Tolerance and immune regulation in rheumatoid arthritis

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Chapter 9

Discussion and general summary

Autoantibodies against post-translationally modified (PTM) proteins are a hallmark of RA. The reason why an immune response starts against PTM proteins is not known. However, it appears crucial to obtain understanding on the breach of tolerance towards PTM proteins as the immune response against these proteins has been intimately implicated in disease-pathogenesis. In this thesis we report that exposure to modified self- and foreign proteins can lead to the generation of anti-modified protein antibodies (AMPA) which are directed against PTMs. Exposure of mice to carbamylated self- and foreign antigens can lead to the formation of self-reactive anti-CarP antibodies (chapter 3) (1). We also found that exposure to carbamylated self-proteins is sufficient to trigger primary immune responses, including autoantibody formation, T cell activation and cytokine production (chapter 4) (2). The studies in chapter 5 describe the observation that vaccinating mice with an acetylated protein leads to the formation of auto-antibodies against carbamylated proteins (anti-CarP antibodies) as well, indicating that different AMPA-responses can evolve from exposure to only one type of modified protein (3). In addition, we found that AMPA from RA-patients purified against one PTM can recognize different classes of PTMs. Chapter 6 describes the relationship between autoantibody status and treatment response. We found that in newly diagnosed RA patients who are receiving methotrexate, autoantibody status was not associated with the chance of achieving early remission. This indicates that methotrexate is effective as initial treatment strategy regardless of autoantibody status (4). The studies described in chapter 7 show that both prophylactic and pre-arthritis treatment strategies lead to a significant reduction of arthritis severity in animal models (5). Chapter 8 describes studies regarding the link between periodontal infection and autoimmunity. In a large cohort of arthritis patients we found that anti-LtxA antibodies were not specifically associated with RA. In addition, there was no association between ACPA or HLA SE alleles among RA patients. Together, these studies highlight that the different anti-modified protein antibody responses present in RA could have a common origin which could be potentially implicated in disease pathogenesis. Regarding the described studies, several issues are worthwhile to be discussed in more detail.

Detection of AMPA responses: specificity and reproducibility

For the detection of AMPA we used anti-carbamylated protein, anti-citrulline and anti-acetylated lysine ELISA antibody techniques (1-3). As these ELISA techniques are predominately used for the identification of these autoantibodies, its accuracy and reproducibility are crucial for the validity of our conclusions. The antigens used for identification are fetal calf serum, serum albumin and fibrinogen. These antigens were chosen as they are readily available, relatively cheap and easily to modify (i.e. carbamylate, citrullinate or acetylate) in a consistent manner. The non-modified counter parts served as

control. After protein modification by carbamylation, citrullination or acetylation, the presence of the modification on the antigens was confirmed by mass-spectrometry. This is a crucial step in order to confirm the solely presence of one particular modification in the absence of other, structurally similar, PTMs. Mass-spectrometry is a very specific method for compound identification as the citrulline and homocitrulline are distinguished based on their position within the protein (they are derived from either an arginine or a citrulline). Furthermore, homocitrulline differs from citrulline by 14 Da. Although mass-spectrometry is a specific technique, it is not a quantitative method. Therefore, we used commercial antibodies recognizing citrullinated, carbamylated or acetylated proteins to quantify the extent of modification present.
We optimized in-house ELISAs to determine the presence of protein modifications and by titrating these antibodies the extent of modifications present can be estimated (although not in absolute terms). To confirm the reproducibility of our assays we performed several internal control experiments. Our previous studies with human sera showed an intra- and inter-assay variability of anti-carbamylated fetal calf serum (anti-Ca-FSC) and anti-carbamylated fibrinogen (anti-Ca-FCS) of around 10-15% (6). Using sera of immunized mice, the intra- and inter-assay variability was more consistent (around 5-10%). In repeated measurements the chance of a false-positive sample in non-immunised mice was generally around 2-5%. It is important to acknowledge that there remains a certain degree of intra assay variation which can be considered as random variations due to the methodology of our assays (e.g. proteins rather than structurally defined peptides).

