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Platelet-Mediated Modulation of Adaptive Immunity: A Communication Link between Innate and Adaptive Immune Compartments

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Summary

Platelets are highly reactive components of the circulatory system with well-documented hemostatic function. Recent studies extend platelet function to modulation of local inflammatory events through the release of chemokines, cytokines, and a number of immunomodulatory ligands, including CD154. We hypothesized that platelet-derived CD154 modulates adaptive immunity. The data reported herein demonstrate that platelets, via CD154, induce dendritic cell maturation, B cell isotype switching, and augment $CD8^+$ T cell responses both in vitro and in vivo. Platelet transfusion studies demonstrate that platelet-derived CD154 alone is sufficient to induce isotype switching and augment T lymphocyte function during viral infection, leading to enhanced protection against viral rechallenge. Additionally, depletion of platelets in normal mice results in decreased antigen-specific antibody production.

Introduction

An innate immune response is often sufficient for pathogen clearance. Frequently, however, adaptive immunity must be initiated to control and resolve infection. The initial response to a challenge by pathogens is inflammation, which involves recruitment and activation of neutrophils, monocytes, and macrophages that in turn release, or provoke, endothelial/epithelial cell release of cytokines, chemokines, and vasoactive substances that further enhance inflammation and leukocyte extravasation (Moser and Loetscher, 2001; Lo et al., 1999). An important consequence of the initial innate inflammatory response is the stimulation of effective antigen presentation by dendritic cells (DC). Maturation of DC is central to the development of adaptive immunity to invading pathogens (Gallucci and Matzinger, 2001), attributed to the observation that DC are uniquely able to activate naive T cells. Through CD154 expression, activated $CD4^+$ T cells provide the signal required for the differentiation of T cell-dependent B lymphocyte responses, a process that includes isotype switching (Yang and Wilson, 1996; Renshaw et al., 1994; Caux et al., 1994). Additionally, CD154 expression on activated $CD4^+$ T cells augments the DC maturation process, a requirement for cross priming and enhancing cytotoxic $CD8^+$ T cell responses induced through the classical pathway (Ridge et al., 1998; Schoenberger et al., 1998; Bennett et al., 1998).

Platelets, upon activation, have been demonstrated to play a large role in initiation and perpetuation of inflammatory responses (reviews by Klinger, 1997; Yeaman, 1997). Recently, platelet expression of CD154 and other immunomodulatory factors was reported, contributing to an expanded understanding of platelet function (Henn et al., 1998, 2001; Ahmad et al., 2001; Diacovo et al., 1994; Bombeli et al., 1998). It is now clear that activated platelets not only function in hemostasis and coagulation but also modulate local inflammatory responses, including contact sensitivity, inflammatory bowel disease, and atherosclerosis through the interaction of platelet-derived immunomodulatory ligands with endothelial cells and infiltrating leukocytes (Henn et al., 1998, 2001; Ahmad et al., 2001; Diacovo et al., 1994, 1998; Bombeli et al., 1998; Geba et al., 1996; Collins et al., 1994; Gawaz et al., 2000; Barry et al., 1998). Given the established importance of CD154 in T and B lymphocyte immunity, we hypothesized that platelets also perform a sentinel function, communicating alterations in homeostasis to B and T lymphocyte compartments. In support of this hypothesis, data reported herein demonstrate that in vitro activated platelets, through CD154, induce dendritic cell maturation and in vivo enhance $CD8^+$ T cell responses and induce isotype switching. Moreover, adoptive transfer studies demonstrate that platelets activated physiologically during adenoviral infection are sufficient, via CD154, for the induction of isotype switching by B cells and augmentation of the $CD8^+$ T cell response during a viral infection that leads to enhanced secondary anti-viral immunity. Furthermore, normal mice depleted of platelets prior to adenovirus injection exhibit decreased efficiency in the production of adenovirus-specific IgG, underscoring the importance of platelets in the generation of an optimal adaptive immune response.

Results

Activated Murine Platelets Express Functional CD154

Previous studies describing the expression and function of platelet-derived CD154 were performed on human

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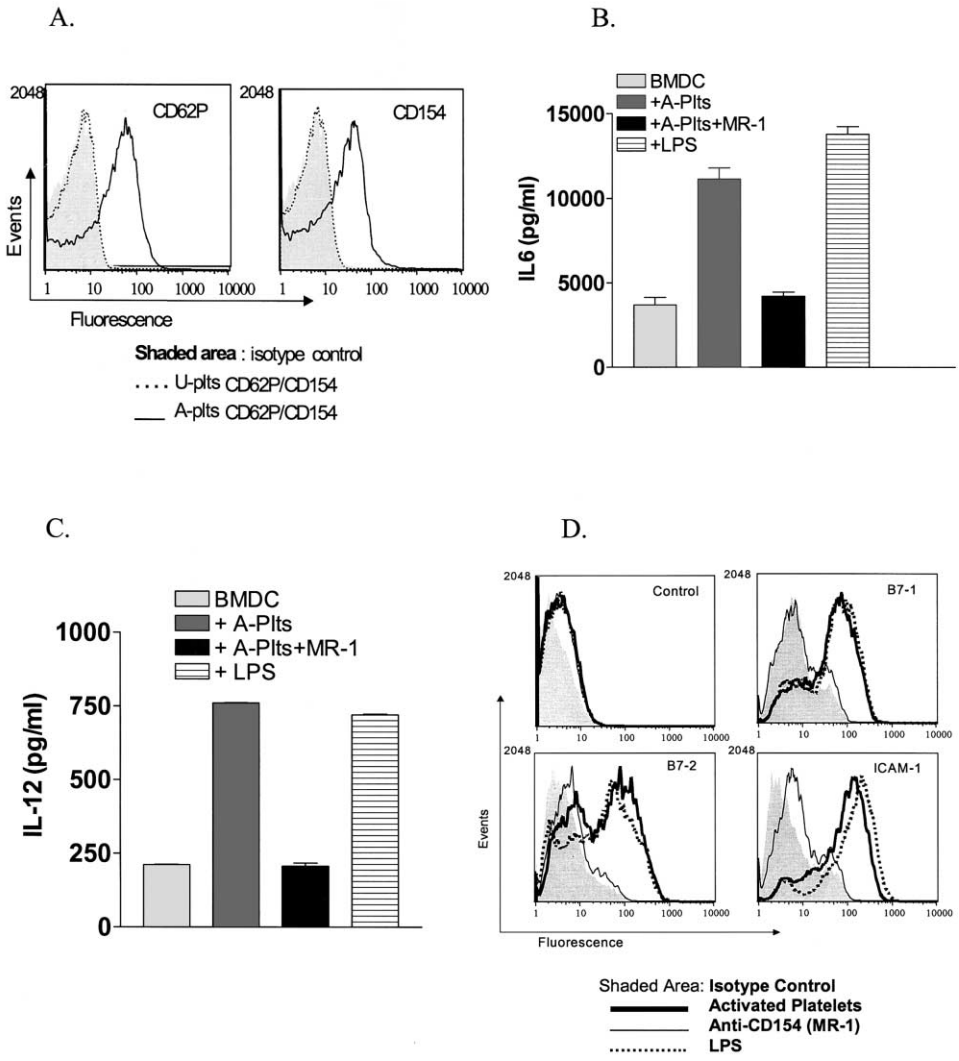

