Beta-2 microglobulin (β2M) is a molecular chaperone for the major histocompatibility class I (MHC I) complex, hemochromatosis factor protein (HFE), and the neonatal Fc receptor (FcRn), but β2M may also have less understood chaperone-independent functions. Elevated plasma β2M has a direct role in neurocognitive decline and is a risk factor for adverse cardiovascular events. β2M mRNA is present in platelets at very high levels and β2M is part of the activated platelet releasate. In addition to their more well studied thrombotic functions, platelets are important immune regulatory cells that release inflammatory molecules and contribute to leukocyte trafficking, activation, and differentiation. We have now found that platelet-derived β2M is a mediator of monocyte pro-inflammatory differentiation through non-canonical TGF-β receptor signaling. Circulating monocytes from mice lacking β2M only in platelets (Plt-β2M<sup>−/−</sup>) had a more pro-reparative monocyte phenotype, in part dependent on increased platelet-derived TGF-β signaling in the absence of β2M. Using a mouse myocardial infarction (MI) model, Plt-β2M<sup>−/−</sup> mice had limited post-MI pro-inflammatory monocyte responses, and instead demonstrated early pro-reparative monocyte differentiation, profibrotic myofibroblast responses, and a rapid decline in heart function compared to WT mice. These data demonstrate a novel chaperone-independent, monocyte phenotype regulatory function for platelet β2M, and that platelet-derived β2M and TGF-β have opposing roles in monocyte differentiation that may be important in tissue injury responses.
Platelet-Derived β2M Regulates Monocyte Inflammatory Responses

Zachary T. Hilt¹, Daphne N. Pariser¹, Sara K. Ture¹, Amy Mohan¹, Pearl Quijada¹, Akua A. Asante², Scott J. Cameron¹, Julie A. Sterling³, Alyssa R. Merkel², Andrew L. Johanson¹, Jermaine L. Jenkins⁴, Eric M. Small¹, Kathleen E. McGrath², James Palis², Michael R. Elliott⁵, Craig N. Morrell¹,⁵

Author Affiliations:

1. Aab Cardiovascular Research Institute, University of Rochester School of Medicine, Box CVRI, Rochester, NY, 14652
2. Center for Pediatric Biomedical Research, Department of Pediatrics, University of Rochester School of Medicine, Rochester, NY, 14652
3. Department of Veterans Affairs, Tennessee Valley Healthcare System. Department of Cancer Biology, Medicine, Division of Clinical Pharmacology, Bone Biology Center, and Biomedical Engineering, Department of Cancer Biology, Vanderbilt University, Nashville, TN, USA
4. Department of Biochemistry, University of Rochester School of Medicine, Rochester, NY, 14652
5. Department of Microbiology and Immunology, University of Rochester School of Medicine, Rochester, NY, 14652

To whom correspondence should be addressed.

Craig Morrell: Craig_Morrell@URMC.Rochester.edu
Box CVRI, Aab Cardiovascular Research Institute
University of Rochester School of Medicine
Box CVRI, Rochester, NY, 14652
Tel: (585) 276-7693
Fax: (585) 276-9921
Beta-2 microglobulin (β2M) is a molecular chaperone for the major histocompatibility class I (MHC I) complex, hemochromatosis factor protein (HFE), and the neonatal Fc receptor (FcRn), but β2M may also have less understood chaperone-independent functions. Elevated plasma β2M has a direct role in neurocognitive decline and is a risk factor for adverse cardiovascular events. β2M mRNA is present in platelets at very high levels and β2M is part of the activated platelet releasate. In addition to their more well studied thrombotic functions, platelets are important immune regulatory cells that release inflammatory molecules and contribute to leukocyte trafficking, activation, and differentiation. We have now found that platelet-derived β2M is a mediator of monocyte pro-inflammatory differentiation through non-canonical TGFβ receptor signaling. Circulating monocytes from mice lacking β2M only in platelets (Plt-β2M−/−) had a more pro-reparative monocyte phenotype, in part dependent on increased platelet-derived TGFβ signaling in the absence of β2M. Using a mouse myocardial infarction (MI) model, Plt-β2M−/− mice had limited post-MI pro-inflammatory monocyte responses, and instead demonstrated early pro-reparative monocyte differentiation, pro-fibrotic myofibroblast responses, and a rapid decline in heart function compared to WT mice. These data demonstrate a novel chaperone-independent, monocyte phenotype regulatory function for platelet β2M, and that platelet-derived β2M and TGFβ have opposing roles in monocyte differentiation that may be important in tissue injury responses.
Introduction

The immune system must continually meet pathogen challenges, maintain tissue homeostasis, and facilitate sterile tissue injury repair. This requires the integrated responses of many cells, including platelets. Platelets have active roles in the regulation of innate and acquired immunity, that are mediated in part by platelet secretion of immune effector molecules acting both locally and systemically, including PF4, RANTES, IL-1β, and TGFβ, amongst many others (1-3). The immune regulatory role for platelets includes the initiation or exacerbation of cardiovascular diseases such as atherosclerosis and myocardial infarction (MI) (4, 5). We have now discovered a novel means by which platelets regulate circulating monocyte inflammatory phenotypes; platelet-derived beta-2 microglobulin (β2M) exerts concentration-dependent, pro-inflammatory effects on monocytes that are opposed by transforming growth factor β (TGFβ).

Platelets, like most cell types except red blood cells (RBCs), express major histocompatibility complex class I (MHC I). MHC I is comprised of a 3 subunit MHCα chain and β2M. β2M is a chaperone for MHCα cell surface trafficking, and β2M stabilizes MHCα once at the membrane. β2M is neither covalently linked to MHCα, nor transmembrane bound, so it easily dissociates from the cell surface and is found at plasma concentrations of 1-2 µg/mL in healthy individuals (6). Plasma β2M has been described as a predictive biomarker for many vascular inflammatory diseases including cardiovascular disease and age associated neurocognitive decline (7-11), but a direct mechanistic role for β2M as an inflammatory molecule has not been demonstrated. β2M mRNA is the highest expressed platelet transcript (12, 13), and proteomic studies
demonstrated that platelets contain abundant β2M protein (14, 15). We have now discovered that platelets are a major source of plasma β2M, and that β2M has critical immune modulatory functions beyond its molecular chaperone roles.

Platelet-derived immune mediators have many direct and indirect effects on monocytes in disease-relevant conditions. Platelet interactions with an inflamed endothelial cell layer at sites of atherosclerotic lesion development lead to the arrest of monocytes at these sites and to the acceleration of atherosclerosis (16). The activation and differentiation of monocytes and macrophages are also directly affected by platelet-derived immune mediators (17, 18). Platelets and monocytes are both activated by MI, and have important roles in MI outcomes (5, 19, 20). Immediately following an MI there are elevated platelet-derived immune mediators and mobilization of pro-inflammatory monocytes that traffic into the infarct region, becoming M1-like pro-inflammatory macrophages. Following the early ischemic tissue injury responses, there is a decline in platelet activation and a shift in circulating monocytes to a pro-reparative phenotype that traffic to become M2-like reparative macrophages that promote infarct wound healing and fibrosis (21). We now demonstrate a direct mechanistic link between post-MI platelet activation, elevated platelet-derived plasma β2M, and platelet β2M-dependent regulation of monocyte phenotypes that affect cardiac injury responses to MI.
Results

