Platelet-derived RANK ligand enhances CCL17 secretion from dendritic cells mediated by thymic stromal lymphopoietin

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Abstract

Dendritic cells (DCs) play an integral role in cellular cascade that initiate and maintain Th2 responses in allergy. In this study, we examined the interaction between platelets and DCs to determine the role of platelets in the intervention of immune responses through modulation of DC functions. Blood-purified myeloid DCs, which had been stimulated with thymic stromal lymphopoietin (TSLP-DCs), formed aggregates with activated platelets. TSLP-DC maturation was induced after the interaction with TRAP6-activated platelets as indicated by an increase in the expression of CD86, CD40, and CD83. In addition, production of a Th2 cell-attracting chemokine, CCL17, was clearly upregulated by coculture of TSLP-DCs with TRAP6-activated platelets. We further found that an expression of RANK ligand (RANKL) on platelets was upregulated by the TRAP6 activation, and that, using the neutralizing antibody against RANKL, the platelet-derived RANKL induces the activation of TSLP-DCs. Thus, activated platelets can intervene in adaptive immune responses through induction of functional modulation of TSLP-DCs. Platelets have the ability to enhance the DC-mediated Th2 response and may contribute to the allergic inflammation. In conclusion, our study provides new insights in platelet functions and the possible mechanism of allergic responses that stem from DCs.

Keywords

CCL17, dendritic cells, platelets, RANK ligand, thymic stromal lymphopoietin

Introduction

Dendritic cells (DCs) are primary antigen-presenting cells distributed throughout lymphoid and non-lymphoid tissues. Accumulating evidence has indicated that DCs are important to orchestrate both innate and adaptive immunity through the cognate and cytokine-mediated mechanisms. Furthermore, DCs are pivotal cells in the linkage of innate and adaptive immune system [1]. From this viewpoint, it has been reported that DCs are involved in the regulation of innate immunity through interaction with various cells [2]. DCs interact with innate lymphocytes such as natural killer (NK), NKT cells, and γδT cells. In these processes, innate lymphocytes and DCs are reciprocally activated by not only cell–cell contacts but also soluble factors [3, 4]. In addition, DCs have been reported to interact with granulocytes, another important member of the innate immune system, through the interaction of CD209 (DC-SIGN) with CD11b. Neutrophils form clusters with immature DCs to induce the maturation of DCs, which enables these DCs to trigger strong T cell proliferation and Th1 polarization [5]. Moreover, after coculture of human granulocytes with DCs, granulocytes activate and secrete elastase and myeloperoxidase in accordance with the increase in the expression of CD50, CD63, and CD64 (the activation markers of granulocytes). This finding suggests the functional and phenotypical activation of granulocytes by interaction with DCs [6].

Furthermore, interactions of DCs with non-lymphoid cells have been observed even in the platelet system. It is well accepted that platelets are one of the key regulators of general inflammation, thrombosis, and atherosclerosis. After platelet activation, they express and release various mediators that possibly intervene in immune responses. Activated platelets induce the maturation of DCs to produce interleukin (IL)-10 in association with significant upregulation of CD83, CD80, and CD86 [7] and platelet-expressed CD40 ligand (CD40L) interacts with DCs to induce DC maturation [8]. In accordance with this finding, Martinson et al. [9] reported that thrombin-activated platelets express CD40L and effectively induce maturation of DCs with a potent immunogenic activity. In the clinical setting, activated platelets enhance interferon-α (IFN-α) secretion by immune complex-stimulated plasmacytoid DCs through a CD40L–CD40 interaction, indicating that the platelet–DC interaction is partly involved in a systemic lupus erythematosus (SLE) pathogenesis [10]. Thus, although DCs are rare while platelets are an abundant component in the blood, both are thought to be mutually critical for the intervention of immune responses. In addition, interactions of DCs and platelets may frequently occur in the blood after activation of platelets, possibly in the case of infection. Although CD40L is thought to be a key platelet-derived activating factor of DCs, there is a report showing that platelets stimulate DCs to polarize Th2 cells in a CD40L-independent manner [11].

In this study, we examined phenotypical and functional modulation of DCs after the DC–platelet interaction. We found that thrombin receptor agonist peptide (TRAP)-activated platelets expressed RANK ligand (RANKL) and induced maturation of myeloid DCs (mDCs) that had been stimulated with thymic stromal lymphopoietin (TSLP), which is critical cytokine...
inducing DC-mediated Th2 inflammation [12]. The activated platelets enhanced the production of Th2-attracting chemokine CCL17 in TSLP-stimulated mDCs through RANKL.