Figure 1. Expression of Functional CD154 by Activated Normal B6 Murine Platelets

(A) Platelets from B6 mice were isolated from peripheral blood as described (Clements et al., 1999) and activated with 1 unit/ml thrombin for 10 min. Activated platelets (A-plt) and unactivated platelets (U-Plts) were tested by flow cytometry for the expression of CD154 or CD62P.
(B and C) Effect of activated platelets on IL-6 and IL-12 production by 5 day BMDC (platelets:cells = 20:1). Cells were incubated for 24 hr ± activated platelets, ± 10 μg/ml of a blocking anti-CD154 mab (MR-1), ± 1 μg/ml LPS, supernatants harvested, and IL-6 (B) and IL-12 (C) concentrations determined by ELISA. Activation status of platelets was verified by flow cytometry (data not shown).
(D) Expression of activation markers on BMDC from (B) as measured by flow cytometry.

platelets (Henn et al., 1998, 2001; Ahmad et al., 2001; Diacovo et al., 1994; Bombeli et al., 1998; Geba et al., 1996; Collins et al., 1994; Gawaz et al., 2000; Barry et al., 1998). To verify that CD154 is expressed on murine platelets, unactivated platelets and platelets activated with thrombin (Figure 1A) or collagen (data not shown) were tested for the surface expression of CD154 or CD62P (P-selectin), the latter representing a marker for α granule release in activated human and murine platelets. The data demonstrate expression of both CD62P and CD154 on murine activated platelets but not unactivated platelets. Further studies demonstrated that surface expression of CD154 persisted on activated platelets for up to 8 hr, and that CD154 also was released into supernatants (data not shown).

To assess the functional capacity of murine platelet-derived CD154 on primary cells, bone marrow-derived dendritic cells (BMDC) were generated and incubated with syngeneic activated platelets, and production of IL-6 and IL-12 was measured. The data show that activated platelets induce IL-6 (Figure 1B) and IL-12 (Figure 1C) production by BMDC in a CD154-dependent manner. Similarly, activated platelets also induced CD154-dependent IL-6 production by a murine monocyte cell line, RAW 264.7 (data not shown). As previously demonstrated for CD154 derived from other sources, platelet-derived CD154 induced expression of maturation markers on BMDC (Figure 1D). Expression of B7.1, B7.2, and ICAM-1 by DC cocultured with activated platelets was enhanced to a level consistent with LPS-induced maturation, and platelet-induced enhancement of each was abrogated by anti-CD154. These data demonstrate that

murine platelet-derived CD154 is a functional ligand capable of inducing CD40-mediated immune function in in vitro assays.

Sufficiency of Platelet-Derived CD154 to Modulate Adaptive Immune Responses

It is well documented that in mice, injection of adenovirus results in splenic and hepatic inflammation (Zhang et al., 2001; Jaffee et al., 1992). Thus, since platelets can modulate inflammation (reviewed by Henn et al., 1998; Yeaman, 1997) and are able to express CD154, experiments were initiated to evaluate the potential of platelets to contribute to adaptive immunity in vivo. Specifically, transfusion studies were performed to assess the ability of platelet-derived CD154 to induce B lymphocyte isotype switching in mice deficient in CD154 ($CD154^{-/-}$) (Yang and Wilson, 1996; Renshaw et al., 1994). The ability of B lymphocytes to switch from the production of IgM/IgD to IgG or other Ig isotypes requires CD40 activation (Renshaw et al., 1994). B cell activation in the absence of CD40 ligation results in hyper IgM syndrome with minimal switching to IgG secretion in humans and mice, making isotype switching a direct indicator of CD154 function (Renshaw et al., 1994). Replication-deficient adenovirus type 5 was injected intravenously (iv) into $CD154^{-/-}$ mice. Immediately after adenovirus injection and 24 hr later, activated platelets from syngeneic normal mice were injected iv, and adenovirus-specific Ig isotypes were measured by ELISA 9 days after immunization (Figure 2A). The transfer of normal activated platelets was sufficient to induce isotype switching, as shown by IgG1 and IgG2b production. Isotype switching was abrogated by anti-CD154, although adenovirus-specific IgM was efficiently produced, linking platelet-derived CD154 to the switch to IgG secretion. Further studies were performed with normal and $CD154^{-/-}$ platelets to confirm the sufficiency of platelet-derived CD154 to induce isotype switching (Figure 2B) in $CD154^{-/-}$ mice. These data corroborate the observations in Figure 2A, demonstrating a switch to adenovirus-specific IgG in $CD154^{-/-}$ mice receiving activated platelets from normal mice but not those receiving activated platelets from $CD154^{-/-}$ mice.

We next determined whether platelet-derived CD154 modulated $CD8^+$ T cell activation as suggested by its ability to induce maturation of BMDC in vitro. Activated platelets from either normal or $CD154^{-/-}$ mice were injected subcutaneously (sc) in normal mice adjacent to the injection site of adenovirus carrying the gene for an ovalbumin/transferin receptor fusion protein (Ad5-mOVA) (Kurts et al., 1998; Siemens et al., 2001). Spleen cells were isolated 10–14 days after immunization and the frequency of ova-specific, IFNγ-producing $CD8^+$ T cells and lytic capacity was determined. The injection of activated platelets from normal, but not $CD154^{-/-}$, mice enhanced the frequency of IFNγ-producing $CD8^+$ T cells (Figure 2C) increasing the total lytic activity (Figure 2D), which confirms the ability of platelet-derived CD154 to augment $CD8^+$ T cell responses in vivo. Taken together, these data demonstrate the sufficiency of platelet-derived CD154 to mediate isotype switching and enhance $CD8^+$ T cell responses; however, the contribution of unactivated circulating platelets to an in vivo immune response to viral challenge remained to be established.

Biological Relevance of Platelet-Derived CD154 in the Modulation of Adaptive Immunity to Viral Challenge

To address the ability of circulating, unactivated platelets to respond to stimuli in vivo and modulate B and T cell responses, a platelet transfusion model was established. To allow measurement by flow cytometry, platelets were labeled with the fluorescent dye CFSE to assess the effect of the transfer on their activation status and persistence in the circulation. Preliminary in vitro studies showed that CFSE labeling did not activate platelets and that CFSE-labeled platelets responded to activation stimuli in a manner identical to unlabeled platelets (data not shown). Subsequently, CFSE-labeled platelets (3×10^8) were transfused into syngeneic hosts and the recovery of $CFSE^+$ platelets determined 24 hr after transfer. The percentage of $CFSE^+$ platelets measured in three separate experiments 24 hr after transfer ranged from 12.5% to 15.6% of the total platelet population in circulation (Figure 3A). CFSE-labeled platelets isolated after transfer did not express CD62P, indicating they were unactivated, and reisolated CFSE-labeled platelets normally expressed CD62P after exposure to thrombin in vitro (Figure 3A). These observations demonstrate that platelets can be adoptively transferred in an unactivated state and retain the capacity to be activated upon exposure to physiological stimuli. Additionally, CFSE-labeled platelets were monitored over time to determine the rate of loss of transferred platelets. Consistent with previously reported clearance rates for murine platelets (Piguet et al., 2001), we found that the percentage of CFSE-labeled platelets decreases with time but detectable numbers remain 4 days after transfer (Figure 3B).