Platelet-derived β2M induces monocyte pro-inflammatory differentiation

Platelets have abundant B2m mRNA and MHC I, but the contribution of platelets to plasma β2M concentrations is not known. To first demonstrate that stimulated platelets release β2M, washed mouse platelets were activated with either ADP or thrombin, and β2M release was quantified by ELISA. Activated platelets released β2M (Fig 1A). Global β2M−/− mice have many immune defects (22), so to explore the role of platelet derived β2M in immune function, we generated platelet-specific β2M knockout mice (PF4-Cre x β2Mfloxflox, Plt-β2M−/−, Fig S1). Washed platelets from WT and Plt-β2M−/− mice were isolated and MHC I surface expression was determined. Platelets from Plt-β2M−/− mice had greatly reduced surface MHC I compared to WT platelets (Fig 1B), but MHC I expression did not differ on white blood cells (WBC), indicating platelet specificity in the β2M−/− mice (Fig 1B, insert). Peripheral blood cell numbers, including CD8+ T cells, were similar in WT and Plt-β2M−/− mice (Fig S2), indicating that, unlike complete β2M−/− mice, a lack of platelet β2M does not grossly affect immune development. A lack of β2M also did not affect platelet activation, as ADP and thrombin similarly activated platelets from WT and Plt-β2M−/− mice based on P-selectin surface expression (Fig 1C), platelet aggregation (Fig 1C), fibrinogen binding (Fig S3), and activated platelet PF4 release (Fig S3). Furthermore, we measured plasma β2M in age-matched WT and Plt-β2M−/− mice and found that plasma β2M in WT mice was very similar to reported human values (11), but plasma β2M was significantly reduced in Plt-β2M−/− mice (Fig 1D), with no difference in other major platelet-derived plasma molecules, including PF4 (Fig S4). These data indicate that platelets are an important source of plasma β2M.
Because β2M has chaperone-independent functions (23), and platelets have important roles in monocyte responses, we asked whether β2M induced monocyte activation. The human THP-1 monocyte cell line was treated with control buffer or recombinant human β2M and IL-8 release was determined in a time- and dose-dependent manner. β2M induced IL-8 release beginning at concentrations consistent with those associated with increased cardiovascular event risk (Fig 2A) (6, 11). Mouse monocytes are broadly classified as pro-inflammatory - Ly6C<sup>hi</sup>, IL-6, KC (mouse IL-8 homologue) secreting - or pro-reparative - Ly6C<sup>lo</sup> and IL-10 secreting. To determine whether β2M induced a primary mouse monocyte pro-inflammatory response, isolated mouse bone marrow monocytes were incubated with β2M for 48 hrs, and their inflammatory phenotype determined by Ly6C expression and cytokine release. Recombinant β2M increased the number of Ly6C<sup>hi</sup> mouse monocytes and the secretion of both KC and IL-6 (Fig 2B-C). Human peripheral blood monocytes were also isolated and incubated with control buffer or recombinant β2M. Characteristic of pro-inflammatory human monocytes (24-26), β2M increased both the number of CD16<sup>++</sup> monocytes and monocyte IL-8 secretion (Fig 2D, confirmed with THP-1 cells Fig S5). These data demonstrated that β2M induced a pro-inflammatory monocyte phenotype.

Platelets have well described roles in both monocyte trafficking and activation (1), but platelet-mediated monocyte effector differentiation is not as well understood. To determine the role of platelet-derived β2M in monocyte effector responses, mouse monocytes were incubated with control buffer, the releasate from activated washed mouse platelets, or platelet releasate and anti-β2M antibody (platelets were thrombin activated and thrombin neutralized with hirudin; monocytes were incubated with platelet
releasate at a ratio of 1 monocyte to releasate from 10 platelets ratio). Consistent with a platelet β2M-mediated pro-inflammatory phenotype, platelet releasate increased Ly6C^{hi} monocytes and KC secretion, but platelet-induced monocyte polarization was attenuated by anti-β2M blocking antibody (Fig 3A and S6). Mouse monocytes were also treated with the releasate from WT or Plt-β2M^{-/-} platelets. In contrast to WT platelets, the releasate from Plt-β2M^{-/-} platelets did not induce a Ly6C^{hi} phenotype or KC secretion (Fig 3B-C). Plt-β2M^{-/-} releasate instead increased monocyte IL-10 release, typical of a pro-reparative monocyte phenotype (Fig 3C). Similar results were noted using platelet releasates from both male and female mice (Fig S7). These findings indicate that in the absence of β2M, platelet activation promotes pro-reparative monocyte differentiation.

Platelets contain and secrete both β2M and TGFβ (27, 28), but TGFβ is associated with inducing a pro-reparative / pro-fibrotic tissue environment and monocyte phenotype. TGFβ and β2M have been noted to be antagonistic in other cellular environments (29, 30), so to determine their effects on monocytes, THP-1 cells were treated with recombinant β2M and TGFβ1 at multiple concentration ratios, and monocyte responses determined after 48 hrs. β2M reduced TGFβ1 mediated IL10 expression (pro-reparative cytokine) and TGFβ1 limited β2M induced monocyte inflammatory cytokine production (IL-8) (Table 1). We therefore determined whether the pro-reparative phenotype induced by β2M^{-/-} platelet releasate was due to increased TGFβ signaling in the absence of β2M. Monocytes were incubated with WT or Plt-β2M^{-/-} releasate in the presence of anti-TGFβ blocking antibody. Blocking TGFβ blunted Plt-β2M^{-/-} releasate-induced IL-10 production (Fig 3D), and increased Plt-β2M^{-/-} releasate-
induced pro-inflammatory responses (Fig S8). There was no difference in platelet releasate TGFβ1 concentration between WT and Plt-β2M<sup>−/−</sup> mice (Fig S9). These data indicate that platelet-derived β2M and TGFβ1 have antagonistic effects on monocyte polarization.

β2M signals through a non-canonical TGFβ receptor pathway

We next sought to determine whether β2M and TGFβ signal through the same receptor, but with different downstream outcomes. To do so, we first pharmacologically inhibited mouse monocyte TGFβR1/TGFβR2 receptor complex activity using the TGFβR1 kinase inhibitor SB431542 (31). TGFβR inhibition blocked β2M-induced KC release (Fig 4A). Treatment of THP-1 cells with SB431542 also blocked β2M-induced upregulation of surface CD16 and IL8 mRNA expression, as well as TGFβ1-induced IL10 expression (Fig S10). As further proof that β2M interacts with TGFβR, we performed surface plasma resonance (SPR). TGFβR1 and TGFβR2 were covalently bound to a sensorchip and either recombinant β2M, TGFβ1, TGFβ3, BSA or FBS were used as the flow through ligands. β2M, TGFβ1 and TGFβ3 bound to the TGFβR1 at ratios similar to their signaling concentrations (Fig 4B, left), consistent with the physiologic observation that β2M signals monocytes at μg/mL concentrations compared to TGFβ signaling at ng/mL concentrations. We confirmed β2M binding to TGFβR2, however at a weaker ratio, compared to TGFβ1 and TGFβ3 (Fig. 4B, right). Negative controls, BSA and FBS, both had negative binding responses indicating β2M did not nonspecifically bind TGFβR (Fig S11). As further proof of TGFβR dependent β2M
monocyte activation, we isolated bone marrow monocytes from WT or myeloid cell-specific TGFβR2−/− mice (LysMCre-TGFβR2flox/flox) (32), treated the monocytes with control buffer or β2M, and determined Ly6C expression 48hrs later. β2M increased Ly6C expression on WT, but not TGFβR2−/−, monocytes (Fig 4C-D).

TGFβR signaling can either be anti-inflammatory (canonical, Smad2/3 dependent) (28), or pro-inflammatory (non-canonical, TAK1/p38/JNK dependent) (33). To explore downstream β2M-TGFβR signaling, THP-1 cells were treated with either a TAK1 (non-canonical pathway) inhibitor or a SMAD3 (canonical pathway) inhibitor prior to β2M treatment. The TAK1 inhibitor completely blocked β2M-induced IL-8 secretion, despite no change in β2M-induced SMAD3 phosphorylation (Fig 5A; P-TAK1 and P-SMAD3 quantified in Fig S12). SMAD3 inhibition only partially blocked β2M-induced IL-8 release (Fig S13), further indicating TAK1-dominant β2M signaling. To explore downstream TAK1 signaling, THP-1 cells were treated with JNK or p38 inhibitors. JNK inhibition strongly blocked β2M signaling, while p38 inhibition only partially blocked β2M inflammatory responses (Fig 5B). Similar results were found using primary mouse monocytes and these inhibitors (Fig S14). Monocytes treated with β2M from WT, but not TGFβR2−/− mice, had increased P-JNK as measured by the change in intracellular P-JNK compared to non-treated controls using intracellular flow cytometry (Fig 5C; TGFβ1 treated negative controls in S15). Similar to the immunoblot data, β2M increased intracellular P-JNK and P-SMAD2/3 in primary mouse monocytes treated with β2M, but this was blocked by the TAK1 and JNK inhibitors (Fig S15). These data together indicate that β2M downstream signaling is in part dependent on TAK1 and JNK.
β2M forms oligomeric complexes in a concentration-dependent manner through cysteine sulphydryl group cross-linking (34). To determine whether β2M signaling is dependent on oligomer formation, we pre-treated β2M with N-ethylmaleimide (NEM) to irreversibly block cysteine dependent oligomer formation and confirmed this by immunoblot after running control and treated β2M on a non-reducing gel (Fig 5D). Control or NEM-treated β2M was then incubated with mouse monocytes. NEM-treated, monomeric β2M did not significantly increase Ly6C<sup>hi</sup> monocytes compared to control β2M (Fig 5D). Treatment of recombinant β2M with NEM did not affect the measurement of β2M by ELISA (Fig S16). These data indicate that β2M signaling may be dependent on β2M in its oligomeric conformation.

