Studies on Understanding the Pathogenic Mechanisms in Arthritis

Experiments Using Joining chain deficient (J-/-) and Ncf1 gene mutated B6N mice

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# Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>H2</td>
<td>Histocompatibility class 2</td>
</tr>
<tr>
<td>J chain</td>
<td>Joining chain</td>
</tr>
<tr>
<td>J-/ or JDTA-</td>
<td>Joining chain deficient mice</td>
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<tr>
<td>Pigs</td>
<td>Polymeric Immunoglobulins</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>PIgR</td>
<td>Polymeric Immunoglobulin Receptor</td>
</tr>
<tr>
<td>SIgA and SIgM</td>
<td>Secretory IgA and IgM</td>
</tr>
<tr>
<td>NALT</td>
<td>Nasal Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Nodes</td>
</tr>
<tr>
<td>TR mice</td>
<td>Mice vaccinated intranasally with CII</td>
</tr>
<tr>
<td>NTR mice</td>
<td>No intranasal vaccination</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen Induced Arthritis</td>
</tr>
<tr>
<td>CAIA</td>
<td>Collagen Antibody Induced Arthritis</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund’s Adjuvant</td>
</tr>
<tr>
<td>CII</td>
<td>Collagen type II</td>
</tr>
<tr>
<td>Anti-CII Abs</td>
<td>Antibodies against collagen type II</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>Bio-CII</td>
<td>Biotinylated collagen type II</td>
</tr>
<tr>
<td>N CII</td>
<td>Native or normal collagen II</td>
</tr>
<tr>
<td>DN CII</td>
<td>Heat denatured collagen II</td>
</tr>
<tr>
<td>CB20</td>
<td>A monoclonal antibody, which recognizes native form of CII</td>
</tr>
<tr>
<td>GB8</td>
<td>A monoclonal antibody specific for denatured collagen</td>
</tr>
<tr>
<td>C57BL/6N</td>
<td>Mice expressing H2b haplotype</td>
</tr>
<tr>
<td>B6N.Ncf1</td>
<td>B6N mice having Ncf1 mutation</td>
</tr>
<tr>
<td>Ncf1</td>
<td>Neutrophil Cytosolic Factor 1</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>NOX2</td>
<td>NADPH Oxidase complex 2</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LM</td>
<td>LipoMannan</td>
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<tr>
<td>i.d</td>
<td>Intradermal injection</td>
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<tr>
<td>i.p</td>
<td>Intraperitoneal injection</td>
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<tr>
<td>i.v</td>
<td>Intravenous injection</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>SA-HRP</td>
<td>Streptavidin Horse Radish Peroxidase</td>
</tr>
<tr>
<td>MMC</td>
<td>Mutated Mouse Collagen</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
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1. Abstract

Joining chain is involved in the polymerization of dimeric IgA and pentameric IgM antibodies, and also for the release of these secretory antibodies at the mucosal surfaces. In this project, joining chain deficient (J-/−) mice were used to develop vaccination strategies for arthritis. Due to the lack of J chain these mice have low levels of polymeric antibodies, which are ineffective for clearance of the antigens. This results in more retention time of the antigen exposure to the immune system. In preventive vaccination experiments, mice were initially exposed to the antigen by intranasal vaccination seven days prior to primary immunization. Vaccinated mice developed less severe arthritis than the non-vaccinated mice. However, in the therapeutic experiments, mice were first immunized and were vaccinated intranasally starting from the day of disease onset. This treatment regimen aggravated the disease in the vaccinated, but not in the non-vaccinated mice. Thus, preventive rather than the therapeutic vaccination was found to be effective against the development of arthritis in J chain deficient mice. We also found that the J-/− mice had decreased IgM and total Ig compared to the control mice. In a parallel project, effect of Ncf1 gene mutation in B6N genetic background was studied. When the mice were immunized with chicken CII in normal CFA containing M. butyricum, control mice developed mild arthritis, but not the B6N.Ncf1 mice. However, the disease severity and incidence was significantly higher in B6N than B6N.Ncf1 mice when they were immunized with chicken CII in CFA containing M. tuberculosi s H37Ra. In another experiment, collagen antibody induced arthritis (CAIA) was induced in B6N.Ncf1 and control mice by intravenous transfer of a cocktail containing four monoclonal antibodies. Again the control mice but not the B6N.Ncf1 mice developed CAIA, implying Ncf1 mutation in contrast to B10 genetic background may not reduce the threshold level for arthritis induction in mice with B6N background. Finally we studied the effect of heterozygosity of the MHC complex (H2q along with H2b) on arthritis in B6N genetic background by inducing CIA. The heterozygous mice (H2q) developed higher disease severity and frequency than the homozygous mice suggesting MHC haplotype interactions as one of the major factors in contributing to arthritis disease development.
2. Introduction

Rheumatoid arthritis (RA) is an inflammatory mediated, systemic autoimmune disease. The etiology of the disease is unknown due to its complex and heterogeneous nature. However, research findings suggest that environment and genes could play an important role in triggering inflammatory pathways. Smoking and MHC class II genes are considered to be one of the major environment and genetic risk factors respectively. Smoking in association with MHC class II HLA-DRB1 can trigger immune reactions in the formation of anti-citrullinated antibodies, which after several years result in RA development. Smoking is also an environmental risk factor in other rheumatic diseases like Systemic Lupus Erythematosus (SLE), where the immune system is triggered to produce antibodies against double stranded DNA (dsDNA). Depending on the genetic context, both the innate and adaptive immune system plays an active role for the initiation and maintenance of chronic RA.

