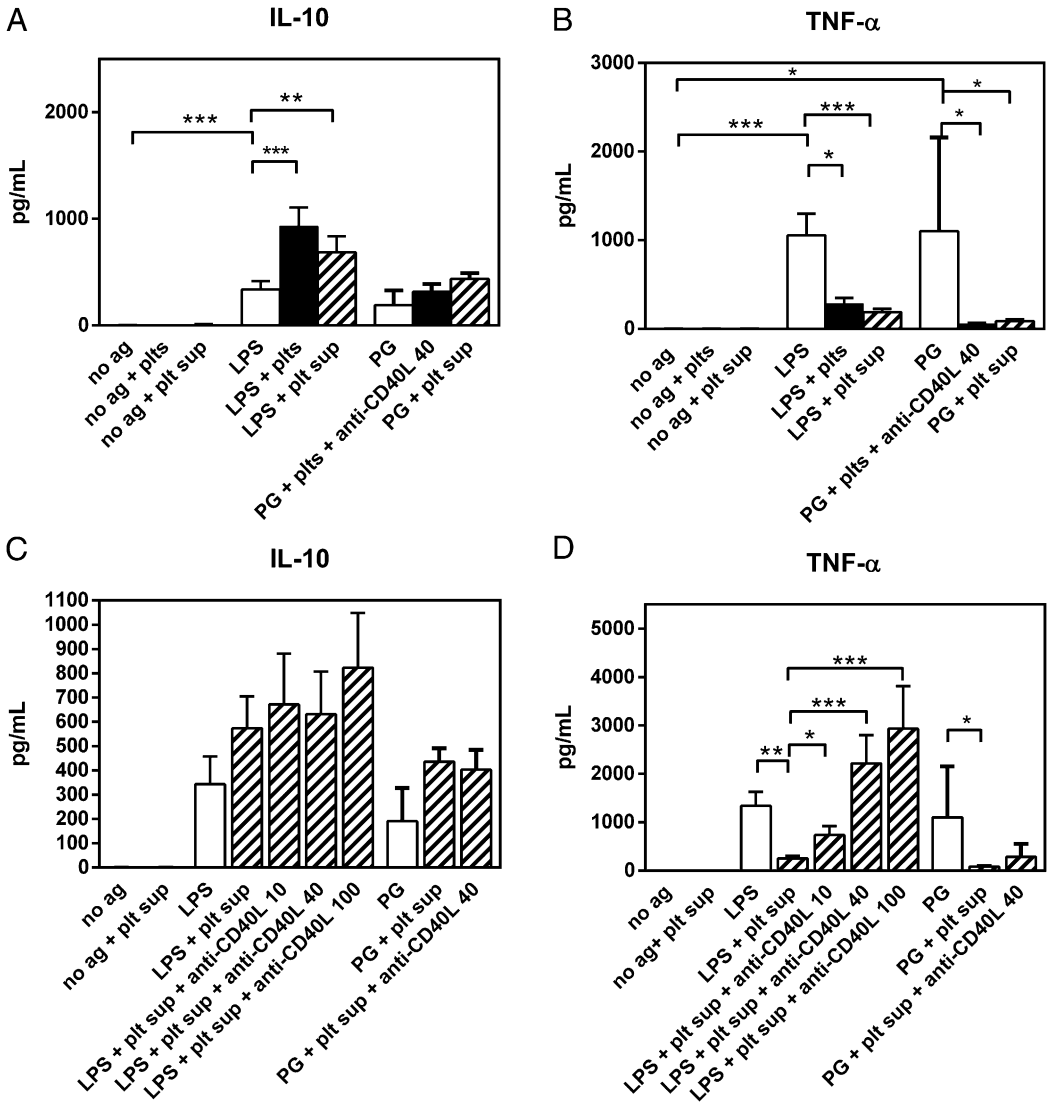


**FIGURE 5.** Influence of soluble platelet factors on LPS-induced PBMC responses. PBMCs were incubated with no Ag (no ag), LPS ($n = 11$), or *P. gingivalis* (PG; $n = 4$), either in the absence of further addition (open bars) or in the presence of TRAP-activated autologous platelets (plts, black bars) or supernatants from TRAP-activated autologous platelets (plt sup, hatched bars). The content of (**A**) IL-10 and (**B**) TNF-α in the cultures after 24 h of incubation is shown as means and SEM ($n = 11$). (**C** and **D**) The corresponding cytokine production in cultures preincubated with CD40L-blocking Ab (0, 10, 40, or 100 μg/ml) before addition of platelet supernatant is shown ($n = 6$ for LPS; $n = 4$ for *P. gingivalis*). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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-