After the validation of the platelet transfusion system, studies were performed to determine whether CD154 derived from platelets activated in response to an adenoviral infection was sufficient to induce isotype switching. Unactivated platelets from normal or $CD154^{-/-}$ mice were transferred into $CD154^{-/-}$ mice, and 24 hr later, mice were challenged iv with adenovirus. Nine days after adenovirus challenge, collected serum was tested for adenovirus-specific IgG (Figure 4A). The results show that switching to IgG occurred in mice receiving normal platelets, whereas mice receiving platelets from $CD154^{-/-}$ mice did not switch to IgG secretion. Adenovirus-specific IgM was produced equally by all groups (data not shown). This platelet CD154-mediated switching was not due to the action of platelets that may have been activated upon injection. $CD154^{-/-}$ mice injected with 10^8 normal in vitro-activated platelets 24 hr prior to adenovirus injection, which is the time interval separating platelet transfusion from adenovirus challenge, did not isotype switch (data not shown). Also, it is unlikely that injection of infectious adenoviral particles directly activates platelets since they are unable to activate platelets in vitro or induce them to express CD62P (Figure 4B). Taken together, these data suggest that circulating platelets, activated through a normal biologic response to viral infection, can induce isotype switching in B lymphocytes through CD154 expression. To estab-

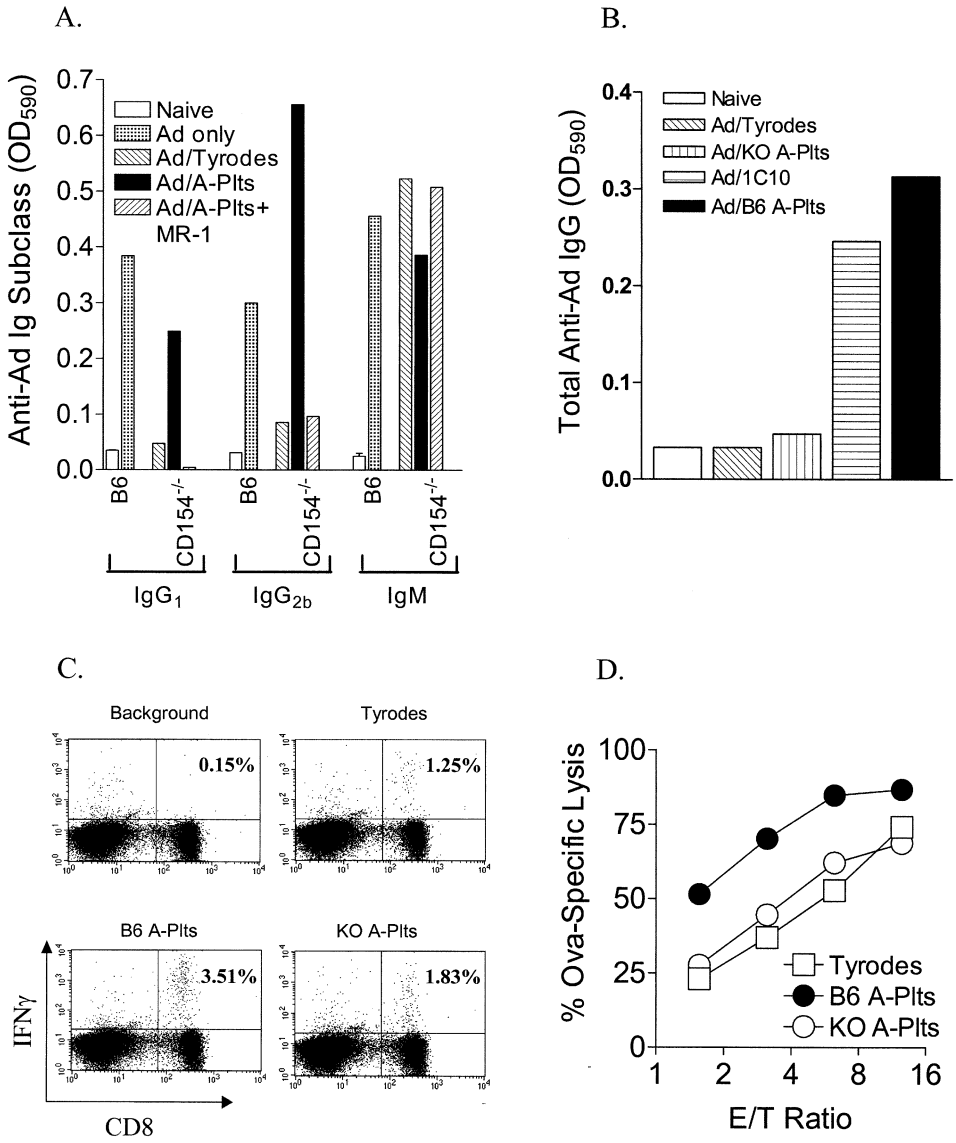

Figure 2. Effect of Platelet-Derived CD154 on Isotype Switching and $CD8^+$ T Cell Activation In Vivo

(A) Normal B6 mice were injected iv with 10^8 pfu adenovirus (Ad only), and $CD154^{-/-}$ mice were injected iv with either 10^8 pfu Ad + Tyrode's buffer (Ad/Tyrodes), 10^8 pfu adenovirus + 10^8 activated B6 platelets (Ad/A-Plts), or 10^8 pfu Ad + 10^8 activated B6 platelets + blocking anti-CD154 (Ad/A-Plts+MR-1). 24 hr later, appropriate groups were injected iv again with 10^8 activated platelets. 100 μg MR-1 was injected into mice 2 days before and the day of adenovirus immunization. Nine days after adenovirus immunization, serum was collected and assayed for adenovirus-specific IgG by ELISA (39). The optical density (OD) readings are reported for a serum dilution 1/200 for B6 mice, and 1/40 for $CD154^{-/-}$ mice. IgG OD values for $CD154^{-/-}$ naive mice were <0.051 for all groups. Data are representative of three separate experiments.

(B) $CD154^{-/-}$ mice were injected with 10^8 pfu adenovirus with either Tyrodes buffer (Ad/Tyrodes), activated $CD154^{-/-}$ platelets (Ad/KO A-plts), activated B6 platelets (Ad/B6 A-plts), or 500 μg agonistic anti-CD40 ab (Ad/1C10) ip. 24 hr later, appropriate groups were again injected iv with 10^8 activated platelets, Tyrodes buffer, or 1C10. OD is reported for a serum dilution of 1/40. The figure is representative of three separate experiments.

(C) Effect of activated platelets (A-Plts) on ova-specific $CD8^+$ T cell frequency. B6 mice were injected sc with 10^8 B6 (B6 A-plts) or $CD154^{-/-}$ (KO A-plts) activated platelets adjacent to the injection site of 10^8 pfu Ad5-mOVA as described (Siemens et al., 2001). 7–10 days after Ad5-mOVA immunization, spleens were collected, and spleen cells were isolated and stimulated in vitro for 4 hr with the ovalbumin-expressing cell line, E.G7, and monitored for intracellular IFNγ. Numbers in the dot plots indicate the percentage of $CD8^+$ T cells that are $IFN\gamma^+$. Backgrounds for each mouse were determined by stimulation with EL4, parental cells for E.G7, which lack OVA expression, and were 0.15% or less for all mice. Data are representative of two separate experiments.

