REVIEW

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Platelet influence on T- and B-cell responses

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Abstract

Understanding the adaptive immune response is an area of research critically important in medicine. Several positive regulators of B- and T-cell activation exist to eliminate pathogens, in which CD40 ligand (CD154) plays a fundamental role. It is well documented that CD154 expressed by CD4 T helper cells can be critical in the proper activation of dendritic cells for the productive stimulation of CD8 T cells and is required for proper T-dependent B-cell immunity. However, platelets are an abundant and systemic source of CD154. While classically known to be important for hemostasis and inflammation, several lines of evidence suggest that platelet-derived ligands can modulate the adaptive immune compartment.

Key words: platelets, CD154, T cells, B cells.

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INTRODUCTION

Understanding how the immune system protects the host from pathogens while avoiding anti-self responses arising from immune dysregulation has been the focus of decades of research (Klinger 1997). At the center of both issues is the context of how the immune response encounters antigen. The innate immune response is the first line of defense that reacts generically to pathogens and will respond in the same manner to subsequent challenges without adaptation or the formation of memory. Adaptive immunity, which is achieved by B and T lymphocytes, is the delayed second line of defense that shows remarkable specificity and the ability to form lasting heightened recall responses against the same antigen. The nature and efficiency of the adaptive response depends almost wholly on the conditions under which antigen is captured and presented to lymphocytes. The triggering of Toll-like receptors (TLRs) on the antigen-presenting cell (APC) via repeated molecular structures common to microorganisms in conjunction with inflammatory signals mediated by chemokines and cytokines is the foundation for forming a robust adaptive response (Iwasaki and Medzhitov 2004). However, if antigen is acquired under non-inflammatory conditions, the adaptive compartment is usually tolerized or regulated through the nonproductive activation of T cells (Redmond and Sherman 2005). A critical control point of this is the activation of dendritic cells (DCs), which are thought to be the only APCs capable of efficiently activating naïve lymphocytes. DCs modulate immunity largely because of their ability to express high levels of major histocompatibility complex (MHC) and costimulatory molecules required to compel resting T lymphocytes to undergo cell cycle progression resulting in productive activation and proliferation (Banchereau and Steinman 1998). Conditions also exist whereby ongoing adaptive immune responses can be shut down by myeloid-derived suppressor cells or T regulatory cells (Nagaraj and Gabrilovich 2007; Vignali et al. 2008).

In addition to inflammatory signals, CD154 is central to the activation of the adaptive immune response (Banchereau et al. 2000; Caux et al. 1994; Renshaw et al. 1994; van Kooten and Banchereau 2000; Yang et al. 1996). Upon activation, CD4 T cells express CD154, which ligates its cognate receptor, CD40, on DCs. CD40 ligation promotes DC maturation and prompts the elevation of costimulatory/adhesion molecule expression and cytokine production, both of which are associated with enhanced antigen presentation to T cells (Caux et al. 1994). Functionally significant CD154 expression was

thought to be restricted to activated CD4 T cells until it was reported that activated platelets also express CD154 (Henn et al. 1998). This raised the possibility that platelet CD154 could influence the adaptive immune response, given the well-known role of platelets in inflammation. Using murine models, we have reported that this is indeed true in B- and T-cell responses (Elzey et al. 2005a; Elzey et al. 2008; Elzey et al. 2003). We found platelets were able to activate DCs *in vitro* and several of our observations have been corroborated *in vitro* using human cells (Czapiga et al. 2004; Kaneider 2003; Martinson et al. 2004; Solanilla et al. 2005). Additionally, other groups have reported an influence of platelets on human DCs that is independent of CD154 (Danese et al. 2004; Hamzeh-Cognasse et al. 2008; Hilf et al. 2002; Kissel K 2006).

PLATELET-DERIVED PRODUCTS AND FUNCTION IN HOST DEFENSE

The classical function of platelets is hemostasis (George 2000). Platelets are abundant in the circulation, with up to $1.5–4\times10^{11}$ cells per liter of human blood, and circulate in an inactivated state. To exert their haemostatic effects, platelets must become activated at the site of injury. Vascular trauma exposes collagen and other extracellular matrix proteins activate platelets as they pass by at high sheer rates and initially tether in a monolayer. Next, stable adhesion and activation cause the release of soluble mediators from platelet intercellular stores, leading to further platelet recruitment and activation, creating a thrombus intended to prevent blood loss (Gresele et al. 2008; Nieswandt and Watson 2003).

