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Studies on the role of alpha enolase as a candidate autoantigen in pristane-induced arthritis and immunological tolerance of citrullinated type II collagen

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ABSTRACT

Rheumatoid Arthritis is a symmetric polyarticular disease, which mainly targets the synovial membrane, cartilage and bone of small diarthrodial joints of hand and feet. The etiology of rheumatoid arthritis remains uncertain. Pristane-induced arthritis in the DA rat is a widely used experimental arthritis model. The mechanisms involved in arthritis development are not known. PIA is MHC class II associated and CD4+ T cells can transfer the disease to naïve recipients. As in human RA the autoantigens driving the joint inflammation are not yet known. In our lab, the immune responses against ubiquitously expressed RNA binding protein hnRNP-A2 (RA33), cartilage specific rat CII and CXI as candidate autoantigens in PIA were studied. In the present study, we investigated the immune responses to the tissue non-specific antigen α-enolase. In my thesis, the first chapter shows the immune response against α-enolase as autoantigen. The MHC dependency and association was assessed in MHC congenics rats. The results obtained will be useful to understand the T cell pathology in PIA and RA.

In the second chapter of my thesis we discuss immune tolerance towards a cartilage specific autoantigen, mouse CII (mCII). The mCII expression in the thymus was studied. From the obtained data two hypotheses of tolerance breakage can be drawn. According to one hypothesis, the CII expressed in the thymus undergo post-translational modification and gets converted into citrullinated CII by the PAD enzymes. The citrullinated CII undergo tolerization and the T cells in the periphery come into contact with native CII in joints and recognize it as foreign. Therefore, the T cells invoke autoimmune response against the native CII in joint. In another hypothesis, the T cells get tolerized towards native CII expressed in thymus. The T cells in the periphery are presented with neoepitopes in the joints, e.g. due to citrullination of CII in joints. This condition elicits the immune response towards citrullinated CII in joints.

CONTENTS

List of abbreviations

APC Antigen presenting cell

CII Type II collagen

CCP Cyclic citrullinated peptide

CIA Collagen-induced arthritis

ConA Concanavalin A

HLA Human leukocyte antigen

IFA Incomplete Freund's adjuvant

IFN Interferon

Ig Immunoglobulin

IL Interleukin

mAbs Monoclonal antibodies

MHC Major histocompatibility complex

OIA Oil-induced arthritis

PAD Peptidyl Arginine Deminase

PBS Phosphate buffered saline

PIA Pristane-induced arthritis

RA Rheumatoid arthritis

RF Rheumatoid factor

SE Shared epitope

TCR T cell receptor

Th T helper cell

TNF Tumor necrosis factor

Treg Regulatory T cell

1. Background:

1.1Rheumatoid Arthritis:

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflammation in the synovial membrane, cartilage and bone. In addition to inflammation in the synovium, which is the joint intimal lining, the aggressive development of pannus formation takes place and it destroys local articular cartilage and ankylosis of the joints [1].

The typical symptoms of patients with RA include swelling, pain and redness in the joints often accompanied with stiffness and fatigue. Rheumatoid arthritis is diagnosed in accordance with 2010 American College of Rheumatology/European League Against Rheumatism criteria for RA [2]. These criteria are drafted on the basis of clinical symptoms, biochemical markers and radiographic changes and the new criteria addresses issues related to early RA. According to these criteria the patients can be confirmed as RA patients if they cover following four areas and establish a point scale, values ranging from 0 to 10. The patient with value of 6 or higher can be classified as RA patient. The four areas are: **1)** joint involvement – depending on the type and number of affected joints showing swelling: up to 5 points **2)** serodiagnosis – depending on titer levels of rheumatoid factors, ACPA determination: up to 3 points **3)** acute phase reaction – elevated erythrocyte sedimentation rate value, elevated C-reactive protein: 1 point **4)** duration of arthritis symptoms like pain, swelling, tenderness – longer than 6 weeks: 1 point.

1.2. Pathogenesis of RA:

RA has long been considered as an autoimmune disease, and is caused by different pathways involving T cells, B cells and autoantibodies, cytokine milieu, fibroblasts and many other cell types [3].

1.2.1 T cells and cytokines:

The role of T cells in the pathogenesis of RA is well elucidated. T cells have been reported to be present in the infiltrates of synovium of RA affected patients [3]. CD4+ αβ T lymphocytes[3,4] are the major number of T cells present in synovium and they express the memory phenotype marker ($CD45RO^+$), activation markers (CD69, CCR4,CD44 & CCR5) and adhesion markers (VLA, CD29, CD44) on their cell surfaces [5,6]. It is hypothesized that the arthritogenic peptides either self or foreign are presented to T cells by antigen presenting cells (APCs – dendritic cells, B cells and macrophages). The APC employs MHC class II molecules for presenting and about 50%-80% of RA patients carry the shared-epitope (SE) of HLA-DRB1 cluster [7,8]. These

alleles encode highly homologous amino acid sequence (QRRAA, QKRAA, RRRAA) in third hyper variable region of HLA-DRβ chain and affect in antigen presentation to T-cell Receptor (TCR).

The synovial T-cells are mainly of Th1 type [9,10] and triggered to secrete IFN-γ and IL-2. The cytokine induces the B cells for production of opsonizing and complement fixing antibodies [11]. The activation of monocytes and macrophages are also observed in synovium [12,13]. It is assumed that IL-17 cytokine plays major role in RA as it is observed in synovium of RA patients [14,15,16] and it has the same function as of IL-1 and TNFα. IL-17 induces release of wide range of molecules, including proinflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α), multiple chemokines, cyclooxygenase-2, prostaglandin E2, and matrix metalloproteinases [17, 18]. IL-17 upregulates the RANKL (receptor activator of NF-κB ligand) expression on chondrocytes and osteoblasts and promotes osteoclastogenesis, which leads to bone erosion in RA.

1.2.1.1 T cell Epitope:

An epitope (antigenic determinant) is the part of antigen capable of eliciting an immune response specifically by B cells or T cells. T cell epitopes are processed and presented on the surface of an antigen-presenting cell (APC), where they are bound to MHC molecules and are required for recognition by specific T cells.

Type II collagen (CII) is main protein component of hyaline cartilage. Many studies have shown T and B cell responses against CII as a possible autoantigen in RA patients [19,20,21]. The immunodominant T cell epitope for CII in CIA is mapped and observed in H2Aq(MHC class II molecule) expressing [22] and DR4 transgenic mice[23]. Strikingly, the same epitope is shared with human RA patients [24]. The T cell epitope is located within the region of the residues 260-270 of CII (CII260-270) [25,26]. This peptide sequence is identical between human, rat bovine and chick CII but differs in one amino acid at position 266 in mouse CII, where a glutamic acid on CII is exchanged for an aspartic acid in mouse [25]. The immunodominant region of CII can undergo post-translational modifications at position 264 and 270 where the lysine gets converted into galactosylated or glucogalactosylated [27], generating several distinct immunodominant epitopes recognized by distinct T cells. Likewise for other MHC class II molecules, it is assumed that arginine residues present in CII could undergo citrullination and form neoepitopes and thereby invoking the immune response.

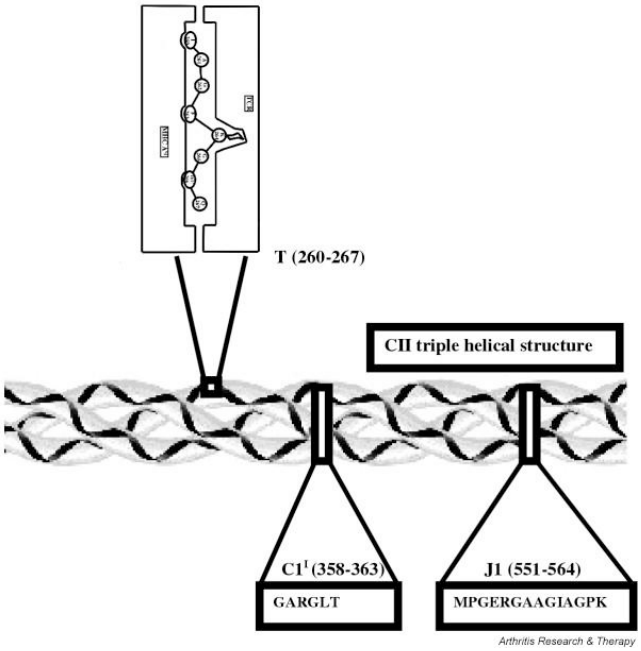

Figure 1:Specific B and T cell Epitopes of Type II collagen (CII)*. Illustration of T cell (residues 260–267) epitopes present on the triple-helical form of the collagen type II[28]*

1.2.2 B cells

B cells can play potentially critical role in the pathogenesis of RA. They may function as APCs by processing and presenting antigenic peptides to the T cells [29,30]. The T cells then proliferate and exert pro-inflammatory activities. It is well known that B cells can bind antigens through their immunoglobulin receptor. The immunoglobulin receptor lies on the surface of the B cell and can bind a very low level of antigen from the environment. The antigen is degraded by the B cell into antigenic peptides. These antigenic peptides are then presented in the groove of the HLA-DR molecules to activate the T cells, which in turn undergo various processes, including proliferation, cytokine production and cell-to-cell interaction, which contribute to the pathogenic process in RA [31].