*Platelet β2M regulates monocyte responses to tissue injury*

Upon tissue trafficking, monocytes polarize and differentiate to macrophage subsets that are most simply defined as pro-inflammatory (M1-like) or pro-reparative (M2-like). Macrophage polarization and differentiation are in part dependent on the trafficking monocyte phenotype. Ly6C<sup>hi</sup> monocytes tend to differentiate to M1-like macrophages that are inflammatory and phagocytic, whereas Ly6C<sup>lo</sup> reparative monocytes differentiate primarily to pro-fibrotic M2-like macrophages that promote fibroblast ECM production (35, 36). To determine whether platelet-derived β2M regulates basal circulating monocyte phenotypes, peripheral blood monocytes were isolated from WT and Plt-β2M<sup>−/−</sup> mice by negative selection, and markers of a pro-inflammatory or pro-reparative differentiation were quantified by qRT-PCR. Compared to WT monocytes, Plt-β2M<sup>−/−</sup> monocytes were skewed to a pro-reparative phenotype
Monocytes isolated from the bone marrow of WT and Plt-β2M−/− mice had similar Ly6C expression and responses to WT platelet releasates, indicating that β2M exerts its effects post-bone marrow development (Fig S17). To determine whether monocytes from Plt-β2M−/− mice are skewed to become pro-fibrotic macrophages, peripheral blood monocytes from WT and Plt-β2M−/− mice were isolated and co-cultured with primary adult cardiac fibroblasts for 72 hrs. When co-cultured with fibroblasts monocytes isolated from Plt-β2M−/− mice expressed gene markers indicative of reparative M2-like macrophages and had increased IL-10 secretion compared to WT mouse-derived monocytes co-cultured with fibroblasts (Fig 6B). There was also increased myofibroblast activation when fibroblasts were co-cultured with monocytes from Plt-β2M−/− mice (Fig 6C). These data indicate that platelet-derived β2M has a major role in post-development monocyte differentiation that affects their pro-fibrotic potential.

Platelets and monocytes are systemically activated and recruited to the heart by acute myocardial infarction (MI) (37). At the time of emergency room presentation plasma β2M was increased in confirmed MI patients compared to healthy controls, demonstrating the association of elevated β2M with acute MI events (Fig 7A). To explore the role of platelet-derived β2M in monocyte responses to MI, we used a mouse permanent ligation of the left anterior descending (LAD) artery model, but induced relatively small MIs to better explore the inflammatory component. WT and Plt-β2M−/− mice had similar tissue perfusion (Fig S18), and like human MI patients, WT mice had significantly elevated post-MI plasma β2M, however, Plt-β2M−/− mice did not (Fig 7B). This demonstrates that platelets are a major source of the increased plasma β2M post-
MI (there was no significant difference in plasma TGFβ1 between WT and Plt-β2M⁻/⁻ mice either pre- or post-MI Fig S19).

There are two phases of mouse monocyte responses to MI; an initial Ly6C<sup>hi</sup> pro-inflammatory monocyte response, followed 4-7 days later by Ly6C<sup>lo</sup> pro-reparative monocytes that promote wound healing and fibrosis (20, 21). To characterize post-MI monocyte responses in WT and Plt-β2M⁻/⁻ mice, blood was collected on multiple days and circulating monocyte Ly6C expression determined (gating strategy, Fig S20). WT mice had an expected early Ly6C<sup>hi</sup> monocyte response, that peaked about d7 before declining (Fig 7C). However, Plt-β2M⁻/⁻ mice had no change in post-MI Ly6C<sup>hi</sup> monocytes compared to baseline (Fig 7C). Cardiac function was assessed by echocardiography. Somewhat surprisingly, compared to WT mice, Plt-β2M⁻/⁻ mice had a rapid decline in post-MI heart function (Fig 7D and S21-S22). By d28 post-MI Plt-β2M⁻/⁻ mice still had worse heart function compared to WT mice, but the EF and FS began to converge (Fig S23). Histologic examination on d15 demonstrated that WT and Plt-β2M⁻/⁻ mice had similar CD68-positive (monocyte lineage marker) infiltrates (Fig 7E), but the CD68-positive cells tended to be focused in areas of fibrosis (trichrome stain) in Plt-β2M⁻/⁻ mice compared to WT mice (representative images, Fig 7E). The area of fibrosis was significantly higher in Plt-β2M⁻/⁻ mice on d15 compared WT mice (Fig 7F). These data are consistent with a pro-reparative skewing of monocytes in Plt-β2M⁻/⁻ mice, leading to an early pro-reparative M2-like macrophage response and increased myofibroblast activation.

To begin to define the early immune responses to MI in WT and Plt-β2M⁻/⁻ mice, we performed a protein array using pooled plasma from mice on d0 and d3 post-MI.
WT mice had an expected post-MI increase in multiple inflammatory molecules including IFNγ, IL-1 and TNFα (Fig 8A). The plasma cytokines elevated in Plt-β2M−/− mice were more indicative of pro-reparative responses, including elevated M-CSF and TIMP1 (Fig 8A). WT and Plt-β2M−/− mice had very similar pre- and post-MI peripheral blood counts, including monocytes (Fig S24), as well as very similar T-cell and neutrophil responses to MI (Fig S25). To better define the early post-MI circulating monocyte differentiation, monocytes were isolated on d0 and d3, and inflammatory cytokines quantified by qRT-PCR relative to their genotype controls. As expected, WT monocytes had increased post-MI expression of Cxcl1, while Plt-β2M−/− monocytes had increased expression of pro-reparative Il10 (Fig 8B), further demonstrating that platelet β2M promotes a pro-inflammatory monocyte response to tissue injury and in its absence a rapid pro-reparative response occurs. To assess the early post-MI macrophage infiltrates in the heart, markers of macrophage phenotype and myofibroblast activation were determined by qRT-PCR on d3 post-MI. Plt-β2M−/− and WT mice had similarly increased pro-inflammatory macrophage markers, but only Plt-β2M−/− mice had greatly increased expression of pro-reparative macrophage gene markers (Fig 9A). WT mice also had more M1-like MHC II hi macrophages in the heart compared to Plt-β2M−/− (Fig S26). We further validated the difference in heart macrophages by histological staining for Arginase-1 (Arg1), a marker for M2-like macrophages. There was a significant increase in post-MI Arg1+ cells in Plt-β2M−/− mice compared to WT mice (Fig S27). Myofibroblast activation markers were also increased in Plt-β2M−/− mouse hearts at this early time point (Fig 9B), suggesting that pro-reparative monocyte responses and M2-like macrophage differentiation in Plt-β2M−/− mice leads to early pro-fibrotic responses to
myocardial injury and an important role for platelets in post-MI monocyte/macrophage responses.