In addition to hemostasis, a role for platelets in innate host defense has been known for decades. Only recently has their influence on adaptive T- and B-cell responses been considered. Platelets contain many bioactive molecules important for inflammation and modulation of the innate immune system. Platelets secrete a number of molecules important for the recruitment of leukocytes, such as interleukin (IL)-8, regulated upon activation normal T-cell expressed and secreted (RANTES), CD62P, platelet factor 4 (PF4), macrophage inflammatory protein-1α, and transforming growth factor-β. Other platelet-derived products are vasoactive to allow the entry of inflammatory cells into tissue, such as serotonin, histamine, prostaglandin E2, and prostaglandin D2, among others, as reviewed in Elzey et al. (Elzey et al. 2005b).

Platelets are also reported to possess direct activity against pathogens such as bacteria, viruses, fungi, and helminths. Platelets can engulf viruses and bacteria (Youssefian et al. 2002) and also aggregate in response to whole bacteria (Boukour and Cramer 2005; Byrne et al. 2003; Kalvegren et al. 2003; Mirlashari et al. 2002). Platelets express TLR2, 4, and 9 (Aslam et al. 2006; Clark et al. 2007). TLR4 activation by lipopolysaccharide (LPS) releases tumor necrosis factor (TNF)-α (Aslam et al. 2006) and soluble CD154 (Cognasse et al. 2008) from platelets, which could enhance APC migration and/or function. In other studies, LPS was shown to inhibit thrombin-mediated activation of platelets (Bucki and Pastore 2006). Because TLR signals are important in the activation of the innate immune compartment and these signals have direct effects on the adaptive immune compartment, it will be interesting to determine the extent to which platelet-TLR signaling and direct activation of platelets by microbes ultimately influence adaptive immunity.

Platelets also produce molecules directly important to the adaptive immune response, i.e. FasL, TNF-related apoptosis-inducing ligand (TRAIL), IL-7, and CD154 (Ahmad et al. 2001; Crist et al. 2004; Elzey et al. 2005b; Soslau et al. 1997). The roles of platelet-derived FasL, TRAIL, and IL-7 have not been studied directly; however, FasL and TRAIL are potent inducers of apoptosis in cancer and virally infected cells (Ahmad et al. 2001; Brincks et al. 2008; Pitti et al. 1996), and local production via platelet activation may be an important immunological regulator. Functional IL-7 has been found in platelets, is released upon platelet activation, and may increase serum concentrations (Damas et al. 2003; Soslau et al. 1997). Since IL-7 is important for the homeostatic proliferation of T cells, platelet activation may contribute to this process.

PLATELET CD154 AND B-LYMPHOCYTE RESPONSES

B cells are responsible for antibody production in the primary and memory responses during infection and immunization. Regarding T-dependent humoral immunity, CD4 T-cell – B-cell communication via CD154 is perhaps the most critical event in normal B-cell mobilization and antibody production. In its absence, germinal centers (GCs) do not form in the spleen or lymph nodes. These are areas of B cells undergoing intense proliferation and are required for isotype switching, somatic hypermutation, and the generation of memory and plasma cells (Caux et al. 1994; Han et al. 1995; Held et al. 1993; Miller et al. 1995; Renshaw et al. 1994). As a result, transgenic mice deficient in either CD40 or CD154 are incapable of producing T-dependent isotype-switched antibodies and do not generate B-cell memory; however, immunoglobulin (Ig) M responses are normal (Renshaw et al. 1994). Clinically, CD40 or CD154 functional deficiencies are referred to as "hyper--IgM syndrome" due to depressed serum levels of IgG, IgA, and IgE but normal or elevated levels of IgM. These patients suffer from recurrent infections of encapsulated bacteria, among several others, which can be fatal (Lougaris et al. 2005).