The infiltrating lymphocytes are seen in inflamed synovial tissues which are in diffused or follicular manner. The follicular infiltrates consists of B cells surrounding the perivascular aggregation of T cells. These B cells can differentiate into plasma cells [32] and take part in the production of auto antibodies.

1.2.2.1 Autoantibodies:

The rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibodies are considered clinically useful as disease markers [33]. The RF is probably the most studied autoantibody in RA. It binds to the Fc region of IgG and contributes to the formation of immune complexes [34].

Antibodies specific to the post-translational modification (citrulline) on proteins like, keratin, filaggrin, fibrinogen, vimentin, fibronectin, α-enolase, CII etc. are referred as anti-citrullinated protein antibodies (ACPAs). ACPAs are rarely found in healthy individuals but in RA patients they seem to be 70–90% and have high disease specificity (90–95%)[35,36]. Proteins are citrullinated during apoptosis and inflammatory process in RA, they are found years before disease onset in the plasma and levels seem to be elevated in the synovial fluid [35].

Citrullination is the post-translational conversion of positively charged peptidylarginine to neutral peptidylcitrulline. The conversion is catalyzed by Peptidyl Arginine Deiminase (PAD) enzyme in the presence of calcium ions. In humans, five PAD isotypes (PAD1, PAD2, PAD3, PAD4 and PAD6) are described with varied tissue expression and only PAD2 and PAD4 have been found to express in inflamed synovial tissue of RA and in other inflammatory arthritides[37].

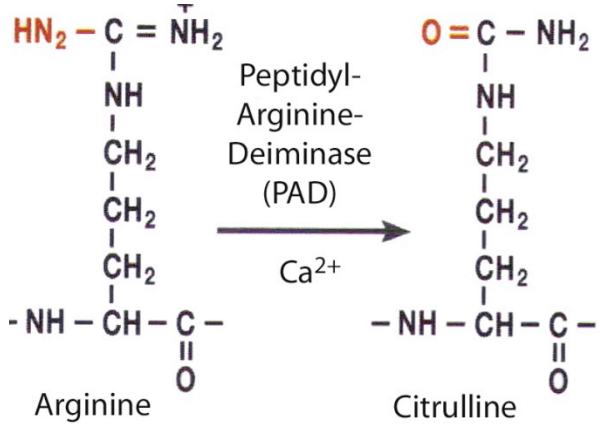

Figure 2: *In citrullination, arginine to citrulline conversion is catalyzed by PAD enzymes in the presence of calcium ions.*

1.3 Risk Factors

The etiology of rheumatoid arthritis is not fully understood but involves a complex interplay of genetic and environmental factors.

1.3.1 Genetic Factors:

Genetic factors also have a substantial impact on pathogenesis of RA. The genetic contribution for the development of RA at the national scale has been studied in monozygotic and dizygotic twin pairs from Finland and United Kingdom. The report suggests a concordance rate in monozygotic twins of 60 % [38]. Multiple loci contribute to genetic risk for RA and among these; the HLA (human leukocyte antigen) locus contributes around 30% to 50% of overall genetic susceptibility to RA. The association between RA and HLA-DR4 (HLA-DRB1*04) was found in 1976 [39].

From the above findings Gregersen et al. established a Shared-Epitope hypothesis. According to this hypothesis, HLA-DR molecules share a conserved motif of amino acid residues (QRRAA, QKRAA, and RRRAA), which could favours certain arthritis-inducing peptides and triggers autoreactive cells [40]. In non-MHC genes, the strongest association was identified with protein tyrosine phosphatase non receptor 22 (*PTPN22*) gene which encodes lymphoid specific phosphatase (Lyp), an important negative regulator of T cell activation [41].

1.3.2 Environmental Factors:

Studies performed for looking out the environmental factors important in RA have identified numerous risk factors. These include smoking and a number of infectious agents.

1.3.2.1 Cigarette Smoking:

Several studies have been implicated the association between cigarette smoking and development of RA [42,43]. It is an important environmental risk factor and the risk is more in HLA-DR4-positive individuals [44,45]. Klareskog et al proposed that both smoking and MHC class II shared-epitope were risk factors for ACPA positive RA. They suggested that smoking, in the presence of HLA-DR SE alleles, initiates an antigen specific autoimmune response to citrullinated self proteins, which triggers the development of RA. Higher levels of ACPA are observed in the cigarette smoking-RA patients [46]. In the bronchoalveolar lavage cells of healthy smokers, an elevated level of

citrullinated protein was found [47]. The increment in frequency of citrullinated protein was associated with elevated expression levels of *PADI2* in the bronchial mucosal and alveolar compartment [47]. The increased levels of *PADI4* expression was also observed in serum of smoker lung cancer patients [48]. However, the mechanism in which the smoking acts on PAD enzymes remains to be clarified. It is also assumed that cyanide present in tobacco contributes the onset of RA [49].

1.3.2.2 Infections:

Infectious agents like *Escherichia coli, Mycobacterium tuberculosis*, *Proteus mirabilis,* Epstein-Barr virus and Parvovirus has been suspected in the development of RA [50,51]. Many mechanisms through which the microbial candidates alter the immune system and cause of autoimmune responses have been proposed. Potential mechanisms for immune response invoked include altered B and T cell repertoire selection and change of Th1 and Th2 cytokine levels [52]. *Porphyromonas gingivalis* has gained attention due to its epidemiologic links between RA and periodontitis[53]. A novel bacterial PAD[54] from *P. gingivalis* has been implicated in citrullination of human fibrinogen and α-enolase peptides and ACPA production in individuals who subsequently develop RA[55]. However, the substantial role of infectious organisms in cause of RA is uncertain.

1.4 Animal models

1.4.1 Pristane Induced Arthritis

Pristane-induced arthritis (PIA) in rats mimics human RA and fulfills the clinical criteria necessary for diagnosis of RA in humans. PIA in rats is induced in susceptible strains such as DA by an intradermal injection of non-antigenic pristane oil (2, 6, 10, 14-tetramethyl pentadecane), resulting in development of severe relapsing arthritis disease course with an erosive and symmetric destruction of peripheral joints after two weeks [56]. The PIA rat model resembles the human disease such as in development of symmetrical disease, presence of serum rheumatoid factors, radiographic changes and chronicity. PIA is T-cell driven and can be transferred with αβ CD4+ T cells [57]. Pristane does not contain peptides that could be presented by MHC molecules. Hence, the characterization of autoantigens was carried out and heterogeneous nuclear ribonucleoprotein (hnRNP) was found to be early target of the autoimmune response in PIA [58]. The disease has been strongly associated with MHC region and dependent on

MHC class II-restricted T cells [57]. However, the association and involvement of the MHC class II genes with PIA is still uncertain[59,60].

1.4.2 Oil Induced Arthritis:

In the oil-induced arthritis model (OIA), incomplete Freund's adjuvant (IFA) is used to induce inflammatory arthritis in DA rats. IFA is a mixture of 85% paraffin oil (Bayol F) and 15% of the emulsifier Arlacel A. The onset of disease is 9-11 days after an intradermal injection of IFA with symptoms of joint inflammation. The disease is mono-phasic and self-remitting and the disease course usually spans 3-4 weeks [61]. As in PIA, susceptibility to OIA is associated with both MHC and non-MHC loci, although only a handful of genes have been conclusively positioned until today [62].

