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CD28 expressing B cells and ACPA+ plasmablasts/-cells of Rheumatoid arthritis patients

Chapter 5


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In press
Abstract

Objective Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation of the synovial membrane of joints and by the presence of auto-antibodies produced by plasmablasts and plasma cells. Recently, expression of CD28 by long lived plasma cells has been described to promote survival and continued antibody production in mice. Considering the central role of B cells, plasma cells and auto-antibodies in RA combined with the fact that treatment with CTLA-4-Ig (abatacept), a drug capable of inhibiting CD28-triggering, is effective for RA, 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 plasmablasts/-cells and ACPA-expressing plasmablasts/-cells at the site of inflammation in RA patients as to determine whether CD28-triggering of (auto-antibody-producing) B cells could be affected by abatacept.

Methods CD28 expression was analysed on B cells as well as plasmablasts/-cells from freshly isolated peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) of RA patients by flow cytometry. As controls, we included PBMCs from SLE patients and healthy donors. The expression of CD28 was also determined on ACPA expressing plasmablasts/-cells derived from SFMC of RA patients.

Results CD28 was expressed on a subset of both B cells and plasmablasts/-cells from peripheral blood and was found to be comparable in RA patients, healthy controls and SLE patients. CD28 was expressed at significantly higher levels on CD20⁺ B cells from RA synovial fluid compared to B cells from peripheral blood. In addition, there was a trend towards higher expression of CD28 on synovial plasmablasts/-cells. CD28 was found to be expressed in similar frequencies on ACPA expressing plasmablasts/-cells derived from synovial fluid of RA patients.

Conclusion Here we show that CD28 is expressed on a subset of B cells, plasmablasts/-cells and ACPA-expressing B cells of RA patients and that CD28 expressing B cells and plasmablasts/-cells are present in higher frequencies in the synovial compartment.
Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized 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 characterized by the presence of auto-antibodies. Anti-citrullinated protein antibodies (ACPA) are among the well-described auto-antibodies in RA. ACPA are highly specific for RA and their presence is associated with a worse prognosis and disease severity [2-4]. ACPA have been implicated in disease pathogenesis [5-7] and have been reported to be enriched in the joints of RA patients as compared to peripheral blood. ACPA present in body fluids have been shown to be produced by plasmablasts/-cells [8-10].

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

CD28 has been known for a long time as a prototypic costimulatory 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 have also been shown to express CD28 [18-20]. CD28 expression by plasma cells is regulated by the transcription factor Paired Box (Pax) 5 [21]. Pax5 represses CD28 expression in B cells, while its loss 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 LLPCs to survive and maintain durable antibody production via interacting with its ligand CD80/CD86 expressed by dendritic cells (DCs) [19]. Deficiency in CD28 expression resulted in a considerable decrease in LLPC numbers and antibody levels.

In addition, CD28 has been revealed to promote the upregulation of B lymphocyte-induced maturation protein-1 (Blimp-1), a regulator of plasma cell differentiation [22]. Although CD28 was found to be expressed on both LLPCs and SLPCs, it only mediated the survival of LLPCs. This differential capacity for survival between LLPCs and SLPCs was shown to be due to signalling downstream of the CD28 Vav motif, which occurred only in LLPCs and not in SLPCs [22].
Abatacept, which is 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 costimulatory molecules CD80 and CD86. Abatacept is used to treat a subgroup of 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 auto-antibodies persist upon treatment with B cell targeting therapies suggests that auto-antibodies are continuously produced, potentially by long lived auto-antibody-producing B cells. As CD28 is also expressed by plasma cells, we questioned whether abatacept could potentially also inhibit CD28-triggering of auto-antibody 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

Peripheral blood (n=19) and synovial fluid (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 (n=11) was obtained for control. Written informed consent was obtained from all donors.

Cell isolation and flow cytometric analysis

PBMCs were isolated from blood of RA patients, SLE patients and healthy donors using Ficoll Paque gradient centrifugation (LUMC Pharmacy). Synovial fluid was obtained from inflamed knee joints of RA patients and first centrifuged to separate the cells from the fluid. Subsequently, SFMCs were isolated using ficoll gradient centrifugation. Cells were surface 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 CCP2 tetramers and a control tetramer as described previously [23]. All samples were measured on a BD LSRFortessa 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 number of cells in peripheral blood was calculated based on the number of collected B cells from a pre-set volume of whole blood per pre-set number of count beads using flow cytometry.
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**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 in blood were compared using a One Way ANOVA. The different cell populations in the SFMCs and PBMCs were compared using a Mann Whitney U test. P-values <0.05 were considered to be significant.

