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CUTTING EDGE

Cutting Edge: T Cells Trigger CD40-Dependent Platelet Activation and Granular RANTES Release: A Novel Pathway for Immune Response Amplification[1]

Silvio Danese,* Carol de la Motte,[†] Brenda M. Rivera Reyes,* Miquel Sans,* Alan D. Levine,* and Claudio Fiocchi[2]*

Platelets, in addition to exerting hemostatic activity, contribute to immunity and inflammation. The recent report that platelets express CD40 led us to hypothesize that CD40 ligand (CD40L)-positive T cells could bind to platelets, cause their activation, and trigger granular RANTES release, creating a T cell recruitment feedback loop. Platelets were cocultured with resting or activated autologous T cells and their activation was assessed by P-selectin expression. RANTES binding to endothelial cells was assessed by confocal microscopy, and its biological activity was demonstrated by a T cell adhesion assay. CD40L-positive T cells induced platelet activation through a contact-mediated, CD40-dependent pathway resulting in RANTES release, which bound to endothelial cells and mediated T cell recruitment. Soluble CD40L induced the same events via p38, but not extracellular signal-regulated kinase, phosphorylation. These results show the existence of a novel platelet-dependent pathway of immune response amplification which brings these non-immune cells close to the level of pathogenic relevance traditionally attributed to classical immune cells.   The Journal of Immunology, 2004, 172: 2011–2015.

R ecent evidence has consolidated the notion that platelets, beside their hemostatic activity, also function as cells that promote immunity and inflammation (1, 2). The proinflammatory activity of platelets occurs through multiple mechanisms, including receptor-mediated cross-talk with and activation of different cells, as well as release of potent biologically active mediators stored in their granules. This interaction is bidirectional as the activated cells in turn activate platelets through a number of receptor-ligand systems (1, 3). The CD40/CD40 ligand (CD40L)[3] pathway plays a particularly important role because of its wide distribution and multiplicity

of actions (4, 5), which go well beyond its postulated function in adaptive immunity (6, 7). At sites of immune reactivity, the CD40/CD40L pathway is invariably activated as shown by the presence of abundant CD40- and CD40L-positive cells, as found in chronic inflammatory and autoimmune disorders (8, 9). This is why blockade of CD40-CD40L interactions is currently under investigation as a new anti-inflammatory therapy (8, 10). CD40 is constitutively expressed on the surface of multiple immune and nonimmune cells, whereas CD40L is expressed predominantly by activated T cells (5). Recently, CD40 identical to the one found on PBMC has been detected on the surface of platelets (11, 12). Because this makes platelets susceptible to stimulation through CD40, we hypothesized that CD40L-positive T cells could directly bind to platelets, activate them, and trigger granular RANTES release, creating a T cell recruitment feedback loop able to amplify the immune response.

Materials and Methods

Reagents and Abs

FITC-conjugated anti-CD40L, PE-conjugated anti-P-selectin (CD62P), PerCP-conjugated anti-CD42a, and FITC- and PE-conjugated mouse IgG1 isotype control were purchased from BD PharMingen (San Jose, CA); PE-conjugated anti-CD3 from DAKO (Carpinteria, CA), anti-CD40 from BD-PharMingen (San Diego, CA), thrombin from Sigma-Aldrich (St. Louis, MO), and the p38 inhibitor SB203580 from Calbiochem (La Jolla, CA). RANTES ELISA kits and human recombinant RANTES were purchased from R&D Systems (Minneapolis, MN) and phospho-p38 and phospho-extracellular signal-regulated kinase (ERK) Abs from Cell Signaling (Beverly, MA). Polystyrene beads coated with murine mAbs to human CD3 and CD28 were obtained from Dynal (Lake Success, NY). Trimeric soluble-CD40L (sCD40L), Abs against CD40 (M2 and M3) and CD40L (M90) were provided by Immunex (Seattle, WA), and the RANTES receptor antagonist met-RANTES by Dr. A. Proudfoot (Serono Pharmaceuticals, Geneva, Switzerland).