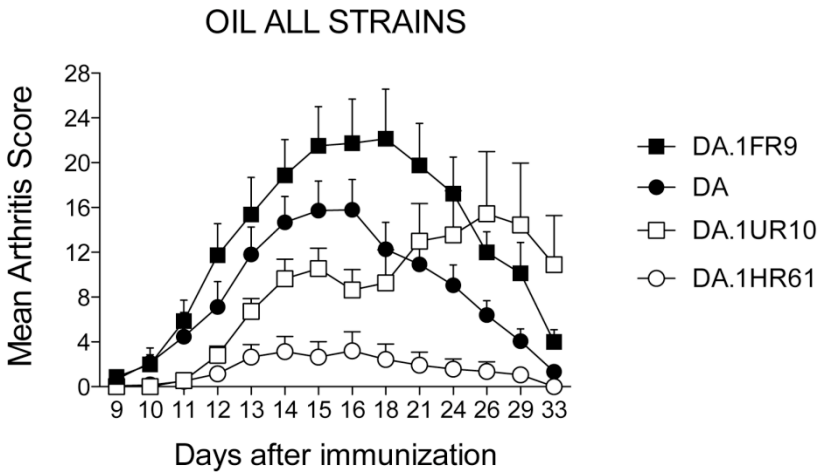

Figure 3: *Suscebtibility to OIA is regulated by MHC II. DA rats congenic for MHC II show variable susceptibility to adjuvant arthritis induced with IFA. Rats with a Brown Norway derived RT1h haplotype (DA.1HR61) are partially protected from arthritis while wild-type (DA) as well as MHC II congenic DA.1UR10 (E3) and DA.1FR9 (LEW.1F) are susceptible. Each group contained 10-12 male rats and the incidence in all groups is 100%.*

2. Chapter 1:

Enolase and its peptides as candidate autoantigens in PIA Animals

2.1 Introduction:

α-enolase belongs to family of cytoplasmic and glycolytic enzyme. It functions as receptor and activator of plasminogen as well as myc-binding protein. The upregulation of α-enolase was found during cell differentiation and proinflammatory conditions. The upregulation was also observed in the joints of RA patients [63]. The native form of α-enolase was described as autoantigen in many autoimmune diseases [64], including RA [65]. However, the autoimmune response against citrullinated α-enolase is specific for RA [66]. In our lab, the immune response against RNA binding protein hnRNP-A2 (RA33) as autoantigen was studied in PIA [58]. The α-enolase was also assumed as autoantigen and antibodies, cytokine response against α-enolase in PIA and OIA were investigated in this thesis. The MHC II dependency and the type of cells involved in the production of cytokines were also analyzed by T cell proliferation and FACS assays.

2.2 Materials and Method:

2.2.1 Animals:

The congenic rat strains DA, DA.1FR9, DA.1UR10 and DA.1HR61 (originating from Zentralinstitut Fur Versuchstierzucht, Hannover, Germany) were bred and maintained in the animal facility of Medical Inflammation Research in a climate-controlled environment with 12-h light/dark cycles and housed in polystyrene cages containing wood shavings and fed standard rodent chow and water ad libitum. The experiments were approved by a local ethical committee.

2.2.2 PIA induction, OIA induction and evaluation of arthritis:

PIA was induced by a single intradermal injection of 100 µl pristane (2,6,10,14-tetramethylpentadecane; Acros Organics, Liège, Belgium) and OIA was induced by injecting 300 µl of IFA (Difco) at the age of 8–12 weeks. Arthritis development was monitored in all four limbs using a macroscopic scoring system. Briefly, 1 point was given for each swollen or red toe; 1 point was given for each swollen mid foot, digit, or knuckle; and 5 points were given for a swollen ankle (maximum score per limb and rat was 15 and 60, respectively).

2.2.3 Antigen ELISA:

The antigen ELISA was carried out by coating 50µl of collagen II (10µg/ml) and human enolase (50µg/ml) on to Costar EIA 96 well plate. The detailed protocol for washing and blocking was provided in the appendix 6.1. The diluted serum of 50 µl per well was added and then washing was carried out. Detection was performed by using biotin conjugated IgG1 antibody (clone RG11/39.4, 2µg/ml, BD Pharmingen, CA, USA) as secondary antibody. Quantitative determination was carried using Eu^{3+}-conjugated streptavidin (Delfia; PerkinElmer) and Delfia® enhancement solution.

2.3.4 T cell stimulation assays & Cytokine detection:

At 10 days after pristane injection, rats were culled and inguinal lymph nodes and spleens were removed. Cells were washed and passed through 40µm strainers. 740,000 cells per well from five of DA, DA.1FR9, DA.1UR10 and DA.1HR61 pristane primed and naive DA rats were stimulated for 72 h at 37°C with 50µg/ml protein or 10 µg/ml peptide in 100 µl of DMEM supplemented with FCS (5%), HEPES (2.4 mg/ml), 2-ME (3.9 µg/ml), and penicillin-streptamycin (104 IU/ml penicillin, 10 mg/ml streptomycin; (Invitrogen Life Technologies). MHC II restriction was determined by adding 20µg/ml OX6 and OX17 (Combined). ConA, 3µg/ml, was used as assay control on all culture plates.

Quantitative measurements of IFN-γ and IL-17 in cell supernatants were performed using the rat IFN-γ(eBioscience) and rat IL-17 BD ELISA protocol (BD Pharmingen) assay) using Eu^{3+}-conjugated streptavidin (Delfia; PerkinElmer) and Delfia® enhancement solution for quantitative determination of absorbance at 450nm. Recombinant proteins were used as positive controls in ELISA.

2.2.5 Antibodies and flow cytometry.

Single-cell suspensions from naïve DA rats were prepared from spleen and cultured with enolase and denatured enolase for 42 hours and Brefeldin-A was added in last 4 hours and cells were stained with mAbs to NK 1.1 (10/78), CD3 (1F4), γδ TCR (V65)and CD4 (OX35) (all antibodies were purchased from Biolegend, La Jolla, CA, USA) and after fixing and permeabilization (BD, San Diego, CA) with mAbs to IFN-γ (DB-1) and IL-17A (polyclonal, R&D). Fluorescence minus one (FMO) were used as controls. Data were collected for 0.2 million live cells on an LSRII (Becton Dickinson) and analyzed with FlowJo software.

2.2.6 Statistics

Graph pad prism 5 was used for statistical analysis. The arthritis score and cytokine measurements were evaluated with the Mann-Whitney *U* test. In all experiments, $p<0.05$ was considered significant.

2.3. Result:

Serum reactivity towards Enolase:

After immunization, the blood serum was collected from PIA rats on day 6, day 10, day 25 and day 94 and OIA rat serum sample was collected on day 25. ELISA was performed to determine immunoglobulin G (IgG) antibody specificity against enolase and collagen II. To carry out this, the ELISA plate was coated with human enolase and rat collagen II and anti-IgG antibody was used to detect the IgG levels. The prominent increase of serum antibody response was observed from day 10 to day 25 (acute phase) than day 94 (chronic phase) as shown in figure 4.A. The MHC association was also assessed in MHC congenic strains but no correlation was found in between serum IgG levels and MHC haplotype (figure 4.D).

Blocking MHC II reduces IFN-γ response in alpha-enolase stimulated Lymph node cells from pristane-primed rats.

To study the MHC class II restriction, the inguinal draining lymph node cells from day 10 post immunized rats were cultured with Anti-MHC class II mAbs OX6 (anti-RT1.B), OX17 (anti-RT1.D) and different proteins and peptides. The different proteins and peptides include rat α-enolase, human α-enolase, rat collagen II, eight different human modified (citrullinated) and non-modified peptides-Eno1, Eno6, Eno12, Eno25, Eno15, Eno66, Eno324 and Pi27-ptm. After culturing for 72 hours, the IL-17 and IFN-γ response was tested with ELISA on cell culture supernatant. IL-17 and IFN-γ has shown elevated response to rat and human enolase (figures 5.A, 6.A) and no response was observed for modified and non-modified peptides (figure 5.D). Where as in MHC blocked cultures, MHC restricted response was observed for IFN-γ (figure 6.B) and no inhibitory effect was observed for IL-17 (figure 5.B).

IL-17 and IFN-γ response to enolase in immunized DR4 transgenic mice and in rats immunized with IFA (OIA) and pristane (PIA).

To test the association between enolase immunity and arthritic response, we assessed the levels of Th1 and Th17 derived cytokines IFN-γ and IL-17 in cultures with T cells from OIA, PIA and healthy controls rats. We further tested T cell immunity in DR4 tg C57/BL6 mice that had been immunized with citrullinated or non-citrullinated enolase. The naive rats showed a greater response to in-vitro challenge with cit and non-cit enolase than IFA and pristane primed rats. In mice, only immunized individuals responded to challenge (figure 7.A). Neither immunized nor naive rats responded to challenge with the highly immunogenic antigen ovalbumin, whereas mice immunized with enolase produced IL-17 but not IFN-γ to the same antigen (figure 7.B). Heat-denaturation of human enolase for 15 min at 56 degrees completely abolished the IFN-γ response in rats but did not affect the IL-17 response. In mice, heat denaturation had no effect on the T cell response (figure 7.C). As enolase is expressed as a recombinant protein and able to trigger response in both primed and non-primed T cells, we used a recombinant irrelevant control protein (DR4 αβ construct) to assess the effect of endotoxin on the rat T cells (this was not tested in mice). Whereas the control antigen could induce an IL-17 response, we could not detect any IFN-γ in these cultures (figure 7.D).

IFN-γ is secreted from NK cells after stimulation with Human α-Enolase:

Lymph node cells from naive DA rats were co-cultured with native enolase over time and the culture supernatant was tested for IFN-γ at indicated time-points. Compared to pan-T cell stimulation (ConA), the response to enolase was delayed and was detected first after 24 hrs (figure 8.A).