(D) Effect of A-Plts on the lytic capacity of $CD154^{-/-}$ $CD8^+$ T cells. Splenocytes were isolated as in (C), but were cultured for 5 days with stimulator E.G7 cells. Viable splenocytes were then harvested and tested as effector cells. Data are representative of three separate experiments.

lish the duration of the platelet CD154-induced switch to IgG, timecourse experiments were performed. $CD154^{-/-}$ mice were transfused with $CD154^{-/-}$ or normal platelets and immunized iv with adenovirus. Plasma was collected at weekly intervals and assayed for the presence of adenovirus-specific IgG. Figure 4C demonstrates that

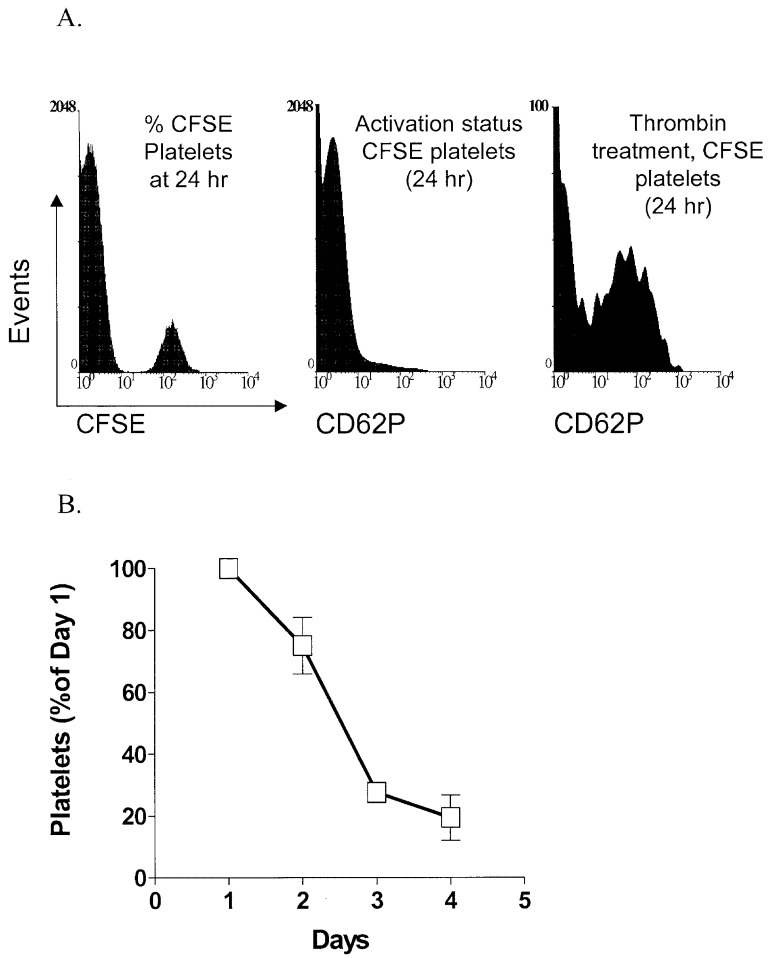

Figure 3. Characterization of the Activation Status and Life Span of Normal Unactivated Platelets after Transfusion into Syngeneic CD154$^{-/-}$ Hosts

(A) CFSE-labeled B6 unactivated platelets (3×10^8) were injected iv into normal mice. Twenty-four hours later, peripheral blood was collected by tail clipping, and the level of CFSE-labeled platelets was determined by flow cytometry. CD62P expression indicated that adoptively transferred platelets were unactivated, and retained the ability to be activated by thrombin in vitro.

(B) Time course of platelet loss was determined by the iv transfusion of 2×10^8 B6 CFSE-labeled platelets into syngeneic CD154$^{-/-}$ hosts. Platelet loss was monitored in the peripheral blood of individual mice (6 mice per group) obtained by daily tail bleeding. Twenty-four hours after platelet transfer, 12% of recovered platelets were CFSE positive, which was established as 100%. Data are representative of three separate experiments.

platelet CD154-induced IgG levels peak at day 7 and return to background by day 28. Consistent with the short kinetic pattern of IgG expression, GC formation was not observed by histology or flow cytometry (B220$^+$ PNA$^+$ events) (data not shown).

Since data from Figure 4 demonstrate that through CD154, platelets can induce isotype switching, we predicted that normal mice depleted of platelets would have diminished IgG production in response to antigen challenge. Using an antibody cocktail (p0p3/4) previously shown to clear platelets (Bergmeier et al., 2000), Figure 5A demonstrates normal mice depleted of platelets before Ad5-mOVA immunization produce very little adenovirus-specific IgG. Additionally, the antibody response to injection of soluble ova protein after platelet depletion was measured. Figure 5B demonstrates a reduced ability in these mice to produce ova-specific IgG1 as well. Upon injection of p0p3/4, platelets are cleared in the spleen and liver (data not shown). Although this is through an Fc-independent mechanism, it is possible that isotype switching was inhibited by B cell Fc receptor binding of platelet-antibody complexes in the spleen, although this is unlikely since the mice were able to produce normal amounts of IgM (data not shown). To address this issue, normal mice injected with p0p3/4 were immunized 24 hr later in the footpad with 10^8 pfu adenovirus to assure isotype switching would be possible only in the draining lymph node (LN) away from the sites of platelet clearance. Indeed, Figure 5C demonstrates that platelet-depleted mice immunized in the footpad are compromised in their ability to produce IgG.

Circulating Platelets Are Sufficient to Modulate CD8$^+$ T Cell Activity during an Immune Response Initiated by Virus Infection

Subsequently, studies were performed to determine whether platelet activation during a normal antiviral response would modulate T cell immunity as observed for B cells in the isotype switching studies. Platelets from either normal or CD154$^{-/-}$ mice were transferred into CD154$^{-/-}$ hosts, and 24 hr later, mice were challenged iv with Ad5-mOVA. Ten days after Ad5-mOVA challenge, spleen cells were isolated, and ova-specific lytic activity was measured. Consistent with previous reports, adenovirus induced cytolytic T lymphocyte (CTL) activity in CD154$^{-/-}$ mice (Yang and Wilson, 1996). Mice receiving CD154$^{+/+}$ platelets exhibited enhanced CTL activity, whereas mice receiving CD154$^{-/-}$ platelets show lytic activity equivalent to mice receiving diluent without platelet transfer (Tyrode's buffer, Figure 6). These findings indicate that platelet-derived CD154 is sufficient to modulate CD8$^+$ T cell responses to an adenovirus challenge; however, it remained to be established that these enhanced primary responses resulted in an en-