**Results**

**CD28 expression on plasmablasts/-cells in peripheral blood of RA and SLE patients and healthy controls**

CD28 has been described to function as a survival molecule for LLPCs. As CTLA-4Ig (abatacept) blocks the binding of CD28 to CD80/CD86 and has been proven to have beneficial effects in a subgroup of RA patients, we investigated whether antibody producing cells of RA patients expressed CD28 and if this CD28 expression was altered compared to SLE patients or healthy controls.

To this end, PBMCs isolated from RA patients, SLE patients and healthy controls were stained with antibodies against CD19, CD20, CD27, CD3, CD14 and CD28 and subjected to flow cytometry. Following exclusion of dead cells and duplets, CD19+ B-cells were visualized by gating on the CD3-, CD14- and DAPI-negative and CD19-positive population. Within this CD19+ cell population; discrimination between B cell subsets was made based on the expression of the markers CD27 and CD20. Plasmablasts/-cells were defined as CD27-high CD20-negative cells and subsequently their CD28 expression was determined (Figure 1A).

The percentage of CD19+ B cells in peripheral blood of RA patients was comparable to the percentages detected in SLE patients and healthy donors (Figure 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 healthy controls were comparable (Figure 1C). Likewise, the percentage and absolute number of plasmablasts/-cells was also not significantly different between the different groups (Figure 1D and 1E). However, we did observe a trend towards an increased percentage and absolute number of plasmablasts/-cells in SLE patients, which is in line with previously published reports [24].

Interestingly, we observed that approximately 5% of plasmablasts/-cells in RA peripheral blood expressed CD28, although the expression was comparable to the CD28 expression on plasmablasts/-cells of SLE patients and healthy controls (Figure 1F and 1G). These results show that plasmablast/-cells of RA patients express CD28 on their surface, although to a comparable extent compared to SLE patients and healthy controls.
Figure 1. CD28 is expressed on plasmablasts in peripheral blood of RA and SLE patients and healthy controls. PBMCs were isolated from peripheral blood of RA patients (n=10), SLE patients (n=10) and healthy controls (HC) (n=11) and analysed for CD28 expression on plasmablasts by flow cytometry. (A) CD19-positive B cells were determined by gating on the CD3-CD14-DAPI-negative and CD19-positive cell population. Within the CD19-positive B cell population discrimination between three different B-cell subsets can be made by their expression.
of CD20 and CD27. Plasmablasts are defined as CD27 high, CD20 negative cells and subsequently their CD28 expression was analysed. (B) A summary of the percentage and absolute number (C) of CD19-positive 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 number of cells per ml of blood. Line represents median.

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 synovial fluid of inflamed joints of RA patients. CD19+ B cells within SFMCs were identified by gating on CD3-CD14-DAPI-negative and CD19-positive cells. Subsequently, CD28 expression was determined on the total CD19+ B cell population, the CD27-high CD20-negative plasmablast/-cell population and the CD20-positive B cell population (Figure 2A). In total, SFMCs of 28 RA patients were compared to PBMCs of 9 RA patients.

The percentage of CD19+ B cells in peripheral blood was significantly higher compared to synovial fluid (Figure 2B). However, the percentage of CD19+ B cells and CD20+ B cells that expressed CD28 was increased in the SFMCs compared to the PBMCs (Figure 2C and E). Furthermore, there was a trend towards an increased percentage of CD28-expressing plasmablasts/-cells in synovial fluid but this did not reach statistical significance when compared to CD28-expressing plasmablasts/-cells from peripheral blood (Figure 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.

CD28 is expressed by ACPA+ plasmablasts/-cells.

ACPA producing B cells are present in the inflamed joints of patients with RA.[9, 10] (and our own unpublished observations). The finding that a fraction of synovial B cells and plasmablasts/-cells displayed high expression of CD28 raised the question whether ACPA+ plasmablasts/-cells derived from the site of inflammation exhibit higher CD28 expression in comparison to total plasmablasts/-cells. To address this question, SFMCs from RA patients were stained for the same markers as described previously and plasmablasts/-cells were defined by high expression of CD27 and absence of CD20 expression. ACPA-expressing B cells were identified using a combination of tetramers as described previously [23]. Subsequently, CD28 expression was assessed and compared to the total plasmablast/-cell population (Figure 3).

ACPA positive plasmablasts/-cells were found to express CD28 although the percentage of CD28 expressing ACPA positive cells was comparable to the total population of CD28 expressing plasmablasts/-cells (Figure 3A and 3B). These results show that CD28 expression can be expressed by
ACPA-expressing plasmablasts/-cells. However, there was no enrichment as compared to the total plasmablast/-cell population from this compartment.