Materials and methods

All procedures were performed under protocols approved by the Ethics Committee at Kansai Medical University.

Platelets

Fresh citrated blood (acidic citrate dextrose) was obtained from healthy volunteers (20 healthy volunteers, aged 24–61 years old). Platelet-rich plasma (PRP) was prepared by centrifugation at 2000 rpm with Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) for 20 minutes at room temperature. Plasma was collected and filtered through a Millex 0.45 μm syringe-driven filter unit (Millipore, Millipore Corporation, Billerica, MA). The platelet-free plasma was used to suspend platelets. Auffy coat was diluted by PBS and centrifuged at 2500 rpm for 10 minutes. The pellet was resuspended in 500 μl plasma and centrifuged at 1800 rpm for 5 minutes. The supernatant was collected as PRP. Platelets in the PRP were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC)-, or biotin-labeled monoclonal antibodies (mAbs) against CD11c, CD14, CD41, CD62p, CD80, CD86, HLA-DR, CD42b, RANK-ligand (receptor activator of NF-κB ligand, RANKL, CD254), LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes, CD258), CD70, TRAIL (TNF-related apoptosis-activating ligand, CD253), CD123 (IL-3 receptor α), CD127 (IL-7 receptor α), CD40L (CD154), OX40L (CD252), 4-1BB, ICOSL (inducible T-cell co-stimulator ligand, CD275), GITRL (glucocorticoid-induced tumor necrosis factor receptor ligand), Fasl (CD178), PD-L2 (programmed cell death-2 ligand, CD273), CD124 (IL-4 receptor α), CD126 (IL-6 receptor), or thromboxane A2 after blood vessel injury by microbial infection), CD40L (CD154) antibody (100 μg/ml) (Biolegend, San Diego, CA.), respectively.

Results

Surface marker analysis of activated platelets

First, we determined the activation profile of platelets stimulated with TRAP6 for 10 minutes at 37°C. As shown in Figure 1, an increase in the CD62P expression was clearly observed as a positive control for platelet activation, indicating that platelets were properly activated under the condition used in this study. We also evaluated other surface markers (Table I), and then newly found that the expression of RANKL was obviously upregulated by the TRAP6 activation (Figure 1A).

Expression of RANK on DCs and aggregation of mDCs with platelets

RANKL is a molecule expressed on activated T cells acting to maintain survival and maturation of DCs [14]. Although freshly isolated mDCs did not express RANK, stimulation of TSLP, but not R848, preferentially induced RANK expression on mDCs for 24 hours (Figure 1B). Thus, it is conceivable that activated platelets interact with TSLP-stimulated mDCs (TSLP-mDCs) via the RANK–RANKL pathway. This type of interaction, probably occurring in blood vessels after platelet stimulation (e.g., through thromboxane A2 after blood vessel injury by microbial infection), may provide new information on the function of mDCs. Thus, we cultured TSLP-mDCs with TRAP6-activated platelets, and an interaction of mDCs and platelets was observed as the DC-platelet aggregates (Figure 2A). Of note is that these aggregates were preferentially seen when both mDCs and platelets were activated by TSLP or TRAP6, respectively. Neither unstimulated nor R848-activated mDCs formed aggregates with stimulated platelets, and unstimulated platelets hardly formed aggregates with TSLP-mDCs (data not shown). Furthermore, such aggregation was partially inhibited by the addition of a neutralizing anti-RANKL mAb, suggesting that the interaction was partly mediated by the RANK–RANKL pathway.

Maturation of mDCs by platelets

We examined the immunophenotypical changes of TSLP-mDCs after the interaction with activated platelets. mDCs were stimulated with TSLP in the presence or absence of unstimulated-platelets or TRAP6-activated platelets for 24 hours. As a result, significant increases in the expression of costimulatory molecules CD40 and CD86, and the DC maturation marker CD83 were observed when mDCs were cultured with TRAP6-activated platelets (Figure 2B and C). Although these increases occurred when mDCs were cocultured with unstimulated platelets, the increases in the expression of these markers were lower.