Heat denaturation of enolase completely abolished the IFN-γ response, suggesting that the enolase driven response is not primarily antigenic and possibly not T cell dependent. We tested this by culturing naive DA derived lymph node cells with native or heat denatured enolase and stained the cells with extracellular markers for T cells and NK cells and after permeabilization with anti-IFN-γ and anti-IL17. As we could show that heat-denaturation of enolase could prevent an IFN-γ mediated response, we used a series of anti-enolase mAbs (developed in our lab, unpublished) to test if any of these would block functional epitope(s). The anti-enolase antibodies were added together with the enolase to the cells and the supernatant was assessed for IL-17 and IFN-γ after 72hrs. None of the antibodies could block IFN secretion and one of the antibodies (Eno 8) rather increased the response (not significant.)(figure 8.B). Intracellular staining of IFN-γ in enolase co-cultured LN cells from naive DA rats showed that NK cells are a significant source of IFN-γ. No detection of IFN-γ or IL-17 was found in gdT cells or abT cells and no detection of IFN-γ was found in cultures with heat-denatured protein (figure 8.C).

2.4. Discussion:

The objective of study was to investigate the overall immune responses of alpha-enolase and its peptides in PIA. Rats immunized with adjuvants, pristane or IFA, develop reactivity to human and rat alpha enolase. Autoantibody response against citrullinated alpha-enolase has been reported in RA patients and the presence was correlated with the severity of the articular destruction [65]. However, in our experiment the serum response precedes the clinical onset and continues to increase during the acute and chronic stages of arthritis. Rats with chronic arthritis display raised level of serum antibodies to enolase although the levels are not increased compared to acute disease. The reason for variation of serum response with disease course may due to change in antigen availability by post-translation modifications. A clear association to any of the tested MHC II haplotype was not found. Although the levels of anti-enolase antibodies in the serum did not correlate with arthritis severity, a difference between the milder OIA and the severe PIA was observed.

Lymph node cells from pristane immunized DA rats congenic for MHC II showed increased cytokine response when cultured with human and rat enolase in vitro. The cellular response to enolase stimulation showed no clear MHC II association but could be suppressed with MHC II specific antibodies. This was particularly clear for the IFN-γ response. The effect of anti-MHC II antibodies on APCs and reason for negative regulation of IFN-γ is matter of investigation.

In vitro cellular response to enolase was not dependent on prior immunization of rats and both the IL-17 and the IFN-γ responses were increased in non-immunized healthy rats. Neither immunized nor naive rats reacted to any of the tested enolase peptides, excluding the as immunodominant epitopes, or to Ova. However, an IL-17 response was raised to the recombinant control protein (DR4 αβ construct) which was comparable to the IL-17 response generated by enolase, suggesting that recombinant human and rat enolase share a non-enolase component that can trigger IL-17 response in naive and pristane immunized rats. Importantly, stimulation with the recombinant control protein did not result in an IFN-γ response indicating that the IFN-γ response is indeed enolase specific. In addition, stimulation with denatured enolase raised an IL-17 but not IFN-γ response. The denaturation of the enolase protein decreased the IFN-γ response in enolase immunized mice suggesting that antigen capture and processing might be reduced after denaturation. Finally, intracellular staining of enolase stimulated naive splenocytes showed increased IFN-γ response by NK cells only.

In conclusion, enolase reactivity occurs in PIA and OIA. Also non-immunized rats showed increased response to enolase and the response can be suppressed by blocking MHC II presentation. NK cells appear to be a source of IFN-γ in enolase stimulated naive immune cells. Citrullinated form of alpha enolase has gained attention as potential biomarker in diagnosis of RA [67], but its pathogenicity is not yet elucidated.

3. Chapter 2:

Where collagen and citrullination meet: a leakage in T cell tolerance

3.1Introduction:

In autoimmunity, the T cell tolerization has a significant role. The thymus is the place where T cell development and maturation takes place. In thymus, the lymphocytes undergo both positive and negative selection. In positive selection, the maturing T cell binds to MHC on thymic epithelial cells and it is saved from programmed cell death. If the T cells fail to recognize MHC then they will undergo cell death. Where as in negative selection, T cells that bind too strongly to self antigens are removed. If the T cells are not removed then they react on the self tissues and cause autoimmune diseases. In RA, the T cell and subsequently B-cell response are found against collagen type II, which is a self protein present in hyaline cartilage of joints.

The presence of anti-citrullinated type II collagen antibodies and citrullinated type II collagen fragments in the articular synovium of RA patients was established by Yoshida *et al*. The site of citrullination and biogenesis of anti-citrullinated type II collagen antibodies still remains a matter of investigation. Accordingly we hypothesize, 1) the immune response against the type II collagen at joints may be due to the formation of neoepitope in the thymus by the process of citrullination and then subsequent tolerization of T cells to this neoepitope or 2) tolerization of T cells to native CII in the thymus and presentation of the citrullinated CII epitope from articular cartilage to the T cells, with subsequent production of autoantibody response against CII by B cells.

Hence, the gene expression pattern of collagen type II and PAD enzymes, which are responsible for citrullination, was studied in naïve mice by quantitative real time PCR (qRT-PCR).

3.2 Materials and Method:

3.2.1Collection of Tissue Samples:

Thymus from 2 days, 4 weeks and 15 months old B10.Q female mice of three in number were collected and placed in RNAlater (Ambion, Inc., Austin, Texas) for mCII gene expression analysis. Whereas for PADI analysis, tissues of new born paw, spinal cord, bone marrow, spleen, thymus, lung, stomach and small intestine were taken from 4 weeks old mice.

3.2.2 RNA isolation and cDNA Synthesis:

The total RNA from above mentioned tissue samples was isolated by using RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions (Appendix 9.1). RNA concentration was analyzed using Nano Drop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, US). cDNA synthesis was carried out using High capacity Reverse Transcription kit (Applied Biosystems Inc, Foster City, CA). The reaction mixture contains 10µl of total RNA, 2 µl oligo(dT) plus random primer, 0.8 µl of dNTP, 0.8 µl of Reverse Transcriptase and 4.4 µl of HPLC water. The conditions used for RT-PCR was 25°C for 10min, 37°C for 120min, 85°C for 5min. cDNA samples were diluted to 1000ng/µl for quantitative amplification.

3.2.3 Real-time PCR:

Relative quantification of gene expression by real-time PCR was performed using ABI 7900HT Fast Real-Time PCR System (Applied Biosystems Inc, Foster City, CA). Each PCR reaction of 20 µl per well was carried out. The 20 µl of master mix contains 1 µl cDNA, 1 µl of primer pair, 1 µl of probe, 7 µl of HPLC water and 10 µl of Taqman Buffer. Samples were loaded in triplicates on ABI 96 well PCR plates and spun down at 4000 rpm for 1 min. The following PCR conditions were used: 95°C for 10 min follow by 40 cycles of 95°C for 15 sec, 56°C for 45 sec, 72°C for 30 sec.

The average C_t value for each sample was normalized against GAPDH and gene expression quantification by comparative C_t value was calculated using $2^{-\Delta\Delta C_t}$.

3.2.4 PCR Amplification:

The PADI (*PADI2*, *PADI4*) genes were PCR amplified from cDNA using a forward primer 5'- CCA AAG GCC CTG ACC GCC TG-3', reverse primer as 5'-AGC AAC TCC GCA GAA CCG CC for *PADI2* and forward primer 5'-CAG CGA GAG CCG GGA CAT GC-3', reverse primer 5'-CCG CGC TGG GAC AAA GCT CA -3' for *PADI4*. The PCR master mix of 20µl is prepared with 10 µl of SYBR Green Supermix (Bio-Rad Laboratories inc., Hercules, CA), 1 µl of Primer, 0.1 µl of Taq Polymerase and 7.9 µl of HPLC water. The PCR conditions used were 95°C for 4.00 min for 1cycle, 95°C for 20 sec, 60°C for 30 sec, 72°C for 30 sec and finally 72°C for 7.0 min for 40 cycles.

3.2.5. Sequencing:

The obtained PAD bands were verified by performing Microcapillary sequencing on ABI 3730 sequencer using a Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The BigDye sequencing reaction mix was run on PCR for chain

termination reaction. The program for sequence reaction of 96°C for 10 sec, 50°C for 5 sec, 60°C for 4min of 25 cycles. The PCR reaction mix contains 0.25 µl of BigDye, 2 µl BigDye Sequencing Buffer, 0 .25 µl of forward primer and 1 µl DNA solution and 6.5 µl of H_2O to a final reaction volume of 10 µl. The sequence reaction was subjected to post cleaning step (Appendix 6.4). After cleaning, the mix was subjected to microcapillary sequencing in ABI3730 sequencer (Applied Biosystems, Foster City, CA).

3.3 Results:

CII expression in thymus:

Mouse CII expression in the thymus was verified by performing qRT-PCR in naïve B10.Q mice of age groups 2 days, 4 weeks and 15 months. β-actin, GAPDH, HPRT and 18s RNA were used as internal control genes for normalizing C_t values(Appendix 6.3). GAPDH was used for calculating fold of gene expression, because of its consistency of C_t values across different age groups. Whereas for the verification of PCR product, the gel run was carried out with mCII and β-actin. The expression of CII in the thymus was found to be age dependent and it gets downregulated as the thymus becomes older (as shown in figure 9).