Figure 2. CD28 is highly expressed on CD20-positive B-cells from synovial fluid of RA patients. SFMCs were isolated from synovial fluid from inflamed knee joints of RA patients and analysed for CD28 expression on B cells by flow cytometry. In total SFMCs of 28 RA patients were analysed and compared to PBMCs of 9 RA patients. (A) CD19-positive B cells were identified by gating on CD3-CD14-DAPI-negative and CD19-positive cells. CD28 expression of the total CD19-positive B cell population was analysed as well as the CD28 expression on the CD27 high CD20 negative plasmablast/plasma cell population and the CD20-positive cell population. (B) Identical
gating strategies were used to determine the CD19-positive B cells population within SFMCs and PBMCs and the summary is depicted. (C) Expression of CD28 by the CD19-positive B-cells (as depicted in B). (D) Expression of CD28 on plasma blasts (CD27 high CD20 negative cells) within SFMCs and PBMCs. (E) Expression of CD28 by CD20+ B cell population (naive and memory B cells together). SFMC; synovial fluid mononuclear cells, PBMC; peripheral blood mononuclear cells. Line represents median. * p<0.05, ns = not significant.

Figure 3

Figure 3. CD28 is expressed by ACPA+ B cells derived from synovial fluid of RA patients. CD28 expression was determined on ACPA expressing plasmablasts/-cells in SFMCs using flow cytometry. (A) CD19+ B cells were gated as depicted in figure 1A and subsequently the plasmablasts/-cells were identified (left). The gate to determine the CD28 positive cells was set on the isotype control (middle) and subsequently the CD28-positive plasmablasts/-cells were identified (right). (B) ACPA-expressing plasmablasts/-cells were defined by a double staining for the citrullinated antigen specific tetramers (left) in combination with a control tetramer. CD28 expression by these ACPA-expressing plasmablasts/-cells was determined using an isotype.

Discussion

This report shows, for the first time, that CD28 expressing B cells and plasmablasts/-cells are present in 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 proportion of CD28+ B cells was significantly higher in synovial fluid as compared to peripheral blood. Likewise, we found that ACPA+ plasmablasts/-cells present in the synovial fluid 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 the (auto-antigen-specific) plasmablast/-cells in RA patients.

We observed a large variation in the proportion of CD28-expressing B cells and plasmablasts/-cells, especially the synovial fluid. 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]and supported by the observation that activated B cells are more prominently present in the inflamed synovium [25, 26].

The reason why B cells start to express CD28 as well as the functional consequences of CD28 expression on human B cells is unknown. However, it is conceivable, based on murine studies [19, 22], that CD28-expression could be involved in the survival and longevity of antibody-secreting cells. 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 [27, 28] and Epstein-Barr Virus (EBV) positive B cells. EBV positive B cells were protected against Fas-induced apoptosis following triggering of CD28 [29]. 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 auto-antibody-producing B cells, a notion that should be verified in future studies.

Although LLPCs reside in the bone marrow, it is possible that these cells are also present in (inflamed) tissues [30-33]. Hence, it is possible that CD28-expressing B cells from the inflamed joint of patients with RA are part of the body’s LLPC population and that they contribute to the chronicity of disease. The absence of enrichment of CD28 expression on ACPA-expressing plasmablasts/-cells when compared to total plasmablasts/-cells does not necessarily suggest that CD28 is not involved in maintaining their survival, as CD28 has been shown to be expressed by both SLPCs as well as LLPCs, however, CD28 was found to have a survival effect only on the LLPC [19]. Hence, CD28 expression on total and auto-antigen-specific plasmablasts/-cells could be comparable, but only auto-antigen-specific plasmablasts/-cells might receive the survival signal, leading to extended survival and higher antibody production.

Because CD28-expressing B cells reside in the inflamed joints of RA patients, it is possible that they are targeted by treatment with CTLA-4-Ig (Abatacept). CTLA-4-Ig leads to disruption of the interaction between CD28 and its ligands CD80/CD86 and is used to treat patients with RA. Abatacept treatment has been shown to result in a decrease in ACPA titres and in a decrease in the percentage of memory B cells in patients with RA [34]. 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. Nonetheless, also other modes of action might be at play. For example, we have recently shown in a pre-clinical model of disease, that abatacept treatment inhibits disease activity in the absence of CD4\(^+\) T cells [35].

These results indicate that the beneficiary effects are also mediated through a CD4\(^+\) T-cell independent action, possibly by inhibiting B cell responses. By showing that in the inflamed synovial compartment, a considerable proportion of B cells, depending on the donor analysed, can express CD28, it is of interest to elucidate whether abatacept can inhibit CD28-triggering of (auto-antibody producing) B cells in RA. Considering the observation that CD28 signalling can promote survival of LLPC, it is tempting to speculate that the beneficial effects of abatacept treatment may be partially a result of a direct effect of Abatacept on plasma cells.

In conclusion, a small fraction of B cells in peripheral blood of RA patients expressed CD28, however, to a comparable extent as the B cells of SLE patients and healthy controls. Interestingly, the proportion of B cells that expressed CD28 was higher on B cells from synovial fluid of RA patients and present on ACPA-expressing B cells in this compartment.
References

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