AMPA responses and cross-reactivity

Since homocitrulline structurally highly resembles citrulline, it is possible that anti-CarP antibodies are cross-reactive to citrullinated antigens. It has been reported that ACPA can bind peptides or proteins containing homocitrulline (7-9), however this does not directly implicate that antibodies in general cannot be specific for these PTMs. Previously, we have shown that human polyclonal antibodies can be specific for either carbamylation or citrullination (10). Additional studies indicate that the specificity towards citrullinated or homocitrulline does exist, but is dependent on the sera used (11). To test for cross-reactivity of polyclonal anti-CarP antibodies with carbamylated proteins, we performed inhibition assays with the same protein used both as antigen and inhibitor (1). This resembles a relevant positive control to show that at the chosen inhibitor concentration, inhibition is indeed present. The relevance of the inhibition assays lies in the comparison to the other modified proteins. Our data show that other, structurally unrelated, modified proteins are also able to inhibit the anti-CarP-response generated.

The studies in chapter 5 describe the exciting observation that vaccinating mice with e.g. an acetylated protein leads to the formation of anti-CarP antibodies as well (3), indicating that different AMPA-responses can evolve from exposure to only one type of modified protein. In our studies we found that the avidity of the antibody response to carbamylated fibrinogen and acetylated fibrinogen was similar after immunization with, respectively, carbamylated ovalbumin (RAI 64.3%) or acetylated ovalbumin (RAI 43.1%) (3). We subsequently found that avidity of the AMPA-response is highest towards the respective modified antigen used for immunization. Discrepancies in the degree of cross-reactivity may be a result of proteins that contain different numbers and locations of PTMs. So far a well-defined chemical construct recognized by anti-CarP antibodies which could be used as a pan inhibitor for inhibition experiments has not been identified so far.

In additional experiments we studied the polyclonal responses present in human serum. We used a biotinylated CCP2 peptide bound column to isolate anti-CCP2 antibodies and biotinylated Ca-FCS for the isolation anti-CarP antibodies in patients serum samples. After purification, ACPA were strongly enriched for reactivity towards both carbamylated and acetylated vimentin peptides (3). Following our studies on the cross-reactivity of purified ACPA from RA patients, our team has studied whether monoclonal ACPA (12) can cross-react to acetylated antigens and other PTM proteins. Interestingly, we recently observed that this human monoclonal ACPA is able to react not only to citrullinated, but also to acetylated fibrinogen (data in preparation). Carbamylated fibrinogen was not recognized. Together these data are important as they show for the first time that ACPA can be cross-reactive towards acetylated proteins as well. The relative cross-reactivity between ACPA, anti-CarP and anti-acetylated lysine antibodies suggests that these autoantibodies may originate from the same B-cell population. Acetylated lysine does not resemble citrulline but bears similarity to homocitrulline except at the side chain terminal amine, which is replaced by a methyl moiety.

In our studies we observed that ACPA cannot only cross-react to some extent to carbamylated proteins, but also to acetylated proteins. In line with our data, others have shown that reactivity to citrullinated vimentin peptides was blocked by preincubation with the soluble citrullinated peptide as expected, with weak inhibition of binding by carbamylated (23%) and acetylated (17%) vimentin peptides. Similar specificity and low cross-reactivity was observed for the anti-acetylated vimentin antibodies as well as for anti-carbamylated vimentin where 32% of the binding was inhibited by preincubation with citrullinated soluble peptide (8). These results might be explained by the notion that AMPA consist of different auto-antibody families that are largely distinct, but that can also display a certain degree of cross-reactivity.

It is interesting to note that a defined antigen-receptor can recognize both citrullinated and acetylated antigens despite the structural dissimilarities of these two antigens. This indicates that different AMPA-responses can evolve from exposure to only one type of

modified protein. These data could represent a paradigm shift explaining the induction of AMPA-responses in RA since they show that the inciting antigen responsible for the induction of e.g. anti-CarP-antibodies does not have to be citrullinated or carbamylated, but could be represented by an acetylated protein. Increasing evidence suggests that mucosal surfaces, specifically the periodontium, the gut and the lungs, as sites of disease initiation of RA and indicate the microbiome as an important driver of the initiation of auto-immunity. In this respect, especially protein–acetylation by bacteria might now also be incriminated in the induction of auto-antibody responses against PTM proteins.
In our future studies it would be interesting to generate and test more monoclonal ACPA for the antigen recognition profile. In addition, it would be of interest to establish a method to purify anti-acetylated protein antibodies to determine if- and to what extent human polyclonal AMPAs are cross-reactive towards different modified antigens. Together with current data, these future experiments will provide valuable insight into the magnitude and extent of the cross-reactive nature of human AMPAs towards three different modified antigens.