In choosing a model to assess the role of platelet CD154 in classical B- and T-cell responses, the murine CD154 gene knockout mouse has proven to be an excel-

lent resource. Since they are incapable of producing appreciable levels of T-dependent isotype-switched antibodies, the impact of platelet CD154 on B-cell antibody production is easily detectable by increased plasma levels of IgG. Studies from our laboratory showed that normal platelets adoptively transferred into CD154--deficient hosts could transiently augment antigen-specific IgG production after adenovirus immunization in a GC-independent manner (Elzey et al. 2003). When adoptively transferred to CD154-deficient mice, relatively low numbers of normal CD4 T cells are unable to generate a significant GC response to adenovirus. However, when normal platelets were co-transferred with the CD4 T cells, sustained IgG production and significantly increased GC formation were detected (Elzey et al. 2005a). Alternatively, when high numbers of normal CD4 T cells were adoptively transferred, platelets did not further augment B-cell responses, indicating that platelet CD154 can indeed contribute to B-cell responses but may not be necessary when CD4 T cell--derived CD154 is abundant, which is the experimental setting of many antigen-specific adoptive transfer models. Although a recent report details the ability of platelets from immune thrombocytopenic purpura patients to prompt autologous self-reactive B cells directly to release antibodies against platelet GPIIbIIIa in a CD154-specific manner (Solanilla et al. 2005), it is unknown exactly how platelets in our model are signaling the B cells *in vivo*. It could be through direct contact with B cells, other cells such as follicular DCs that in turn stimulate B cells, or by particulate/soluble mediators released by the platelets.

Upon activation, platelets discharge microparticles and exosomes, collectively termed platelet-derived microvesicles (PDMVs) (Heijnen et al. 1999). These PDMVs bud off from the platelet and contain components of the platelet, including the CD154 protein (Baj--Krzyworzeka et al. 2002; Otterdal et al. 2004). We hypothesized that PDMVs are sufficient to deliver the platelet-derived CD154 signal. To test this, we developed a novel assay to determine the bioactivity of CD154 in the products of platelet activation. It is known that CD154 and TNF-α induce monocyte chemotactic protein (MCP)-1 production in the murine MS-1 endothelial cell line. By using the different platelet fractions in conjunction with anti-TNF-α, the specific activity of CD154 from the different platelet preparations could be determined by measuring MCP-1 mRNA levels using quantitative real time PCR. Like intact platelets, isolated PDMVs increased the MCP-1 message specifically through CD154 activity. Intriguingly, the PDMVs from activated platelets were able to induce MCP-1 mRNA despite extremely low levels of total protein, but true soluble CD154 did not, suggesting that CD154 in PDMVs may be the biologically active form. Subsequently, we determined that CD154-sufficient PDMVs were able to induce proliferation of primary B cells *in vitro*. When PDMVs were the singular source of CD154, IgG production was augmented and GC formation was re-established *in vivo* (Sprague et al. 2008). Physiologically, this implicates a possible role for PDMVs as mediators of disease states in which platelets are major contributors, including heart disease. Clinically, this could have significance for acute transfusion reactions arising from platelet transfusion that is attributed to platelet CD154 (Cognasse et al. 2008).

PLATELET CD154 AND T-LYMPHOCYTE RESPONSES

In addition to the important role of CD154 in B-cell function, CD154 is important in T cell-mediated responses. In the absence of CD40-CD154 interaction, primary T-cell responses are depressed and memory responses are compromised (Bennett et al. 1998; Borrow et al. 1996; Schoenberger et al. 1998).

CD4 T cells and platelets have recently been shown to be co-involved in hepatic ischemia/reperfusion injury with some involvement of CD154, although in an antigen-independent fashion (Khandoga et al. 2006). $CD3^+$ T cells have been shown to activate platelets through CD40/CD154 interactions to secrete RANTES to further mediate T-cell recruitment (Danese et al. 2004). PF4, solely produced by platelets in the circulation, can inhibit human T-cell activation *in vitro* (Fleischer et al. 2002) as well as inhibit human regulatory T-cell activity (Liu et al. 2005). Because platelet-associated CD154 and circulating soluble CD154 are increased in many autoimmune diseases (Bigalke et al. 2006; Cabeza et al. 2004; Danese et al. 2006; Danese et al. 2007; Zietkowski et al. 2008), further investigation in the ability of platelets to modulate CD4 T-cell responses may have clinical significance. The mechanism of this impact is unknown, but since platelets do not express requisite costimulatory molecules for activating naïve CD4 T cells, a reasonable hypothesis is that this modulation is accomplished via effects on DCs, not by direct contact with T lymphocytes.