Because platelet-derived β2M and TGFβ have opposing monocyte differentiation effects \textit{in vitro}, we determined whether the \textit{in vivo} Plt-β2M\textsuperscript{−/−} pro-reparative are due to enhanced TGFβ signaling. Plt-β2M\textsuperscript{−/−} mice were treated with a TGFβ blocking antibody to reduce plasma TGFβ1 (Fig S28) and subjected to ligation of the LAD coronary artery. On d4 post-MI, Plt-β2M\textsuperscript{−/−} mice treated with TGFβ blocking antibody had similar monocyte pro-inflammatory Cxcl1 expression as WT mice (Fig 10A). Markers of cardiac macrophage differentiation and myofibroblast activation were also analyzed; increased M2-like pro-fibrotic macrophage and myofibroblast activation markers were attenuated in Plt-β2M\textsuperscript{−/−} mice treated with anti-TGFβ antibody (Fig 10B-C). WT mice treated anti-TGFβ antibody had no significant change in macrophage or myofibroblast activation markers at d4 post-MI compared to genotype controls (Fig S29) indicating that at this early time point TGFβ signaling is only dominant in Plt-β2M\textsuperscript{−/−} mice. These \textit{in vivo} data indicate that while platelet-derived β2M polarized monocytes towards pro-inflammatory responses, TGFβ acts to promote monocyte pro-fibrotic responses in response to MI.
Discussion

Taken together, our data demonstrate that platelet-derived β2M has a direct role as a central regulator of monocyte inflammatory responses. We have found that β2M and TGFβ may both signal monocytes through the same receptor, but with different downstream signaling pathways and physiologic outcomes. This implies that robust platelet activation, such as occurs post-MI, leads to elevated concentrations of plasma β2M that promote pro-inflammatory monocyte responses. However, after the immediate infarct event, the amount of ongoing platelet activation, and thus plasma β2M concentrations, eventually decline below the β2M signaling threshold. There is then a shift to pro-reparative monocytes that may be facilitated by a decline in β2M, allowing for TGFβ signaling that occurs at much lower concentrations than β2M. Latent TGFβ also becomes activated post-MI, perhaps accounting for a delay in TGFβ dominant signaling. Our results point to the important role of platelets as master regulators of monocyte differentiation responses in ischemic tissue injury, including platelet regulation of monocyte activation and inflammatory phenotype differentiation in a time post tissue injury-dependent manner. How β2M and TGFβ both signal through a common receptor, but with different outcomes, is not clear. TAK1 signaling is in part ubiquitination-dependent (38, 39), but ubiquitination of SMAD4 can limit SMAD2/3 nuclear trafficking (40). It is possible that ubiquitination-dependent events downstream of β2M and TGFβ receptor binding may regulate the different signaling outcomes, but more work is needed to demonstrate this.

It is interesting to note that while platelets have been associated with promoting inflammatory effects in the context of myocardial infarction and other acute tissue injury
models, platelet-derived TGFβ has been shown to contribute to fibrosis in models of cardiac hypertrophy using the mouse trans-aortic constriction (TAC) model (27, 41). TAC promotes cardiac remodeling and fibrosis by producing pressure overload, and results in less platelet activation than does MI. Mice lacking TGFβ only in platelets had less TAC-induced cardiac fibrosis (27) and mice depleted of platelets after TAC, had a reduction in the pro-reparative cytokine IL-10 compared to control TAC mice (41). This indicates that in more chronic vascular disease models, TGFβ signaling may dominate and produce a pro-reparative monocyte phenotype due to its lower signaling threshold compared to β2M. Therefore, injury size and tissue damage may dictate the degree of platelet activation response, which in turn through β2M and TGFβ concentrations, may direct monocyte differentiation responses.

Although this study examined β2M in the context of MI, the results may also be extended to other disease contexts. Elevated β2M is a biomarker of both age- and HIV-associated neurocognitive decline (11, 42), although the β2M cell source and its role in the disease process beyond a simple biomarker is not known. Platelet activation is also linked to HIV-associated neurocognitive decline (43), but more research must be conducted to evaluate if this occurs through a β2M-dependent mechanism. In addition, TGFβR is expressed by many types of cells. Our studies only examined β2M-TGFβR signaling in monocytes. Similar interactions may occur in other cell types, but more work is needed to determine whether our findings are monocyte specific, likely extending these studies into many other fields of biologic research.

Here we demonstrated a novel mechanism of platelet-mediated monocyte responses and differentiation. Platelet-derived β2M and TGFβ function in opposition to
each other to regulate monocyte pro-inflammatory vs pro-reparative differentiation. In the absence of either immune mediator the inflammatory response balance is disrupted and adverse outcomes may occur. Therefore, as therapeutic strategies targeting platelets in an inflammatory context are contemplated, these counter-balancing effects must be considered along the post-tissue time continuum, emphasizing the complex and still poorly understood roles for platelets in immune responses.
Materials and Methods

Reagents

- Anti- mouse APC MHC I [H-2Db] (17-5999-80/28-14-8), mouse PE-Cy5 MHC II [I-A/II-E] (50-141-32/[M5/114.15.2]), mouse APC CD4 (17-0041-82/GK1.5), mouse APC CD9 (17-0091-82/KMC8), mouse PE CD9 (12-00-91-81/KMC8) antibodies were purchased from eBioscience. Antibodies to mouse PE F4/80 (123110/BM8), mouse FITC CD3 (100306/145-2C11), mouse FITC Ly6C (128006/HK1.4), mouse/human APC CD11b (101212/[M1/70]), mouse PerCP/Cy5.5 CD115 (135526/AFS98), Mouse PE CD182 (149303/SA044G4), mouse PerCP/Cy5.5 CD184 (146510/L276F12), human BV421 CD14 (325627/HCD14), human APC CD16 (302012/3G8), human APC/Cy7 CD68 (333821/[Y1/82A]), human BV605 CD163 (333615/[GHI/61]) and mouse APC IgG2a (400219/MOPC-173) were purchased from BioLegend. Mouse FITC P-selectin/CD62P (553744/RB40.34), mouse FITC CD45 (553080/30-F11), mouse PE CD8a (553033/53-6.7), mouse FITC Ly6G (551460/1A8), human BV786 CD83 (565336/HB15e), mouse AlexaFluor 647 phospho-JNK (562481/N9-66) and mouse PE phospho-SMAD2/3 (562586/O72-670) antibodies were purchased from BD Bioscience. Human fibrinogen with Oregon Green 488 Conjugate (F-13192) was purchased from Thermo Fisher. Rabbit Anti-CD68 (ab125212) for immunohistochemistry, rabbit anti-TGFβ (ab92486) and rabbit AlexFluor 488 anti-β2M (ab195298) antibodies for immunoblot were purchased from Abcam. Rabbit monoclonal antibodies to phospho-Smad3 (9520S/C25A9), total Smad3 (9513S), phospho-Tak1 (4531S), total Tak1 (5206S) and Arginase-1 (93668S/D4E3M) were purchased from Cell Signaling Technology. ELISA kits for human IL-8 (DY208), human IL-6 (DY206), mouse IL-6
(DY406), mouse KC (DY453), mouse IL-10 (M1000B), mouse TGFβ1 (DY1679), mouse PF4 (DY595) and human β2M (KGE019) were purchased from R&D Systems.

Proteome profiler mouse cytokine array kit, panel A (ARY006) was purchased from R&D Systems and performed according to manufacturer instructions. ELISA kit for mouse β2M (LS-F14141) was purchased from LifeSpan BioSciences.

Lipopolysaccharides (LPS, L6529) from Escherichia coli O55:B5 was purchased from Sigma Aldrich. Human recombinant β2M (BDB551089) was purchased from BD Bioscience. Human β2M isolated from human urine (PRO-553) was purchased from ProSpec. Human recombinant TGFβ1 (78067) and TGFβ3 (78131) was purchased from STEMCELL Technologies. Recombinant mouse TGFβ1 (14-8342-62) was purchased from Thermo Fisher Scientific. 2-Methylthioadenosine diphosphate trisodium salt (ADP, 1624) and TAK1 inhibitor (5Z)-7-Oxozaeaelol (3604) were purchased from Tocris Bioscience. ALK-5 inhibitor SB431542 (61-646-15MG) was purchased from Thermo Fisher Scientific. P38 inhibitor SB202190 and JNK inhibitor SP600125 were purchased from Fisher Scientific. Human thrombin and Smad3 inhibitor SIS3 were purchased from Cayman Chemical. Neutralizing antibody to TGFβ and β2M were purchased from Bio X Cell. Recombinant human TGFβ receptor 1 (ALK-5) and human TGFβ receptor 2 Fc chimera proteins were purchased from R&D Systems. Series S CM5 chip for surface plasmon resonance was purchased through Fisher Scientific.