Platelet-T cell cocultures

Platelets were isolated from blood of healthy donors, resulting in a >99% pure population as assessed by expression of CD42b (13). Peripheral blood T cells (PBT) were cultured in RPMI 1640 with 10% FBS and activated for 16 h with

IL-2 and anti-CD3 plus anti-CD28 Abs to induce CD40L (14). Resting platelets were mixed with various ratios of resting or activated autologous PBT, CD40L-negative Jurkat, or CD40L-positive D1.1 cells. In some experiments, T cells were fixed in 1% paraformaldehyde. In other experiments, platelets or T cells were preincubated with 10 μg/ml M2, M3, or M90 blocking Abs or 10 μg/ml SB203580. Platelet-T cell adhesion was assessed by flow cytometry measuring the concomitant expression of CD3 and CD42b in the aggregates. After 30 min at 37°C, cocultured cells were fixed in 1% paraformaldehyde and stained for flow cytometry (13). As a control, platelets were stimulated with sCD40L (0.01, 0.1, 1.0, and 10 μg/ml) or thrombin (0.5 U/ml) for 30 min at 37°C.

Flow cytometric analysis

For CD40, CD62P, and CD42b expression, platelets were fixed in 1% paraformaldehyde, stained with the appropriate Ab, examined by a Coulter Epics XL Flow Cytometer (Beckman Coulter, Fullerton, CA), and results were analyzed using Winlist (Verity Software House, Topsham, ME). For CD40L or CD3 expression, T cells were stained with FITC-conjugated anti-CD40L or PE-conjugated anti-CD3, respectively (13).

Assessment of phosphorylated p38 and ERK mitogen-activated protein (MAP) kinases and RANTES granules release

Induction of phosphorylated p38 and ERK MAP kinases was assessed by Western blotting using phospho-specific Abs. RANTES was measured in supernatants of paraformaldehyde-fixed platelets cocultured with T cells or sCD40L-stimulated platelets using a commercial ELISA (15).

Binding of platelet-derived RANTES to endothelial cells and T cell adhesion assay

Cell-free supernatants from degranulated platelets or recombinant RANTES (0.5 μg/ml) were added to human intestinal microvascular endothelial cell (HIMEC) monolayers for 60 min at 37°C (16). After washing, RANTES was detected on the HIMEC surface by flow cytometry and confocal microscopy (17).

MOLT4 T cells were used for the adhesion assay (16). Confluent HIMEC monolayers were left alone or stimulated with cell-free supernatants from 100×10^6 activated platelets or 0.5 μg/ml recombinant RANTES. In some experiments, MOLT4 cells were preincubated for 30 min at 37°C with 1 μg/ml met-RANTES. After 1 h, calcein (Molecular Probes, Eugene, OR)-labeled MOLT4 cells were overlaid and 1 h later nonadherent cells where removed by gentle washings with PBS. Adherent cells in duplicate wells were quantified using Image Pro Plus (Media Cybernetics, Silver Spring, MD) connected to a digital camera (Optronics, Olympus, Tokyo, Japan) and results were expressed as adherent cells per square millimeter.

