

Identification and characterisation of anti-citrullinated protein antibody (acpa)-producing b cells in patients with rheumatoid arthritis (ra) Kerkman, P.F.

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Chapter **6**

CD28 is expressed by synovial fluid B cells in rheumatoid arthritis

Priscilla F. Kerkman, Diahann T.S.L. Jansen, H. El Bannoudi, Ellen I.H. van der Voort, Tom W.J. Huizinga, René E.M. Toes, Hans U. Scherer

Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

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Abstract

Objectives: Rheumatoid arthritis (RA) is an autoimmune disease characterised by inflammation of the synovial membrane of joints and the presence of autoantibodies produced by plasmablasts and plasma cells. Recently, expression of CD28 by long-lived plasma cells was found to promote survival and continued antibody production in mice. Considering the central role of B cells, plasma cells and autoantibodies in RA combined with the clinical efficacy of inhibiting CD28-triggering with CTLA4-Ig (Abatacept), we investigated the expression of CD28 by plasmablasts/-cells in peripheral blood of RA patients, systemic lupus erythematosus (SLE) patients and healthy controls. Likewise, we investigated the expression of CD28 by synovial fluid plasmablasts/-cells specific for citrullinated antigens.

Methods: B cells and plasmablasts/-cells obtained from freshly isolated peripheral blood and synovial fluid mononuclear cells (PBMC/SFMC) of RA patients were stained for CD28-expression by flow cytometry. PBMC from SLE patients and healthy donors were included as controls. The expression of CD28 by ACPA-expressing plasmablasts/-cells derived from SFMC of RA patients was determined by antigen-specific tetramer staining.

Results: CD28 was expressed on a subset of both B cells and plasmablasts/cells from peripheral blood and was comparable in RA patients, healthy controls and SLE patients. CD28 was expressed at significantly higher levels on CD20⁺ B cells derived from SFMC compared to B cells from PBMC. There was a trend towards higher expression of CD28 on synovial plasmablasts/-cells. In addition, CD28 was also expressed by ACPAexpressing plasmablasts/-cells derived from synovial fluid of RA patients.

Conclusions: CD28 is expressed on a subset of B cells, plasmablasts/cells and ACPA-expressing B cells of RA patients. CD28-expressing B cells and plasmablasts/-cells are present in higher frequencies in the synovial compartment.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by inflammation of the synovial membrane of joints, leading to severe cartilage damage, bone erosion and disability if left untreated. RA affects approximately 1 % of the population[1] and is often characterised by the presence of autoantibodies. Anti-citrullinated protein antibodies (ACPA) are among the best-described autoantibodies in RA. ACPA are highly disease-specific and their presence associates with disease severity and worse prognosis[2-4]. ACPA have been implicated in disease pathogenesis[5-7] and are enriched in the joints of RA patients as compared to peripheral blood. ACPA-secreting plasmablasts/-cells have been found in both peripheral blood and in the synovial compartment[8-10].

Upon activation, naïve or memory B cells can differentiate into antibodysecreting plasmablasts and plasma cells. Based on their lifespan, plasma cells can be divided into two subsets; short lived plasma cells (SLPC) and long lived plasma cells (LLPC)[11]. Short-lived plasma cells are generated shortly after exposure to an antigen and have a relatively limited lifetime. In contrast, LLPC can persist for a much longer time period, sometimes lifelong, and provide long lasting humoral protection[12-14]. Most LLPC reside in survival niches in the bone marrow[15].

CD28 has been known for a long time as a prototypic co-stimulatory molecule for T cell activation. In conjunction with TCR activation, CD28 triggering leads to enhanced T cell activation, function and survival[16, 17]. The expression of CD28, however, is not confined to T cells, as plasma cells can also express this marker[18-20]. In fact, CD28-expression by plasma cells is regulated by the transcription factor Paired Box (Pax)5[21]. Pax5 represses CD28-expression in B cells, while loss of active Pax5 in plasma cells leads to the induction of CD28-expression. Recently, compelling evidence has been provided for the involvement of CD28 in long-term survival and function of plasma cells in mice. CD28 was found to function as an intrinsic factor that confers the capacity to LLPC to survive and maintain durable antibody production via interacting with its ligand CD80/CD86 expressed by dendritic cells (DC)[19]. Deficiency in CD28-expression resulted in a considerable decrease in LLPC numbers and antibody levels. In addition, CD28 promoted the up-regulation of B lymphocyte-induced maturation protein-1 (Blimp-1), a regulator of plasma cell differentiation [22]. Although CD28 was expressed on both LLPC and SLPC, it only mediated the survival of LLPC. This differential capacity for survival between LLPC and SLPC was shown to depend on downstream signalling of the CD28 Vav motif, which occurred only in LLPC and not in SLPC[22].

