Cellular & Molecular Immunology (2015) 12, 700–707
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RESEARCH ARTICLE

Platelets promote allergic asthma through the expression of CD154

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Platelet activation is associated with multiple immune responses and the pathogenesis of various immune-related diseases. However, the exact role and the underlying mechanism of platelets in the progression of allergic asthma remain largely unclear. In this study, we demonstrate that during antigen sensitization, platelets can be activated by ovalbumin (OVA) aerosol *via* the upregulation of CD154 (CD40L) expression. Platelet transfer promoted allergic asthma progression by inducing more severe leukocyte infiltration and lung inflammation, elevated IgE production and strengthened T helper 2 (Th2) responses in asthma-induced mice. Accordingly, platelet depletion compromised allergic asthma progression. *Cd154*-deficient platelets failed to promote asthma development, indicating the requirement of CD154 for platelets to promote asthma progression. The mechanistic study showed that platelets inhibited the induction of Foxp3$^+$ regulatory T cells both *in vivo* and *in vitro* at least partially through CD154, providing an explanation for the increase of Th2 responses by platelet transfer. Our study reveals the previously unknown role of platelet CD154 in the promotion of asthma progression by polarizing Th2 responses and inhibiting regulatory T-cell generation and thus provides a potential clue for allergic disease interventions.
Cellular & Molecular Immunology (2015) **12,** 700–707; doi:10.1038/cmi.2014.111; published online 24 November 2014

Keywords: allergic asthma; CD154; platelet; regulatory T cells; Th2 response

INTRODUCTION

Platelets are small anucleate fragments derived from bone marrow megakaryocytes and have traditionally been viewed as crucial mediators of coagulation and thrombosis. However, increasing evidence suggests that platelets also have important roles in regulating immunity and inflammation through various mechanisms.[1] Platelets express a variety of sensors, including pattern recognition receptors, through which they can rapidly recognize and capture invading bacteria.[2] In addition, they are equipped with a large number of intracellular bioactive granules (including δ-, α- and λ-granules), which are rapidly mobilized and released upon platelet activation. These released granules contain a variety of inflammatory cytokines, chemokines and adhesive molecules that can then recruit and activate neutrophils and monocytes, as well as other leukocytes, thereby amplifying local antimicrobial defense. Moreover, platelets can physically interact with adjacent monocytes and dendritic cells (DCs) *via* receptor-ligand interactions, particularly CD154/CD40 crosstalk, contributing to enhanced antigen presentation and adaptive immune responses.[3,4]

CD154 (CD40 ligand, CD40L) is a membrane bound protein belonging to the TNF superfamily. Engagement of CD40 by its ligand CD154 plays a central role in mediating the interaction between antigen-presenting cells (APCs) and lymphocytes.[5] Particularly, CD154 derived from activated platelets is involved in multiple immune processes, including endothelial cell reactions,[6] germinal center formation,[7] T-helper cell priming[8,9] and cytotoxic T-cell activation.[10,11] However, the detailed mechanism how platelet CD154 influences the development of different T-cell subsets and consequently shapes the outcome of adaptive immune responses remains unclear.

By using different animal models, studies have shown the indispensable role of platelets in the initiation and progression of inflammatory and autoimmune diseases, including sepsis,[12] atherosclerosis,[13] autoimmune myocarditis,[14] systemic lupus erythematosus,[15] rheumatoid arthritis[16–19] and so on. Allergic asthma is a chronic inflammatory pathological condition with critical involvement of T helper 2 (Th2) cells that is harmful to human health throughout the world.[20] Identifying the role of platelets in the development of allergic asthma will not only

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Received: 7 August 2014; Revised: 12 October 2014; Accepted: 13 October 2014

benefit our understanding of the molecular and cellular mechanisms of allergic asthma but may also provide potential targets for therapy of this disease. Although previous studies showed evidence of platelet activation,[21–23] as well as of the involvement of CD154[24] in asthma, the exact role of platelet activation and the underlying mechanisms in the progression of allergic asthma remain elusive.

In this study, we found that allergens can directly activate platelets and upregulate the expression of CD154 by platelets. By platelet transfer or depletion experiments, we showed that platelets promote the progression of allergic asthma. Using *Cd154*-deficient ($Cd154^{-/-}$) mice, we found that CD154 is required for the function of platelets in promoting asthma. Finally, we demonstrate that platelets inhibit the differentiation of regulatory T cells *via* CD154 and consequently polarize the Th2 response, adding new insights into the pathogenesis of allergic asthma.

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 mice were from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China). $Cd154^{-/-}$ mice were from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained under pathogen-free conditions and used at 6–8 weeks of age. All animal experiments were carried out according to National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University (Shanghai, China).