Statistical analysis

Data were analyzed by GraphPad (San Diego, CA) using the Student t or ANOVA tests followed by the appropriate post hoc test and expressed as mean ± SEM. Significance was set at $p < 0.05$.
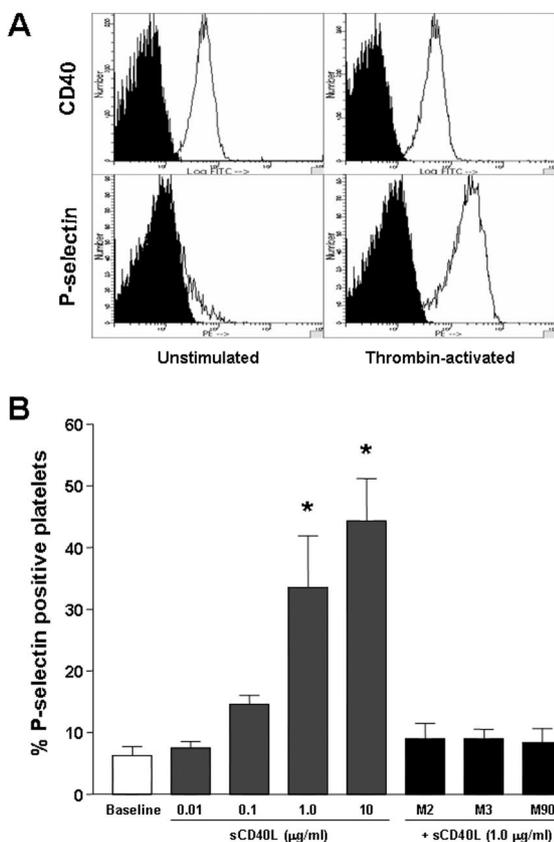

FIGURE 1. sCD40L-induced platelet activation through constitutively expressed CD40. *A,* Flow cytometric analysis of CD40 and P-selectin surface expression by resting and activated platelets. Platelets were left untreated or activated with thrombin for different time periods (2, 5, 10, 15, and 30 min), after which they were stained with the appropriate mAb. The black curve represents the background signal from the isotype control. This figure is representative of five separate experiments (30 min). *B,* Induction of platelet P-selectin expression by sCD40L stimulation. Platelets were stimulated with increasing concentration of sCD40L and stained for P-selectin expression. Data represent the mean ± SEM of five separate experiments. *, $p < 0.001$ for sCD40L-stimulated compared with unstimulated platelets.
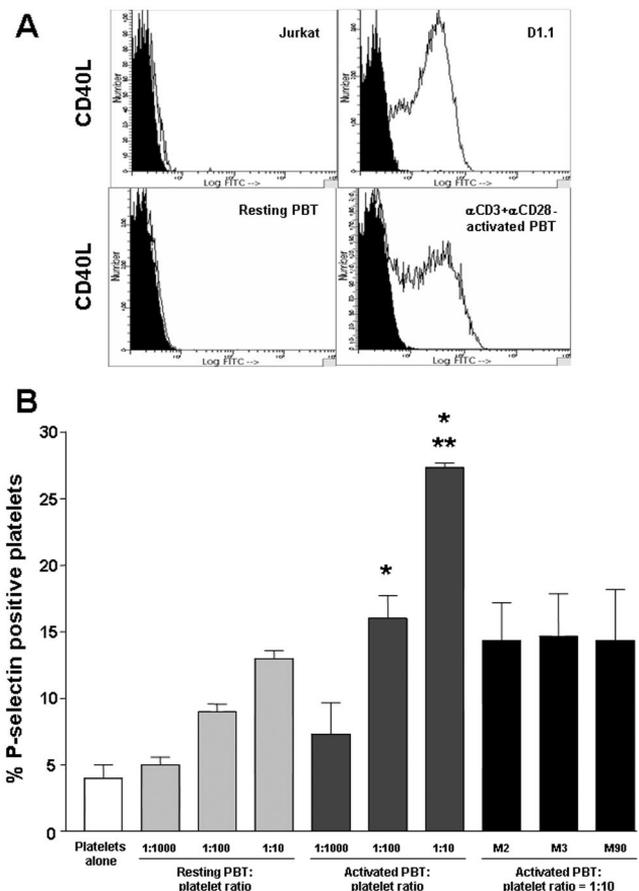
FIGURE 2. Platelet activation by CD40L-positive T cells. *A,* Flow cytometric analysis showing constitutive and CD3+CD28-induced expression of CD40L by D1.1 cells and PBT, respectively. This figure is representative of four separate experiments. *B,* Up-regulation of platelet P-selectin expression by T cell membrane-bound CD40L. Increasing ratios of resting and activated T cells were cocultured with a fixed number of autologous platelets, and platelet P-selectin expression was assessed by flow cytometry. Data are mean ± SEM of four separate experiments. *, $p < 0.05$ for activated vs resting PBT (1:100) and for activated vs blocking Ab-treated PBT (1:10); **, $p < 0.01$ for activated vs resting PBT (1:10).