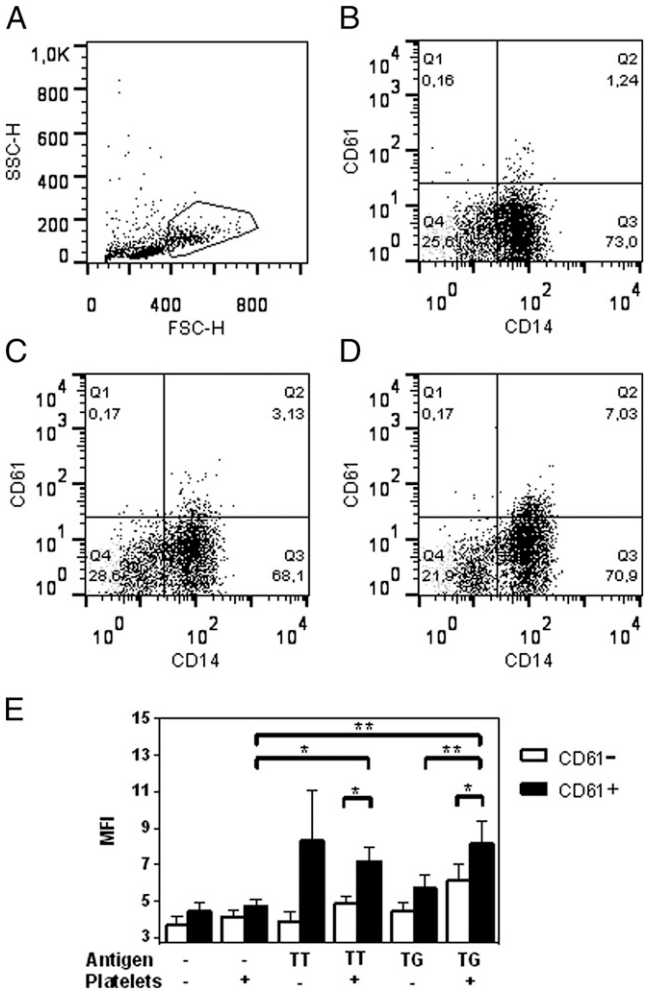


**FIGURE 6.** Adherence of platelets to monocytes. PBMCs were stimulated with TG, TT, or no Ag in the absence or presence of TRAP-activated autologous platelets (plts). After 16 h of incubation, the secretion of IL-10 by $CD14^+CD4^{low/int}$ monocytes was measured by flow cytometry using a bispecific anti-CD45/anti–IL-10 capture reagent. Monocytes with adherent platelets were identified as $CD61^+$ events within this gate. (**A–D**) Representative flow cytometry dot plots showing PBMCs cultured without Ag or platelets (A, B), or stimulated with TG in the absence (C) or presence (D) of TRAP-activated autologous platelets. (A) Identification of monocytes on the basis of forward/side scatter characteristics is illustrated. (B–D) The staining, within this gate, for the monocyte marker CD14 and the platelet marker CD61 and the corresponding quadrant statistics is shown. Monocytes with adherent platelets ($CD14^+CD61^+$ events) are displayed in the upper right quadrants. (**E**) Enhancement of monocyte IL-10 secretion by adhesion of platelets (closed bars) is shown. IL-10 secretion is illustrated as MFI. The *x*-axis intersects the *y*-axis at an MFI of 2.67, which corresponds to the autofluorescence of unmarked cells. Means and SEM of five experiments are shown. $*p < 0.05$, $**p < 0.01$.

ditional experiments, supernatants from activated platelets proved efficient in inhibiting $CD4^+$ T cell proliferation induced by TT or *P. gingivalis* ($p < 0.05$ for both Ags; data not shown).

The inhibitory effect of platelets on $CD4^+$ T cell proliferation could not be abrogated by addition of Abs against IL-10 (Fig. 7B), CD40L (Fig. 7C), or TGF-βR1 (data not shown) to cultures stimulated with *P. gingivalis*. Moreover, the inhibitory effect of platelets on $CD4^+$ T cell proliferation could not be mimicked by addition of rTGF-β to *P. gingivalis*–stimulated cultures without platelets (data not shown).