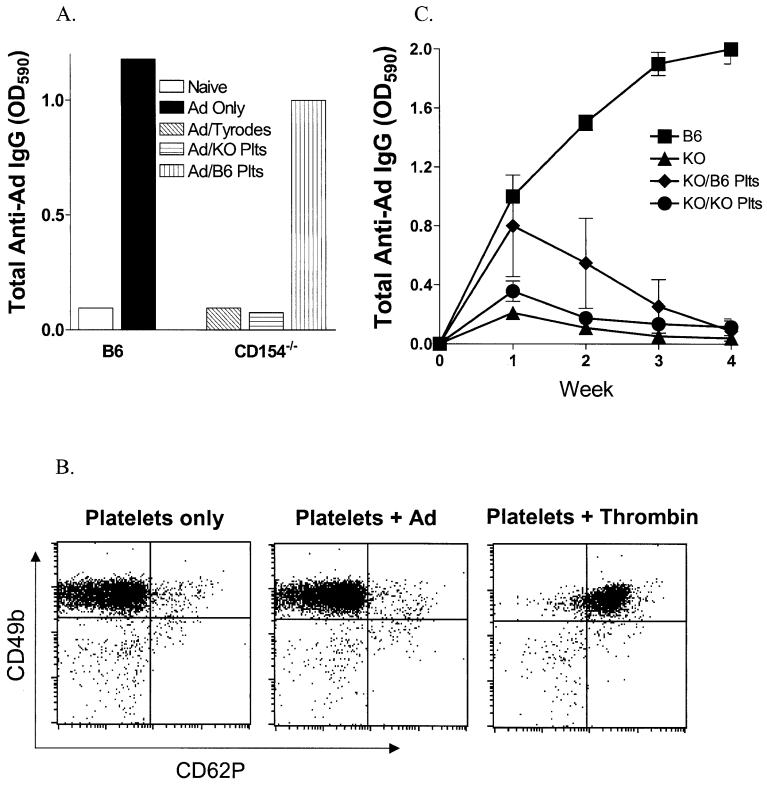

Figure 4. Ability of Circulating, Unactivated Normal Platelets Transferred into CD154$^{-/-}$ Mice to Induce Isotype Switching in Response to Adenovirus Challenge

(A) Unactivated platelets, either CD154$^{-/-}$ (Ad/KO Plts) or normal (Ad/B6 Plts), were isolated and transferred (3×10^8 per recepient) into CD154$^{-/-}$ mice. Twenty-four hours later, 10^8 pfu adenovirus were injected iv into appropriate groups. Serum was collected for determination of isotype switching 9 days after adenovirus challenge. IgG levels were determined by ELISA as in Figure 2. Data are representative of two separate experiments. OD is reported for serum dilutions of 1/200 for normal CD154$^{+/+}$ mice, and 1/40 for CD154$^{-/-}$ mice.

(B) Platelets were harvested from B6 mice and 5×10^6 were incubated in 1 ml of Tyrodes buffer $\pm$ 10^7 pfu adenovirus for 30 min. Positive control platelets were treated with 0.5 U/ml thrombin for 5 min. CD62P expression versus CD49b (platelet marker) was measured by flow cytometry.

(C) To assess duration of platelet-induced isotype switching, appropriate CD154$^{-/-}$ mice were injected with 3×10^8 normal (KO/B6 Plts) or CD154$^{-/-}$ (KO/KO Plts) platelets and 24 hr later immunized iv with 2×10^8 pfu of adenovirus. Plasma was harvested from mice at weekly intervals and analyzed as in Figure 2. Data are representative of two separate experiments.

hanced secondary response against adenoviral rechallenge.

Normal Platelets Enhance CD154$^{-/-}$ Secondary Immune Response against Adenovirus

As stated above, the platelet transfusion model achieved 12.5%–15.6% donor platelets in the circulation of recipients 24 hr after adoptive transfer. We hypothesized that if host platelets were depleted before receiving a platelet adoptive transfer, a much greater percentage of donor platelets in the circulation could be attained, further enhancing the ability of the platelets to modulate the immune response. Initial titrations determined the appropriate dose of iv injection of p0p3/4 that

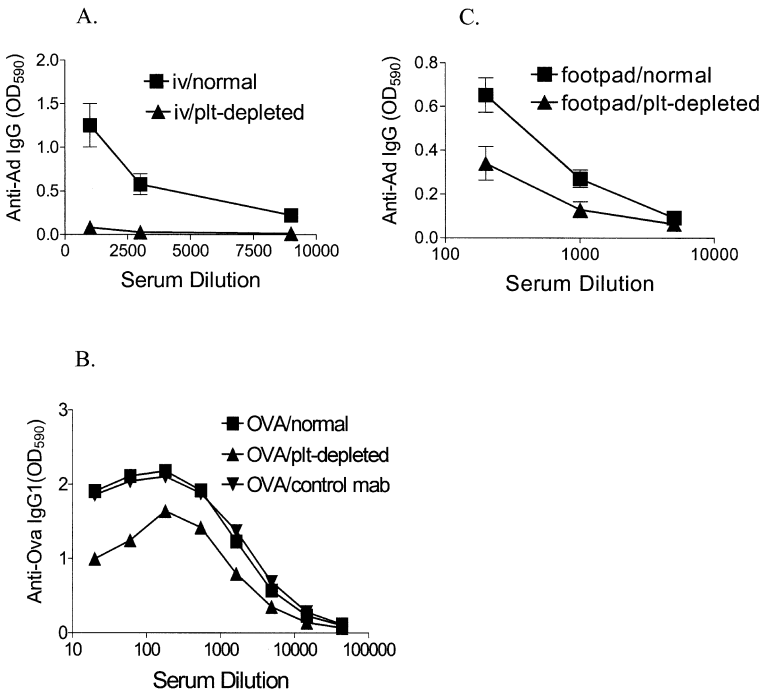

Figure 5. Effect of Platelet Depletion in Normal Mice on Adenovirus-Specific Antibody Production

(A) Five B6 mice/group were injected iv with 100 μg p0p3/4 (plt-depleted) or not (normal) on day 0, and 50 μg on day 5. Mice were immunized iv with 10^8 pfu adenovirus on day 1. Serum was harvested on day 9 and tested for adenovirus-specific IgG as in Figure 2.

(B) Four-six B6 mice/group were injected iv with 10 μg p0p3/4 (plt-depleted) or not (normal) on days 0, 3, 6, and 9. Mice were immunized ip with 200 μg ovalbumin in PBS on day 1. Serum was harvested on day 14 and tested for ova-specific IgG1. Data are representative of two experiments.

(C) Five B6 mice/group were injected iv with 100 μg p0p3/4 (plt-depleted) or not (normal) on day 0, and 50 μg on day 5. Mice were immunized sc in the footpad with 10^8 pfu adenovirus on day 1. Serum was harvested on day 9 and tested for adenovirus-specific IgG as in Figure 2. Data are representative of two separate experiments.

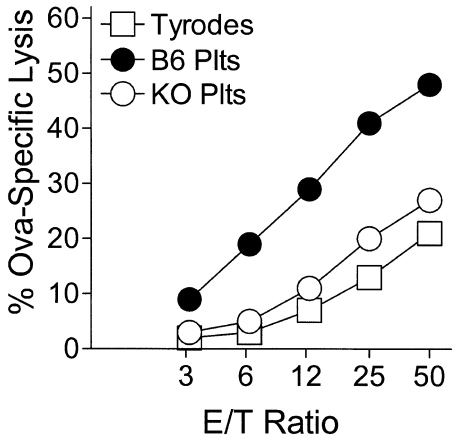

Figure 6. Ability of Circulating Normal Platelets Transferred into CD154$^{-/-}$ Mice to Augment CD8^{+} T Cell Activation in Response to Adenovirus Challenge

CD154$^{-/-}$ mice were injected with Tyrode's buffer (Tyrodes), normal platelets (B6 Plts), or CD154$^{-/-}$ platelets (KO Plts), and were immunized iv with 10^8 pfu Ad5-mOVA 24 hr later. After 10 days, splenocytes were collected for determination of ova-specific lytic capacity as in Figure 2D. The data are representative of two separate experiments.