Joining chain (J chain) is a protein of molecular weight 15kD, which is responsible for the polymerization of immunoglobulins. IgM and IgA exist in pentameric and dimeric forms respectively. Joining chain structure is highly conserved in vertebrates, its synthesis is mainly important for assembly and transcytosis of IgM and IgA. Plasma cells at the mucosal site secrete polymeric antibodies containing J chain. Joining chain provides the binding site for the attachment of the polymeric immunoglobulin receptor (pIgR), expressed on the basolateral surface of the glandular epithelial cells. This interaction between J chain and pIgR aids in the uptake of polymeric antibodies and transport them to the luminal surface, where they are released into the glandular lumen. During the transcytosis, some part of the pIgR is subjected to proteolytic cleavage and gets associated with the pIgs, known as secretory component (SC). The dimeric IgA and pentameric IgM bearing the secretory component are called as secretory antibodies. These sIgA and sIgM are the key immune players at the mucosal surfaces, which help in opsonisation of the pathogens. Also the secretory component protects the antibodies from proteolytic degradation. The requirement of the J chain in transport of pIgs was investigated by using antibodies developed against the human J chain. Anti-J chain antibodies inhibited the binding of the pIgs to the pIgR resulting in no transcellular transport of the secretory antibodies.

To study the role of J chain in arthritis, J chain deficient mice (J/-) were used. This mouse was generated by inserting the diphtheria toxin A gene into the J chain locus. J/- mouse had reduced serum IgM levels, but increased IgA levels compared to wild type mice. Collagen induced arthritis and vaccination experiments were performed on J/- mice to study and compare the disease severity and effect of intranasal vaccination.

In a parallel project, collagen induced arthritis (CIA) a model for human RA, was induced in arthritis-resistant C57BL/6 or B6N mice. In mice, susceptibility to CIA varies depending on the MHC haplotype and the genetic background. Generally mice having MHC haplotypes (H2k or H2d) are highly susceptible for CIA, when compared to the mice with H2b haplotype. B6N mice have H2b haplotype, which makes them resistant for CIA. According to published articles, resistance for CIA in B6N mice is mainly due to the defect in the T cell and anti-CII antibody response after the booster immunization. Induction of disease in B6N mice also depends on the maturation of the dendritic cells (DCs), which can be achieved by the high doses of Mycobacterium tuberculosis. However some studies report that CIA can be induced in B6N mice with chicken type II collagen that is dependent...
on the immunization conditions, adaptive immune response and the role of non-MHC genes\textsuperscript{19,20}.

Transgenic mouse expressing mutated mouse collagen (MMC) has strong T cell tolerance towards the self antigens and is resistant to CIA. Introduction of \textit{Ncf1} mutation into the MMC mouse resulted in tolerance break down and induction of arthritis due to the lower production of reactive oxygen species (ROS) \textsuperscript{21}. In a similar approach, CIA resistant H2\textsuperscript{b} B6N mice are bred with \textit{Ncf1} mutated mice to introduce \textit{Ncf1} mutation and see whether it can reduce threshold for disease induction. \textit{Ncf1} gene encodes the neutrophil cytosolic factor protein, a component of the NADPH oxidase complex. This complex is important for the production of reactive oxygen species (ROS), which are highly reactive. Superoxides (O\textsubscript{2}\textsuperscript{-}) are the free radicals generated by the transfer of electron from NADPH to the oxygen molecules (O\textsubscript{2}). ROS are very important for the active functioning of the immune system and they help in the clearance of the harmful pathogens. Phagocytic cells like macrophages, neutrophils and dendritic cells release ROS through NADPH complex\textsuperscript{22}.

Defects in the subunits of the NADPH oxidase complex results in non-functional oxidase complex, ultimately resulting in negligible or lower ROS production. This in turn led to recurrent pathogenic infections resulting in chronic granulomatous disease (CGD) \textsuperscript{23}. The finding of natural polymorphic mutation in \textit{Ncf1} gene encoding neutrophil cytosolic factor or p47phox was attributed to the lower ROS production in rats which developed severe arthritis\textsuperscript{24}. In a similar finding, truncated \textit{Ncf1} protein was generated due to a splice site mutation in the mouse \textit{Ncf1} gene resulting in pronounced arthritis and encephalomyelitis due to reduced oxidative burst\textsuperscript{25}. Initially ROS produced from the NADPH oxidase 2 (NOX2) complex was thought to be mediators of inflammatory responses causing destruction of cells and tissues. Based on the recent findings they are believed to have immune regulatory functions, which are important in the mechanisms of autoimmunity. Research from the rodent studies suggests that ROS can suppress the auto-reactive T cells against self antigens and helps in modulating the severity of immune diseases like arthritis. These scientific findings of autoimmunity provide the targets for the development of new treatment strategies to cure the chronic autoimmune diseases\textsuperscript{26–28}.