Although neither IL-10, TGF-β, nor CD40L thus seemed to account for the inhibitory effect of platelets on $CD4^+$ T cell proliferation, TNF-α blockade by etanercept impaired $CD4^+$ 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 PMBC 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 not the likely 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

Table II. Frequencies of monocyte-platelet aggregates defined as the percentage of $CD4^{int}CD14^{+}CD61^{+}$ cells in the PBMC population

| | Mean ± SD, $n = 5$ | | | | | |
|---|---|---|---|---|---|---|
| Stimulating Ag | No Ag | No Ag + Platelets | TT | TT + Platelets | TG | TG + Platelets |
| $CD4^{int}CD14^{+}CD61^{+}$ % of total PBMCs | 1.84 ± 0.73 | 2.82 ± 1.05[a] | 2.45 ± 1.09 | 4.58 ± 4.5[a] | 3.91 ± 2.71 | 7.95 ± 4.24[b] |

[a] $p < 0.05$ (compared with no Ag).
[b] $p < 0.01$ (compared with TG alone or no Ag).

(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

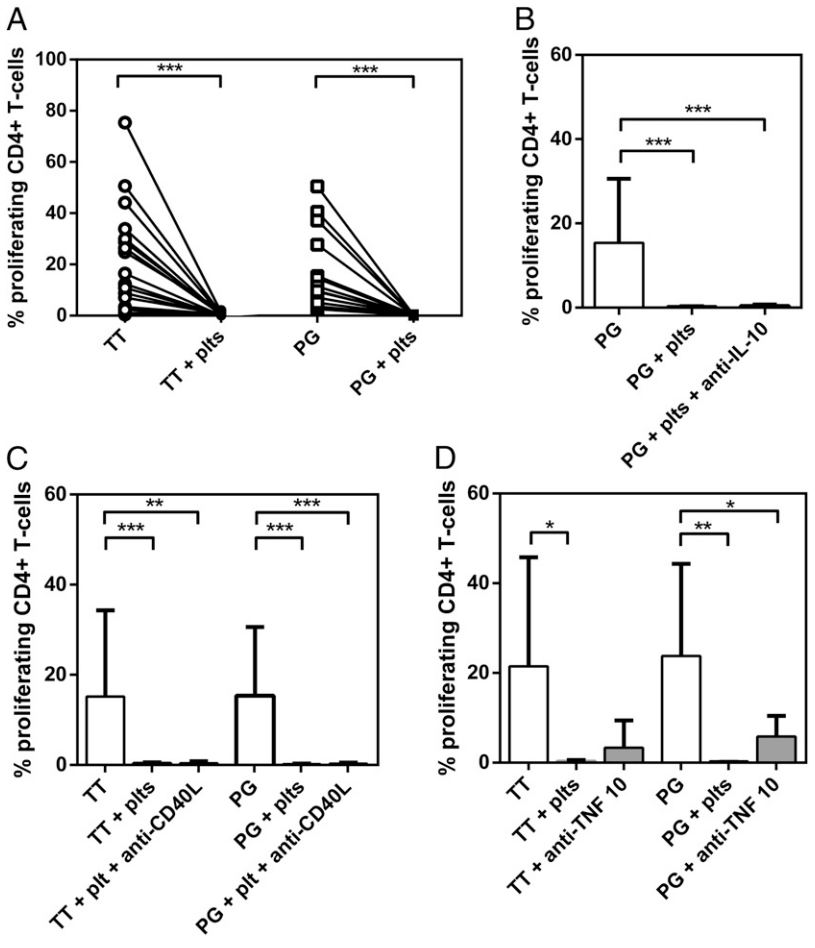


**FIGURE 7.** Influence of platelets on TT- or TG-induced $CD4^{+}$ T cell proliferation. PBMCs from healthy donors were labeled with CFSE and stimulated with TT or *P. gingivalis* (PG) for 7 d in the presence (+ plts, closed symbols) or absence (open symbols) of TRAP-activated autologous platelets. 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. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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, IL-10, 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-elicited 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 Rigshospitalet) for excellent technical assistance in the laboratory, Morten Bagge Hansen (Department of Clinical Immunology, Copenhagen University Hospital Rigshospitalet) 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