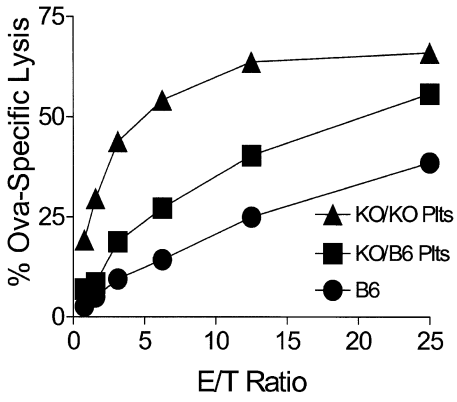

Figure 7. Normal Platelets Enhance Secondary Immune Responses to Adenovirus in CD154$^{-/-}$ Mice

CD154$^{-/-}$ mice were depleted of endogenous platelets and transfused 24 hr later with normal (KO/B6 Plts) or CD154$^{-/-}$ platelets (KO/KO Plts). Four hr later, plt-transfused CD154$^{-/-}$ and normal mice (B6) were immunized iv with 10^7 pfu of adenovirus. Eleven days later, they were challenged sc on the flank with 2×10^7 pfu Ad5-mOVA. After 10 days, splenocytes were collected for determination of ova-specific lytic capacity as in Figure 2D. The data are representative of two separate experiments.

depleted 95%–99% of host platelets but not platelets adoptively transferred 24 hr later (data not shown). It was determined that depletion of host platelets allowed transfused platelet levels to reach up to 82% of those in the circulation 4 hr after transfusion, and up to 45% after 24 hr (data not shown). It is from this platform that we tested the ability of platelets to contribute to enhanced viral immunity.

To determine if the normal platelet-enhanced B and T cell responses in CD154$^{-/-}$ mice resulted in an increased ability to respond to a secondary viral challenge, it was necessary to demonstrate that these mice, when previously immunized with adenovirus, more efficiently eliminated a second adenovirus challenge when compared to CD154$^{-/-}$ mice transfused with CD154$^{-/-}$ platelets. To this end, platelet-depleted CD154$^{-/-}$ mice were transfused with either 3×10^8 normal or CD154$^{-/-}$ platelets and primed with 10^7 pfu of adenovirus. Eleven days later, they were challenged sc with 2×10^7 pfu of Ad5-mOVA (sc injection was necessary to avoid neutralizing IgM in the CD154$^{-/-}$ mouse circulation [data not shown]). After 10 days, ova-specific CTL responses were measured. If the normal platelets transfused into CD154$^{-/-}$ mice were able to enhance the initial anti-adenovirus response, it was predicted that these mice should have lower ova-specific CTL versus CD154$^{-/-}$ mice transfused with CD154$^{-/-}$ platelets. As previously demonstrated, lowered CTL activity would be attributable to a more efficient secondary immune response to adenovirus resulting in an inefficient primary response to ova. (Siemens et al., 2001). Indeed, Figure 7 shows that platelet-depleted CD154$^{-/-}$ mice transfused with normal platelets have reduced anti-ova CTL activity as compared to platelet-depleted CD154$^{-/-}$ mice transfused with CD154$^{-/-}$ platelets. This indicates that normal platelets, through CD154, can contribute to an enhanced immune response against a pathogen rechallenge. This platelet CD154-mediated response was not due to the action of platelets that may have been activated upon injection. CD154$^{-/-}$ mice depleted of endogenous platelets and injected with 10^8 normal in vitro-activated platelets 4 hr prior to adenovirus injection did not isotype switch (data not shown).

Discussion

Previously, the main functional role attributed to platelets was hemostasis and maintenance of the integrity of the vessel wall. However, more recently it has become apparent that platelets also play a prominent role in modulating inflammation. Upon activation, platelets release chemokines and cytokines, including IL-8, RANTES, MIP-1α, MCP-3, cationic proteins, and adhesive proteins, and upon binding to endothelial cells, induce the production of a broad array of inflammatory molecules including IL-6, IL-8, GM-CSF, and MCP-1 (reviewed by Boehlen and Clemetson, 2001; Klinger, 1997). Platelets also have been reported to express the immunomodulatory molecules ICAM-2, CD95L, and CD154 (Henn et al., 1998; Ahmad et al., 2001; Diacovo et al., 1994). In light of the known importance of CD154 in adaptive immunity and the published in vitro studies demonstrating platelet-derived CD154 activation of endothelial and dendritic cells (Henn et al., 1998; Hilf et al., 2002), we hypothesized that platelet-derived CD154 also modulated adaptive immune responses. Our results clearly show murine platelet-derived CD154 to be sufficient for the induction of isotype switching and augmentation of primary CD8^{+} T cell responses. Moreover, platelets appear necessary for optimal IgG production, and platelet-derived CD154 is important to the enhanced secondary anti-adenoviral immune response, suggesting a novel role for platelets in adaptive immunity.

Initial studies were performed in vitro to verify expression and function of CD154 in murine platelets, as previous studies demonstrating expression and function were performed with human platelets (Henn et al., 1998). Figure 1 shows expression of CD154 after platelet activation by either thrombin or collagen as was described for human platelets. In addition, murine platelet-derived CD154 was shown to induce the activation/maturation of DC as measured by increased expression of cell surface markers and enhanced IL-6 and IL-12 production. These data are consistent with recent observations demonstrating human platelet-derived CD154 to upregulate CD62E, CD54, and CD106 on cultured human umbilical vein endothelial cells and induce the secretion of MCP-1 and IL-8 (Henn et al., 1998). Likewise, human platelets induce maturation of Langerhans cells in in vitro cultures (Gatti et al., 2000). These data, which uniformly show platelet-mediated activation of CD40 on various cell types, are contrasted by a recent report demonstrating that platelets bind the heat shock protein, gp96, and attenuate gp96-mediated DC maturation in vitro (Hilf et al., 2002). In studies evaluating the effects of platelets on gp96-induced cytokine production by DC, the authors did not observe in vitro DC activation by platelets in the absence of gp96. Based on previous reports and our data demonstrating platelet-mediated DC activation, the reason for this is unclear, but may involve platelet/DC ratios or cell densities in culture. Regardless, our data, which utilize genetically modified mice to probe platelet function, extend the observations on platelet function to the in vivo setting and demonstrate that circulating platelets are sufficient to enhance adaptive T and B cell responses to adenovirus challenge in a CD154-specific manner.