Analyses of DCs

To analyze the surface expression, the cultured DCs were stained with PE-labeled anti-RANK Ab, FITC-labeled anti-CD40, PE-labeled anti-CD83, and PE-or APC-labeled anti-CD86 mAbs, and then analyzed by the FACScalibur. CCL17 in DC culture supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) after 24 hours of culture (R&D Systems, Minneapolis, MN).

Inhibition assay

To inhibit the RANK-RANKL or CD40-CD40L interaction in vitro, DCs stimulated with TSLP and the activated platelets in the presence of a neutralizing anti-human RANKL antibody (125 μg/ml) (R&D Systems, Minneapolis, MN) and/or anti-human CD40L (CD154) antibody (100 μg/ml) (Biolegend, San Diego, CA.), respectively.
As shown in Figure 2D and E, the increase in the expression of CD86 on TSLP-DCs induced by the activated platelets was downregulated by the addition of neutralizing mAb to RANKL or CD40L. This result indicates an involvement of RANK–RANKL and CD40–CD40L pathways in the TSLP-DC maturation through DC–platelet adhesion. Furthermore, an increase in the expression of CD86 was observed after the coculture with the supernatant of TRAP6-activated PRP (as a platelet supernatant) (Figure 2F and G). This finding suggests that soluble factors such as CD40L and/or RANKL released from TRAP6-activated platelets can also contribute to the maturation of TSLP-DCs. However, the presence of TRAP6-activated platelets themselves much effectively induces the maturation of DCs and, therefore, cell–cell (DC-platelet) adhesion is likely to be a required process to efficiently induce the maturation of TSLP-DCs.

It is noteworthy that an increase in the number of viable mDCs was also observed after coculture with TRAP6-activated platelets (data not shown). This observation indicates that the interaction of mDCs with platelets might be involved in the maintenance of mDCs after their maturation.
Increase in production of DC-derived Th2 chemokine CCL17 induced by platelets

To examine functional modulation of mDCs after the interaction with platelets, TSLP-DCs were cocultured with or without unstimulated or TRAP6-activated platelets for 24 hours, and their production of CCL17 (also known as thymus and activation regulated chemokine, TARC), a Th2 attracting chemokine, was determined by ELISA. As shown in Figure 3, although CCL17 production was observed in the cultures of TSLP-DCs, but not R848-stimulated mDCs, culture containing platelets significantly enhanced CCL17 production. Notably, TRAP6-activated platelets facilitated higher production of CCL17 than did unstimulated platelets. Neither activated platelets nor unstimulated platelets alone produced CCL17. The functional modulation of mDCs induced by platelets was partially inhibited by the blocking mAbs to RANKL and CD40L (Figure 4), further indicating the
importance of RANK–RANKL and CD40–CD40L pathways in the mDC–platelet interaction.

Discussion

It has been clarified that DCs are involved in the initiation of Th2 responses [15], and TSLP equips DCs with the programming role for Th2 polarization [12]. CD11c+ DCs activated by TSLP express OX40 ligand (OX40L), which prime naive Th cells to differentiate into Th2 cells that produce IL-4, IL-5, and IL-13 [16]. In addition, TSLP-activated DCs produce Th2 cell-attracting chemokines, CCL17 and CCL22 (also known as...
macrophage-derived chemokine, MDC) and induce homeostatic expansion of CRTH2+ memory T cells, which play a critical role for the maintenance of the allergic condition [17]. Thus, DCs activated by TSLP participate in pathogenic inflammatory Th2 cell responses in allergy [18]. In this study, we clarified a capability of platelets to enhance the TSLP-DC functions through the platelet-derived RANKL.

Platelets are abundant components of the blood and intervene immune responses by expressing various immune mediators after their activation. Indeed, platelets have been implicated in the development of immune-related disorders such as asthma and atopic dermatitis [19–23]. Therefore, platelets and immune system might affect each other, and DCs as a key component of immune responses might be functionally modulated through interactions with platelets.

We clarified that both maturation of TSLP-DCs and upregulation of their CCL17 production are induced after interaction with activated platelets. It is well known that CD40L, which induces DC activation [24], is derived from activated platelets. In addition, we newly identified that RANKL is another platelet-derived activation molecule, which enhances TSLP-DC activation. This finding provides a plausible explanation for the previous report showing that there is a CD40L-independent mechanism of platelets in activating DCs with Th2-skewing property [11]. Based on our results, TSLP-DCs stimulated with activated platelets might have further potency to facilitate the attraction of memory Th2 cells to the inflammatory sites by enhanced production of CCL17, which maintains Th2 cell-mediated allergic condition.