Results and Discussion

Constitutively expressed surface CD40 mediates platelet activation

We initially investigated whether activation could modulate the constitutive expression of CD40 on the surface of platelets. Stimulation with thrombin for up to 30 min revealed that, in contrast to the marked up-regulation of P-selectin, the expression of CD40 remained constant as demonstrated by flow cytometric analysis (Fig. 1A). These results were confirmed using two different Abs (M2 and M3) and by Western blot analysis (data not shown).

To assess whether engagement of surface CD40 could promote activation, platelets were exposed to escalating doses of sCD40L and their activation state was monitored by P-selectin expression. Flow cytometric analysis revealed a dose-dependent increase in the number of P-selectin-positive platelets which was completely abrogated by blockade of either CD40 with M2 or M3 or CD40L with M90 (Fig. 1B). None of these blocking Abs altered baseline or thrombin-induced P-selectin expression levels (data not shown).

CD40L-positive T cells induce RANTES release by activated platelets

Having demonstrated that the platelet CD40 receptor is functional, we next investigated whether direct binding by CD40L-bearing T cells, as it may happen in immune-mediated or inflammatory conditions, could induce platelet activation. For this purpose, we cocultured platelets with different numbers of autologous resting or activated PBT expressing CD40L induced by anti-CD3 plus anti-CD28 stimulation. D1.1 and Jurkat cells were used as controls (Fig. 2A). As measured by P-selectin expression, resting PBT induced moderate, dose-de-

pendent platelet activation, whereas CD40L-positive T cells triggered a significantly higher degree of activation (Fig. 2B). The effect of activated T cells was inhibited by blocking CD40 on platelets or CD40L on T cells. In cocultures with activated PBT, the number of platelet-T cell aggregates was three times higher than that present in cocultures with resting PBT and decreased by 40–50% when M2, M3 (anti-CD40), or M90 (anti-CD40L) blocking Abs were added. CD40L-positive D1.1 cells mimicked the P-selectin up-regulation effect of activated PBT, whereas CD40L-negative Jurkat cells reproduced the results obtained with resting PBT (data not shown). The same results were also obtained when T cells were prefixed in 1% paraformaldehyde (data not shown).

Up-regulation of P-selectin is not merely a phenotypic marker of activation but also implies the functional release of platelets' granular stores (1). Among multiple mediators, stores are particularly rich in RANTES, a chemokine critical for recruitment of T cells at sites of inflammation (18, 19). As shown
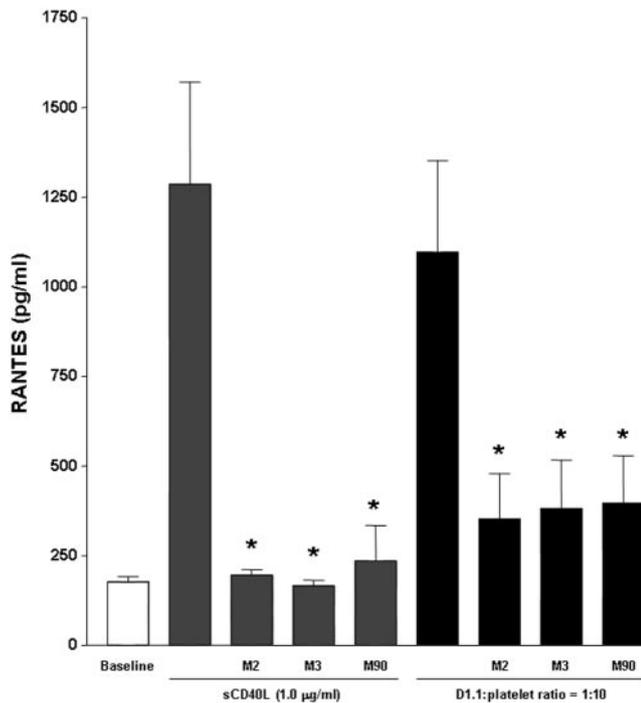

