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IL-17A increases ADP-induced platelet aggregation

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Abstract

The increased risk of thromboembolism and higher incidence of cardiovascular disorders are among the most common causes of morbidity in patients suffering from autoimmune diseases. In this study we tested the hypothesis that IL-17A, a key pro-inflammatory cytokine involved in the development of autoimmune diseases, exerts pro-aggregant effects on both human and mouse platelets. Human or murine platelets were incubated with IL-17A for 2 min at 37 °C prior the addition of the stimuli. Aggregation was monitored in a light transmission aggregometer measuring changes in turbidity with continuous observation over a 5-min interval after the addition of the stimuli. IL-17A, CD42b and CD62P expression as well as fibrinogen bindings were measured by FACS while Erk-2 phosphorylation was analyzed by western blot using phospho-specific antibodies. Pre-incubation with IL-17A increased ADP-, but not collagen-induced platelet aggregation and accelerated CD62P expression and exposure of fibrinogen binding sites. These effects were associated with a faster kinetic of ADP-induced Erk-2 phosphorylation and were lost in platelets deficient in the IL-17 receptor. Together these results unveil a novel aspect of the inflammatory nature of IL-17A suggesting, at the same time, that therapeutic strategies targeting this cytokine might provide further benefit for the treatment of autoimmune diseases by reducing the risk of cardiovascular-related pathologies.

Keywords

Interleukin-17A; platelet aggregation; autoimmune diseases; inflammation

Introduction

Interleukin-17A (IL-17A) belongs to a new family of cytokines that have recently taken center stage in autoimmunity [1; 2]. This cytokine plays a pivotal role in the development of various models of chronic immune inflammatory diseases including collagen-induced arthritis [3] and MRL/*lpr* [4] mice (lupus-like diseases mouse model). These experimental findings have been confirmed by clinical studies showing a positive correlation between plasma levels of IL-17A and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [5; 6] or the rheumatoid arthritis Disease Activity Score (DAS) [7].

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One common feature of patients suffering from chronic autoimmune inflammatory diseases is the development of cardiovascular complications including an increased risk of thromboembolism, an important causes of morbidity and mortality in these immune pathologies [8]. Intriguingly, these studies have suggested that this phenomenon is independent of an intrinsic alteration in the character and function of platelets and most likely due to the sustained systemic inflammatory status typical of these pathologies [9]. However, the molecular and cellular mechanisms underlying these effects are poorly understood.

In this study we tested the hypothesis that IL-17A might influence platelet responsiveness and activation. Our results report the first evidence that this cytokine acts as a pro-aggregant agent increasing platelet responses to ADP, thus revealing another piece of evidence of the complex crosstalk between systemic inflammation and risk of cardiovascular pathologies in autoimmune diseases.

Material and methods

Mice

C57BL/6 and IL-17RA-null [10] (on C57/BL6 background) mice (24-28 g) were housed under standard conditions and maintained in accordance with United Kingdom Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act 1986) and of the European Union directives.

Human blood

Blood donors were 20- to 35-year-old healthy men and women who were tested to be negative for HIV, hepatitis B virus, and hepatitis C virus. Further exclusion criteria were manifest infections during the last 4 weeks, fever, symptomatic allergies, abnormal blood cell counts or increased liver enzymes. All healthy volunteers did not receive anti-platelet or anticoagulant therapy and gave oral and written consent. Cell collection and separation was covered by ethical approval 05/Q0603/34 (East London and The City Research Ethics Committee 1).

Reagents

ADP and collagen were obtained from Instrumentation laboratory (Cheshire, UK). Prostacyclin was purchased from Biomol (Exeter, UK) and recombinant mouse IL-17A from R&D System (Abingdon, UK). Unless otherwise stated, all the other reagents were from Sigma-Aldrich Co. (Dorset, UK).

Platelet aggregation assay

Both human and mouse platelets were prepared as previously described [11; 12]. Human and murine platelet aggregation was monitored in a 4-channel APACT 4004 light transmission aggregometer measuring changes in turbidity with continuous observation over a 5-min interval after the addition of either ADP (3 μ M) or collagen (5.0 μ g/ml). To test the effects of IL-17 (0.07-2.0 μ g/ml) on aggregation, platelets were incubated with the cytokine for 2 min at 37 °C prior the addition of the stimuli.

Flow cytometry

Platelets (3 $\times$ 10⁵ platelets/ μ l) were resuspended in FACS buffer (PBS containing 5% foetal calf serum and 0.02% NaN₂) containing CD16/CD32 Fc γ IIIR-blocking antibody (1:500; clone 93; eBioscience) for 30 min. Thereafter, cells were incubated for 1 h with the following FITC or PE-conjugated antibodies: CD42b (1:200; clone HIP, eBioscience),

CD62P (1:400; clone AK-4, Serotec), fibrinogen (1:200; clone HIP2, Dako) and IL-17RA (1:200; clone J10MBS, eBioscience). For intracellular staining, platelets were permeabilized in FACS buffer containing 0.3% saponin for 1 h and then fixed with 2 % of paraformaldehyde for further 30 min. At least 1×10^5 cells were analyzed per sample, and positive and negative populations were identified based on the staining obtained with specific (see above) and irrelevant IgG isotypes. Data were analyzed with FlowJo software.