AMPA responses in mice

In chapter 4 we showed that stimulation of spleen cells with dendritic cells pulsed with carbamylated mouse albumin led to the induction of a strong T cell response, cytokine production and proliferation. Stimulation of spleen cells with homocitrulline containing peptides identified by mass spectrometry resulted in a PTM specific T-cell response. These findings suggest that posttranslational modification of self-proteins can result in 'new' antigens for which immune tolerance does not exist. It was recently shown by others that, sera of mice exposed to tobacco smoke contains anti-CarP antibodies and increased amounts of carbamylated vimentin (13). Although the formation of PTMs seems to promote tolerance loss and autoimmunity in RA, it is still unknown whether AMPA responses are directly pathogenic or a marker for inflammation.
ACPA are considered to be a highly important serological marker in RA patients, however, their role and importance in mouse models of arthritis remains far less clear. In mouse models of RA the antibody dependent collagen-induced arthritis (CIA) and K/BxN model clearly require B cells, and the sera can be used to transfer disease, confirming a direct pathogenic role of humoral immunity in arthritis (14, 15). In the CIA model, inflammatory arthritis is induced in genetically susceptible mice by immunization with type II collagen (CII) (16) which subsequently leads to the induction of autoreactive anti-CII antibodies (17, 18). There is conflicting evidence whether ACPA are involved in CIA (19). It has been reported that ACPA can be found in sera of mice with arthritis that did not receive additional vaccines with citrullinated antigens (20-22). However, data whether ACPA can induce or exacerbate

symptoms are limited as the majority of animal studies show that passive transfer of ACPAs alone does not induce arthritis (21, 23-25).
Our team has previously shown that anti-CarP antibodies, but not anti-CCP2 antibodies, are present in sera of DBA-1 mice and C57BL/6 with CIA. It was shown that the onset of arthritis was preceded by an increase of anti-CarP antibodies (26). These data are in line with the data that in asymptomatic blood bank donors there is a rise in anti-CarP levels prior to clinical onset of RA (27). For future studies it would be interesting to examine whether anti-acetylated lysine antibodies are also present in sera of mice with arthritis. We recently studied the role of anti-CarP antibodies in arthritis using our monoclonal anti-CarP antibody (data in preparation). In a pilot experiment we tested whether exposure to anti-CarP antibodies could exacerbate arthritis. DBA-1 mice were injected with CFA and CII according to protocol and later received additional anti-CarP antibodies. In these first experiments, we observed no exacerbation of symptoms upon transfer of anti-CarP antibodies. It would be interesting in the future to study the role of AMPA in arthritis using additional monoclonal antibodies such as anti-acetylated lysine antibodies.
In summary, the hypothesis that AMPA responses play a key role in the pathogenesis of RA needs solid and repeatable animal data. The identification of additional PTM responses besides ACPA, such as anti-carbamylate and acetylated protein antibodies, enables us to study the induction and role of these AMPA in animal models of arthritis.

Autoantibodies in the pathogenesis of RA

Despite many years of intensive research, the pathogenesis of RA remains elusive. It appears that breach of tolerance towards PTM proteins and the generation of AMPA is intimately implicated in disease-pathogenesis. Genetic predisposition and environmental factors such as smoking are thought to contribute to this break of tolerance. However, asymptomatic individuals can harbour autoantibodies without developing RA for many years, which suggests that the sole presence of an autoantibody is insufficient to trigger the onset of RA (27).
Previous studies have shown that ACPA-producing B-cells are found to be enriched in synovial fluid (28, 29), suggesting that ACPA can be produced locally and directly contribute to synovial inflammation. So far, little information is currently available on the nature of carbamylate proteins that are present in the (inflamed) joint. Using mass-spectrometry we have been able to identify carbamylated serum albumin (1) and alfa-1-anti trypsin (30) in the synovial compartment of RA patients. Recently, other carbamylated proteins have been identified in patients with renal disease (31, 32), indicating that it is likely that also other carbamylated proteins can be carbamylated in RA patients. It will be interesting to see whether the nature and location of carbamylated proteins in the synovial compartment is similar or distinct from the citrullinated proteins found in the joint. Studies in chapter 6