Tissue expression of *PADI2* and *PADI4*

Expression of *PADI2* and *PADI4* was verified in the mouse tissues like spinal cord, bone marrow, spleen, thymus, lung, stomach and small intestine from 4 week B10.Q mice and also in cDNA of new born paw of B10.Q mouse. The expression of *PADI2* was found in all the above mentioned tissues except bone marrow. *PADI4* expression was also verified in spleen, thymus and spinal cord and the bands were obtained in the gel (as shown in figure 12). The amplified bands obtained were excised from gel excluding the bands formed due to self primer dimers and cross primer dimers. Microcapillary sequencing was done for both *PADI2* and *PADI4*. The band was confirmed for *PADI2*, but for *PADI4* sequencing result showed anomaly due to technical error.

3.4 Discussion:

The citrullinated autoantigens have gained recent attention as potential targets of immune response in RA [67]. Understanding the mechanism involved in generation of autoantigens and patterns of citrullination may have significant application in development of novel therapies. The expression of *PADI2* and citrullinated proteins has been correlated with the development of clinical symptoms in RA patients [68,69]. However; sites and circumstances for citrullination of autoantigens *in vivo* in RA are still matter of investigation [70]. In our study, we found the expression of type II collagen in thymus and *PADI2* expression in both thymus and bone marrow of the mouse. *Per se*,

these data suggests the two possible ways of tolerance breakage and generation of ACPA.

According to one hypothesis, the formation of ACPA is due to tolerization of T cells towards citrullinated CII formed in the thymus by PAD and then presentation of native CII epitopes at the joint cartilage level in the periphery, causing autoimmune responses towards the native CII in joints. The other hypothesis for ACPA generation involves citrullination of CII in the joints, originating neoepitopes as the T cells were tolerized towards native CII in the thymus, causing immune response in joints. The peptides bearing post translational modifications, e.g. citrullination, may be presented by MHC molecules eliciting highly specific T cell responses, leading to the immunological tolerance breach. These neoepitopes may not be expressed in the thymus during lymphocyte selection. The tolerance breakage due to post-translational modification of a potential T cell determinant was reported in celiac disease [71].

However, the citrullinated type II collagen is found to be present in the joints of RA patients and anti-citrullinated type II collagen antibodies are observed in circulation and as well as in synovia of RA patients[72]. Our assumptions are based on the fact that ACPA response occurs years before disease onset [73] and the reason may be due to the tolerance breakage. The accelerated citrullination of proteins and production of ACPA in RA patients may be triggered by smoking or infection [73]. Infection of *P. gingivalis* causes periodontitis and it is assumed that the epitope spreading in small proportion of periodontitis patients might lead to RA. In epitope spreading, increase or shift in antigen recognition occurs during the course of an immune response[74] i.e. the specific citrullinated peptides generated outside the bone joint may spread to other host citrullinated proteins in the affected joint, leading to immune responses with ACPA and RF [75].

As mentioned before, inhibiting citrullination would ameliorate RA disease. The treatments like glucocorticoids[76] and PAD inhibitors[77] are still in developmental stages. To get the full advantage from these treatment approaches the complete knowledge of ACPA biogenesis should be known.

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6.Appendix:

6.1ELISA:

The indirect ELISA technique was used for studying the presence and type of antibodies present in the serum of OIA and PIA. The blood from rats was collected in eppendorf tube and the centrifugation was carried at 500g for 10 mins to obtain serum. The ELISA technique is as followed.

Coating:

1. The first step was coating the ELISA plates with antigens and for this the coating was performed with CollagenII (Stock-4.8 mg/ml) of 50µg/ml and Human Enolase (3.5 mg/ml) of 50µg/ml. The coating was carried on Costar EIA 96 well plates by preparing 5ml of above solutions.52 µl of collagen and 71µl of Human enolase were added into 5ml PBS buffer separately. As PBS is isotonic diluant.

2. The Costar plates were labeled and then solution was decanted into petri plate and multi channel pipettor was used to pipette the antigen solution into plates. The plates were shake gently to ensure uniform level of solution on plates.

3. The plates were sealed with film and then they were kept for incubation at room temperature for 2hours for adsorption of Antigen with the polystyrene plate.

4. The washing of plates for one time was carried out and then plates were strike on tissue paper to remove the excess PBS.

Blocking:

1. The blocking was carried out by using 5% BSA in 0.01% of Tween in PBS. The preparation of this blocking solution was done with 100ml of 0.01 Tween PBS was mixed with 5g of BSA (Sigma Aldrich) and then mixed vigorously. BSA is non-reacting protein and it gets coated on the areas other than the antigens.

2. The solution was taken in petriplate and 200µl of BSA was pipetted into ELISA plate wells.

3. The plates were sealed with film and then kept for 1hour incubation in Room temperature.

4. Then, plates were washed with 1x 0.01 Tween PBS for 4times.

Serum samples:

1. The labeled serum samples were taken and then the dilutions were performed as indicated i.e; in first well 276 µl of Tween 1% BSA, PBS and 24µl of serum were taken and from these wells 150 µl of serum was added into the consecutive wells which were

containing 150µl of Tween 1% BSA PBS. The samples used were A2,J2,J0,E4,C4,H2,A3,G0,D2,G1,D4 and H4.Along with these,U1,U2,U3,U5,U6 duplicates of 1:50 dilutions were added.

2.50µl of samples were taken and pipetted into ELISA plate wells.

3. The plates were kept for incubation of 1hour at room temperature.

4. The antibodies present in the serum binds to the antigen coated into wells. And unbounded antibodies were removed by washing with PBS for 4 times and then striking the plates onto the paper plates to remove PBS.

Secondary antibody:

1. The 0.01% of Tween in 1% BSA in PBS was taken in 10 ml tube and the Biotinylated Anti rat IgG antibodies from Rat ELISA BOX present in fridge was taken.

2. The ratio of Dilutions, 1:5000, Hence 2µl of Antibody was added into 10 ml tube.

3. The tube was inverted for proper mixing and then the solution was poured into petriplates.

4. The washed plates were loaded with the antibody solution of 50ul /well immediately after washing.

5. The plates were kept for incubation for a period of 1 hour. Washing was performed for 4times.

Detection:

1. The assay buffer of 10ml was taken and to it 10 µl of Eu labeled streptavidin was added. The Assay Buffer containing Streptavidin binds to the biotin on the Biotinylated antibodies. The streptavidin have Europium which is lanthanide. This lanthanide fluorescence and it was measured in Synergy 2 (Gen5) plate reader.

2. 50 µl/well of Assay buffer was added into the wells and kept for 30 mins incubation at room temperature.

Development:

1. The Eu^{3+}-Enhancement solution enhances the fluorescence. Hence 50µl/well of enhancement solution was added and kept for 5mins.

2. The Gen5 reading project was opened with europium protocol and the plates were kept in the order for reading.

6.2RNA Isolation:

1. The tissue from RNAlater was taken and made into small pieces. These pieces were taken in the ceramic bead tubes and added with 350 µl of Total RNA lysis solution.
2. The tube with beads was subjected to shredding for 4mins and it was spun at 3000 rpm for 5mins
3. Now the lysate without tissue was taken in new eppendorf tube and 350 µl of 70 % Ethanol was added to each sample and mixed gently.
4. The solution was transferred into the column (Qiagen RNeasy mini Kit): approx 700 µl at one go.
5. Centrifuged at 9000 rcf for 30 s at room temperature. Flow-through was discarded.
6. 500 µl of **RW1 Buffer** was added to the column and spun at 9000 rcf for 30 s at room temperature, Flow-through was discarded.
7. 500 µl of **RPE Buffer** was added to the column and spun at 9000 rcf for 30 s. Flow-through was discarded. This step was carried out twice.
8. The column was spun at 9000 rcf for 1min without adding anything to drain out excess **RPE Buffer**.
9. Column was transferred to 1.5 ml safelock eppendorf tube and 50 µl **HPLC water** was added to column and centrifuged at 9000rcf for 1 min.
10. Eppendorf tube was transferred to freezer at -20 °C.

6.3 PCR Primers

For normalizing delta C_t value, many housekeeping genes were verified and the primer sequences for them are as follows.