Reagents

RPMI medium 1640 and fetal bovine serum were purchased from PAA Laboratories (Les Mureaux, France). Ovalbumin (OVA, grade V) was purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-mouse CD4-coated magnetic beads were from Miltenyi Biotech (Gladbach, Germany). FITC-conjugated antibodies to mouse CD154, CD40 and P-Selectin, an APC-conjugated antibody to mouse CD41, PerCP-Cy5.5-conjugated antibodies to mouse CD4, and anti-IFN-γ, anti-IL-4 and anti-mouse Foxp3 staining sets were from eBioscience (San Diego, CA). The rabbit anti-mouse thrombocyte antibody (AIA31440) was from Accurate Chemical & Scientific Corporation (NY, USA). Recombinant human TGF-β was from PeproTech. Mouse IL-4, mouse IL-13 and mouse TGF-β ELISA kits were from R&D (MN, USA).

Regulatory T-cell induction

Regulatory T cell (Treg) induction was carried out according to methods previously described[25] with minor modifications. $CD4^+$ T cells were enriched from C57BL/6 splenocytes *via* positive selection with magnetic beads, and the purity of enriched cells was confirmed to be over 90% by FACS. $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (5 μg/ml), soluble anti-CD28 (2 μg/ml), rhTGF-β (10 ng/ml), anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml) in the absence or presence of platelets from wild-type or $Cd154^{-/-}$ mice for 72 h. Intracellular staining of Foxp3 was then performed.

Flow cytometry

For cell surface staining, the single-cell suspensions were incubated with the antibody cocktails for 20 min at 4 °C. Intracellular Foxp3 staining was then performed with an anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Data were obtained on an LSR II and analyzed with FACSDiva software (both from BD Biosciences, CA, USA).

Cytokine assays

Levels of IL-4, IL-13 and TGF-β were detected using ELISA kits according to the manufacturer's protocols (R&D).The serum concentration of OVA-specific IgE was determined by ELISA. Briefly, 96-well plates (Nunc, Rochester, NY, USA) were coated with 10 μg/ml OVA overnight, incubated with diluted serum samples (1 : 200) for 2 h and then incubated with anti-mouse IgE-HRP (eBioscience) for 1 h. Plates were washed five times with wash buffer between each step. The results were analyzed spectrophotometrically at 450/590 nm. All incubation steps occurred at room temperature.

Isolation and activation of murine platelets

Murine platelets were prepared as previously described.[6] In brief, peripheral blood from C57BL/6 mice was collected *via* cardiac puncture into syringes containing 0.5 ml acid-citrate dextrose buffer (10.0 g/l *D*-glucose, 12.5 g/l Na-Citrate, and 6.85 g/l citric acid), and pipetted into tubes containing 5 ml PIPES buffer (150 mM NaCl and 20 mM PIPES, pH 6.5). Blood samples were centrifuged at 100*g* for 10 min, and the supernatant was collected and mixed with 1 U/ml apyrase and 1 M prostaglandin E1. The supernatant was centrifuged at 1000*g* for 10 min. The platelet pellet was resuspended in Tyrodes buffer (134 mM NaCl, 20 mM HEPES, 12 mM $NaHCO_3$, 2.9 mM KCL, 0.34 mM Na_2PO_4, 1 mM $MgCl_2$, 5 mM glucose and 0.5 mg/ml BSA, pH 6.5). To activate platelets, 0.5 U/ml thrombin was added to platelets in Tyrodes buffer. All steps were performed at room temperature.

Induction of allergic asthma

BALB/c mice were immunized intraperitoneally with 100 μg OVA emulsified in 100 μg aluminum hydroxide on days 1, 8 and 15. On days 22, 24 and 26, mice were aerosol-challenged for 30 min with 1% OVA diluted in 5 ml PBS delivered by a PARI-Boy nebulizer (PARI GmbH, Starnberg, Germany). Platelets (from either wild-type or $Cd154^{-/-}$ mice) or anti-platelet antibodies were given intratracheally 30 min before each OVA aerosol challenge. Control mice were not immunized with OVA, but received the normal OVA aerosol challenge. After the last aerosol challenge, mice were sacrificed and analyzed for bronchoalveolar lavage (BAL) fluid platelet activation, leukocyte infiltration and Th2 cytokine expression, serum OVA-specific IgE levels and lung histology.

Histological analysis

Histology analysis was carried out according to previously described protocols.[26] In brief, lungs were inflated with 4%

paraformaldehyde, embedded in paraffin, sectioned and stained with H&E.