Abatacept, a fusion protein consisting of an extracellular domain of human cytotoxic T lymphocytes associated antigen 4 (CTLA-4) and a part of the human IgG Fc region, binds with high affinity to the co-stimulatory molecules CD80 and CD86. Abatacept is used to treat RA patients, and it is widely believed that its effectiveness is attributed to the ability to prevent T cell activation by blocking the binding of CD80/CD86 to CD28 expressed by T cells. The observation that autoantibodies persist upon treatment with B cell targeting therapies suggests continuous production of autoantibodies, potentially by LLPC. Indeed, we have



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recently shown that the synovial compartment in RA is equipped to function as an inflammatory niche that promotes survival of ACPA-producing plasma cells[23]. As CD28 is also expressed by plasma cells, we questioned whether Abatacept could potentially also inhibit CD28-triggering of autoantibody producing B cells. Therefore we investigated the expression of CD28 on B cells, plasmablasts/-cells and ACPA-expressing plasmablasts in RA patients.

Materials and methods

Patients

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Peripheral blood (n=19) and synovial fluid (SF, n=28) samples were obtained from ACPA-positive RA patients visiting the outpatient clinic of the Department of Rheumatology at the Leiden University Medical Center, the Netherlands. Patients were diagnosed with RA according to the 1987 classification criteria. Peripheral blood from SLE patients (n=10) and healthy donors (HD, n=11) was obtained for control. Written informed consent was obtained from all donors.

Cell isolation and flow cytometric analysis

PBMC were isolated from blood of RA patients, SLE patients and HD using Ficoll Plaque gradient centrifugation (LUMC Pharmacy). SF was obtained from inflamed knee joints of RA patients and first centrifuged to separate the cells from the fluid. Subsequently, SFMC were isolated using ficoll gradient centrifugation. Cells were stained with the following antibodies; CD3 Pacific Blue (UCHT1) or CD3 Alexa Fluor 700 (UCHT1), CD14 Pacific Blue (M5E2), CD19 APC-Cy7 (Sj25C1), CD20 PerCP (L27), CD27 PE-Cy7 (M-T271), CD28 PE (L293) or CD28 PE-CF594 (CD28.2) from BD Biosciences and CD20 Alexa Fluor 700 (2H7) from Biolegend. Dead cells were excluded with the use of 4',6-diamidino-2-phenulindole (DAPI; Molecular Probes). ACPA-expressing B cells were identified with a combination of differentially labelled CCP2 tetramers and a control tetramer, as described previously[24]. All samples were measured on a BD Fortessa or a BD LSRII cell analyser (BD Biosciences) and analysed using BD FACSDIVA software (BD Biosciences) and FlowJo version 7.6.5 (Tree Star Inc).

Absolute numbers of cells in peripheral blood were calculated based on the number of B cells from a pre-set volume of whole blood per pre-set number of beads using flow cytometry.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software Inc.). Percentages and absolute numbers of CD19⁺ B cells, plasmablasts/-cells and CD28-expressing plasmablasts/-cells were compared using One Way ANOVA. The different cell populations in SFMC and PBMC were compared using Mann Whitney U test. p Values <0.05 were considered significant.



Results

Circulating plasmablasts in peripheral blood of RA patients, SLE patients and healthy donors express CD28

CD28 can function as survival molecule for LLPC[22]. As CTLA-4Ig (Abatacept) blocks the binding of CD28 to CD80/CD86 and has beneficial therapeutic effects in a subgroup of RA patients, we investigated whether antibody secreting cells of RA patients express CD28, and whether this CD28-expression is altered compared to SLE patients or HD. To this end, PBMC isolated from RA patients, SLE patients and HD were stained with antibodies against CD19, CD20, CD27, CD3, CD14 and CD28 and subjected to flow cytometry. Following exclusion of doublets, B cells were visualised by gating on the CD3⁻CD14⁻DAPI⁻CD19⁺ cell population. B cell subsets were discriminated based on the expression of CD20 and CD27, and plasmablasts/-cells were defined as CD27⁺⁺CD20⁻ cells. (figure 6.1A). The percentage of CD19⁺ B cells in peripheral blood of RA patients was comparable to the percentages detected in SLE patients and HD (figure 6.1B). In line with this finding, the absolute numbers of CD19⁺ B cells, as determined by number of cells per ml of blood, in RA compared to SLE patients and HD were comparable (figure 6.1C). Likewise, the percentage and absolute number of plasmablasts/-cells was not significantly different between the different groups (figure 6.1D-E), although we did observe a trend towards an enhanced frequency of plasmablasts/-cells in SLE patients, in line with previous reports [25, 26]. Interestingly, we observed that approximately 5% of plasmablasts/-cells in RA peripheral blood expressed CD28. This expression was comparable to the CD28-expression on plasmablasts/-cells of SLE patients and HD (figure 6.1F-G). Together, these results show that a fraction of plasmablast/-cells of RA patients express CD28, although to a comparable extent compared to SLE patients and HD.