Several lines of evidence point to a role for platelets in CD8 T-cell responses. We were the first to show that depletion of platelets lowers the generation of adenovirus-specific cytotoxic T lymphocytes (CTLs) and that CD154 on platelets plays a role in intravenous or subcutaneous adenoviral immunization (Elzey et al. 2008; Elzey et al. 2003). When mice were immunized with low doses of a recombinant replication-deficient adenovirus expressing the ovalbumin transgene (Ad5-mOVA) in the presence of collagen (a strong platelet agonist), CD8 T-cell frequency and lytic function were augmented. Moreover, only the mice immunized in the presence of collagen were protected from OVA-recombinant virulent *Listeria monocytogenes* challenge. Administration of blocking antibodies against the platelet-specific collagen receptor GPVI before immunization with adenovirus/collagen significantly inhibited the generation of antigen-specific CD8 T cells. This indicates that platelet responsiveness to collagen is the key factor in CTL gen-

eration to subcutaneous antigen and not enhanced transgene expression, as is possible with delivery of the vector in collagen. Additionally it was found that platelet CD154 was also required for collagen-mediated CTL augmentation (Elzey et al. 2008). How platelet CD154 affects the T-cell response is currently unknown, although it is easy to hypothesize that it is through conditioning of DCs and not direct T-cell contact since platelets lack costimulatory molecules, and it has been reported that human platelets are unable to induce allogeneic CD8 T-cell responses *in vitro* (Gouttefangeas et al. 2000). After our initial report that platelet CD154 could mature DCs *in vitro*, several others have corroborated those findings, although there are studies that report platelets can inhibit DC function, as noted above. However, it is difficult to envision how intact platelets could contact DCs in the draining lymph nodes responsible for T-cell clonal expansion. One possibility is the release of PDMVs that could be transported to the draining lymph nodes via transmigrating Langerhans cells. A recent report details the *in vitro* ability of neutrophil transmigration across epithelial cells, which enables platelet transmigration as well and could explain how intact platelets could gain entry to the subcutaneous space to be activated by the injected collagen (Weissmüller et al. 2008).

In two different models of hepatitis, CD8 T cells mediate viral clearance but are also the pathogenic effectors leading to critical organ damage (Iannacone et al. 2005). In the hepatitis B virus (HBV) transgenic model of acute viral hepatitis it was found that platelet depletion with anti-GPIbα diminished lesion severity and decreased the infiltration of adoptively transferred HBV-specific CTLs in the liver. Importantly, CTLs isolated from normal or thrombocytopenic mice retained function *in vitro*. Similar results were also seen in a lacZ model of acute viral hepatitis, which involves endogenous CTL generation with recombinant adenovirus instead of CTL adoptive transfer. In mice that were reconstituted with prostaglandin E1-inhibited platelets (Iannacone et al. 2005) or administered aspirin/clopidogrel to inhibit platelet function (Iannacone et al. 2007), hepatic accumulation of CTLs was also reduced. These studies suggest that platelet activation is important for CTL migration into the liver but not for effector function, although CTL frequency in secondary lymphoid organs and *in vivo* CTL function were not addressed.

Other studies demonstrated that platelets play a role in CTL responses to lymphocytic choriomeningitis virus (LCMV). LCMV-infected mice show a decrease in platelet numbers and reduced aggregation to collagen and ADP. Mice depleted of platelets and infected with LCMV generate approximately 80% fewer CTLs and have higher viral titers than un-depleted mice but have equal *in vivo* lytic capacity by eight days after infection. Reconstitution of platelet-depleted mice with platelets three days after LCMV infection have increased CTL numbers in the spleen and liver, suggesting that platelets are important for the expansion of antigen-specific CTLs. This group also investigated the role of platelet-derived CD154 in this system. Adoptive transfer of $CD154^{-/-}$ platelets three days after LCMV infection was indeed able to partially increase CTL numbers in platelet-depleted mice, suggesting that other platelet-derived molecules are also important in this process (Iannacone et al. 2008).

Conversely, platelet-derived products have recently been shown to modulate CD8 T-cell migration and CD8 T cell-mediated disease. The role of vasoactive serotonin has been investigated in the LCMV model. Mice that were devoid of CD8 T cells had reduced platelet aggregation in the liver upon LCMV infection, suggesting that CD8 T cells recruit platelets. $Thp1^{-/-}$ mice, which cannot synthesize serotonin, had reduced hepatocyte injury and fibrosis compared with wild-type (WT) controls upon LCMV infection. WT and serotonin-deficient mice develop similar numbers of interferon-γ-producing CD8 T cells in the spleen and liver, but fewer CD8 T cells migrated into the intralobular region of the liver in $Thp1^{-/-}$ mice. WT mice treated with exogenous serotonin had delayed CTL responses, decreased hepatic damage, and increased viral titers. This suggests that platelets, via serotonin release, hinder CD8 T-cell infiltration of the liver, allowing increased viral replication, and may ultimately cause chronic hepatitis (Lang et al. 2008), although acute pathology is less severe.