There has been an increase in the prevalence of asthma and allergic diseases such as atopic dermatitis and allergic rhinitis [25]. These allergic disorders are characterized by inflammatory processes in which the Th2 cells are crucial for the initiation and maintenance of allergic inflammation [26]. Cytokines secreted by Th2 cells such as IL-4, IL-5, and IL-13 are important mediators in the developmental process of these allergic diseases, leading to increased IgE concentrations, mast cell degranulation, and eosinophil-mediated inflammation [27]. In this pathogenic setting, TSLP from the epithelium (e.g., in atopic dermatitis) may induce epithelial and dermal mDCs to TSLP-DCs, and activated platelets leaked from microvessels by scratching in the inflammatory sites could further activate TSLP-DCs. TSLP also play an important role in the pathogenic condition of asthma. TNF-α-mediated induction of TSLP is observed in human bronchial epithelial cells, lung fibroblasts, and airway smooth muscle [28], and TSLP expression is increased in the airways of patients with asthma and is correlated with the Th2-attracting chemokines and disease severity [29–31]. Also diapedesis of platelets in areas of allergic inflammation is found in intra-alveolar space or extra-vascularly in bronchial tissues in patients with asthma [32,33] and in allergen-challenged mice [34,35], indicating a possibility of platelet migration in a direct response to allergen. Thus, it is feasible that the extravasated platelets in the inflammatory sites can activate TSLP-DCs to facilitate the enhanced production of CCL17, as shown in this study. This would lead to attraction of memory Th2 cells that further drives the pathogenic allergic spiral enhancing aggravation of the allergic state. It has been reported that CCL17 induces platelet activation and aggregation via its receptor, CCR4 expressed on platelets [36–38]. Thus, upregulation of CCL17 can induce platelet activation, which in turn leads to further activation of TSLP-DCs and to acceleration of this allergic spiral. In fact, the plasma level of CCL17 has been reported to be elevated in patients with atopic dermatitis and to show significant correlation with severity scoring of atopic dermatitis (SCORAD) index [39]. Platelets in these patients are increased and activated to produce platelet-derived microparticles and soluble P-selectin, both of which are markers of platelet activation [20]. Thus, in addition to DCs, platelets are important players to make allergic condition through DC activation.

TSLP is an IL-7-like cytokine expressed mainly by epithelial cells, and is capable of affecting various types of cells including DCs, naïve CD4+ T cells, NKt cells, basophils, B cells, and regulatory T cells [40]. Basophils can be activated by TSLP, and they produce TSLP after stimulation with active papain or FcεR triggering by IgE crosslinking [41]. This finding indicates a possibility that blood mDCs can be stimulated with TSLP from activated basophils under physiological condition and that the resultant TSLP-DCs can be further stimulated by activated platelets in the inflammatory microenvironment of the blood. Thus, our study of the interaction of TSLP-DCs with activated platelets may provide new insights into platelet functions and the possible mechanism of allergic responses that stem from DCs.

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Declaration of interest

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References

Platelet-derived RANKL induces TSLP-DC activation

22. Pitchford SC, Yano H, Lever R, Riffo-Vasquez Y, Ciferri S,
21. Tamagawa-Mineoka R, Katoh N, Ueda E, Takenaka H, Kita M,
15. Pulendran B, Tang H, Manicassamy S. Programming dendritic cells
DOI: 10.3109/09537104.2014.920081

Platelet-derived RANKL induces TSLP-DC activation

25. Devereux G. The increase in the prevalence of asthma and allergy:
production by dendritic cells
142(3):587–593.

24. Georas SN, Guo J, De Fanis U, Casolaro V. T-helper cell type-2

27. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T


31. Zhou B, Comeau MR, Smedt TD, Liggitt HD, Dahl ME, Lewis DB, Gyarmati D, Ayte; Campbell DJ, Ziegler SF. Thymic


33. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsies in asthma. An ultrastructural, quantitative study and

34. Pitchford SC, Riffo-Vasquez Y, Sousa A, Moni S, Gresele P, Spina D, Page CP. Platelets are necessary for airway wall


strongly potentiates the ability of the chemokines MDC, TARC, and


40. Zhang Y, Zhou B. Functions of thymic stromal lymphopoietin

41. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for