Mouse Studies

All mice used in this study were on a C57/Bl6 background. After verification that male and female WT and β2M⁻/⁻ platelets induced similar monocyte responses (Fig S7),
male mice were used for all studies. Platelet-specific \( \beta 2M \) knockout mice were generated by crossing PF4-Cre mice (Jackson Laboratory) with \( \beta 2M^{\text{flox/flox}} \) mice (Cyagen Biosciences) to obtain a PF4-Cre-\( \beta 2M^{\text{flox/flox}} \). The \( B2m \) gene (GenBank accession number: NM_009735, Ensembl: ENSMUSG00000060802) is located on mouse chromosome 2. Exon 1 contains the translation initiation codon, Exon 3 contains the stop codon; exons 2~3 were selected as the conditional knockout region flanked by LoxP sites (Extended data 1). The targeting vector was generated using BAC clones from the C57BL/6J library and will be transfected into the C57BL/6 ES cell line. The conditional KO allele was obtained after Flp-mediated removal of the Neo selection marker. Cre mediated recombination deleted exons 2~3 of \( B2m \). Myeloid TGF\( \beta R2^{-/-} \) and control WT mice were provided by Julie Ann Sterling (32).

Myocardial infarction was induced by ligation of the left anterior descending (LAD) coronary artery and echocardiography performed (VisualSonics, Toronto, Canada) by standard trans-sternal approach as we have described in past studies(5). Mice were perfused with methylene blue and hearts were collected and sectioned to indirectly quantify size of infarcted zone.

Complete blood counts (CBCs) were performed using an Abaxis VetScan HM5 on mouse blood obtained by retro-orbital collection into EDTA coated tubes (Greiner Bio One). Mouse plasma was isolated from blood collected in EDTA tubes and stored at -20°C.

Mouse hearts were collected at day 3 post-MI and immediately placed into digestion buffer at 37°C for 1 hour. Digestion buffer consisted of 1x PBS (Corning), 305 U/mg collagenase II (Worthington), and 100 mM CaCl\(_2\) (Sigma-Aldrich). The digested
hearts passed through a 100 µm mesh nylon strainer and then centrifuged at 1200 x RPM for 5 minutes. The pellet was resuspended into PBS and stained for flow cytometry.

Mice treated with anti-TGFβ antibody (BioXcell) received initial injection (300 µg) the day before LAD ligation. A second injection (100 µg) was given on d2 post-MI. Mouse hearts and peripheral blood were collected at d4 as previously described.

Immunohistochemistry

WT and PF4^{Cre+}β2M^{floxfloxflox} mouse hearts were collected and immediately placed into fixative (60% Methanol, 10% Acetic Acid, 30% dH₂O). Hearts were cross sectioned, paraffin embedded, and sectioned onto slides at a thickness of 5 µM.

For immunostaining, slides were deparaffinized and rehydrated and placed into 3% H₂O₂ for 15 minutes. Slides were washed with TBS 3 times. In a pressure cooker slides were incubated in Dako Target Retrieval Solution (S1699) for 15 minutes washed in PBS and then incubated in Dako Protein Block (X0909) for 30 minutes. Anti-CD68 (Abcam, 125212) was diluted 1:500 into Dako Antibody Diluent (S0809), Anti-Arginase1 was diluted 1:500 (Cell Signaling Technology, 93668S) and incubated overnight at 4°C. Slides were rinsed in PBS and biotinylated anti-rabbit antibody (Vector Laboratories, BA-1000, 1:250 in Dako Antibody Diluent) incubated for 30 minutes at room temperature. Slides were rinsed and incubated with VECTASTAIN® Elite® ABC-HRP Kit (Vector Laboratories, PK-6100) for 30 minutes, again and DAB Peroxidase (HRP) Substrate (Vector Laboratories, SK-4100) added for 5 minutes. Slides were washed in
dH20 for 5 minutes, counterstained, and coverslip added. As a negative control rat IgG2b was used in the primary antibody step.

Paraffin embedded mouse heart sections were mounted and stained with Masson’s trichrome reagent. Slides were deparaffinized, rehydrated and placed in Bouin’s Fix Solution (Fisher Scientific, 11-201) in the oven for 15 minutes. Slides were washed until yellow color disappeared. Equal parts of Welgerts Hematoxylin A and B (Fisher Scientific, 50-317-75, 50-317-79) were mixed and solution was added to each section for 5 minutes. Slides were rinsed with tap water for 5 minutes then quickly rinsed with dH20. Belbrich Scarlet Fuchsin (Sigma-Aldrich, HT151) was added to samples for 5 minutes. Slides were rinsed with dH20 for 5 minutes. One part Phosph tungstic Acid (Sigma-Aldrich, HT152), one part Phosphomolybdic Acid (Sigma-Aldrich, HT153) and two parts dH20 were mixed and the Phosph tungstic/Phosphomolybdic solution added to each slide for 5 minutes. The solution was tapped off and Aniline Blue (Sigma-Aldrich, HT154) was added to each slide for 5 minutes. Slides were then rinsed with 1% acetic acid (diluted in dH20) for 1 minute and dehydrated to Xylene and mounted with a coverslip.

All IHC images analyzed were taken under 10x magnification, using a BX41 microscope and imaged with SPOT camera and SPOT Basic imaging software. Trichrome slides were quantified using a previously published method (44) that utilizes ImageJ with the colour deconvolution (version 1.5) plug-in. The Masson Trichrome vector was selected, and the green component was used for analysis. Each samples threshold was adjusted to upper slider 0, lower slider 200 to distinguish areas of collagen deposition. The images were measured with pixel intensity and normalized to
surface area of tissue for each section. Slides stained for Arginase-1 were analyzed using ImageJ were images were converted to 8-bit and threshold was adjusted to upper slider 0, lower slider 160 to differentiate between positive cells (brown) and negative (blue). The adjusted images were measured for pixel intensity and normalized to tissue surface area.

Platelet Studies

Mouse platelets were obtained by retro-orbital bleed into heparinized Tyrodes solution. Washed WT and PF4-Cre-β2M^{floxflox} mouse platelets were isolated as previously described(45). For activation platelets were incubated with thrombin or ADP for 30 minutes. Surface CD62P, MHC I and Fibrinogen binding were analyzed by FACS. Platelet releasate collection, washed platelets resuspended in the monocyte culture media were stimulated with 1 U/mL of thrombin for 20 mins and thrombin neutralized with hirudin (1U/mL). Platelets were pelleted by centrifugation and the supernatant collected to treat primary mouse cells in culture. TGFβ1 and PF4 release were measured by ELISA. The ratio of platelets to monocytes was at a 100:1 or 10:1 ratio respective to the experiment.

For platelet aggregation, whole mouse blood was centrifuged for 15 mins at 1000 rpm, platelet rich plasma (PRP) was isolated and samples washed and resuspended in plasma at a concentration of 50× 10^7 platelets/mL. Samples were incubated with a 1:100 dilution of anti- CD9-APC or CD9-PE (Abcam) antibodies for 15 mins. Labeled platelets were mixed 1:1 and agonist-stimulated at 37°C while shaking as we have published (46). Double positive platelets were then quantified by flow cytometry.
Human plasma was collected from myocardial infarction patients under the University of Rochester School of Medicine Institutional Review Board (IRB) approved protocol RSRB00061784, and normal healthy controls under IRB protocol RSRB00028659.

Cell Culture

THP-1 cells (ATCC) were seeded in 24-well plates with RPMI media and 1X glutamax (Thermo Fisher), 2% penicillin/streptomycin (Invitrogen), 10% fetal bovine serum (FBS) (Life Technologies), 1X Sodium Pyruvate (Life Technologies), 1X non-essential amino acids (Life Technologies) and 1X minimum essential medium vitamins (Life Technologies). Cells were incubated with SB431542 (10 μM, Thermo Fisher Scientific), (5Z)-7-Oxozeaenol (1 μM, Tocris Bioscience), SIS3 (10 μM, Cayman Chemical), or SP600125 (10 μM, Fisher Scientific) 1 hour prior to treatment with β2M (5 μg/mL) or TGFβ1 (10 ng/mL). CD16 surface expression was measured through FACS. IL-8 release was determined through ELISA (R&D Systems, Duoset). Transcripts for Il8 and Il10 were analyzed by qRT-PCR. THP-1s were treated with β2M (0, 1, 2, 5, 10 μg/mL) and/or TGFβ1 (0, 1, 5, 10 μg/mL) for 48 hours. IL-8 release was measured through ELISA and Il10 transcripts through qRT-PCR.