Western blotting

Platelets (6×10^7 platelets/sample) were stimulated with ADP ($3 \mu\text{M}$) in presence or absence of IL-17A ($0.2 \mu\text{g/ml}$) for different times and immediately lysed adding $200 \mu\text{l}$ of 6 $\times$ SDS sample buffer. Samples were subjected to electrophoresis on SDS-12% polyacrylamide gel as previously described [13]. Briefly, samples were subjected to standard SDS-polyacrylamide gel electrophoresis and electrophoretically blotted onto polyvinylidene difluoride membranes (Millipore, Watford, UK). Membranes were incubated with primary antibodies in Tris-buffered saline solution containing Tween 20 and 5% (w/v) non fat dry milk overnight before 1.5h incubation period with horseradish Peroxidase-conjugated secondary antibody (Dako, Cambridgeshire, UK). Proteins were detected using the enhanced chemiluminescence (ECL) detection kit and visualized on Hyperfilm (Amersham Biosciences). The primary antibodies used were mouse monoclonal anti-phospho Erk2 and anti-Erk2 (Santa Cruz Biotechnology).

Statistics

Data were analyzed by paired or unpaired Student's t-test or, when appropriate, one way ANOVA followed by Bonferroni's test for multiple comparisons. All assays were repeated at least in triplicate and the results are expressed as means $\pm$ S.E.M. Values of $P < 0.05$ were considered significant.

Results

Several studies have shown that stimulation of platelets with a variety of physiologic stimuli, including ADP, initiates the primary reversible phase of aggregation characterized by shape change and pseudopod formation. Using stronger stimuli, such as collagen, platelet secretion takes place, with the release of platelet granule proteins and recruitment of additional platelets. This gives rise to the secondary irreversible phase of aggregation [14]. To investigate the effects of IL-17A on platelet we first tested the effects of this cytokine on ADP-induced aggregation.

ADP can evoke either reversible or irreversible platelet aggregation depending on the concentration applied [15]. Based on preliminary results, we have chosen a concentration of ADP ($3 \mu\text{M}$) that caused a partial ($29.5 \pm 1.2\%$) and reversible aggregation. When human platelets were incubated with IL-17A at concentrations known to exert an inflammatory response [16] (0.07 - $2.0 \mu\text{g/ml}$), there was an increased response to ADP (Figure 1A) whilst IL-17A alone did not cause any effects (data not shown). We observed that IL-17A induced an increased magnitude of the response to ADP and in few cases the aggregation became irreversible ($\sim 8\%$; not shown). Cumulative analysis in several donors showed that this effect was concentration-dependent, with a peaked ($+40\%$) at $0.2 \mu\text{g/ml}$ IL-17A (Figure 1A) and was not observed with denatured (90°C for 5 min) cytokine (data not shown).

To test the effects of IL-17A on irreversible aggregation, we next stimulated platelets with collagen. As shown in Figure 1B, IL-17A did not influence collagen-induced aggregation ($68.0 \pm 5.5\%$) indicating a specific effect of this cytokine on the reversible phase of the aggregation response of human platelets.

To confirm the activating effects of IL-17A on platelets, we next measured membrane expression of CD62P and exposure of fibrinogen-binding sites on the α IIb β 3 integrin by FACS. Consistent with results obtained in the aggregation assay, platelets pre-treated with IL-17A (0.2 μ g/ml) displayed faster kinetics of CD62P externalization, and binding to fibrinogen, compared to platelets stimulated with ADP alone, over a period of 5 min (Figure 1C, top and bottom panels, respectively).

We next determined if platelets would express a functional IL-17A receptor, termed IL-17RA, using a double staining protocol with anti-CD42. As reported in Figure 2A, CD42-gated unpermeabilized platelets showed a discrete IL-17RA staining compared to IgG control stained cells (top panels; MFI 4.7 vs. 3.1, respectively). Analysis in permeabilized platelets (bottom panels) confirmed these results with an increase in MFI compared to unpermeabilized platelets (MFI 6.5 vs. 4.7, respectively) suggesting that a proportion (~50%) of IL-17RA is stored intracellularly, likely available for externalization upon platelet activation.

To demonstrate a functional role of IL-17RA in platelets we measured Erk phosphorylation as the main signalling pathway triggered by this receptor and relevant for platelet function. Consistent with previous studies, stimulation with ADP induced a time-dependent increase in Erk-2 phosphorylation that peaked at 5 min [17; 18]. Conversely, pre-incubation with IL-17A induced a faster kinetic that peaked at 0.5 min and declined after 5 (Figure 2B).

The causal involvement of IL-17RA in the observed effects was obtained by testing the cytokine on platelets from wild-type and IL-17RA^{-/-} mice. IL-17RA^{-/-} platelets displayed no difference in response to ADP compared to wild-type control platelets (data not shown). However, costimulation in presence of IL-17A showed an increase in aggregation in the latter but not the former genotype (Figure 2C).