1. George, J. N. 2000. Platelets. *Lancet* 355: 1531–1539.
2. Frojmovic, M. M., and J. G. Milton. 1982. Human platelet size, shape, and related functions in health and disease. *Physiol. Rev.* 62: 185–261.
3. Jurk, K., and B. E. Kehrel. 2005. Platelets: physiology and biochemistry. *Semin. Thromb. Hemost.* 31: 381–392.
4. Weyrich, A. S., and G. A. Zimmerman. 2004. Platelets: signaling cells in the immune continuum. *Trends Immunol.* 25: 489–495.
5. Sprague, D. L., B. D. Elzey, S. A. Crist, T. J. Waldschmidt, R. J. Jensen, and T. L. Ratliff. 2008. Platelet-mediated modulation of adaptive immunity: unique delivery of CD154 signal by platelet-derived membrane vesicles. *Blood* 111: 5028–5036.
6. Blair, P., and R. Flaumenhaft. 2009. Platelet alpha-granules: basic biology and clinical correlates. *Blood Rev.* 23: 177–189.
7. von Hundelshausen, P., and C. Weber. 2007. Platelets as immune cells: bridging inflammation and cardiovascular disease. *Circ. Res.* 100: 27–40.
8. Naldini, A., E. Morena, M. Fimiani, G. Campoccia, V. Fossombroni, and F. Carraro. 2008. The effects of autologous platelet gel on inflammatory cytokine response in human peripheral blood mononuclear cells. *Platelets* 19: 268–274.
9. Gleissner, C. A., P. von Hundelshausen, and K. Ley. 2008. Platelet chemokines in vascular disease. *Arterioscler. Thromb. Vasc. Biol.* 28: 1920–1927.
10. Semple, J. W., and J. Freedman. 2010. Platelets and innate immunity. *Cell. Mol. Life Sci.* 67: 499–511.
11. Clark, S. R., A. C. Ma, S. A. Tavener, B. McDonald, Z. Goodarzi, M. M. Kelly, K. D. Patel, S. Chakrabarti, E. McAvoy, G. D. Sinclair, et al. 2007. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat. Med.* 13: 463–469.
12. Hagihara, M., A. Higuchi, N. Tamura, Y. Ueda, K. Hirabayashi, Y. Ikeda, S. Kato, S. Sakamoto, T. Hotta, S. Handa, and S. Goto. 2004. Platelets, after exposure to a high shear stress, induce IL-10-producing, mature dendritic cells in vitro. *J. Immunol.* 172: 5297–5303.
13. Kissel, K., S. Berber, A. Nockher, S. Santoso, G. Bein, and H. Hackstein. 2006. Human platelets target dendritic cell differentiation and production of proinflammatory cytokines. *Transfusion* 46: 818–827.
14. Wang, Y., and J. Niu. 2008. Platelets inhibit in vitro response of lymphocytes to mitogens. *Immunol. Lett.* 119: 57–61.
15. Elzey, B. D., J. Tian, R. J. Jensen, A. K. Swanson, J. R. Lees, S. R. Lentz, C. S. Stein, B. Nieswandt, Y. Wang, B. L. Davidson, and T. L. Ratliff. 2003. Platelet-mediated modulation of adaptive immunity. A communication link between innate and adaptive immune compartments. *Immunity* 19: 9–19.
16. Elzey, B. D., J. F. Grant, H. W. Sinn, B. Nieswandt, T. J. Waldschmidt, and T. L. Ratliff. 2005. Cooperation between platelet-derived CD154 and CD4+ T cells for enhanced germinal center formation. *J. Leukoc. Biol.* 78: 80–84.
17. Gerdes, N., L. Zhu, M. Ersoy, A. Hermansson, P. Hjemdahl, H. Hu, G. K. Hansson, and N. Li. 2011. Platelets regulate $CD4^+$ T-cell differentiation via multiple chemokines in humans. *Thromb. Haemost.* 106: 353–362.
18. Fleischer, J., E. Grage-Griebenow, B. Kasper, H. Heine, M. Ernst, E. Brandt, H. D. Flad, and F. Petersen. 2002. Platelet factor 4 inhibits proliferation and cytokine release of activated human T cells. *J. Immunol.* 169: 770–777.
19. Ford, T. C., and D. Rickwood. 1990. A new one-step method for the isolation of human mononuclear cells. *J. Immunol. Methods* 134: 237–241.
20. Nielsen, C. H., R. G. Leslie, B. S. Jepsen, M. D. Kazatchkine, S. V. Kaveri, and E. Fischer. 2001. Natural autoantibodies and complement promote the uptake of a self antigen, human thyroglobulin, by B cells and the proliferation of thyroglobulin-reactive CD4(+) T cells in healthy individuals. *Eur. J. Immunol.* 31: 2660–2668.
21. Nielsen, C. H., L. Hegedüs, K. Rieneck, A. C. Moeller, R. G. Leslie, and K. Bendtzen. 2007. Production of interleukin (IL)-5 and IL-10 accompanies T helper cell type 1 (Th1) cytokine responses to a major thyroid self-antigen, thyroglobulin, in health and autoimmune thyroid disease. *Clin. Exp. Immunol.* 147: 287–295.