Statistical analysis

The statistical significance of differences for paired samples was analyzed by two-tailed Student's *t*-tests. Statistical significance was determined as $P<0.05$.

RESULTS

Ovalbumin aerosols induce platelet activation in the airway

Platelets can be activated by various physiological and pharmacological stimuli such as thrombin, adenosine diphosphate and arachidonic acid, among others. To address whether airway platelets can be activated by allergens during antigen sensitization, we induced allergic asthma in BALB/c mice with OVA aerosol challenge and analyzed platelet activation in BAL fluid. CD41 is a platelet-specific marker, whereas CD154 is inducibly expressed by activated platelets. We found that platelets harvested from OVA-exposed mice had significantly increased expression of the activation marker CD154, whereas those from control mice did not express CD154 (Figure 1). Thus, OVA aerosol is sufficient to induce platelet activation in the airway.

Platelet transfer exaggerates allergic asthma, whereas platelet depletion ameliorates allergic asthma

We next investigated whether platelets could affect the development of allergic asthma through the adoptive transfer of platelets or depletion of platelets (administration of an anti-platelet antibody) before each OVA inhalation. The number of $CD41^+$ platelets in BAL fluid significantly increased after platelet transfer and decreased after platelet depletion over an 8-h period (Figure 2a). Giemsa staining of BAL fluid showed increased numbers of eosinophils, macrophages, lymphocytes and neutrophils after platelet transfer and decreased numbers after platelet depletion in asthma-induced mice (Figure 2b). Consistently, lung histology showed increased mononuclear infiltration and tissue inflammation after platelet transfer but decreased infiltration after platelet depletion (Figure 2c). Serum IgE is closely associated with the severity of asthma.

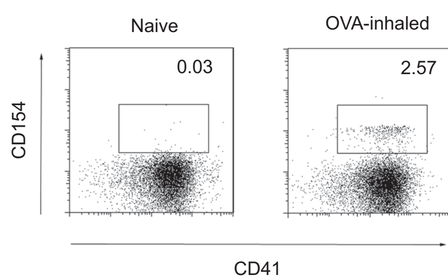

Figure 1 OVA induces platelet activation in the airway. BAL fluids collected from BALB/c mice, either untreated (naive) or challenged with OVA aerosol (OVA-inhaled), were analyzed for CD41 and CD154 expression by FACS. Data are representative of three independent experiments with similar results. BAL, bronchoalveolar lavage; OVA, ovalbumin.

We found that platelet transfer increased the expression of serum OVA-specific IgE, whereas platelet depletion decreased the expression (Figure 2d). Asthma is characterized by enhanced Th2-type responses. We also found that platelet transfer increased IL-4 and IL-13 levels in BAL fluid, whereas platelet depletion decreased those levels (Figure 2e). Together, we found more severe disease progression of allergic asthma in asthma-induced mice after platelet transfer and, accordingly, reduced disease severity of allergic asthma after platelet depletion. Thus, platelets promote the progression and severity of allergic asthma.

CD154 is required for platelets to promote asthma development

Activated platelets release various immune modulatory factors and upregulate a series of membrane bound ligands, which contribute to the regulation of immune responses. Platelet-derived CD154 has been shown to affect adaptive immune responses. However, the exact role of CD154 in allergic asthma remains unknown. Unlike wild-type platelets, which enhanced lung leukocyte infiltration (Figure 3a and b), serum OVA-specific IgE levels (Figure 3c), and lung IL-4 and IL-13 expression (Figure 3d), platelets from $Cd154^{-/-}$ mice failed to enhance asthma development (Figure 3). Therefore, *Cd154* deficiency blocks the function of platelets to promote asthma progression. Thus, CD154 is required for platelets to promote asthma development.

Platelets inhibit Treg generation *via* CD154

We next wondered about the mechanism by which platelet CD154 promotes asthma progression. It was shown that the inhibition of Tregs was associated with enhanced Th2-type inflammation in asthma, so we studied the impact of platelet activation on Treg generation. Under Treg polarization conditions *in vitro*, Foxp3 expression was considerably reduced in the presence of platelets, suggesting that platelets inhibit Treg induction *in vitro* (Figure 4a). Moreover, $Cd154^{-/-}$ platelets had a reduced ability to inhibit Treg induction compared with wild-type platelets, suggesting that platelets inhibit Treg generation at least partially *via* CD154 expression (Figure 4a). We next examined the effect of platelets in Treg generation *in vivo*. We found a reduced frequency of Tregs in the mediastinal lymph nodes of asthma-induced mice after platelet transfer. Consistently, $Cd154^{-/-}$ platelets had a reduced ability to inhibit Treg generation *in vivo* compared with wild-type platelets (Figure 4b). Together, platelets inhibit Treg induction both *in vitro* and *in vivo* at least partially *via* CD154. Because we did not include APC in our *in vitro* culture system, the above data suggest a direct inhibitory role of platelets on Treg differentiation. In addition to the abundant CD154 expression, platelets also have high expression of other immune regulatory molecules such as P-Selectin, CD40 and TGF-β. However, there was no significant difference in the expression of P-Selectin, CD40 and TGF-β between these two strains (Figure 4c and d), indicating that the reduced inhibitory capacity of $Cd154^{-/-}$