The requirement for CD154 in adaptive T and B lymphocyte responses is well established. CD154/CD40 interaction is critical in the generation of thymus-dependent B cell antibody production. Stimulation of naive B cells through the B cell receptor (signal 1) induces the production of IgM, but CD154-mediated activation of CD40 by $CD4^+$ T cells (signal 2) is necessary for the initiation of isotype switching (Xu et al., 1994; Renshaw et al., 1994). Data reported herein show via transfusion of normal platelets into $CD154^{-/-}$ mice that platelet-derived CD154 is sufficient to initiate isotype switching to adenoviral antigens in $CD154^{-/-}$ mice.

In addition to isotype switching, germinal center formation also is dependent on CD40/CD154 signaling. In germinal centers, B cells undergo somatic hypermutation, producing antibodies of higher affinity, and differentiate into memory or plasma cells (reviewed by Rudin and Thompson, 1998). Indeed, $CD154^{-/-}$ or $CD40^{-/-}$ mice develop a hyper-IgM syndrome, lacking both isotype switching and germinal center formation, which can be independent events (Xu et al., 1994; Kawabe et al., 1994). Although we have demonstrated that normal platelets are sufficient to induce isotype switching in $CD154^{-/-}$ mice, we have not observed the formation of germinal centers in these mice. Figure 4C demonstrates that the IgG produced in response to platelet CD154 is transient, consistent with an extrafollicular response. However, Figure 5 demonstrates the requirement for platelets in the generation of robust IgG, presumably from GCs. Previous studies suggest that the B cells that produce high-affinity IgM shortly after infection are also those that go on to produce IgG in the GC (Baumgarth et al., 1999). Additionally, rare GC can be observed as early as day 3 in an immune response, long before T cells would be able to provide CD154 stimulation (Szakal et al., 1990). Although it is well established that CD154 expressed by $CD4^+$ T cells is required for GC formation (reviewed by Parker, 1993), platelets express CD154 within minutes of activation, which could provide the signal necessary for early GC formation. Our data, when combined with these reports, are consistent with a model in which the naive B cell receptor recognizes adenoviral repetitive structures and signal 2 is provided by platelet-expressed CD154 resulting in extrafollicular IgG production. These B cells then begin to form GC that are subsequently stabilized/matured by antigen-specific T cells and a mature B cell response ensues. Without platelets to inititate and T cells to sustain, GC formation is compromised, consistent with the observation of a lack of GC in $CD154^{-/-}$ mice transfused with normal platelets and compromised IgG production in normal mice depleted of platelets. Conversely, it may be that the continual clearance of platelets by the spleen and liver results in a hindrance of splenic B cell IgG responses; however, this is unlikely since mice continually depleted of platelets produce normal amounts of IgM and inhibition of IgG production also occurs in draining LN, a site distinct from platelet clearance.

In addition to GC dysfunction, T cell responses in $CD154^{-/-}$ mice have been reported to be impaired as well. After adenoviral immunization, $CD154^{-/-}$ $CD4^+$ T cells failed to proliferate or produce cytokines in response to viral antigens (Yang and Wilson, 1996; Yang et al., 1996). Additionally, $CD154^{-/-}$ $CD8^+$ T cell adenovirus-specific lytic responses were significantly reduced, but were restored to near-normal levels by administration of agonistic anti-CD40 monoclonal antibody. Furthermore, $CD154^{-/-}$ mice had a markedly reduced ability to eliminate adenoviral transgene (β-galactosidase) expression in the liver as compared to normal B6 mice (Yang and Wilson, 1996). Likewise, in $CD154^{-/-}$ mice LCMV-specific $CD8^+$ T cell memory responses also were impaired (Borrow et al., 1996, 1998). The mechanism for these deficiencies is uncertain; however, the currently accepted paradigm suggests that optimal $CD8^+$ T cell activation is dependent on CD154-mediated APC maturation induced by $CD4^+$ T cells. The in vivo data presented herein not only demonstrate the sufficiency of platelet-derived CD154 to partially reconstitute isotype switching but also show platelet-derived CD154 to enhance $CD8^+$ T cell responses in both normal and $CD154^{-/-}$ mice. Initial studies in which activated platelets were injected showed augmentation of the frequency of adenovirus-specific T cells and also demonstrated enhanced cytotoxic T cell function, suggesting the sufficiency of platelets for supporting T cell responses. Further studies investigating T cell responses during adenoviral infection in which circulating normal platelets were transfused into $CD154^{-/-}$ mice showed that platelet-derived CD154 was sufficient to enhance $CD8^+$ T cell responses to an adenovirus antigen. The enhancement of T cell responses is associated with increased protection against pathogens and tumors. Figure 7 demonstrates that only $CD154^{-/-}$ mice trans-

fused with normal platelets, but not CD154$^{-/-}$ mice or those transfused with CD154$^{-/-}$ platelets, possess enhanced secondary immunity against Ad5-mOVA in that prior immunity to adenovirus reduces the T cell response to ova. This enhanced immunity to adenovirus could be due to the presence of platelet CD154-induced IgG (Figures 4A and 4C) at the site of sc injection that neutralizes the virus, or to enhanced T cell responses (Figure 6) that eliminate Ad5-mOVA quickly enough to reduce the primary response to ova, or both.

The mechanisms by which platelet-derived CD154 communicates with T and B lymphocytes are not known. Data presented herein show that platelet-derived CD154 provides the necessary second signal for maturing the antibody response prior to expansion of the helper T cell compartment, which traditionally provides the CD154 signal and enhances CD8^{+} T cell responses. Since the iv injection of adenovirus induces splenic inflammation (Zhang et al., 2001), platelet expression of CD154 in the spleen may act locally to induce isotype switching and induce maturation of splenic DC. Alternatively, during infection or injury, it may be that as resident Langerhans cells (LC) migrate through the site, they contact CD154 on activated platelets which initiates/perpetuates their maturation process on the way to the draining lymph node. In support of this concept, activated platelets have been reported to cause maturation of human LC in vitro (Gatti et al., 2000). Alternatively, soluble CD154 and other platelet-derived immunomodulatory ligands (Henn et al., 2001; Ahmad et al., 2001) could be released into the lymph and/or circulatory systems and function at sites distant to the point of platelet activation. The observations reported herein do not distinguish between these two alternatives; however, in vitro studies show that platelet-free supernatants from activated platelets induce DC maturation, whereas the platelet pellet has no effect (data not shown). Furthermore, preliminary in vivo studies suggest that platelet-free supernatants from activated platelets induce isotype switching to adenoviral antigens, suggesting the potential for modulation of adaptive immunity at sites distant from the point of activation through the release of CD154 by activated platelets. While the findings reported herein clearly demonstrate platelet-mediated modulation of adaptive immunity, further studies are needed to determine whether platelet-derived products function locally or at sites distant to the point of activation and to determine the biological role of platelets during normal immune responses. Also, since the CD154$^{-/-}$ mice described in this report receive only one platelet transfusion prior to the onset of inflammation, it is unknown how the continued presence of normal platelets from multiple transfusions may influence their immune response.

That platelets can have a large influence on early innate inflammatory responses is a well-established concept (reviewed by Klinger, 1997; Yeaman, 1997). The data presented herein extend that literature base and suggest a role for platelets in the initiation and/or the expression of immunity to invading microorganisms. We hypothesize that the low activation threshold of platelets allows them to serve a sentinel function, responding to early inflammatory or traumatic events associated with invasion, and that platelet activation forms a communication link via CD154 between innate and adaptive immune compartments, transmitting early signals of alterations in homeostasis to B lymphocytes and via DC maturation to naive T cells.