There are also conflicting data regarding the role platelets and platelet-associated products play in allograft rejection. Xu et al. found that soluble and platelet-derived CD154 contribute to allograft rejection by an unknown mechanism (Xu et al. 2006). Given that CD8 T cells are important in this process (Clarkson and Sayegh 2005), it is likely that platelets could be modulating the activation or generation of these cells. On the other hand, there is evidence that platelets may suppress CD8 T-cell function in a murine model of transfusion-related immunomodulation. Transfusion of MHC class I-bearing platelets allows for prolonged allograft survival (Aslam et al. 2008), although specific CD8 T-cell function and the involvement of CD154 were not addressed in this study. These reports indicate that platelets could have a tremendous clinical role in transplantation.

PLATELETS AND CLINICAL DISEASE

Due to their ubiquity in the circulation and very high numbers, platelets and their inflammatory/immune mediators are well suited to participate in the formation of many biologic responses. Several autoimmune inflammatory diseases with T- and B-cell components, including inflammatory bowel disease, arteriosclerosis (Mach et al. 1998; Schonbeck and Libby 2001), diabetes (Danese and Fiocchi 2005; Varo et al. 2005), immune thrombocytopenia (Solanilla et al. 2005), Kawasaki disease (Wang et al. 2003), and systemic lupus erythematosus (Delmas et al. 2005; Sidiropoulos and Boumpas 2004), have been linked

with increased platelet activation and surface expression of CD154. Elevated plasma CD154 is also detected and is considered a marker for many diseases of which platelets are thought to be the source. Currently it is unknown whether increased platelet CD154 is a result or a cause of these conditions. The connection between human disease and platelet influence on autoimmune T and B cell activity is speculative, but an intriguing hypothesis nonetheless. Should this hypothesis prove to be correct, it will be important to determine how to modulate CD154 expression on platelets.

Our group has begun to study the regulation of CD154 in the platelet progenitor cell, the megakaryocyte. In studying the regulation of CD154 expression in platelets, the megakaryocyte becomes an important consideration. Platelets have very limited transcriptional ability. Although known to take up and sequester a limited number of proteins from the serum, such as fibrinogen and immunoglobulin (Klinger 1997), the fact that the predominant form of CD154 in the inactivated platelet is the full-length membrane-bound form provides strong evidence that CD154 is expressed in megakaryocytes and subsequently packaged into proplatelets during thrombocytogenesis (Otterdal et al. 2004). Furthermore, in immune thrombocytopenic purpura, in which CD154 is up-regulated in megakaryocytes, CD154 mRNA was detected by *in situ* hybridization (Solanilla et al. 2005). We recently reported that megakaryocytes do indeed express CD154 during differentiation and maturation as they progress from an early hematopoietic progenitor cell to a mature megakaryocyte phenotype (Crist et al. 2008). It was further demonstrated that, like transient CD154 expression in activated T cells, calcium mobilization and protein kinase C activation mediated both differentiation-dependent and extracellular agonist-mediated CD154 expression in megakaryocytes (Crist et al. 2008). Overall, these studies were the first to show a regulatory pathway in megakaryocytes that potentially could alter the inflammatory and immunomodulatory activity of platelets. When these data are considered along with the observations that levels of circulating soluble and platelet-associated CD154 are elevated in several autoimmune and chronic inflammatory diseases, the potential of systemic feedback on the immunomodulatory and pro-inflammatory activity of platelets becomes evident.

CONCLUSION

In addition to supporting thrombosis, platelets release a significant array of immunologic and inflammatory mediators. Their high number in the bloodstream enables them to efficiently respond at the initiation of chronic or acute circulatory perturbations and inflammation. While this appears detrimental in the case of cardiovascular disease, it may have tremendous significance in the area of host defense. Platelets can assist the innate immune response by releasing chemotactic and vasoactive substances which attract and facilitate leukocyte extravasation into surrounding tissues and by activating the endothelium to do the same. Additionally, *in vitro* and *in vivo* evidence suggest platelets also enhance antigen presentation, resulting in a more robust adaptive immune response. Because of animal studies indicating that platelets can influence T- and B-cell responses, and inflammatory clinical diseases that appear to be autoimmune in nature, it becomes important to determine what, if any, role platelets may have in activating lymphocytes against self antigens.

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