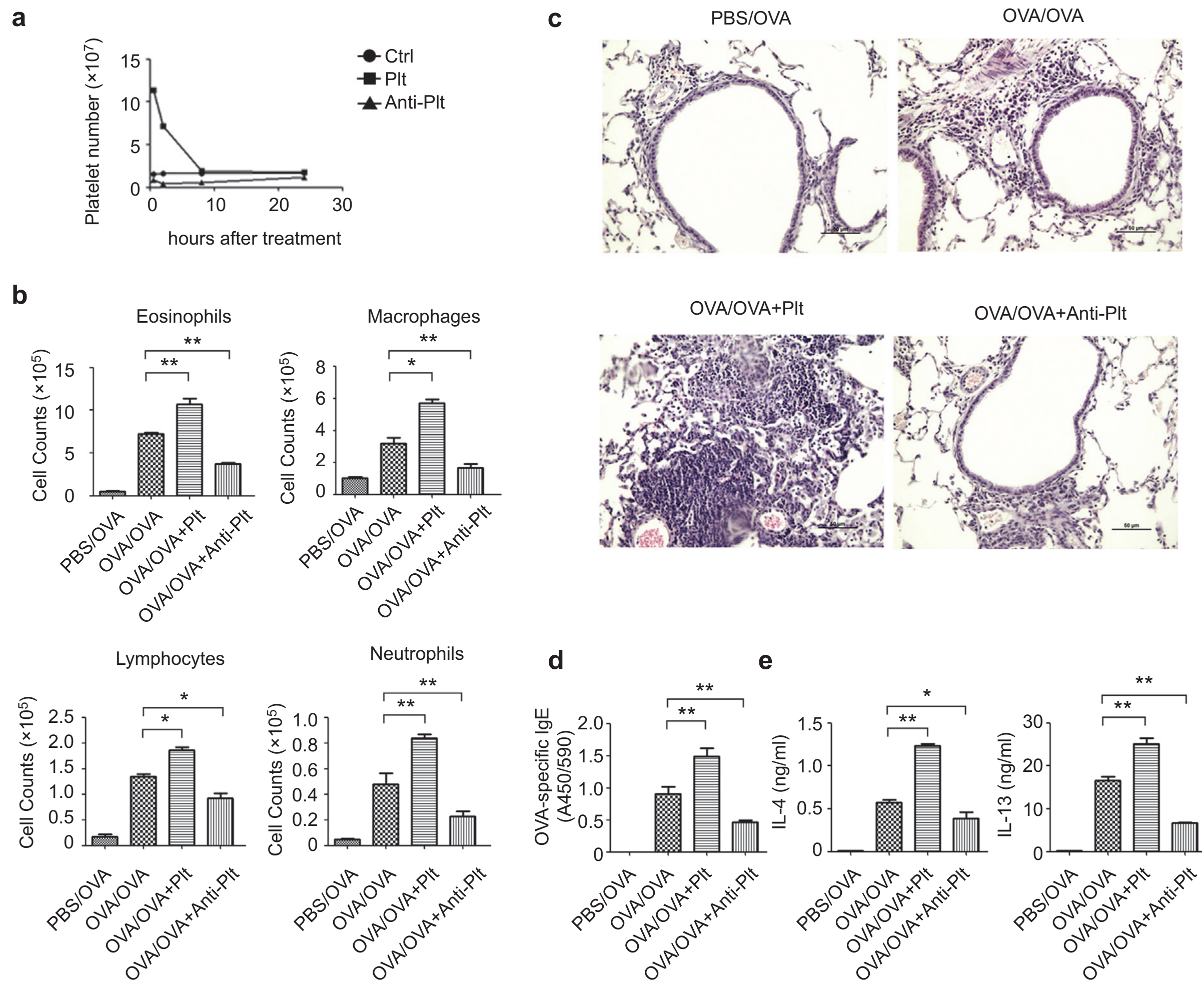

Figure 2 Platelet transfusion enhances the progression of allergic asthma, whereas platelet depletion alleviates disease progression. (**a**) Changes of platelet numbers in BAL fluid after platelet transfer or depletion. Mice were left untreated (Ctrl) or were administered platelets (Plt) or anti-platelet antibody (Anti-Plt). (**b**–**e**) BALB/c mice were administered platelets (OVA/OVA+Plt), anti-platelet antibody (OVA/OVA+Anti-Plt) or PBS (OVA/OVA) before exposure to OVA-aerosol. Control BALB/c mice were not immunized but received OVA aerosol challenge (PBS/OVA). After the last OVA inhalation, the mice were killed for disease progression analyses. BAL fluid was collected and stained with Giemsa for leukocyte counts (**b**). Lung tissues were prepared for paraffin-embedded sectioning and H&E staining. Scale bars=50 μm (**c**). Sera were collected, and OVA-specific IgE was measured by ELISA (**d**). BAL fluid was collected, and the concentrations of IL-4 and IL-13 were assessed by ELISA (**e**). *$P<0.05$ and **$P<0.01$ (two-tailed Student's *t*-test). Data are from three independent experiments (**a**, **b**, **d**, **e**; the mean and s.d. of five determinants) or are representative of three independent experiments with similar results (**c**). BAL, bronchoalveolar lavage; OVA, ovalbumin.

platelets on Treg differentiation is not due to the altered expression of these molecules.

DISCUSSION

Platelets are classically viewed as crucial mediators of thrombosis and coagulation. However, increasing evidence has demonstrated the regulatory role of platelets in various immune processes and in the development of various autoimmune diseases. It had been noticed that platelet activation was related to the progression of asthma, yet the exact role and underlying mechanisms of platelets in asthma progression remained elusive. In our study, we demonstrated that platelets promote the progression of allergic asthma through their expression of CD154, thus providing a new mechanistic explanation for the immunological mechanism of allergic asthma and outlining attractive intervention strategies for allergic diseases.

Asthma is a chronic airway inflammatory disorder associated with Th2 overactivation, elevated IgE production and strengthened mast cell and eosinophil recruitment. Upon exposure to airway allergens, APCs trigger the initiation of innate immune signaling, undergo maturation and migration, and subsequently activate and regulate T cell-dependent adaptive immune responses. The pulmonary microenvironment can also induce the generation of regulatory DCs, which contribute to the maintenance of immune homoeostasis and the control of lung inflammation.[27] In particular, Th2-type cytokines are the major driver of asthma through the induction of Th2 cell survival (IL-4), IgE production (IL-4 and IL-13), and mast cell and eosinophil recruitment, activation and maturation