Primary mouse monocytes were isolated from femurs and tibias of mice. The bone marrow was flushed with isolation buffer (1X PBS, 2% FBS, 1mM EDTA, Gibco) using a 20-gauge needle (BD Bioscience) and RBCs lysed with ACK Lysis Buffer (Gibco). Cell suspension was passed through a 100 μm mesh nylon strainer and monocytes were isolated using EasySep™ Mouse Monocyte Isolation Kit (STEMCELL Technologies). Circulating monocytes were collected by retro-orbital bleed into EDTA
coated tubes and spun to collect a WBC rich buffy coat. RBCs were lysed and monocytes isolated by the described negative selection kit. Isolated monocytes were cultured in DMEM with 1X Glutamax (Thermo Fisher), 10% FBS, 2% penicillin/streptomycin. Primary mouse monocytes were treated with recombinant β2M (5 µg/mL) or recombinant TGFβ1 (10 ng/mL) for 48 hours. Isolated mouse monocytes treated with platelet releasate were pretreated with control buffer, anti-TGFβ antibody (10 µg/mL), or anti-β2M antibody (10 µg/mL) for 1 hour prior to treatment with platelet releasate. Recombinant β2M (5 µg/mL) or PBS was pretreated overnight at 37°C with N-ethylmaleimide (Thermo Fisher, NEM, 10 µM) or control buffer under constant rocking; Primary mouse monocytes were then treated with the co-incubated PBS or β2M +/- NEM for 48 hours. Ly6C surface expression was measured through FACS.

Human peripheral blood was obtained from the New York Blood Center (New York, New York). The buffy coat was isolated from whole blood using Ficoll-Paque Plus (GE Healthcare) density centrifugation with SepMate™-50 (STEMCELL Technologies) according to the manufacturer’s instructions. From the buffy coat, primary human monocytes were isolated using EasySep™ Human Monocyte Isolation Kit (STEMCELL Technologies). Monocytes were incubated in the RPMI media described. Human monocytes were treated with purified β2M derived from human urine (10 µg/mL).

Adult cardiac fibroblasts were isolated from C57/Bi6 mice hearts. Hearts were excised from the mouse chest and the aorta and atria were removed prior to cutting the ventricular myocardium into 1mm pieces in 1xPBS. Tissue was then digested in 10 mg of Collagenase II (Worthington Biochemical Corporation; Lakewood, NJ) diluted in 10 mLs of PBS using a gentleMACS™ Octo Dissociator with Heaters (Miltenyi Biotec;
Auburn, CA). Digested tissue was then placed through a 100 mm strainer with full media (High glucose DMEM media containing 10% FBS and 1% Penicillin/Streptomycin) and centrifuged at 1000 rpm for 10 minutes. Pelleted cells were then plated on plastic culture dishes in full media for 5 hours to allow for the adherence of fibroblasts. After 5 hours, media was removed and adherent fibroblasts were washed twice with PBS before replacing cells with fresh full media. Media changes occurred once a day until fibroblasts reached 80% confluency. Isolated peripheral monocytes were then added and co-cultured for 72 hours.

Surface Plasmon Resonance

Surface plasmon resonance (SPR) measurements were performed on a General Electric (GE) Biacore T200 instrument, utilizing a CM5 sensor chip. TGFβR1/Alk-5 was covalently surface immobilized to the CM5 sensor chip at a concentration of 30 µM/mL. Experiments were performed using a running buffer of 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005 % Tween 20, 1% DMSO that was of 0.2µ filtered and degassed. Kinetics/affinity binding experiments for TGFβ1 (80 nM), TGFβ3 (80 nM), β2M (40 µM), BSA (7 µM), and FBS (10%) were run at a flow rate of 30 µL/min. Injections had a contact time of 90 sec, dissociation time of 120 sec, and binding was measured in relative response units (RU) that were calculated based on the difference between the immobilized protein flow cell and control flow cell to subtracted blank injections of buffer alone. The regeneration buffer consisted of 2 M NaCl and was injected after each ligand were flowed. Regeneration buffer had a contact time of 30 seconds, flow rate of 30 µL/min, and stabilization period of 60 sec.
TGFβR2 was covalently surface immobilized to the CM5 sensor chip at a concentration of 60 µM/mL. Affinity binding experiments for TGFβ1 (20 nM), TGFβ3 (20 nM), β2M (40 µM), BSA (7 µM) and FBS (10%) were run at a flow rate of 30 µL/min. Injections had a contact time of 60 sec, dissociation time of 120 sec, and binding was measured in relative response units (RU) that were calculated based on the difference between the immobilized protein flow cell and control flow cell to subtracted blank injections of buffer alone. The regeneration buffer consisted of glycine pH 2.0 (GE Healthcare) and was injected after each ligand was flowed. Regeneration buffer had a contact time of 30 seconds, flow rate of 30 µL/min, and stabilization period of 60 sec.

Analysis of results were performed using GE Biacore T200 evaluation software version 3.0. Results were graphed in GraphPad Prism as an average of replicates.

Quantitative Real-Time PCR

Hearts from mice were collected d0 or d3 post-MI and placed into RNAlater RNA Stabilization Reagent (Qiagen). Isolated hearts were homogenized using a Tissue-Tearor (BioSpec). Isolated primary mouse monocytes from peripheral blood, whole blood platelets and/or primary heart fibroblasts were pelleted and resuspended in RLT lysis buffer. Cultured THP-1 cells were pelleted and resuspended in RLT lysis buffer. RNA was extracted using RNeasy Mini Kit (Qiagen). Concentration of RNA was determined using Nanodrop (Thermo Fisher Scientific). The isolated RNA was turned into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time PCR analysis was performed using the TaqMan® Gene Expression Master Mix Protocol on a BioRad iCycler. TaqMan Gene expression primers were purchased from ThermoFisher Scientific.
Immunoblotting

Cells were lysed with 1X cell lysis buffer (Cell Signaling Technology) mixed with protease inhibitor cocktail (Sigma-Aldrich) per manufacture instructions. Protein samples were mixed 1:1 with 2x Laemmlı buffer and loaded into Mini-PROTEAN® TGX Gels (BioRad). The gels were run at 100V, in 1X Tris/Glycine/SDS Buffer. Protein was transferred from the SDS-PAGE gel to a nitrocellulose membrane (BioRad) in transfer buffer at 110V for 1 hour with an ice pack. Blots were blocked in blocking buffer consisting of 3% BSA (Sigma Aldrich) dissolved in Tris-buffered saline (Fisher Scientific) with 0.1% Tween-20 (TBS-T) for 1 hour at room temperature. Primary antibody was diluted 1:1000 in blocking buffer and incubated overnight at 4°C. Anti-rabbit HRP was used as the secondary antibody (GE Healthcare) and diluted 1:10000 in 5% milk for 1 hours at room temperature under constant rocking. Immunoblots were developed with Supersignal West Pico (Thermo Fisher) using the BioRad ChemiDoc MP chemiluminescence settings.

Non-Reducing Gel Electrophoresis

Recombinant β2M (10 µg/mL) was pretreated overnight at 37°C with NEM (10 µM) or control buffer under constant rocking. Samples were mixed 1:4 into 4x non-reducing loading buffer (bromophenol blue, xylene cyanol, glycerol in H2O). Samples were loaded into Mini-PROTEAN® TGX Gels (BioRad). The blots were run at 100V, in 1X Tris/Glycine Buffer. Protein was transferred from the non-reducing gel to a nitrocellulose membrane (BioRad) in transfer buffer at 100V for 1 hour with an ice pack.
Blots were blocked in blocking buffer consisting of 3% BSA (Sigma Aldrich) dissolved in Tris-buffered saline (Fisher Scientific) with 0.1% Tween-20 (TBS-T) for 1 hour at room temperature. FITC-tagged β2M antibody was diluted 1:1000 in blocking buffer and incubated overnight at 4°C. Blots were developed using BioRad ChemiDoc MP Alexa 488 setting.