As already stated, RA is a multifactorial, complex and heterogeneous disease. Different genes and effect of environment and their interactions are responsible for triggering immune response. In order to better understand and elucidate the underlying pathogenesis, there is a need for animal models. These models are the basis for conducting investigations, as they resemble or similar in clinical phenotype to the human RA. Also the animal models helps in identifying the candidate genes and inflammatory components responsible for the disease. This in turn helps in developing preventive or therapeutic treatment strategies against a target and also in testing the efficacy of the developed drug or combination treatment\textsuperscript{29–31}. There are different kinds of animal models available for different human diseases and as well for RA like induced models, spontaneous or gene targeted models. Among all the animal models, collagen induced arthritis (CIA) is the common and widely used model to study the disease mechanisms in RA. In this model the disease is induced by injecting collagen type II (CII) molecule along with Complete Freund’s Adjuvant (CFA). This model helps to better understand the contribution of different MHC genes, antigen epitopes and various components of innate and adaptive immune system\textsuperscript{32}.
3. Materials and Methods

3.1 Mice

In all the experiments either 8 to 12 weeks old male J-/- mice or B6N.Ncf1 mice of both gender were used. As control groups in J-/- and B6N.Ncf1 experiments, B10.Q and B6N mice were used respectively. J-/- mice were obtained from Prof. Tomas Leanderson, Lund University and B6N.Ncf1 mutated mice (6N) were obtained from the Jackson Laboratory (Bar Harbor, ME), USA and backcrossed to B6N mice for further four generations. In one of the vaccination experiment, Cia9i sub-congenic mice derived from B10.Q-NOD Cia9 congenic mice reported earlier were used as controls for J-/- mice. Due to very low susceptibility of B10.Q mice in our experiment, we decided to use Cia9i mice as positive controls for the CIA experiment as they can develop more severe arthritis. All the mice were kept in the specific pathogen free (SPF) animal house facility, Medical Inflammation Research lab, Department of MBB, Karolinska Institute. Mice were housed in individually ventilated cages (IVC) and were fed with standard rodent chow and water ad libitum, having twelve hours of light and dark cycles and at room temperature (20°C to 25°C). All the animal experiments were performed according to the Swedish Animal Ethical Laws. N66/10 and N169/10 are the ethical permits used for arthritis experiments and for ear or toe markings respectively.

3.2 Antigen preparations

Rat and chicken collagen type II proteins were used in the immunization/vaccination experiments. Pepsin digested and purified swarm chondrosarcoma was the source for rat CII as described previously. Chicken CII was prepared from the sternum of chicken as described earlier. CII was dissolved in 0.1 M acetic acid and stored at 4°C in refrigerator to maintain its triple helical native form, which is important for immunization efficacy.

3.3 Collagen Induced Arthritis (CIA)

CIA in J-/- mice was induced by injecting 100 µg of rat CII emulsified with Complete Freund’s Adjuvant (CFA, Difco Laboratories, Detroit, USA) in a total volume of 100 µl per mouse as a primary immunization (day 0). Mice were given booster immunization on day 35, with 50 µg of CII in Incomplete Freund’s adjuvant (Difco) in a total volume of 50 µl per mouse. In B6N.Ncf1 mice, CIA was induced with chicken type II collagen. Mice were primarily immunized on day 0 with 200 µg of CII emulsified with CFA in a total volume of 100 µl per mouse. On day 21, mice were boosted with 100 µg of CII in IFA in a total volume of 50 µl per mouse. All the injections were done intradermally at the tail base.

3.4 Collagen Antibody Induced Arthritis (CAIA)

CAIA was induced in B6N.Ncf1 and B6N mice by transfer of monoclonal antibodies intravenously. Four monoclonal antibody cocktail of 1 mg each, containing antibodies against the major epitopes on the CII molecule were used to induce the disease. Monoclonal antibodies CIIC1, M2139, CIIC2 and UL1 are specific against the C1, J1, D3 and U1 epitopes on CII respectively. CII-specific antibody secreting B cell hybridomas were previously developed and characterized in the lab. The hybridomas were cultured in CL-1000 flasks and the antibodies were purified from culture supernatants as described in detail earlier. Three hours after the antibody transfer, mice were given 40 µg of lipomannan intraperitoneally as an enhancement. Lipomannan (from Mycobacterium
*smegmatis*, InvivoGen, USA) was used to stimulate the immune system by activation of TLR2 receptors\(^4^2\). Mice were scored daily for up to day 27.

### 3.5 Arthritis scoring and assessment

Experimental mice were scored and weighed thrice weekly. Appearance of clinical arthritis features of joints like redness, inflammation and edema were considered as disease onset. Single score was given to an inflamed digit, 5 points for inflamed midpaw and 5 points for inflamed hind paw. The same scores were given for the forelimbs. On total for each fully inflamed paw would get 15 points and total 60 points/mouse\(^2^5\). Arthritis severity and incidence data were collected for all the CIA and CAIA experiments.