CD28-expression on B cells and plasmablasts/-cells from synovial fluid of RA patients

As phenotype and presence of immune cells in peripheral blood is not necessarily representative for cells present at the site of inflammation, we subsequently investigated the expression of CD28 on B cells and plasmablasts/-cells present in SF of inflamed joints of RA patients. CD28-expression was determined on the total CD3⁻CD14⁻DAPI⁻CD19⁺ B cell population, the CD27⁺⁺CD20⁻ plasmablast/-cell population and the CD20⁺ B cell population (figure 6.2A). In total, SFMC of 28 RA patients were compared to PBMC of 9 RA patients. The percentage of CD19⁺ B cells in peripheral blood was significantly higher compared to SF (figure 6.2B). However, the percentage of CD19⁺ B cells and of CD20⁺ B cells that expressed CD28 was increased in SFMC compared to PBMC (figure 6.2C and E). Furthermore, there was a trend towards an increased percentage of CD28-expressing plasmablasts/-cells in SF but this did not reach statistical significance when compared to CD28-expressing plasmablasts/-cells from peripheral blood (figure 6.2D). These findings show that B cells and plasmablasts/-cells expressing CD28 are enriched at the site of inflammation in a part of RA patients.



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Figure 6.1: CD28 is expressed on plasmablasts in peripheral blood of RA patients, SLE patients and healthy donors. PBMC were isolated from peripheral blood of RA patients (n=10), SLE patients (n=10) and healthy donors (HD) (n=11) and analysed for CD28 expression by flow cytometry. (A) CD19⁺ B cells were determined by gating on the CD3⁻CD14⁻DAPI⁻CD19⁺ cell population and further subdivided in subsets based on the expression of CD20 and CD27. Plasmablasts were defined as CD27⁺⁺CD20⁻ cells. (B) Summary of the percentage and absolute number (C) of CD19⁺ B cells. (D) Percentage and absolute number (E) of plasmablasts for the different groups. (F) Summary of the percentage and absolute number (G) of plasmablasts expressing CD28. Absolute numbers are provided as number of cells per ml of blood. Lines represent median values.



Figure 6.2: CD28 is highly expressed on CD20⁺ B cells in synovial fluid of RA patients. SFMC were isolated from synovial fluid obtained from inflamed knee joints of RA patients and analysed for CD28-expression by B cells using flow cytometry. In total, SFMC of 28 RA patients were analysed and compared to PBMC of 9 RA patients. (A) CD28-expression on total B cells and on B cell subsets was analysed as in figure 6.1. (B) Identical gating strategies were used to determine the CD19⁺ B cells in SFMC and PBMC, and the summary is depicted. (C) Expression of CD28 by CD19⁺ B cells (as depicted in (B)). (D) Expression of CD28 by CD20⁺ B cells (naive and memory B cells combined). Lines represent median values. * p<0.05, ns = not significant.

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Figure 6.3: CD28 is expressed by ACPA⁺ B cells in synovial fluid. CD28-expression was determined on ACPA-expressing plasmablasts/-cells in SFMC using flow cytometry. (A) CD19⁺ B cells were gated as depicted in figure 6.1A followed by identification of plasmablasts/-cells (left). Cut-off for CD28-expression (right) was set on the isotype control (middle). (B) ACPA-expressing plasmablasts/-cells were defined by a double staining for citrullinated antigen specific tetramers (left) in combination with negativity for a control tetramer.

CD28 is expressed by ACPA-expressing plasmablasts/-cells

ACPA-secreting cells are present in the inflamed joints of patients with RA[9, 10]. The finding that a fraction of synovial B cells and plasmablasts/-cells displayed high expression of CD28 raised the question whether ACPA-expressing plasmablasts/-cells derived from the site of inflammation exhibit higher CD28-expression in comparison to total plasmablasts/-cells. To address this question, SFMC from RA patients were stained for the presence of citrullinated antigen-specific B cells that were identified using a combination of streptavidin tetramers as described previously[24]. Subsequently, CD28-expression was assessed and compared to the total plasmablast/-cell population (figure 6.3). ACPA-expressing plasmablasts/-cells were found to express CD28 although the percentage of CD28-expressing ACPA-expressing cells was comparable to the total population of CD28-expressing plasmablasts (figure 6.3A-B). These results show that CD28-expression can be expressed by ACPA-expressing plasmablasts/-cells.