22. Assenmacher, M., M. Lohning, and A. Radbruch. 2002. Detection and isolation of cytokine secreting cells using the cytometric cytokine secretion assay. *Curr. Protoc. Immunol.* Chapter 6: Unit 6.27.
23. Byrne, A., and D. J. Reen. 2002. Lipopolysaccharide induces rapid production of IL-10 by monocytes in the presence of apoptotic neutrophils. *J. Immunol.* 168: 1968–1977.
24. Nielsen, C. H., M. P. Galdiers, C. J. Hedegaard, and R. G. Leslie. 2010. The self-antigen, thyroglobulin, induces antigen-experienced CD4+ T cells from healthy donors to proliferate and promote production of the regulatory cytokine, interleukin-10, by monocytes. *Immunology* 129: 291–299.
25. Solanilla, A., J. M. Pasquet, J. F. Viallard, C. Contin, C. Grosset, J. Déchanet-Merville, M. Dupouy, M. Landry, F. Belloc, P. Nurden, et al. 2005. Platelet-associated CD154 in immune thrombocytopenic purpura. *Blood* 105: 215–218.
26. Pircher, R., P. Jullien, and D. A. Lawrence. 1986. Beta-transforming growth factor is stored in human blood platelets as a latent high molecular weight complex. *Biochem. Biophys. Res. Commun.* 136: 30–37.
27. Grainger, D. J., D. E. Mosedale, and J. C. Metcalfe. 2000. TGF-beta in blood: a complex problem. *Cytokine Growth Factor Rev.* 11: 133–145.
28. Bruserud, O. 2013. Bidirectional crosstalk between platelets and monocytes initiated by Toll-like receptor: an important step in the early defense against fungal infections? *Platelets* 24: 85–97.
29. Furie, B., and B. C. Furie. 1995. The molecular basis of platelet and endothelial cell interaction with neutrophils and monocytes: role of P-selectin and the P-selectin ligand, PSGL-1. *Thromb. Haemost.* 74: 224–227.
30. Miner, J. J., L. Xia, T. Yago, J. Kappelmayer, Z. Liu, A. G. Klopocki, B. Shao, J. M. McDaniel, H. Setiadi, D. W. Schmidtke, and R. P. McEver. 2008. Separable requirements for cytoplasmic domain of PSGL-1 in leukocyte rolling and signaling under flow. *Blood* 112: 2035–2045.
31. Rinder, H. M., J. L. Bonan, C. S. Rinder, K. A. Ault, and B. R. Smith. 1991. Activated and unactivated platelet adhesion to monocytes and neutrophils. *Blood* 78: 1760–1769.
32. Smyth, S. S., R. P. McEver, A. S. Weyrich, C. N. Morrell, M. R. Hoffman, G. M. Arepally, P. A. French, H. L. Dauerman, and R. C. Becker; 2009 Platelet Colloquium Participants. 2009. Platelet functions beyond hemostasis. *J. Thromb. Haemost.* 7: 1759–1766.
33. Stinchcombe, J. C., and G. M. Griffiths. 2007. Secretory mechanisms in cell-mediated cytotoxicity. *Annu. Rev. Cell Dev. Biol.* 23: 495–517.
34. Chen, Y., J. Chen, Y. Xiong, Q. Da, Y. Xu, X. Jiang, and H. Tang. 2006. Internalization of CD40 regulates its signal transduction in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 345: 106–117.
35. Xia, C. Q., and K. J. Kao. 2003. Effect of CXC chemokine platelet factor 4 on differentiation and function of monocyte-derived dendritic cells. *Int. Immunol.* 15: 1007–1015.
36. Thaher, F., S. Plankenhorn, and R. Klein. 2011. Differential effects of the tumor necrosis factor alpha-blocker infliximab and etanercept on immunocompetent cells *in vitro*. *Int. Immunopharmacol.* 11: 1724–1731.
37. Brown, G. R., E. L. Lee, and D. L. Thiele. 2003. TNF enhances CD4+ T cell alloproliferation, IFN-gamma responses, and intestinal graft-versus-host disease by IL-12-independent mechanisms. *J. Immunol.* 170: 5082–5088.
38. Naldini, A., E. Morena, I. Filippi, A. Pucci, M. Bucci, G. Cirino, and F. Carraro. 2006. Thrombin inhibits IFN-gamma production in human peripheral blood mononuclear cells by promoting a Th2 profile. *J. Interferon Cytokine Res.* 26: 793–799.
39. Fujimi, S., M. P. MacConmara, A. A. Maung, Y. Zang, J. A. Mannick, J. A. Lederer, and P. H. Lapchak. 2006. Platelet depletion in mice increases mortality after thermal injury. *Blood* 107: 4399–4406.