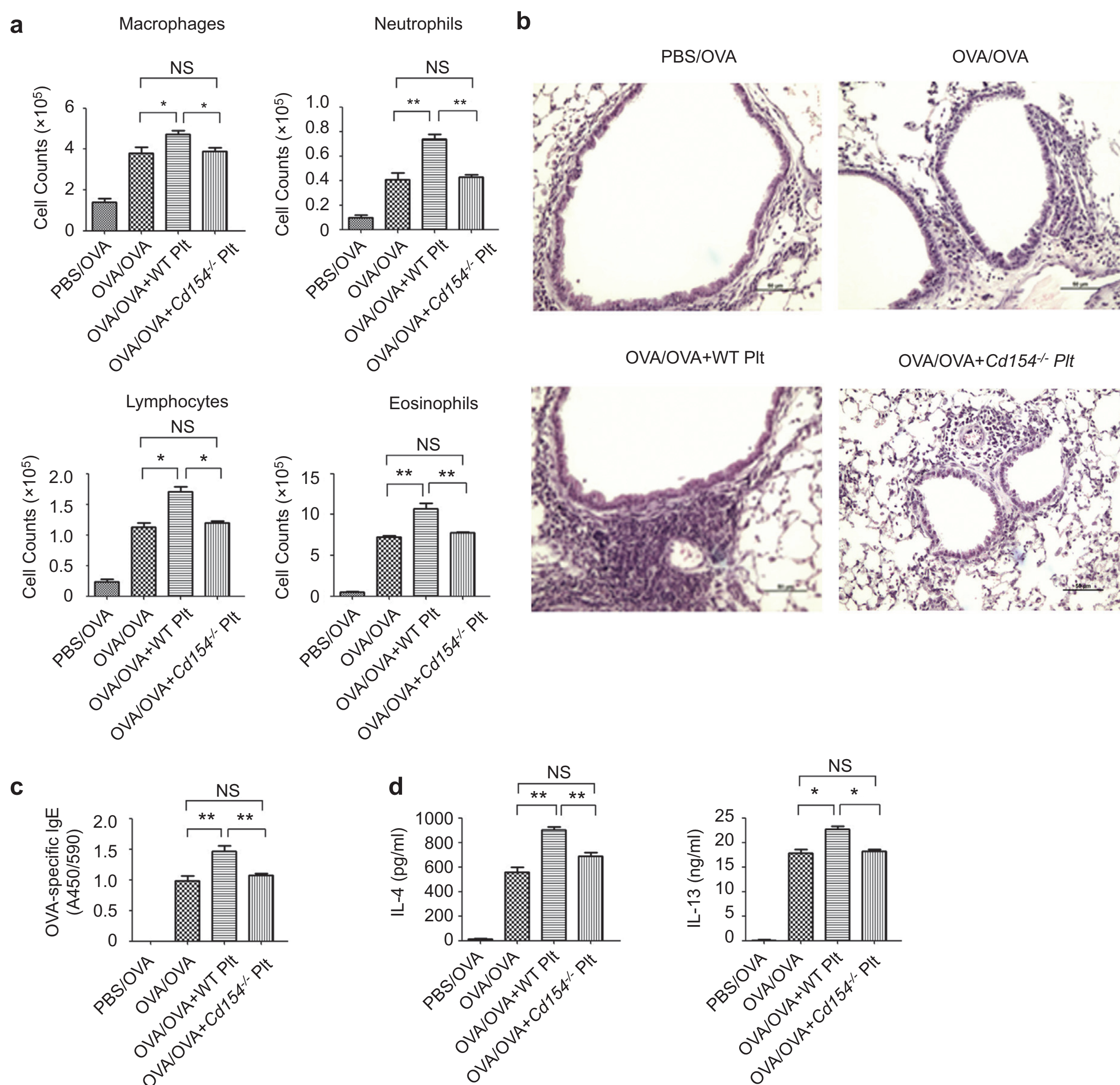

Figure 3 CD154 is required for platelets to promote asthma. BALB/c mice were administered platelets derived from wild-type mice (OVA/OVA+WT Plt) or *Cd154*$^{-/-}$ mice (OVA/OVA+*Cd154*$^{-/-}$ Plt) or were administered PBS (OVA+PBS) before exposure to OVA-aerosol. Control BALB/c mice were not immunized but received OVA aerosol challenge (PBS/OVA). After the last OVA inhalation, the mice were sacrificed for disease progression analyses. (**a**) BAL fluid was collected and stained with Giemsa for leukocyte counts. (**b**) Lung tissues were prepared for paraffin-embedded sectioning and H&E staining. Scale bars=50 μm. (**c**) Sera were collected, and OVA-specific IgE was measured by ELISA. (**d**) BAL fluid was collected, and the concentrations of IL-4 and IL-13 were assessed by ELISA. NS, not significant, *P<0.05 and **P<0.01 (two-tailed Student's *t*-test). Data are from three independent experiments (**a**, **c**, **d**; the mean and s.d. of five determinants) or are representative of three independent experiments with similar results (**b**). BAL, bronchoalveolar lavage; OVA, ovalbumin.

(IL-3, IL-5, IL-9, IL-13 and GM-CSF).[28] Considerable efforts have been made to develop strategies to treat allergic inflammation through the modulation of innate and adaptive immunity. However, clinical trials with inhibitors of IL-4, IL-13 and GM-CSF have been successful in some studies focusing on asthma therapy but not in others.[29] Therefore, it would be more effective to treat asthma by simultaneously suppressing multiple cytokines and disconnected pathological processes. In this study, we show that platelet depletion and *Cd154* depletion could significantly attenuate asthma progression by inhibiting IL-4, IL-13 and IgE production and leukocyte infiltration, suggesting a promising strategy for the treatment of allergic asthma.

The CD40–CD154 interaction was shown to promote the production of IgE and pro-inflammatory mediators in asthma. For example, engaging CD40 on epithelial cells promoted asthma progression,[30] while silencing *Cd40* gene expression in epithelial cells resulted in reduced disease progression.[31] CD40–CD154 crosstalk between B cells and mast cells was shown to induce airway inflammation and remodeling in allergic asthma.[32] Moreover, *Cd40* gene polymorphisms were