Data Analysis

Flow cytometers were either Accuri C6 or BD LSR II. All FACS samples were analyzed using FlowJo version 7.6. Proteome profiler mouse cytokine array kit, panel A was analyzed using ImageJ version 1.50i. Real-time PCR was analyzed in Microsoft Excel using calculations for fold change 2(-delta delta CT) using Gapdh as reference gene and normalized to genotype controls. Relative protein expression was determined using BioRad Image Lab version 6.0.

Statistical Overview

All statistical tests were done using GraphPad Prism. Standard student t-test, 2 tailed, was used for when two independent samples were compared with P-value <0.05 considered significant. One-way ANOVA with Bonferroni Correction was used when comparing more than two independent groups. A P-value of <0.05 was considered statistically significant and represented graphically by 1 star; any p-value <0.01 was represented by two stars. All data represents mean ± SEM.

Study Approval

All animal studies were approved by the University of Rochester Institutional Animal Care and Use Committee (Rochester, NY) under protocol number 2009-022.
Human studies were approved by the University of Rochester (Rochester, NY) Institutional Review Board under protocol number RSRB00061784. All subjects provided informed consent prior to their participation.
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45. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, et al. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell*. 2003;115(2):139-50.

Author Contributions: Zachary Hilt and Craig Morrell planned, performed, and analyzed experiments and wrote the manuscript. Daphne Pariser, Sara Ture, Amy Mohan, Pearl Quijada, Akua Asante, and Andrew Johanson all performed and analyzed experiments. James Palis, Kathleen McGrath, Scott Cameron, Michael Elliott, Jermaine Jenkins, Julie Ann Sterling, Alyssa Merkel and Eric Small all provided experimental input, reagents, and analyzed data.

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Table 1. β2M and TGFβ are antagonistic to each other. THP-1 cells were co-incubated with multiple concentrations of recombinant β2M or TGFβ and IL10 mRNA or IL-8 release determined. β2M blunted TGFβ-induced IL10 and TGFβ reduced β2M-mediated IL-8 release, demonstrating antagonistic roles for these molecules in monocyte effector responses (Both tables, N=3).
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IL10 Fold Change
Figure 1.

A. [Graph showing [\(\beta_2M\)](ng/mL) levels in control, ADP, and thrombin treatments.

B. [Graph showing CD45+ cell distribution by MHCI expression in WT and \(\beta_2M^{-/-}\) platelets.

C. [Graph showing P-selectin (MFI) expression in WT and Plt-\(\beta_2M^{-/-}\) platelets in response to varying ADP concentrations.

D. [Graph showing fold change in double positive platelet aggregation in response to varying thrombin concentrations in WT and Plt-\(\beta_2M^{-/-}\) conditions.

** and *** denote statistical significance.
Figure 1. Platelets are a major source of plasma β2M.  

A) Activated platelets release β2M. Mouse platelets were isolated and treated with control buffer, ADP (10 µM) or thrombin (1 U/mL). β2M release was measured by ELISA (N=4, *P<0.05, **P<0.01, One-way ANOVA with Bonferroni Correction).  

B) Platelets, but not WBC, from Plt-β2M−/− mice lack MHC I surface expression. Anti-MHC I or control IgG was incubated with circulating platelets or CD45+ cells (insert) from WT and Plt-β2M−/−. MHC I was quantified by flow cytometry (N=3, *P<0.01, One-way ANOVA with Bonferroni Correction).  

C) Platelets from WT and Plt-β2M−/− mice had similar activation and aggregation. Washed WT and Plt-β2M−/− platelets were stimulated with ADP and surface P-selectin measured. Platelets were also labeled with APC or PE antibodies, thrombin stimulated and platelet aggregates determined as double positive cells by flow cytometry (N=4 for both panels, One-way ANOVA with Bonferroni Correction).  

D) Platelets are a major source of plasma β2M. Concentration of plasma β2M in 10 wk old WT and Plt-β2M−/− mice was determined by ELISA (N=5 WT, N=6 Plt-β2M−/−, *P<0.05 vs WT, Unpaired two-tailed t-test with Welch’s correction).
**Figure 2. β2M induced monocyte inflammatory responses.**  

**A)** THP-1 cells were treated with β2M and IL-8 release was determined by ELISA. β2M induced inflammatory molecule production in a time and dose dependent manner (N=4, *P<0.05, **P<0.01 vs 0, One-way ANOVA with Bonferroni Correction).  

**B-C)** β2M induced a primary mouse monocyte pro-inflammatory phenotype. Mouse bone marrow monocytes were isolated and treated with control PBS or β2M (5 μg/mL).  

**B)** 48 hrs later Ly6C surface expression was measured by flow cytometry (N=9 control, N=8 β2M, **P<0.01, Unpaired two-tailed t-test with Welch’s correction)  

**C)** KC and IL-6 release determined by ELISA (N=9 control, N=8 β2M, **P<0.01, Unpaired two-tailed t-test with Welch’s correction).  

**D)** β2M induced a human monocyte inflammatory phenotype. Human peripheral blood monocytes were isolated and treated with control PBS or β2M (10 μg/mL). CD16 surface expression was measured by flow cytometry (right panel, N=4, *P<0.05, Unpaired two-tailed t-test with Welch’s correction) and IL-8 release by ELISA (Left panel, N=4 control, N=7 β2M, *P<0.05, Unpaired two-tailed t-test with Welch’s correction). All figures were representative graphs of experiments that were repeated at least twice.
Figure 3.

A. Ly6Ch^hi (%) vs. control, releasate, and releasate + anti-β2M Ab.

B. Ly6Ch (%) vs. control, WT, and β2M^/-.

C. [KC] ng/mL vs. control, WT, and Plt-β2M^/-.

D. Interleukin-10 (pg/mL) vs. control, WT, Plt-β2M^/-, and anti-TGFβ Ab.
Figure 3. Platelets induce a monocyte pro-inflammatory phenotype in a β2M dependent manner. A-B) Mouse monocytes were incubated with control buffer, the releasate from WT platelets +/- anti-β2M antibody, or B) Plt-β2M-/- mouse platelet releasate (10:1 platelet:monocyte ratio). Control WT platelet releasate induced Ly6C hi monocytes more than A) anti-β2M antibody (N=4 control, N=6 releasate +/- anti-β2M Ab, *P<0.05 vs Control, One-way ANOVA with Bonferroni Correction) or B) Plt-β2M-/- mouse platelet releasate (N=4 control, N=3 WT, Plt-β2M-/-, *P<0.05 vs Control, One-way ANOVA with Bonferroni Correction). C) Releasate from β2M-/- platelets did not induce mouse monocyte KC, but instead induced IL-10 release (Both panels, N=4; *P<0.01 vs Control, One-way ANOVA with Bonferroni Correction). D) Plt-β2M-/- releasate induced IL-10 is TGFβ dependent. 10 µg/mL of anti-TGFβ antibody blocked Plt-β2M-/- releasate induced IL-10 production (N=4; *P<0.05, One-way ANOVA with Bonferroni Correction). All graphs and tables are representative from experiments repeated at least twice.
Figure 4. β2M pro-inflammatory phenotype signaling is through non-canonical TGFβ receptor signaling. A) Inhibition of TGFβR signaling ameliorated β2M induced monocyte activation. Mouse monocytes were incubated with control buffer, β2M, or β2M and a TGFβRI kinase inhibitor (SB431542). KC production was determined 48 hrs later (N=4; **P<0.01, One-way ANOVA with Bonferroni Correction). B) β2M binds to TGFβR1 and TGFβR2. Fc-TGFβR1 or Fc-TGFβR2 were immobilized on a sensor chip and β2M, TGFβ1 or TGFβ3 binding determined by SPR. C-D) WT and TGFβR2−/− monocytes were incubated with control buffer or β2M. C) β2M induced WT, but not TGFβR2−/− monocyte, Ly6C hi phenotype. D) Quantification. (N=4; *P<0.05, One-way ANOVA with Bonferroni Correction).
Figure 5.

A. 

B. 

C. 

D.
**Figure 5.** β2M signals through a non–canonical TGFβ receptor signaling mechanism.  

A) Inhibition of TAK1 (7-ox, 1µM) blocked β2M induced THP-1 IL-8, but had no effect on β2M induced SMAD3 activation (P-SMAD3). THP-1 cells were treated with TAK1 inhibitor or control buffer prior to β2M. IL-8 was determined by ELISA (N=4; **P<0.01 vs control, One-way ANOVA with Bonferroni Correction) and P-SMAD3 and total SMAD3 determined by immunoblot (representative image quantified in supplemental data).  