Discussion

This report shows, for the first time, that CD28-expressing B cells and plasmablasts/-cells are present in peripheral blood of patients with RA and SLE. In peripheral blood, CD28 was expressed at comparable levels by plasmablasts/-cells of RA patients, SLE patients and healthy controls. Importantly, the



X Y proportion of CD28⁺ B cells was significantly higher in SF as compared to peripheral blood. Likewise, we found that ACPA-expressing plasmablasts/-cells present in the SF of patients with RA can express CD28, but that the level of CD28-expression is similar in ACPA-expressing plasmablasts/-cells compared to total plasmablasts/-cells. Hence, CD28-expression is present, but not enhanced on (autoantigen-specific) plasmablast/-cells in RA patients.

We observed a large variation in the proportion of CD28-expressing B cells and plasmablasts/-cells, especially in SF. Here, 0 to up to 30% of plasmablasts/cells were found to express CD28. The reason for this variation is not known, nor is it known why CD28-expressing B cells are more prominent in the synovial compartment. The latter could be related to the notion that CD28-expression by B cells could be activation-dependent, as suggested by others[18]. Also, activated B cells are more prominently present in the inflamed synovium[27, 28].

The reason why B cells start to express CD28 and the functional consequences of CD28-expression by human B cells are unknown. However, it is conceivable, based on murine studies, that CD28-expression could be involved in the survival and longevity of antibody-secreting cells[19, 22]. Recently, it has been demonstrated that CD28 on LLPCs has a crucial role in maintaining antibody production and survival. Although CD28 is expressed on both LLPCs and SLPCs, it promotes survival in only LLPCs. Likewise, CD28 has been shown to be involved in the survival of multiple myeloma cells within the bone marrow[29, 30] and of Epstein-Barr Virus (EBV)-positive B cells. EBV-positive B cells were protected against Fas-induced apoptosis following triggering of CD28[31]. As also a proportion of ACPA-expressing B cells express CD28, it is tempting to speculate that CD28-signalling is involved in the longevity of these autoantibody-producing B cells, a notion that should be verified in future studies.

Although long-lived plasma cells reside in the bone marrow, it is possible that these cells are also present in (inflamed) tissues[32-35]. Hence, it is possible that CD28-expressing B cells from the inflamed joint of RA patients are part of the body's LLPC population and that they contribute to the chronicity of disease. Lack of enrichment of CD28-expression on ACPA-expressing plasmablasts/cells when compared to total plasmablasts/-cells does not exclude that CD28 is involved in maintaining their survival, as CD28 has been shown to be expressed by both SLPC as well as LLPC, while it has a survival effect only on LLPC[19]. Hence, CD28-expression on total and autoantigen-specific plasmablasts/-cells could be comparable, but only autoantigen-specific plasmablasts/-cells might receive the survival signal, leading to extended survival and higher antibody production. In addition, it is possible that these cells are susceptible to treatment with CTLA-Ig (Abatacept). CTLA-4Ig leads to disruption of the interaction between CD28 and its ligands CD80/CD86 and is used to treat patients with RA. Intriguingly, Abatacept treatment results in a decrease in ACPA titres and in the percentage of memory B cells in patients with RA[36]. It is still not fully clear how Abatacept exerts its beneficial effects in RA, although its effects on the prevention of T cell activation are widely accepted. Other modes of action, however, might also be at play. For example, we have recently shown in a preclinical model of disease, that Abatacept treatment inhibits disease activity in



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the absence of CD4⁺ T cells[37]. These results suggest that the beneficiary effects are also mediated through a CD4⁺ T cell independent mechanisms, possibly by inhibiting B cell responses. As a considerable proportion of B cells in SF, depending on the donor analysed, can express CD28, it will be important to elucidate whether Abatacept can inhibit CD28-triggering of (autoantibodyproducing) B cells in RA. Together, it is tempting to speculate that the beneficial effects of Abatacept treatment may in part be the result of a direct effect of Abatacept on plasmablasts/-cells.

In summary, a small fraction of B cells in peripheral blood of RA patients expresses CD28. The proportion of B cells that express CD28 is higher on B cells in SF. And finally, APCA-expressing B cells in this compartment can also express this marker.

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