### 3.6 Measurement of total serum anti-CII antibody levels

The ELISA costar flat bottom plates (Immunochemistry Technologies, Bloomington, USA) were coated with 10 µg/ml of type II collagen and incubated overnight at 4\(^0\)C. Plates were washed and 50 µl of five-fold diluted serum samples were incubated for 1 hour at room temperature. For detecting total anti-CII Ig levels, HRP conjugated goat anti-mouse Ig (H+L) (Southern Biotech, Alabama, USA) at 1:4000 dilution or anti mouse-kappa light chain antibody (home made antibodies from the clone 187.1 at 1:1000 dilution) was used. For detecting anti-CII IgM, IgG, IgG1, IgG2c, IgG2b respective HRP conjugated goat anti-mouse secondary antibodies at 1:4000 dilution were used (Southern Biotech, Alabama, USA). Fifty µl of secondary antibody was added and the plates were incubated for 1 hour at room temperature. As a detection system for biotinylated secondary antibody, streptavidin peroxidase (Invitrogen, NY, USA) at 1:2500 dilutions were added and incubated for 45min at room temperature. Finally as the substrate for the enzyme, 50 µl of ABTS (for 1 plate= one 5 mg tablet + 500 µl ABTS buffer + 4.5 ml of distilled water) (Roche Diagnostics Corporation, Indianapolis, USA) solution was added and incubated for 30 minutes in dark at room temperature. Plates were read using Wallac 1420 software (version 3.00) at 405nm in Wallac victor model 1420-002 multi-label micro-plate reader (Perkin Elmer life and analytical sciences, Turku, Finland).

### 3.7 Collagen biotinylation

To study the *in vivo* uptake of CII, the collagen molecule was biotinylated. 1.5 mg/ml of CII dissolved in 0.1M acetic acid was initially dialyzed into PBS of pH 7.5. Then 1 mg of NHS-LC biotin (Thermo Scientific, Rockford, USA) was dissolved in 1 ml of distilled water and 180 µl of this solution was added to CII in PBS and the reaction mixture was incubated on a rotator at room temperature for 4 hrs. After the reaction, CII was dialyzed against 0.1 M acetic acid with three changes and stored at 4\(^0\)C until used.

### 3.8 Biotinylated collagen concentration and efficiency of biotinylation determination

Quality and concentration of biotinylated CII was determined by ELISA. To determine the biotinylation efficiency, ELISA plates were coated in duplicates with 50 µl of avidin (10 µg/ml) (Sigma-Aldrich, St. Louis, USA) and incubated overnight at 4\(^0\)C. Ten µg/ml concentration of each of the biotinylated, native, denatured CII were loaded in duplicates onto avidin coated plates and also they were coated directly to the wells. Biotinylated antibody was used as positive control and the samples were incubated for 1 hour at room temperature. CII was denatured by heating CII in a water bath at 60\(^0\)C for 25 mins. Fifty µl
of CB20 (5 µg/ml) and GB8 (1 µg/ml) monoclonal antibodies were used as primary antibody to detect native and denatured CII respectively. As a secondary antibody HRP conjugated goat anti-mouse IgG (H+L) chain 1:5000 dilution (The Jackson laboratory, Bar Harbor, USA) was used. ABTS (Roche Diagnostics Corporation, Indianapolis, USA) was added as enzyme substrate for color development after 30 min incubation at room temperature. Plates were read in Wallac reader (Perkin Elmer). To determine the concentration of biotinylated CII, similar ELISA protocol was used except a change in antigen coating. Native and biotinylated CII were first denatured at 60°C for 25 min and then coated at 1 µg/ml in duplicate wells. Denatured CII was used as a standard to obtain the standard graph to calculate the concentration of biotinylated CII. Both samples were diluted five times from the starting well up to seven steps. GB8 (1 µg/ml) was used as primary antibody for detection of denatured form of CII.

3.9 Immunohistochemistry

Biotinylated CII was used to study its uptake by different immune cells in the nasal associated lymphoid tissue (NALT) in vivo. J-/- and B10.Q mice were vaccinated intranasally with biotinylated or normal CII at different concentrations, 50 µg and 100 µg. Twenty four hours later NALT and cervical lymph nodes were collected and fixed in cryo-moulds using TissueTek OCT compound (Sakura Finetek Inc, Torrence, CA, USA). Cryosections were stained as follows: Sections were dried overnight and fixed in ice cold acetone for 5 min. Sections were then washed in 0.05% PBS-Tween buffer and endogenous peroxidase was blocked by incubating the sections in hydrogen peroxide solution for 30 minutes. Sections were then washed in PBS and streptavidin peroxidase at 1:500 dilution were added onto each section and incubated for 45 min at room temperature. Freshly prepared DiAmino Benzidine (DAB, Vector Laboratories, CA, USA) was used as substrate for peroxidase. Sections were then counterstained with haematoxylin and mounted with cover slip using pertex mounting medium (Leica Microsystems GmbH, Wetzlar, Germany).