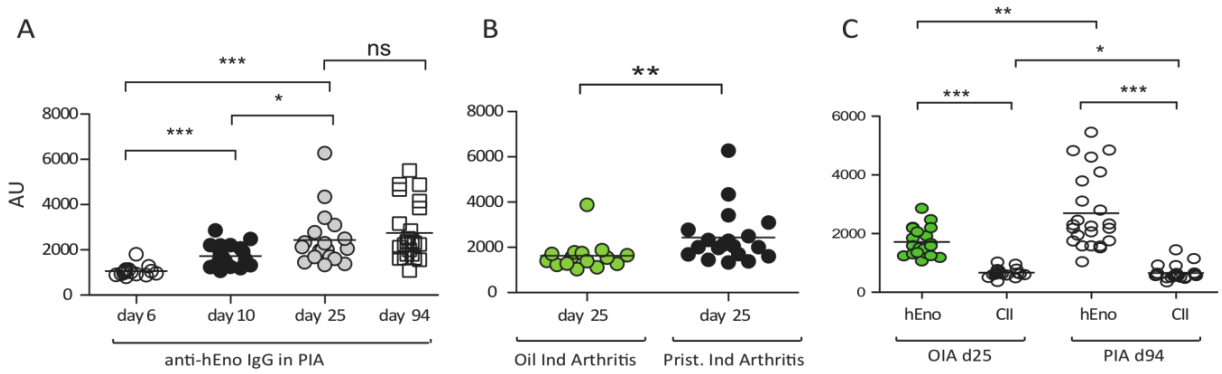
Primer	**Nucleotide sequences**
β-Actin	5'- GCCCCTGAGGAGCACCCTGT-3'(forward) 5'- TGTGGGTGACCCCGTCTCCG-3'(reverse)

HPRT	5'-AAACTTTGCTTTCCCTGGTTA-'(forward) 5'-AGGCTTTGTATTTGGCTTTTC-3'(reverse)
18s RNA	5'-GTAACCCGTTGAACCCCATT -3'(forward) 5'- CCATCCAATCGGTAGTAGCG-3'(reverse)
GAPDH	5'-AGGCCGGTGCTGAGTATGTC -3'(forward) 5'- TGCCTGCTTCACCACCTTCT-3'(reverse)

6.4 Post cleaning Step:

1. The sephadex powder is applied to hydrophilic filter plate and 300 µl of H_2O was added and kept for incubation at 8 °C.
2. The water from the plate was drained out by spinning it at 900g for 5mins.
3. Again the 100 µl of water was added to plate and centrifugation was performed as above.
4. The Bigdye PCR mix was added to hydrophilic plate and centifugation was carried out at 900g for 5mins by putting a new PCR plate underneath the hydrophilic plate.
5. This plate is fixed with septum and white holder for robotic arm.

6.5 Figures: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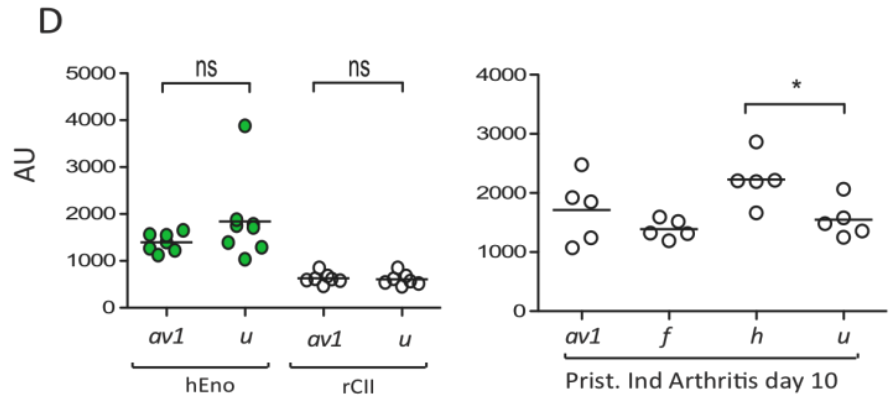
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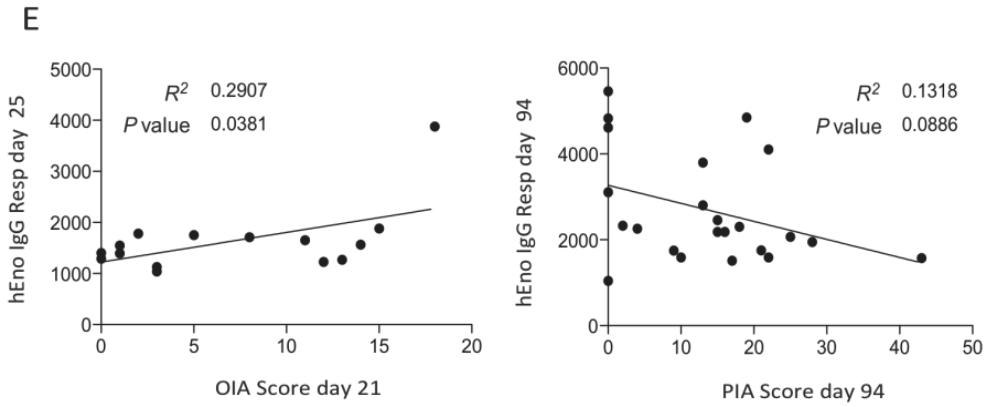
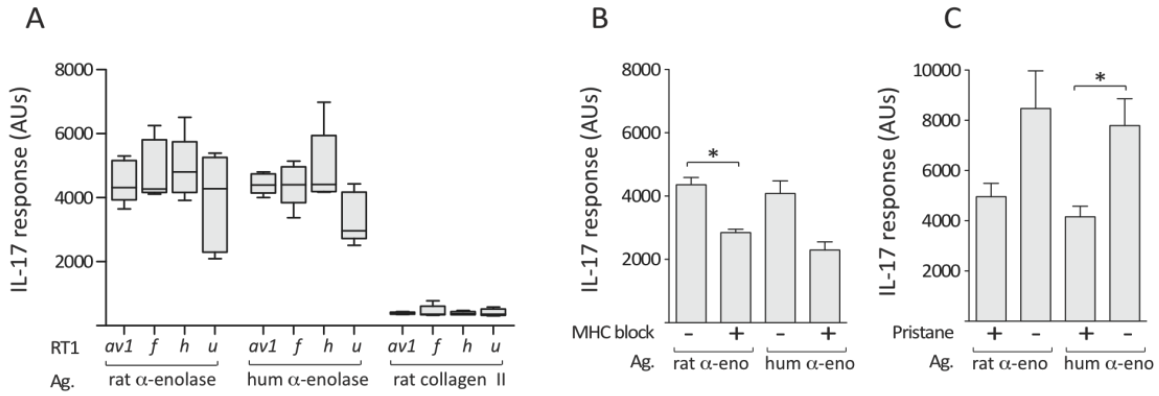


Figure 4: Serum reactivity to alpha-enolase in PIA and OIA A*) IgG response to human enolase at indicted time-points after immunization with pristine (pooled data from several experiments with MHC II congenic DA rats).* ***B****) Comparison IgG response to enolase day 25 after IFA and Pristane injection.* ***C****) Comparison between anti-enolase and anti-CII response in early OIA and chronic PIA.* ***D)*** *MHC II association of anti-enolase response in OIA days 25(left) and PIA day 10(right).* ***E****) Correlation enolase IgG and arthritis in OIA day 25 and PIA day 94.*



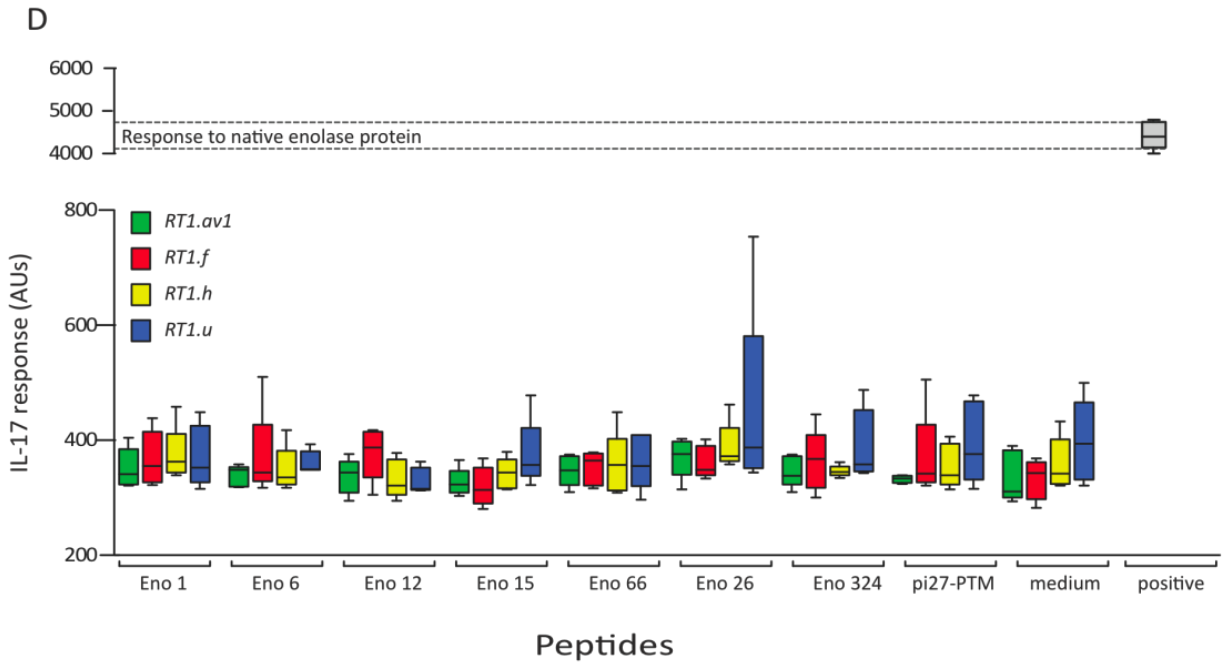


Figure 5: MHC II independent IL-17 response to alpha-enolase in rat.