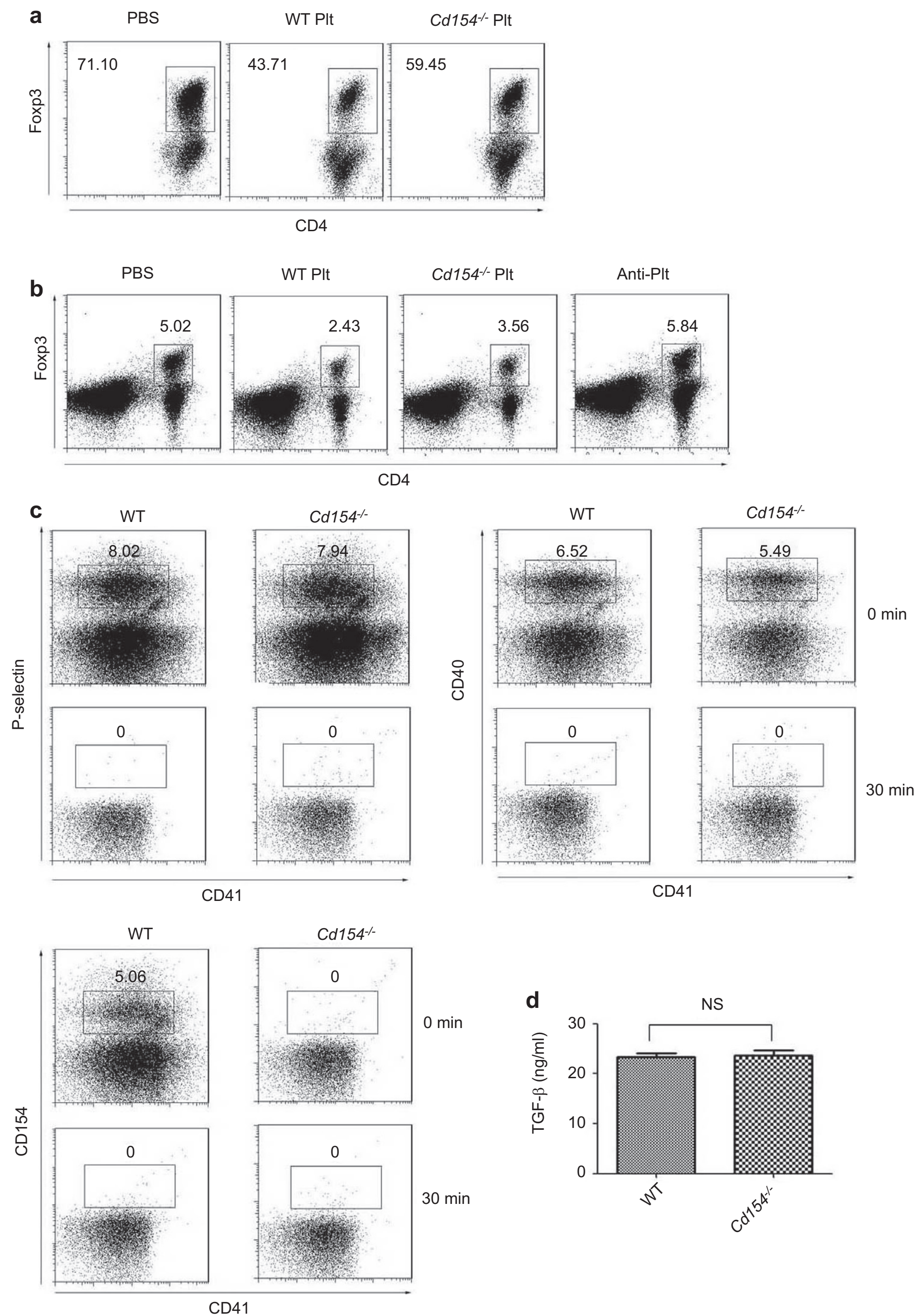

Figure 4 Platelets inhibit the induction of Treg generation *in vitro* and *in vivo* via CD154. (**a**) $CD4^+$ T cells were cultured under Treg polarization conditions in the presence of platelets from wild-type (WT Plt) or *Cd154*$^{-/-}$ mice (*Cd154*$^{-/-}$ Plt) or PBS for 72 h and analyzed for Foxp3 expression. Numbers indicate percentages of $Foxp3^+$ cells in the $CD4^+$ gate. (**b**) BALB/c mice were administered platelets from wild-type mice (WT Plt) or *Cd154*$^{-/-}$ mice (*Cd154*$^{-/-}$ Plt), PBS (PBS) or anti-platelet antibody (Anti-Plt) before exposure to OVA-aerosol. Mediastinal lymph nodes were collected after the last OVA inhalation and were analyzed for Foxp3 expression. Numbers indicate percentages of $Foxp3^+$ cells of the total cells. Data are representative of three independent experiments with similar results. (**c**) Expression of P-Selectin, CD40, CD154 on BAL platelets from OVA-sensitized WT or *Cd154*$^{-/-}$ mice at 0 (0 min) or 30 min (30 min) after OVA sensitization. Data are representative of three independent experiments with similar results. (**d**) Platelets harvested from WT or *Cd154*$^{-/-}$ mice via cardiac puncture. A total of 2.5×10^8 platelets were resuspended in 1 ml Tyrodes buffer and activated with 0.5 U thrombin. Supernatants were collected and assayed for TGF-β. NS, not significant (two-tailed Student's *t*-test). Data are from three independent experiments (**d**; the mean and s.d. of three determinants) or are representative of three independent experiments with similar results (**a**–**c**). BAL, bronchoalveolar lavage; OVA, ovalbumin; Treg, regulatory T cell.