Discussion

A growing number of evidences gathered over the last few years indicate that IL-17A might play a key role in the development of autoimmune diseases. One common feature of these pathologies is the systemic inflammatory response driven primarily by cytokines and their contribution to activation of the coagulation cascade. As a result, patients suffering from autoimmune diseases often have an elevated risk of thrombosis as well as an increased susceptibility to the development of cardiovascular diseases.

In this study we have tested the effects of IL-17A on platelet aggregation. Our results show that IL-17A alone does not influence platelet activation while it amplifies the primary reversible phase of ADP-induced platelet aggregation. Interestingly, these results are in agreement with studies in other systems showing that IL-17A *per se* poorly stimulates signalling pathways such as Erk or NF- κ B while it has a remarkable stimulatory effect on these pathways when used in conjunction with other stimuli [19]. Consistently, IL-17A was found to synergize with TNF- α for the induction of GM-CSF [19], and with CD40-ligand for the release of IL-6, IL-8, RANTES and MCP-1[20]. It therefore seems that, also in platelets, IL-17A remains faithful to its role as a co-stimulant hence likely endowed with important modulatory functions in inflammatory settings.

It is well-established that platelet adhesion is mediated via glycoprotein GPIb receptors through interaction with the von Willebrand factor and that further physiologic activation of platelets via intracellular signaling pathways leads not only to an increased expression of the GPIIb/IIIa receptor complex, but also to a conformational change and exposure of the fibrinogen binding site. Subsequent fibrinogen bridging allows firm attachment of adjacent

platelets [21]. This process is a prerequisite for platelet aggregation and thrombus formation. Similarly, the increased expression of CD62P is predictive for an elevated risk of circulating platelet-leukocyte aggregates that are typically considered predictive of thrombus formation [22] and observed in patients with acute myocardial infarction [23] as well as in patients suffering autoimmune diseases [24; 25; 26]. Our results showing a faster kinetic of ADP-induced CD62P expression and fibrinogen binding in platelets pre-treated with IL-17A suggest that this cytokine might facilitate their adhesion to damaged endothelium as well as to other circulating leukocytes ultimately leading to thrombus formation.

Interestingly, a recent study has shown that incubation of platelets with supernatants of primary endothelial cells stimulated with IL-17A induced a strong aggregatory response [27]. These results provide further evidence for the pro-thrombotic action of IL-17A and suggest an indirect mechanism linking IL-17A and the increased risk of atherosclerosis in patients suffering autoimmune diseases.

In conclusion, the findings of this study unveil a novel and unpredicted aspect of the inflammatory nature of IL-17A and further highlight the importance of this cytokine in the context of autoimmune diseases. Recent studies indicate that the IL-17A-specific human monoclonal antibody AIN457 could be a promising therapy for the treatment of psoriasis, rheumatoid arthritis and chronic uveitis in Phase II trials [28]. Therefore, our results might prompt further investigations on the possibility that therapies targeting IL-17A might provide an extra beneficial therapeutic effect in autoimmune diseases by limiting the risk of cardiovascular diseases associated with thromboembolic events.

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Figure 1. IL-17A increases ADP-induced platelet activation

(A) IL-17A-induced increase in platelet response to ADP (3μM) or (B) collagen (5μg/ml). Values are means ± S.E.M of 5 experiments with different donor platelets and expressed as percentage (%) increase of control aggregation to ADP ($29.5 \pm 1.2\%$) and to collagen ($68.0 \pm 5.5\%$); * $P < 0.05$, ** $P < 0.01$ vs. ADP alone. (C) Histograms for CD62P expression and fibrinogen binding of human washed platelets pre-incubated with PBS vehicle control or IL-17A (0.2μg/ml) for 2 min prior to stimulation with ADP. Data are representative of 5 different experiments with different donor platelets.

Figure 2. Platelets express a functional IL-17RA

(A) Representative histograms for IL-17RA expression in intact (top panel) or permeabilized (bottom panels) human platelets. Human washed platelets were stained with FITC-anti-CD42P, PE-anti-IL-17RA antibodies or FITC- or PE-conjugated IgG as detailed in Materials and Methods. Numbers in the histograms indicate the MFI of IL-17RA and IgG control staining in CD42-positive (R2 gate) platelets. Data are representative of 5 different analyses with different donor platelets. (B) Western blotting analyses of p-Erk2 and total Erk in human platelets stimulated with ADP (3 μ M) or ADP plus IL-17A (0.2 μ g/ml) incubated for the indicated minutes. Data are representative of 3 experiments with different donor platelets. (C) Representative graphs showing the % of increase of aggregation in murine platelet obtained from wild-type (C57/BL6) or IL-17R null (IL-17R^{-/-}) mice stimulated with ADP (3 μ M) plus IL-17A (0.2 μ g/ml) for 2 min. Values are means $\pm$ S.E.M of 3 separate experiments; ** P <0.05 vs. ADP alone.