A*, MHC II congenic DA rats were primed with pristane and analyzed for immune response to rat and human alpha-enolase and to rat collagen type II, before onset of clinical disease(d10).* ***B,*** *Addition of rat specific mAbs to RT1.B (OX6) and RT1.D (OX17) showed no clear reduction in response to neither rat nor human enolase.* ***C,*** *IL-17 response was significantly reduced in rats injected with pristane.* ***D,*** *Immune response was not raised against enolase derived peptides as tested by stimulating pristane primed T cells from MHC II congenic strains with 8 different human modified or non-modified enolase peptides*

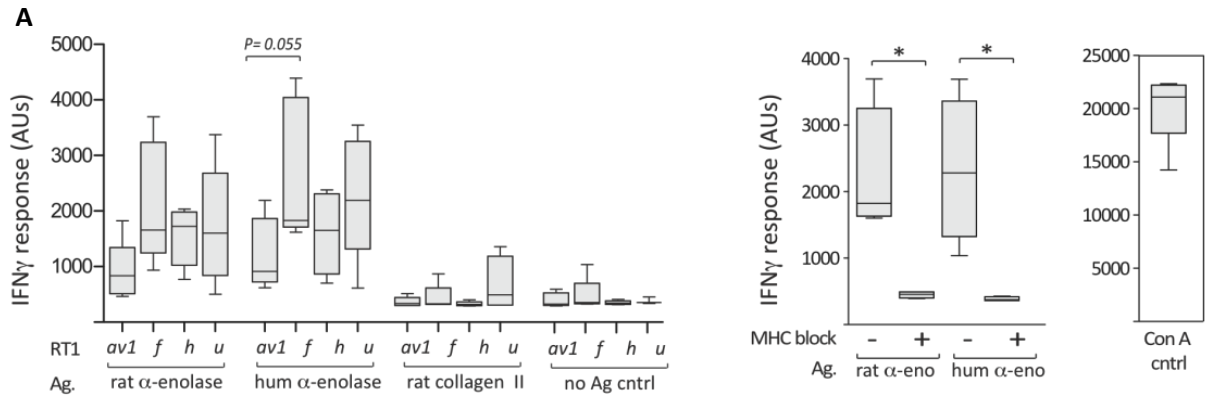


Figure 6.Blocking MHC II reduces IFN-γ response in alpha-enolase stimulated LN cells from pristane-primed rats.

A, *MHC II association to enolase immunity in PIA, n=5.* ***B,*** *Addition of anti-RT1.B (OX 6) and RT1.D (OX17) blocks IFN-γ response to enolase re-stimulation in vitro.*

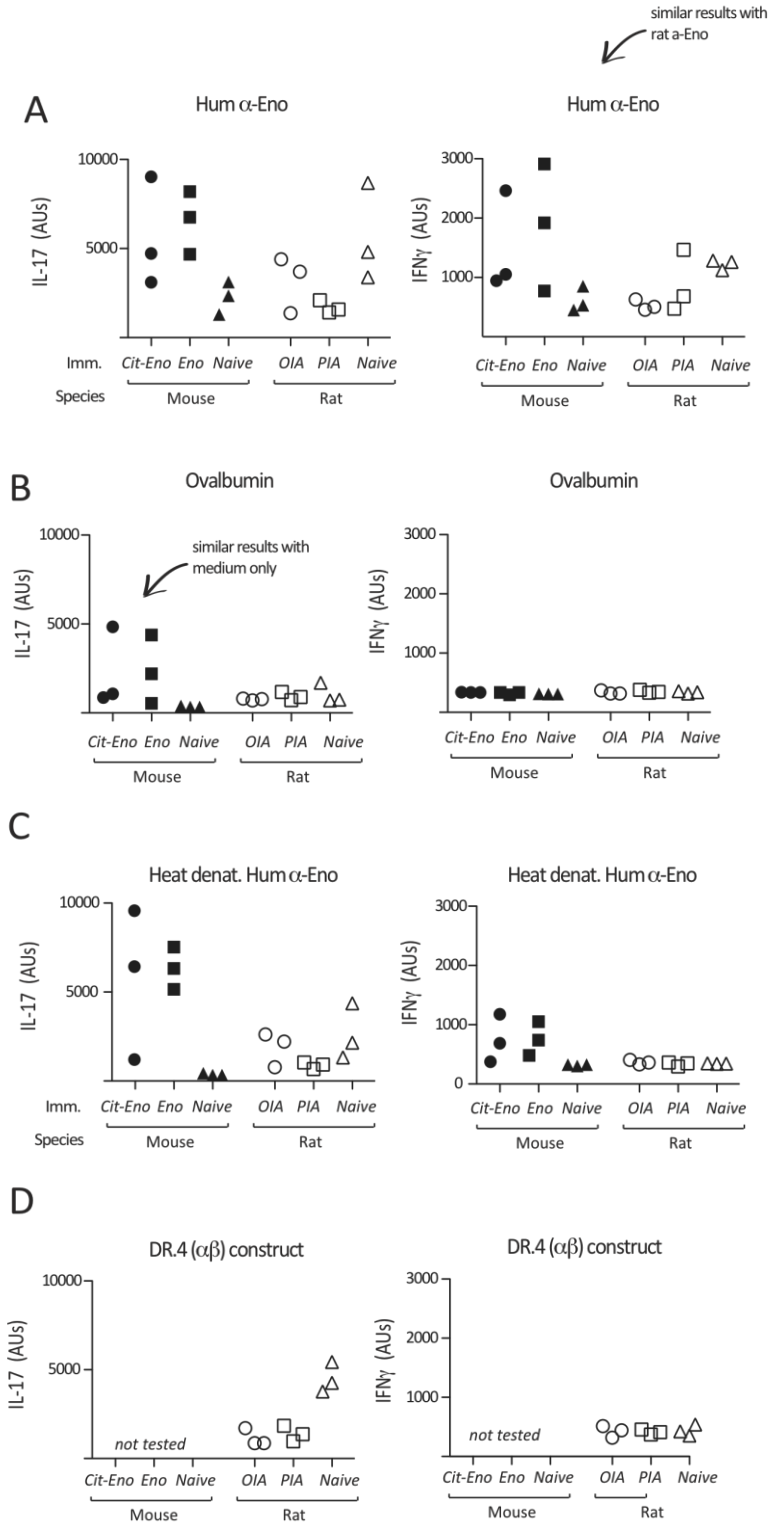


Figure 7:TH1 (IFN-γ) driven response to alpha-enolase in the rat is conformation dependent and down-regulated after immunization. *To test whether enolase immunity in the rat is associated with an arthritic response, we assessed the levels of TH1 and TH17 derived cytokines IFN-γ and IL-17 in cultures with T cells from OIA, PIA and healthy controls rats. We further tested T cell immunity in DR4 tg C57/BL6 mice that had been immunized with citrullinated or non-citrullinated enolase. (A) Naive rats showed a greater response to in-vitro challenge with cit and*

non-cit enolase than IFA and pristane primed rats. In mice, only immunized individuals responded to challenge. (B) Neither immunized nor naive rats responded to challenge with the highly immunogenic antigen ovalbumin, whereas mice immunized with enolase produced IL-17 but not IFN-γ to the same antigen. (C) Heat-denaturation of human enolase for 15 min at 56 degrees completely abolished the IFN-γ response in rats but did not affect the IL-17 response. In mice, heat denaturation had no effect on the T cell response. (D) As enolase is expressed as a recombinant protein and able to trigger response in both primed and non-primed T cells, we used a recombinant irrelevant control protein (DR4 AB construct) to assess the effect of endotoxin on the rat T cells (this was not tested in mice). Whereas the control antigen could induce an IL-17 response, we could not detect any IFN-γ in these cultures.