3.10 Genotyping

MHC haplotypes of B6N.Q 10N1i mice were identified by genotyping using microsatellite marker primer pairs on ABI 3730 analyzer (AB Applied Biosystems, CA, USA). Tail samples were collected from the mice and DNA was extracted by adding 50 mM NaOH and boiling the tissue sample at 95°C for 90 min. Digested samples were vortexed and centrifuged at 13200 rotations per minute for 15 min. 40 µl of the supernatant was mixed with 10 µl, 1M Tris-HCl to neutralize the DNA product. The MHC haplotypes qq, qb, bb were determined by using the D17MitNds3, D17Mit47 and D17Mit24 (Eurofins MWG Operon, Ebersberg, Germany) primer pairs. Individual primer master mix was made by adding distilled water, 10x PCR buffer, dNTPs, Taq polymerase (all products from New England BioLabs, UK), primer mix constituting 9 µl plus 1 µl of the neutralized DNA for PCR setup. DNA was amplified by performing PCR run at TCL 60 program (parameters: denaturation at 94°C for 1 min, annealing temperature 60°C for 2 mins, 72°C for 2 min for 35 cycles and finally 60°C for 10 min). Later the PCR product was diluted in 50 µl distilled water to perform capillary electrophoresis. For this 9 µl of Gene Scan™ 600 LIZ size standard (AB applied biosystems) was mixed in 850 µl of HiDi solution (AB applied biosystems). Then 8 µl of mixed HiDi solution and 1 µl of diluted PCR product were added to the ABI barcode plates. Plates were sealed with septa and the well products were vortexed and the plate was subjected to the fragment denaturation before continuing on to electrophoresis. Finally the plate was loaded onto the ABI machine to run capillary
electrophoresis. The results were analyzed in genemapper software (version 3.7, AB applied biosystems, Foster city, CA, USA).

4. Results

4.1 Experiments with J chain deficient mice

4.1.1 J chain deficient mice are susceptible to arthritis

CIA was induced in J-/-(n=15) and B10.Q (n=13) mice according to the standard protocol to study the contribution of joining chain deficient antibodies during arthritis development. We monitored arthritis development (disease severity and incidence) for 90 days by scoring them three times per week. As shown in figure 1, there is no significant difference between J-/ and control B10.Q mice either in disease severity or incidence indicating a minor role for polymeric antibodies in collagen induced arthritis (Figure 1).

4.1.2 Therapeutic vaccination strategy with type II collagen in CIA

CIA was induced in J-/ and B10.Q mice and both groups were sub-grouped as follows: treated (TR) and non-treated (NTR). On the day of the disease onset (considered as day 0), mice in the treated groups were vaccinated intranasally with 100 µg of CII. Arthritis was monitored for 20 days post-vaccination by scoring them 3 times per week. As shown in Figure 2, mean disease scores did not significantly differ between treated and non-treated groups either for J-/ or B10.Q mice. This data shows that the deficiency in sIgA or IgM at the mucosal site, do not have any effect on arthritis disease course, when therapeutic approach was used as a vaccination strategy (Figure 2).

4.1.3 Preventive vaccination strategy with type II collagen in CIA

CIA was induced in J-/ and B10.Q mice and both the groups were sub-grouped as follows: treated (TR) and non-treated (NTR). Before the disease initiation, at day-7, J-/ (n=8) and B10.Q (n=9) mice in treated sub-groups were vaccinated intranasally with 100 µg of CII, whereas mice in control sub-groups of J-/ (n=12) and B10.Q (n=13) mice were left untreated. All the mice were immunized with CII emulsified in CFA on day 0 and were boosted with CII in IFA on day 35. Mice were scored for 80 days. As shown in Figure 3, mucosal vaccination in J-/ mice had a suppressive effect on CIA disease severity compared to non-treated littermate controls. Such an effect was not observed in B10.Q mice. The mice were bled three times on day 0, day 35 and on day 90 during the experiments. Blood samples were collected by cheek bleeding technique and sera collected were used to analyze anti-CII antibody levels (Figure 3).

4.1.4 J-/ mice have compromised IgM and total antibody response to CII during arthritis development

To investigate how arthritis disease severity correlates to CII reactive immunoglobulin level during CIA, we bled all the experimental mice used in mucosally vaccinated mice in the preventive experiment, before and after arthritis induction at days 35 and 90. We measured IgM and total Ig (using anti-kappa light chain specific antibodies) immune responses specific to CII using ELISA. As shown in figure 4, J-/ mice have significantly lower levels of CII-specific total antibodies at days 35 and 90 compared to J-chain sufficient B10.Q
mice in both treated and untreated sub-groups. CII reactive IgM isotype specific ELISA shows that both experimental groups mount antigen specific IgM response after the immunization, but IgM response was significantly compromised in treated and untreated sub-groups of J/-/ mice compared to B10.Q controls at both the time points tested, days 35 and 90 (Figure 4).

4.1.5 Modified Therapeutic vaccination experiment

Therapeutic vaccination experiment (modified version) was performed on J/-/ and Cia9i mice by vaccinating the mice with 100 µg CII intranasally on three time points i.e. day of disease onset (day 0), days 2 and 4. In this experiment both mice groups were immunized on day 0, with 100 µg of CII in CFA and boosted on day 35, with 50 µg of CII in IFA. The mice were scored daily after booster immunization to monitor the day of disease onset. On the day of disease onset mice were grouped into treated and non-treated groups. Mice in the treated group were vaccinated with 100 µg of CII on three alternate days 0, 2, and 4. As shown in figure 5, we distributed the mice in different groups that had similar mean arthritis score on the day of disease onset. Surprisingly, mice in the vaccinated group got more disease compared to the non-treated J/-/ mice, inferring that the treatment has actually enhanced the disease. Serum anti-CII IgM levels were significantly reduced in the J/-/ mice compared to the J chain sufficient wild type Cia9i mice at 1:100 dilutions. Unfortunately, the day of disease onset for control mice were missed. Hence, they were excluded from the experiment (Figure 5).