B) JNK inhibitor (SP600125, 10 µM) greatly attenuated THP-1 IL-8 production, but p38 inhibitor (SB202190, 10 µM) only partially attenuated IL-8 (Both panels; N=4, **P<0.01 vs control, One-way ANOVA with Bonferroni Correction).  

C) β2M-induced WT, but not TGFβR2−/− monocyte, p-JNK. WT and TGFβR2−/− monocytes were incubated with control buffer or β2M and intracellular p-JNK determined by flow cytometry. (N=4, *P<0.05, Unpaired two-tailed t-test with Welch’s correction).  

D-E) Blocking β2M oligomer formation with NEM inhibited β2M induced monocyte inflammatory phenotype. β2M was pre-treated with buffer or NEM and then added to mouse monocytes for 48 hrs. D) NEM reduced β2M oligomer formation (non-reducing gel, top), and E) Ly6C^hi^ monocytes (Bottom panel, N=4, *P<0.05, One-way ANOVA with Bonferroni Correction).
Figure 6.

A. Inflammatory

- **Cd86**
- *Fcgr1*
- **Chil3**
- **Arg1**

B. Inflammatory

- **Cd86**
- *Nos2*
- **Fcgr1**

Reparative

- **Chil3**
- **Arg1**
- **Il10**

C. Postn

- **Col1a2**
- **Acta2**
- **Col3a1**
Figure 6. Platelet β2M regulates circulating monocyte differentiation. A) Circulating monocytes were isolated from WT and Plt-β2M<sup>−/−</sup> mice and pro-inflammatory and pro-reparative monocyte gene markers were quantified by qRT-PCR. Plt-β2M<sup>−/−</sup> mouse monocytes had pro-reparative monocyte gene expression (All panels, N=4; *P<0.05, Unpaired two-tailed t-test with Welch’s correction). B-C) Monocytes from Plt-β2M<sup>−/−</sup> mice differentiate to pro-reparative, fibroblast activating, macrophages <i>in vitro</i>. Peripheral blood monocytes from WT and Plt-β2M<sup>−/−</sup> mice were co-incubated with cardiac fibroblasts for 72 hrs and B) macrophage differentiation and C) fibroblast activation determined by qRT-PCR, and IL-10 secretion determined by ELISA. Plt-β2M<sup>−/−</sup> derived monocytes had increased pro-reparative differentiation and IL-10 secretion and induced more fibroblast activation compared to WT mouse monocytes (All Panels of B and C, N=4; *P<0.05, **P<0.01 vs WT, Unpaired two-tailed t-test with Welch’s correction).
Figure 7.

A. 

B. 

C. 

D. 

E. 

F.
Figure 7. Platelet derived β2M mediates monocyte inflammatory responses to myocardial infarction.  A) Plasma β2M is elevated in humans post-MI. Plasma was isolated from healthy subjects and confirmed MI patients. β2M was measured by ELISA (N=22 Control, N=55 MI, *P<0.05 vs Control, Unpaired two-tailed t-test with Welch’s correction). B) WT, but not Plt-β2M−/− mice had increased post-MI plasma β2M. β2M measured pre and d1 post-MI by ELISA (N=5; *P<0.05 vs Plt-β2M−/−, One-way ANOVA with Bonferroni Correction). C) WT mice had increased circulating Ly6C hi monocytes post-MI, but Plt-β2M−/− mice had no change from d0 (N=3 WT 0,2, Plt-β2M−/− 0,2,3 days; N=4 WT 3,18, Plt-β2M−/− 18 days, N=5 Plt-β2M−/− 7 days, N=6 WT 7 days; *P<0.05, **P<0.01, One-way ANOVA with Bonferroni Correction). D) Plt-β2M−/− mice had a rapid post-MI decline in heart function compared to WT mice (Both panels, N=5, *P<0.05 vs Plt-β2M−/−, Unpaired two-tailed t-test with Welch’s correction). E) WT and Plt-β2M−/− mice had similar post-MI monocyte lineage infiltrates (CD68+), but monocytes are associated with areas of ECM deposition (trichrome) in Plt-β2M−/− mice. Representative images 20X images, from d15 post-MI. F) Fibrosis quantification (N=3; Control; N=4 D15 Post-MI, *P<0.05, One-way ANOVA with Bonferroni Correction).
Figure 8.

A. WT Control vs Plt-β2M−/− Control

WT MI vs Plt-β2M−/− MI

Positive

CXCL13 C5a G-CSF GM-CSF CCL1 CCL11 sICAM-1 IFN-γ IL-1a IL-1b IL-1ra IL-2
IL-3 IL-4 IL-5 IL-6 IL-7 IL-10 IL-13 IL-12 p70 IL-16 IL-17 IL-23 IL-27
CXCL10 CXCL11 CXCL1 M-CSF CCL2 CCL12 CXCL9 CCL3 CCL4 CXCL2 CCL5 CXCL12
CCL17 TIMP-1 TNF-α TREM-1

PBS

Increased WT MI vs Control
Increased Plt-β2M−/− MI vs Control

B. Cxcl1 and Il10 Expression

Fold Change

WT Plt-β2M−/− WT Plt-β2M−/−
Figure 8. Plt-β2M−/− mice have an early pro-reparative skewed monocyte response to MI.  

A) Plasma inflammatory protein array. WT mice had a d3 post-MI increase in pro-inflammatory plasma proteins, whereas Plt-β2M−/− mice had a greater increase in pro-reparative plasma proteins.  

B) Circulating monocytes were isolated from WT and Plt-β2M−/− mice pre and d3 post-MI and qRT-PCR performed for Cxcl1 and Il10. Monocytes from Plt-β2M−/− mice had less Cxcl1 and increased Il10 expression post-MI compared to WT mice. (Both panels; N=4, **P<0.01, One-way ANOVA with Bonferroni Correction).
Figure 9.

A. 

![Graphs showing fold change for Cd86, Nos2, and Fcgr1.](image)

B. 

![Graphs showing fold change for Chil3, Arg1, and Il10.](image)

C. 

![Graphs showing fold change for Postn, Fn1, Col1a2, and Col3a1.](image)
Figure 9. Plt-β2M⁻/⁻ mice have an early reparative and fibrotic response to MI. Cardiac mRNA was isolated from control and d3 post-MI hearts. A) Plt-β2M⁻/⁻ mouse hearts had increased post-MI markers of pro-reparative macrophage phenotype (N=4; normalized to genotype control, **P<0.01, One-way ANOVA with Bonferroni Correction) and B) increased fibroblast activation compared to WT mice (N=4; normalized to genotype control, *P<0.05, One-way ANOVA with Bonferroni Correction).
Figure 10.

A. 

**Cxcl1**

- WT
- Plt-β2M^+/−
- Plt-β2M^+/− + αTGF-β Ab

Fold Change

B. 

**Il10**

- WT
- Plt-β2M^+/−
- Plt-β2M^+/− + TGFβ Ab

**Arg1**

- WT
- Plt-β2M^+/−
- Plt-β2M^+/− + TGFβ Ab

Fold Change

C. 

**Postn**

- WT
- Plt-β2M^+/−
- Plt-β2M^+/− + TGFβ Ab

**Fn1**

- WT
- Plt-β2M^+/−
- Plt-β2M^+/− + TGFβ Ab

Fold Change
Figure 10. Plt-β2M−/− early pro-reparative responses to MI are at least in part TGFβ dependent. Plt-β2M−/− mice were treated with anti-TGFβ antibody prior to MI. D4 post-MI, monocyte and macrophage inflammatory phenotypes and myofibroblast activation were determined. Blocking TGFβ in Plt-β2M−/− mice A) increased circulating monocyte inflammatory cytokine expression (N=4, **p<0.01, One-way ANOVA with Bonferroni Correction) and B) decreased cardiac pro-reparative macrophages (N=4, **p<0.01, One-way ANOVA with Bonferroni Correction) and C) decreased myofibroblast activation compared to control Plt-β2M−/− mice (N=4, **p<0.01, One-way ANOVA with Bonferroni Correction).