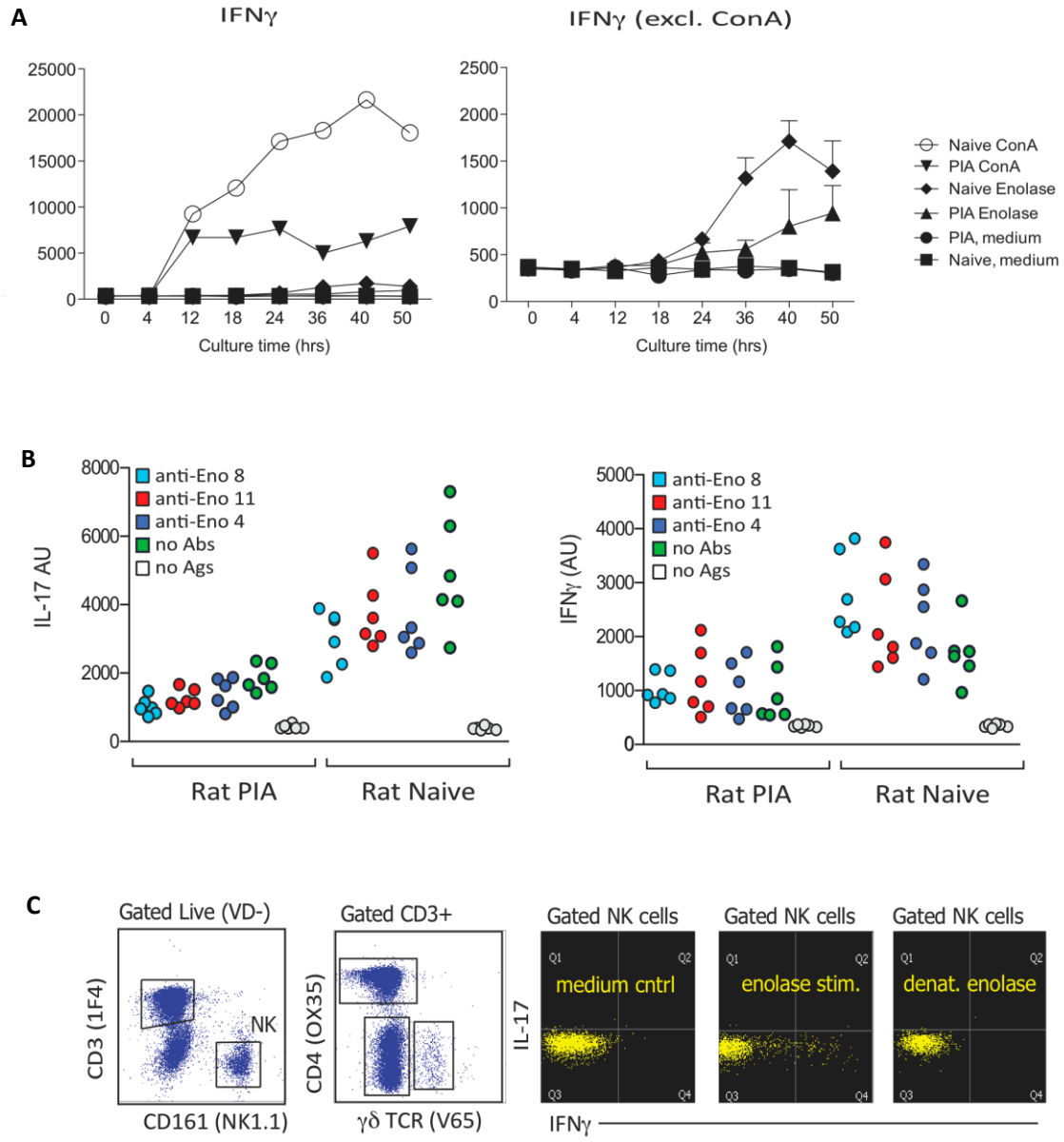


Figure 8: NK cells secrete IFN after stimulation with human alpha enolase.

NK cells, not T cells, exposed to native enolase produce IFN-γ. Heat denaturation of enolase completely abolished the IFN-γ response, suggesting that the enolase driven response is not primarily antigenic and possibly not T cell dependent. We tested this by culturing naive DA derived LN cells with native or heat denatured enolase and stained the cells with extracellular markers for T cells and NK cells and after permeabilization with anti-IFN-γ and anti-IL17. ***A)*** *LN cells from naive DA rats were co-cultured with native enolase over time and the culture SN was tested for IFN-γ at indicated time-points. Compared to pan-T cell stimulation (ConA), the response to enolase was delayed and was detected first after 24 hrs.* ***B)*** *As we could show that heat-denaturation of enolase could prevent an IFN-γ mediated response, we used a series of anti-enolase MAbs (developed in our lab, unpublished) to test if any of these would block functional epitope(s). The anti-enolase antibodies were added together with the enolase to the cells and the SN was assessed for IL-17 and IFN-γ after 72hrs. None of the antibodies could block IFN secretion and one of the antibodies (Eno 8) rather increased the response (not sign.).* ***C)*** *Intracellular staining of IFN-γ in enolase co-cultured LN cells from naive DA rats showed that NK cells are a significant source of IFN-γ. No detection of IFN-γ or IL-17 was found in gdT cells or abT cells and no detection of IFN-γ was found in cultures with heat-denatured protein.*

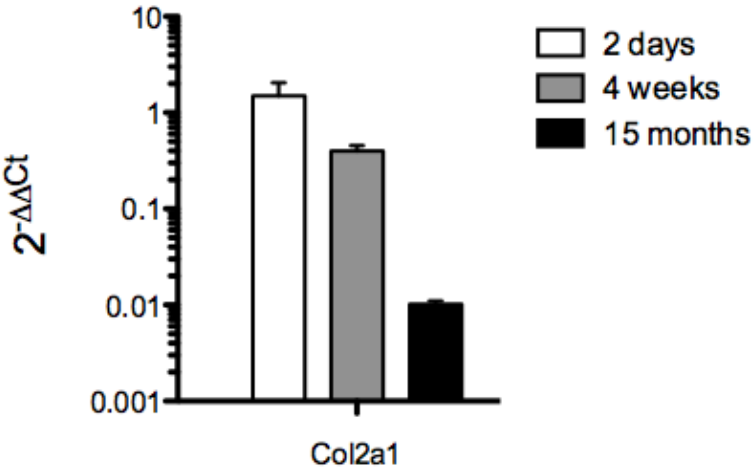


Figure 9: *Age wise gene expression of type II collagen (Col2A1)*

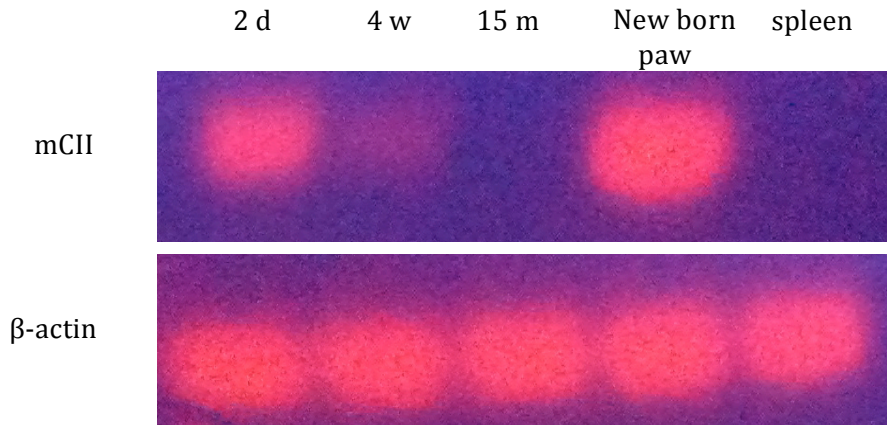


Figure 10*: Gene expression of CII and corresponding β-actin expression.*

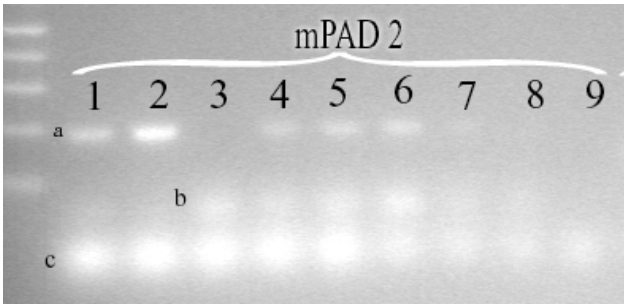


Figure 11: PAD 2 expression of Mice *1. New Born Paw 2.Spinal Cord 3.Bone marrow 4.Spleen 5.Thymus 6.Lung 7.Stomach 8.Small Intestine 9.NTC. a) PADI2 bands were observed according to their size. b) Cross primer dimer at ~50bp c) Self primer (hair pin loop) at ~25bp.*

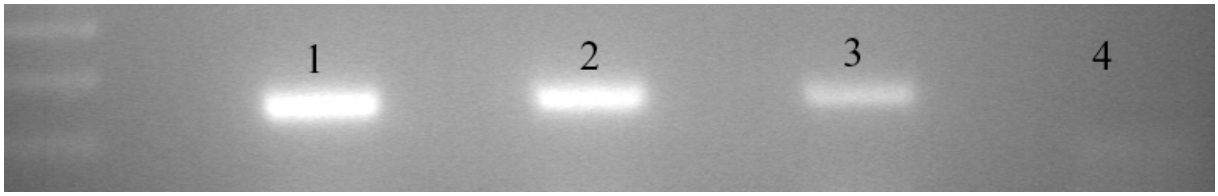


Figure 12: PADI4 expression of Mice *1. Spleen 2.Thymus 3.Spinal cord 4.NTC*