identified in patients with asthma and were related to elevated serum IgE levels in asthma patients.[33] Consistent with these previous studies, our study provides the first evidence, through the use of *Cd154*$^{-/-}$ mice, that CD154 on platelets promotes IgE production, leukocyte infiltration and Th2-type responses in a mouse model of allergic asthma.

However, another study focusing on the CD40–CD154 interaction in asthma progression yielded paradoxical results. In *Cd40* deficient mice, asthma progression was more severe, as demonstrated by elevated airway hyperactivity, eosinophilia and predominant Th2 cell accumulation in airway, indicating a suppressive role of CD40 in both asthma and Th2 differentiation.[34] The detailed mechanism and biological significance for this controversial function of the CD40–CD154 interaction in asthma requires further investigation. It might be caused by different functions of CD40–CD154 in different cell types or at different phases of the immune response. In this respect, studies with cell type-specific overexpression or silencing of *Cd40* or *Cd154* could provide valuable evidence to discern the cellular mechanisms involved in this discrepancy.

Both naturally occurring thymus-derived $CD4^+CD25^+Foxp3^+$ Tregs and inducible Tregs suppress the development of allergies through multiple mechanisms, including the inhibition of other effector Th1, Th2, and Th17 cells, eosinophils, mast cells, basophils, inflammatory DCs and inflammatory cell migration to tissues.[35] The effect of CD154 on Treg induction and activation has been elusive. It was shown that soluble CD154 induced the activation of the immune-regulatory enzyme IDO, IL-10 production and programmed death-1 expression by T cells, and the expansion and maintenance of Tregs and therefore, played an immunosuppressive role in HIV infection and cancer.[36,37] Furthermore, B cells activated by CD40 are more potent in inducing and expanding Tregs than immature DCs.[38] On the contrary, activated platelets expressing CD154 were found to promote atherogenesis and inflammation by inhibiting the recruitment of Tregs.[39] Therefore, it is important to clarify the cell intrinsic role of CD154 in Treg induction. In our study, we showed that CD154 on platelets directly inhibits the induction of Tregs both *in vitro* and *in vivo*, providing a mechanistic explanation for the promotion of inflammatory responses and disease progression by CD154. However, further study is required to determine how platelet CD154 regulates Treg induction. It remains unknown whether CD154 is involved in the transcriptional or translational control of Foxp3 expression, whether CD154 exerts an inhibitory function in Treg differentiation through CD40/CD154 signaling or through other unknown mechanisms, and whether antigen-presenting cells such as DCs or monocytes are involved in this process. These issues should be addressed in the future.

Further investigation is also required to determine the reason for the enhanced Th2-type responses after platelet transfer in asthma-induced mice. This might be due to the inhibition of Tregs by CD154 expression in platelets, as we have demonstrated in this study. This possibility could be assessed by Treg transfer experiments in the future. However, it may also be related to the elevated function of antigen-presenting cells or expression of chemotactic factors or to disordered Th1/Th17 cell activation. The direct effect of platelet CD154 in the differentiation of Th1, Th2 or Th17 cells is another intriguing issue. One study found that activated platelets promoted production of Th1 cytokines including IFN-γ and TNF-α but not Th2 cytokines such as IL-4 and IL-5.[40] CD40 was also shown to be involved in TCR expression in the periphery through interactions with RAG1 and RAG2.[41] Therefore, it remains of interest to clarify more detailed mechanisms behind the regulatory role of platelets and their activation marker CD154 in the context of the complex immune network of antigen-presenting cells and different types of T cells.

AUTHOR CONTRIBUTIONS

XC designed and supervised the research; JT and TZ conducted the experiments; ZG contributed reagents and analytical tools; JT, JL and XC analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

We thank R Zhang and N Dong for their technical assistance. This work was supported by the National Key Basic Research Program of China (2013CB530502) and the National Natural Science Foundation of China (31270966, 81123006).

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