4.1.6 Modified preventive vaccination experiment in J/-/ and B10.Q mice

J/-/ and B10.Q mice were grouped into treated and non-treated groups. On day 0, CIA was induced with 100 µg of rat CII in a stronger adjuvant, CFA containing H37RA. On days 6 and 34, mice in the treated group were vaccinated with 100 µg of CII intranasally following another published vaccination protocol \(^{44}\). Mice were boosted on day 35 and were scored weekly thrice to compare arthritis severity and incidence between the vaccinated and non-vaccinated mice groups. As shown in Figure 6, J/-/ treated mice developed less severe disease compared to the non-treated mice, but the difference was not significant. In B10.Q mice, vaccinated group responded well and got significantly low disease scores compared to the non-treated mice group, which developed severe disease. This data shows the suppressive effect of CII vaccination in B10.Q mice (Figure 6).

4.1.7 Biotinylation quality and concentration determination by ELISA

Biotinylation of CII was done to determine the nature (native vs. denatured forms) and differential retention time of CII in NALT of J/-/ and B10.Q mice. Quality and concentrations of biotinylated CII were determined by ELISA. Briefly, biotinylated collagen was either loaded onto avidin coated plates or coated directly into the wells. Native and denatured collagen coated wells served as controls for the CB20 and GB8 primary antibodies respectively. As shown in figure 7, the signals detected on avidin-coated wells correspond to the biotinylated CII and the biotinylated antibody was used as a positive control for the determination of biotinylation efficiency. The signals on the direct-coated wells correspond to the native and denatured forms of CII samples as we have used the primary antibodies CB20 and GB8 that recognized native and denatured CII as positive controls \(^{43}\) (Figure 7).
4.1.8 *In vivo* study of collagen uptake in nasal mucosa

B10.Q mice were intranasally vaccinated with biotinylated and normal CII. After 24 hours NALT and cervical lymph nodes were collected to study the uptake of CII by the immune cells in nasal mucosa. NALT sections were stained for biotinylated collagen using streptavidin peroxidase. As shown in figure 8, sections were not stained positive for biotinylated CII. Negative staining might be due to absence of freely distributed CII in the tissue. Furthermore, the APCs present at the mucosal site might have taken up CII, for internal processing to present peptides on MHC molecules (Figure 8).

4.2 Effect of *Ncf1* mutation on arthritis induction in B6N genetic background

4.2.1 CIA induction in B6N,*Ncf1* and B6N mice with chicken collagen in normal CFA

In order to study whether *Ncf1* can reduce the threshold for arthritis induction, as in B10.Q mice in less arthritis susceptible B6N genetic background, we immunized B6N mice with or without *Ncf1* mutation with chicken CII as described in materials and methods. Mice were scored for arthritis development thrice per week after the booster immunization. As shown in figure 9, surprisingly, B6N,*Ncf1* mice did not develop arthritis, but control mice developed mild disease with low frequency. To assess the serum anti-CII antibody levels, mice were bled on days -1, 20 and 56. Anti-CII antibody levels were similar in both the groups. On day 57, mice were given 25 µg of LPS (*E.coli* 055:B5, Sigma Aldrich) in 200 µl of PBS per mouse to see whether there will be any enhancement in disease severity. LPS is normally used to enhance the incidence and severity of antibody induced arthritis. B6N,*Ncf1* mice developed disease with low scores but with an increased frequency (Figure 9).

4.2.2 Stronger CFA is needed to induce disease in B6N mice with chicken CII

In this experiment, disease was induced in the B6N and B6N,*Ncf1* mice with chicken CII emulsified in a stronger adjuvant (CFA containing *Mycobacterium tuberculosis* strain, H37RA). Mice were scored thrice weekly for arthritis development. As shown in figure 10, B6N mice developed severe disease with a high frequency, but B6N,*Ncf1* mice had fewer score and also lower disease incidence. Anti-CII antibody concentrations were significantly higher in B6N mice than B6N,*Ncf1* mice on day 57. Total anti-CII IgM and IgG levels and also the IgG subclass analysis of IgG1, IgG2b and IgG2c in day 20 serum had no significant differences between the groups (Figure 10).

4.2.3. *Ncf1* gene mutation has minimal effect at the effector phase of arthritis in B6 background

To investigate the effect of *Ncf1* mutation at the effector phase of disease, CAIA was induced in B6N,*Ncf1* and B6N male mice by intravenous transfer of a cocktail of monoclonal antibodies. Four monoclonal antibody cocktail containing antibodies against the major epitopes on the CII molecule were used to induce the disease. Three hours after the antibody transfer, mice were given 40 µg of lipomannan (InvivoGen) intraperitoneally as an enhancement to stimulate the innate immune system by TLR2 pathway. As shown in figure 11, B6N,*Ncf1* mice didn’t develop disease, but B6N mice developed disease with high frequency (Figure 11).
4.2.4 CIA experiment in B6N.Q-10N1i mice with chicken CII