FIGURE 4. Involvement of p38 MAP kinase in platelet CD40 signaling. *A,* Immunoblotting of CD40-induced p38 phosphorylation in sCD40L-stimulated platelets. Platelets were stimulated with sCD40L and then lysed at the indicated time points. Immunoblotting was performed with Abs specific for phosphorylated p38 and ERK. This figure is representative of three separate experiments. *B,* Inhibition of CD40-dependent platelet activation by SB203580. Platelets were activated with sCD40L with or without pretreatment with the p38 MAP kinase inhibitor SB203580 and then submitted to flow cytometric analysis for P-selectin expression. The black curve represents the background signal from the isotype control. The presence of DMSO, used for SB203580 dilution, had no effect on P-selectin expression. Data represent the mean ± SEM of three separate experiments. *, $p < 0.01$ for inhibitor-treated compared with sCD40L-stimulated platelets. *C,* Inhibition of CD40-dependent RANTES release by SB203580. Platelets were stimulated with sCD40L in the presence and absence of SB203580, and RANTES content in the supernatants was measured by ELISA. Data are mean ± SEM of three separate experiments. *, $p < 0.01$ for inhibitor-treated compared with sCD40L-stimulated platelets.

FIGURE 3. CD40-dependent RANTES release by platelets. RANTES release by soluble and membrane-bound CD40-stimulated platelets. Platelets were stimulated either with sCD40L or paraformaldehyde-fixed D1.1 cells (1:10), and RANTES content in the supernatants was measured by ELISA. Data are mean ± SEM of three separate experiments. *, $p < 0.01$ for sCD40L- and D1.1 cell-stimulated platelets vs blocking Ab treatment.
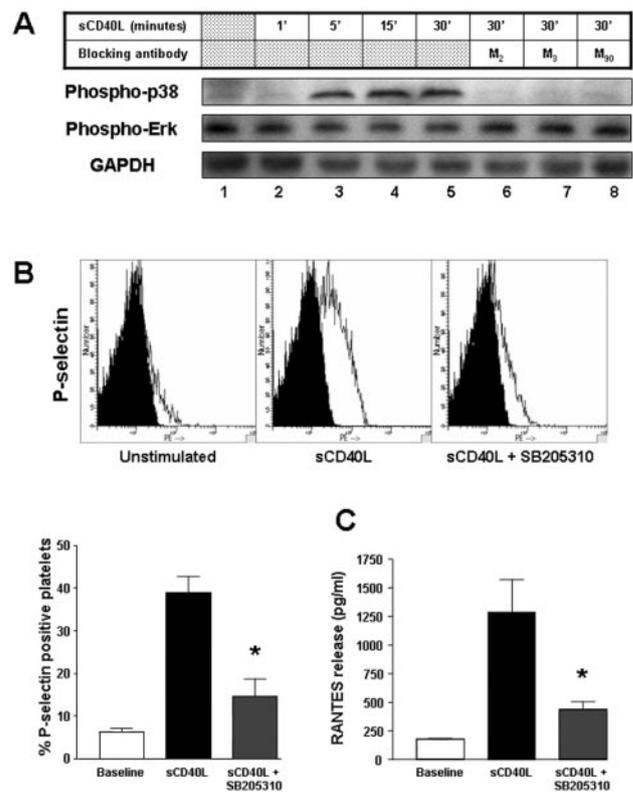
in Fig. 3, large amounts of platelet-derived RANTES were released by both soluble and paraformaldehyde-fixed D1.1 membrane-bound CD40L, an effect that was abrogated by CD40 or CD40L blockade.