Although our data show that activated platelets are sufficient to induce DC maturation in vitro, it is unclear how they may contribute in vivo in relation to other stimuli known to induce DC maturation, including pathogen-associated molecular patterns and CpG dinucleotides and endogenous factors generated by cellular stress and necrotic cell death such as tumor necrosis factor (TNF) and interleukin 1 (IL-1) (Gallucci and Matzinger, 2001). Further studies are necessary to begin to understand the interrelationships among these factors in the activation of a mature immune response.

Experimental Procedures

Mice

Normal C57Bl/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). CD154 gene knockout mice (CD154$^{-/-}$) (B6 background) were purchased from Jackson Laboratories (Bar Harbor, ME).

Reagents

The intracellular cytokine staining kit, IL-6 and IL-12 ELISA kits, and all flow cytometry antibodies were purchased from PharMingen (San Diego, CA). 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE) was purchased from Molecular Probes, Inc. (Eugene, OR). LPS, thrombin, apyrase, PGE_1, and mitomycin C were purchased from Sigma (St. Louis, MO). The CD154 blocking antibody MR-1 and agonistic anti-CD40 antibody 1C10 were generous gifts from Thomas Waldschmidt (University of Iowa, Iowa City, IA). The platelet-depleting antibody (a mixture of clones p0p3 and p0p4 (p0p3/4)) was generated as previously described (Bergmeier et al., 2000). All adenovirus vectors were produced by the Gene Transfer Vector Core at the University of Iowa.

Isolation and Activation of Murine Platelets

Murine platelets were isolated essentially as previously described (Clements et al., 1999). In brief, mice were anesthetized and bled by cardiac puncture. Blood was collected into syringes containing 0.5 ml ACD (12.5 g/L Na Citrate, 10.0 g/L D-glucose, and 6.85 g/L citric acid), added to 5 ml PIPES (150 mM NaCl and 20 mM PIPES [pH 6.5]), and spun at 100 $\times$ g for 15 min. The platelet-rich supernatant was collected and 1 U/ml apyrase and 1 μM PGE_1 (final concentrations) were added and spun at 1000 $\times$ g for 10 min. The platelet pellet was resuspended in Tyrodes buffer (134 mM NaCl, 2.9 mM KCL 0.34 mM Na_2PO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose, and 0.5 mg/ml BSA [pH to 6.5]), and counted using a Coulter Particle Counter (Coulter Corp., Miami, FL). To activate platelets, 0.5 U thrombin/ml was added to platelets in Tyrode's buffer. All platelet manipulations were performed at room temperature.

Flow Cytometry

Cells or platelets were incubated with pre-titrated amounts of appropriate antibodies for 30 min at 4°C in the dark, washed 2$\times$ in FACS buffer (2% FCS and 0.02% NaN_3 in PBS), and resuspended in 2% paraformaldehyde. Intracellular IFNγ was measured according to kit protocol. Events were collected on a FacScan (Beckman, San Jose, CA) and analyzed using Cellquest software.

Generation and Treatment of BMDC

Bone marrow from B6 mice was harvested from femurs and tibias as previously described (Warren et al., 2000). In brief, RBC were lysed with ACK buffer, B cells were eliminated by panning, and remaining cells were cultured overnight in 24-well plates at 5 $\times$ 10^5/well in complete RPMI (RPMI + FCS, 2-ME, Na Pyruvate, HEPES, and antibiotics). Nonadherent cells were then collected and cultured for 5 days in 1000 U/ml recombinant murine (rmu) IL-4 (Peprotech, Rocky Hill, NJ) and rmuGM-CSF (R&D Systems, Minneapolis, MN).

At this time, BMDC were used for experiments using MR-1 anti-CD154 (10 μg/ml) and LPS (1 μg/ml).

CFSE Labeling and Injection of Platelets

Ten milliliters of 2×10^7 platelets/ml in Tyrodes buffer was incubated for 10 min at 37°C in 2 μM CFSE, washed 2×, and injected retroorbitally into mice.

Measurement of Anti-Adenoviral Antibody Production

Serum was collected from mice 7–14 days after immunization with adenovirus. Ninety-six-well plates were coated overnight at 4°C with 10^9 Ad5-βgal particles per well in 50 μl 0.1 M $NaHCO_3$ (pH 9.2). Wells were then washed and blocked 2–4 hr at room temperature with 3% BSA in 0.01% TWEEN 20 and 0.02% NaN_3. Blocking solution was decanted and 100 μl diluted plasma incubated per well for 2–4 hr at room temperature. After six washes, 100 μl peroxidase-labeled secondary antibody was incubated for 1–2 hr at room temperature per well. Wells were washed seven times and 100 μl fresh substrate (OPD in 0.04 M Na_2HPO_4 and 0.02 M citric acid [pH 5.0]) added and samples incubated at room temperature in the dark for 30 min. The reaction was then stopped by adding 25 μl 4.5 M H_2SO_4 per well. Sample absorbances were measured at 490 nm.

Measurement of Anti-Ova Antibody Production

Four to six mice per group were treated with 10 μg anti-platelet antibody (p0p3/4) or isotype control at days 0, 3, 6, 9, and 12. Mice were immunized intraperitoneally (ip) with 200 μg ova in PBS on day 1. After 14 days, serum was harvested and anti-ova antibody levels were measured by ELISA. ELISA plates were coated overnight at 4°C with 100 μg/mL OVA in 0.1 M $NaHCO_3$ (pH 9.2) and blocked for 2 hr at room temperature with 10% FCS in PBS. Samples diluted with 10% FCS-PBS were added and incubated for 2 hr at room temperature. After washing, HRP- conjugated isotype specific goat anti-mouse Ig (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each well and incubated for 1 hr. Plates were washed and developed with substrate (BD pharmingen) for 10–30 min. Samples absorbances were read at 450 nm and readings at 570 nm were subtracted.

Lytic Assays and $CD8^+$ T Cell Frequency Quantitation

Mice were immunized subcutaneously (sc) with 10^8 pfu of control adenovirus or Ad5-mOVA. Ten to 14 days later, spleens were harvested, ground between two frosted microscope slides, treated with ACK lysis buffer, and washed. For $CD8^+$ intracellular staining of IFNγ, 3×10^5 splenocytes per well were incubated for 4 hr in a humidified 5% CO_2 37°C incubator in 96-well flat-bottom plates with 1.5×10^5 E.G7 cells per well (OVA-expressing EL4 cells) or EL4 cells as background controls, and treated according to kit instructions. $CD8^+$ $IFN\gamma^+$ events were measured by flow cytometry. For lytic assays, 5×10^6 splenocytes per well were incubated with 10^5 mitomycin C-treated E.G7 cells per well in 24-well plates in a humidified 5% CO_2 37°C incubator. Five days later, viable splenocytes were recovered and incubated with ^{51}Cr-labeled E.G7 or EL4 targets in 96-well round-bottom plates. Four hours later, the supernatants were harvested and counted. Percent lysis for each sample was calculated using the formula (sample CPM–spontaneous release CPM)/(max CPM–spontaneous CPM) × 100.

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