Arthritis can easily be induced with mice having H-2q haplotype. Hence, we introduced this MHC haplotype into B6 genetic background from B10.Q mice and backcrossed for 10 generations. We intercrossed the heterozygous mice to get all the three combinations of MHC haplotypes H2b (homozygous), H2q (homozygous) and H2qb (heterozygous) mice and tested them for arthritis induction. As shown in figure 12, heterozygous (H2qb) mice developed severe disease with a high disease incidence compared to (H2q) and (H2b) littermate controls. Disease severity was high in (H2q) than (H2b) mice, but disease frequency was same in both the groups. Serum anti-CII antibody levels on day 56 was also higher in (H2qb) mice which correlated with disease severity, but similar antibody levels were observed in (H2q) and (H2b) mice (Figure 12).
5. Discussion

The main objective of the project was to develop preventive and therapeutic vaccination treatment regimens for arthritis in joining (J) chain deficient (J-/−) mice, which lack pentameric IgM and dimeric IgA antibodies. Joining chain protein helps in the polymerization of IgM and IgA antibodies, which are functionally important in defense mechanisms at mucosal surfaces9. J chain is important for epithelial transcytosis of pIgs by the pIgR into the luminal surface9. Investigations show that IgM and IgA can polymerize even in the absence of J chain, but they have less stability and thus are inefficient for pIgR mediated transport46. Many investigations emphasized the importance of joining chain for pIgR mediated transcytosis of pIgs into the glandular secretions by using antibody directed against the J chain and also in joining chain deficient mouse models12,46,47.

In a preliminary experiment we investigated whether J-/− mice can develop arthritis by inducing CIA. Joining chain sufficient B10.Q mice were used as controls. Both the J-/− and B10.Q mice developed arthritis when CIA was induced. Mean disease severity and incidence in J-/− mice were similar to that of B10.Q mice. This result infers that there is only a minor role for joining chain in arthritis development.

Many studies were performed on animal models of various autoimmune diseases to develop treatment protocols. In most of the autoimmune diseases, the possible candidate auto-antigens were used for tolerance induction. Collagen type II, Myelin Basic Protein (MBP), porcine insulin and Acetylcholine Receptor (AChR) were the candidate auto-antigens used to induce tolerance via orally or intranasally in animal models of arthritis, experimental autoimmune encephalomyelitis (EAE), diabetes and experimental Myasthenia Gravis48–51. Nasal administration of antigen over oral route is beneficial due to low doses of antigen because it lacks enzymatic digestion of antigen as in oral and also due to reduced B and T cell responses towards the antigen52. Hence, we investigated the role of J chain in the mucosally induced tolerance mechanisms to develop vaccination regimens for arthritis.

Both preventive and therapeutic vaccination approaches were studied using J-/− and B10.Q mice. In the J-/− mice the IgA and IgM antibodies are less effective as they can’t form polymers and also lacks pIgR mediated transport into the mucosal and glandular secretions12,46,47. Due to this, the retention time of the intranasally applied antigen can be increased which can result in initiating tolerance mechanisms towards the autoantigen by the autoreactive cells. Tolerance can be induced in autoreactive cells either by total suppression or by anergy, a state of unresponsiveness depending on the dosage of the antigen used in vaccination53.

Initially preventive and therapeutic vaccination experiments were performed based on the standard protocols. In therapeutic vaccination, CIA was induced in the mice on day 0 and the mice were vaccinated when they developed clinical symptoms of the disease i.e. on the day of disease onset. J-/− treated mice had low disease severity compared to the non-treated group, but the difference was not significant due to the less number of mice in the groups. B10.Q treated mice didn’t respond to the vaccination and both the groups had same disease severity.

In preventive vaccination experiment, mice were initially vaccinated before inducing arthritis. J-/− mice responded well to the vaccination and they didn’t develop arthritis compared to the J-/− mice in untreated group, which developed arthritis. In the B10.Q
controls, mice in the treated group developed more disease scores than the mice in non-treated group. These results infer that the preventive vaccination in vaccinated J-/ mice resulted in prevention of disease, which was not seen in the B10.Q mice.

In the preventive experiment, anti-CII IgM levels in both the treated and non-treated J-/mice group were significantly lower compared to the B10.Q controls. This was same for the total anti-CII Ig levels among the groups. There was no difference in IgM and total Ig antibody levels in treated and non-treated J-/ and B10.Q mice when compared within the groups. This data doesn’t correlate with vaccination and can’t explain the vaccination effect in J-/ mice. Earlier investigations documented that J chain deficient mice had reduced levels of IgM and IgG, but increased levels of IgA in serum. Hence, further experiments are needed to explain the vaccination effect in J-/ mice.

Vaccination experiments were performed by modifying the above protocols, to obtain conclusive data. In the therapeutic vaccination experiment, arthritic mice were treated with CII three times on alternative days, starting from day of disease onset. Surprisingly, J-/ mice in the treated group developed more disease than the non-treated mice inferring that the vaccination actually enhanced the disease. This vaccination effect might be due to the multiple treatment regimens followed in the established disease.

In modified preventive vaccination experiment, mice were vaccinated twice following the earlier protocol. J-/ treated mice responded well to the vaccination and they developed less disease when compared to the non-treated J-/ mice. Interestingly, B10.Q treated mice also responded well to the vaccination and developed less disease than the non-treated group. Cellular studies are further warranted to explain this anomaly.