describe the relationship between autoantibody status and remission in newly diagnosed RA-patients treated with first-line methotrexate. Methotrexate is the most widely used anti-rheumatic drug in clinical practice (33). We found that in newly diagnosed RA patients who are receiving methotrexate, autoantibody status (ACPA and/or rheumatoid factor) was not associated with the chance of achieving early remission (4). In the future, patients may benefit from treatment tailored to “autoantibody status”.

It has been hypothesised that very early treatment initiation can prevent the development of RA, a so called “window-of -opportunity". Animal models provide a model to study the developing (auto)immune response at a very early disease phase. The translation of different stages of experimental arthritis to the evolution of human disease might provide valuable information regarding possibilities of disease prevention. Based on different studies in animal models we found that both prophylactic and pre-arthritis treatment strategies lead to a significant reduction of arthritis severity, chapter 7 (5). Autoantibody formation against type II collagen can be used as a marker to characterize different stages of disease. The time period in which auto-immunity is present and arthritis is still absent was referred to as the pre-arthritis period. Current animal research mainly focusses on testing anti-rheumatic drugs in established disease and preventive treatment strategies are not studied frequently. In an ideal experiment, different interventions should be studied side-by-side in different disease phases and with a similar treatment schedule to be able to compare efficacy.

Protein modification and periodontal disease appear to be relevant for the pathogenesis of RA, however, how these processes may be mechanistically related remains poorly defined. The periodontal pathogen Aggregatibacter actinomycetemcomitans (Aa) seems to represent a link between periodontal infection and autoimmunity. It was previously shown by others that Aa is capable to induce neutrophil hypercitrullinations through the secretion of leukotoxin A (LtxA), a bacterial pore-forming toxin that induces calcium influx and subsequent hyperactivation of PAD enzymes in the neutrophil. The studies in chapter 8 describe that in a large cohort of arthritis patients, anti-LtxA antibodies (used as surrogate marker of Aa infection) were not specifically associated with RA but could also be identified in other forms of inflammatory arthritis (34). In addition, we found no association between ACPA or HLA SE alleles in contrast to the previous study (33). Differences in cohorts and methodology may account for the difference in effect size. In our cohort the anti-CCP positivity is 58% (Leiden) versus 77% (Baltimore). The Leiden Early Arthritis Clinic includes patients with recent onset arthritis in whom definitive diagnosis are established after1 year of follow up. In contrast, the Baltimore cohort includes a wide range of disease durations, form early disease to decades. Regarding methodology, the cut-off for positivity was based on the lowest point of the linear part of the standard curve (2000 arbitrary units/ml, 75% specificity). It remains important to emphasize that despite not affecting comparisons of

median antibody concentrations between groups, a degree of uncertainty in cut-off selection can alter any interaction analyses.
Many bacterial species are able to acetylate proteins (35), including bacteria proposed as link between periodontal infection and RA (36). Disturbances of the microbiome, for example during infection, could lead to the increased formation of acetylated proteins which are detected by the immune system and thereby contribute to the induction of AMPA-responses. Microbe-specific T cells could initially help the B cells recognize the microbe-derived modified protein and thereby contributing to isotype-switching and somatic hypermutation.

Final conclusions

In conclusion, we showed that exposure to modified self- and foreign proteins can lead to the generation of AMPA. The observation that exposure to an acetylated protein leads to the formation of auto-antibodies against carbamylated proteins as well, indicates that different AMPA-responses can evolve from exposure to only one type of modified protein. Understanding the full AMPA response, the triggers that drive AMPA production, their mutual cross-talk and the pathways by which AMPA and/or AMPA expressing B cells possibly contribute to RA might – in the long run- allow interventions that prevent disease development, a highly desirable goal in the quest against rheumatic diseases.

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