Since platelet CD40 expression is constitutively stable, the degree of T cell activation appears to control the platelet activation and degranulation process. In addition, since paraformaldehyde-fixed T cells were just as effective as live cells in inducing activation, direct cell-cell contact through membrane-bound CD40L is clearly sufficient to trigger platelet activation independently on the release of its soluble form by T cells. In vivo, at sites of active vascular inflammation, such as the atherosclerotic plaque or the mucosa of inflammatory bowel diseases (14, 20), CD40L-positive cells are abundant and may stimulate platelets through both cell-cell contact and the release of sCD40L, a synergistic effect that can potentiate tissue injury.

Critical involvement of p38 MAP kinase in RANTES release by CD40-activated platelets

It has been recently reported that the activation of platelets through the CD40 pathway is calcium independent (12), but no information exists on what signaling molecules are triggered downstream of the receptor. Since MAP kinases are involved in CD40-activated immune and nonimmune cells (21), we investigated the possible phosphorylation of p38 and ERK in platelets upon sCD40L ligation. Phosphorylated p38 was undetectable in resting platelets, but its induction was obvious in sCD40L-treated platelets and completely prevented by exposure to CD40 (M2 and M3) or CD40L (M90) Abs (Fig. 4A). In contrast, resting platelets exhibited a constitutive level of ERK phosphorylation which failed to be up-regulated by CD40-mediated signaling or inhibited by blocking Abs. The selectivity of this differential signaling was confirmed when thrombin was used instead of sCD40L, resulting in the expected up-regulation of both p38 and ERK phosphorylation (data not shown).

To corroborate the involvement of p38 in CD40-dependent platelet activation, the selective inhibitor SB203580 was used. A marked inhibition of P-selectin up-regulation was observed ($p < 0.001$; Fig. 4B), pointing to a key role of p38 in platelet activation through CD40 engagement. Specificity was further confirmed by lack of inhibition of P-selectin up-regulation by SB203580 when thrombin was used (data not shown).

Based of these results, we investigated whether blocking p38 could prevent RANTES release. As shown in Fig. 4C, SB205380 markedly suppressed the release of RANTES by sCD40L-stimulated platelets. MAP kinases play a key role in multiple cell types involved in immune and inflammatory responses, and their inhibition is increasingly being considered as a valid therapeutic approach (22). In this regard, the novel identification of p38 as an essential signal transduction molecule in CD40-dependent platelet activation offers a potential new strategy to block platelet proinflammatory effects.

Platelet-derived RANTES enhances T cell recruitment

Leukocyte recruitment at sites of inflammation is crucially dependent on chemokine presentation by local microvascular endothelial cells (19). We investigated whether platelet-derived RANTES could be immobilized on the surface of HIMEC and mediate T cell retention. Confocal microscopy revealed that after exposure to activated platelet-derived supernatants RANTES was readily detected on their surface, similarly to recombinant RANTES (Fig. 5A). Results were confirmed by flow cytometry (data not shown). We then tested whether RANTES released by platelets was able to mediate recruitment of T cells. Few MOLT4 cells bound to unstimulated HIMEC, but their number significantly ($p < 0.01$) increased after HIMEC exposure to activated platelet-derived or recombinant RANTES. Pretreatment of T cells with met-RANTES, a RANTES receptor antagonist, markedly reduced ($p < 0.05$) the number of adherent T cells (Fig. 5B).
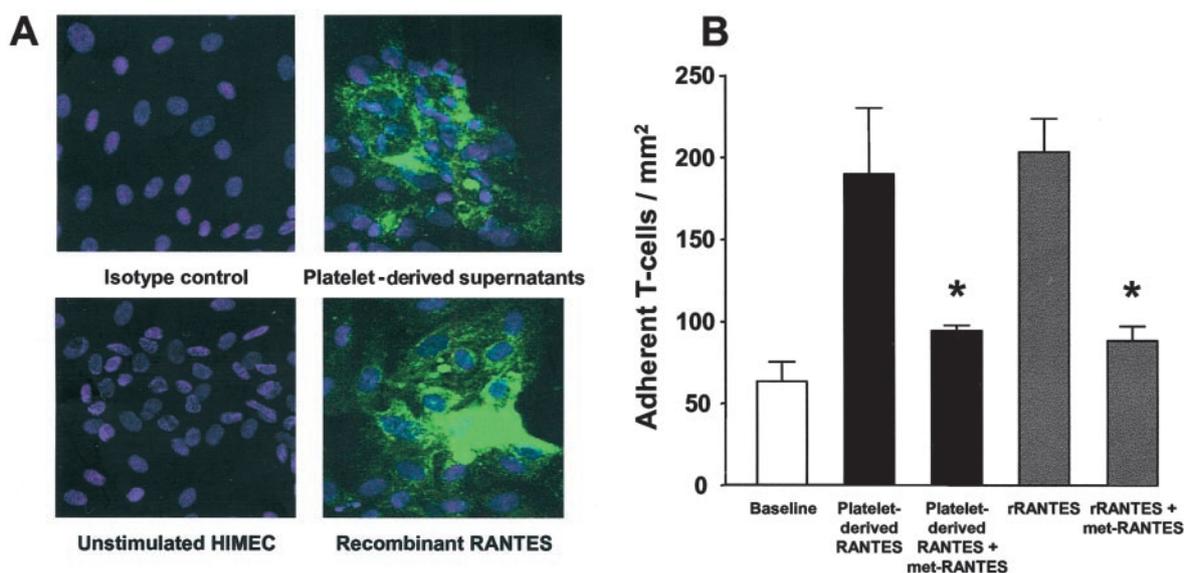

FIGURE 5. Platelet-derived RANTES mediates increased T cell recruitment. *A,* Detection of platelet-derived and recombinant RANTES on the surface of HIMEC by confocal microscopy. Confluent HIMEC monolayers were exposed to degranulated platelet-derived or recombinant (r) RANTES and fluorescently labeled for detection of RANTES (Alexa 488 secondary Ab, green) and nuclei (DAPI, blue). This figure is representative of four separate experiments. *B,* Platelet-derived RANTES-mediated T cell adhesion to HIMEC and inhibition by met-RANTES. HIMEC monolayers were left untreated (baseline) or exposed to degranulated platelet-derived or recombinant RANTES. MOLT4 cells, preincubated or not with met-RANTES, were added to the HIMEC monolayers. The number of adherent cells in each experimental condition was expressed as mean ± SEM of four separate experiments. *, $p < 0.05$ for met-RANTES-pretreated compared with untreated MOLT4 cells.

RANTES, a CC chemokine for memory T cells and monocytes (18), when produced by extravascular cells, needs to reach the lumenal surface through transcytosis to directly elicit leukocyte migration from the blood (23). In contrast, when derived from intravascular cells, as in the case of platelets, RANTES can instantly bind endothelial surfaces and promptly induce leukocyte retention (24). Considering their number and their ability to swiftly release massive amounts of preformed RANTES, platelets may mediate a first line and highly effective mechanism for leukocyte recruitment, particularly under inflammatory conditions, when both their activation state and number are greatly increased (1).

To the best of our knowledge, this is the first study demonstrating that activated T cells can stimulate platelets directly, resulting in CD40-mediated stimulation. Platelets are first activated through direct contact with T cells and, through the release of RANTES, secondarily recruit more T cells that lead to further platelet activation, creating an amplification loop that promotes leukocyte recruitment to sites of immune reactivity. Moreover, since P-selectin is up-regulated in activated platelets and facilitates binding to T cells constitutively expressing P-selectin glycoprotein ligand 1 (25), this could further contribute to intensification and maintenance of immune and inflammatory responses. These results reinforce the contribution of this unique cell type in immunity, bringing platelets close to the level of pathogenic relevance traditionally attributed to classical immune cells.

Acknowledgments

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