Hence more studies are needed to clarify the importance of joining chain in mucosal tolerance induction as a vaccination for RA. Published data demonstrates that whole CII, conjugates of CII and different collagen peptides can prevent or ameliorate the disease when administered intranasally either before primary immunization or after established disease.

To further understand the mechanisms of collagen uptake during vaccination, biotinylated and normal CII were used. However, NALT and lymph node sections stained negative for streptavidin peroxidase staining. This infers the absence of free CII in the NALT tissue, implying that the injected CII was already taken up by the antigen presenting cells present at the mucosal site for antigen processing and presentation and further time points are needed.

In a parallel project, experiments were performed on Ncf1 gene mutated B6N mice. B6N mice are less susceptible for arthritis, due to the MHC haplotype H2b. Susceptibility of mice for arthritis induced by CII depends on the MHC complex and the type of anti-CII antibody response generated. Mice with MHC H2b haplotype are considered to be highly susceptible due to the development of high anti-CII antibody levels. Susceptibility for CIA also depends on the magnitude of IgG2a antibody subclass production, interactions of various MHC and non-MHC genes and availability of complement factors. It is also shown that the resistance for CIA in B6N mice is due to the defect in secondary immune response in the cell mediated and humoral immune response against CII after secondary immunization.
In rats it was first discovered that mutation in Ncf1 gene led to more susceptibility and severe arthritis due to less production of oxygen radicals\textsuperscript{24}. In mouse models of CIA, it was shown that Ncf1 mutation enhanced arthritis, experimental autoimmune encephalomyelitis and also in mice expressing mutated mouse collagen it breaks tolerance for CII to induce disease\textsuperscript{21,25}. Therefore, we studied the effect of introducing Ncf1 gene mutation in B6N genetic background with H2\textsuperscript{b} haplotype. CIA was induced in B6N, Ncf1 and B6N mice with chicken CII in normal CFA. In this experiment both the B6N and B6N, Ncf1 mice developed very mild disease incidence but only two mice in B6N group had mean disease scores of 20. Majority of the mice had single inflamed digit with one score, but after LPS treatment there was increase in disease incidence in B6N, Ncf1, which had mean scores between 5 to 10 points. Analysis of anti-CII antibody levels infers that there is no difference among the groups in both primary and secondary immune response. In another experiment (data not shown) CII source was changed and the mice were immunized with rat CII to study whether the T cell epitopes on rat CII molecule can induce disease by interacting with H2\textsuperscript{b} haplotype. Mice in neither of the groups developed any disease, inferring that strong affinity interactions between the antigen (CII) and MHC haplotype is needed for triggering anti-CII immune response. It has already been shown that susceptibility to develop arthritis depends on the ability to generate immune response against autologous collagen or heterologous CII. Mice with H2\textsuperscript{q} and H2\textsuperscript{r} MHC haplotypes are most efficient in generating anti-CII immune response against different species of CII\textsuperscript{15,57}.

It was shown that to induce disease in B6N mice, the dosage of Mycobacterium tuberculosis was important for the maturation of dendritic cells to activate the accessory immune cells\textsuperscript{18}. So, we immunized the mice with high concentration of CFA containing M. tuberculosi s H37RA. In this experiment also the B6N mice developed disease with high incidence and severity. Surprisingly, the B6N, Ncf1 mice developed very mild disease with low frequency and arthritis. Mice were bled at regular intervals to assess the total anti-CII antibody levels during the course of the experiment. The primary anti-CII antibody response was similar between the groups, but there was significant difference in the secondary anti-CII antibody levels. A further level of different subtypes and IgG subclass analysis was performed on the day 20 serum. IgG antibody levels were higher than the IgM in both groups and were similar. Even the IgG1, 2c, 2b levels between the groups were similar. Secondary antibody response was high in B6N compared to the B6N, Ncf1 mice, which correlated well with the disease phenotype. Increase in the secondary anti-CII antibody response is responsible for the high disease severity in B6N mice, but the antibody levels remained low in B6N, Ncf1 mice. To further explain the observed data, cell mediated immune responses are to be analyzed in both the groups. Attempts were made in this direction but the assays (determination of IL-2 and IFN-\gamma levels after stimulation of splenocytes with chicken CII) did not work as expected.

We also investigated the effect of Ncf1 mutation at the effector phase of CAIA in the B6N genetic background. In CAIA also, the B6N mice developed higher disease frequency, but the severity was very mild and the B6N, Ncf1 mice didn’t develop any disease. This experiment infers that there is minimal effect of Ncf1 gene mutation at the effector phase in the B6N genetic background. These experimental results collectively emphasize that Ncf1 gene mutation in B6N genetic background has minor influence to break the threshold for arthritis induction in contrary to the B10.Q mice bearing H2\textsuperscript{q} MHC haplotype. Also in a heterogeneous type of autoimmune disease it is difficult to predict how the different genes interact with each other in initiating a disease phenotype. It is believed that in complex
diseases when a gene gets mutated and lost its function, there will be many other genes acting to compensate the functional defect. Another possibility is the existence of the modifier genes in the B6N genetic makeup, which have the epistasis function as observed in the systemic lupus erythematosus (SLE) disease. In SLE, it is has been reported that Sles1 disease modifier gene can completely suppress the disease caused by the susceptibility Sle1 locus in B6N genetic background\textsuperscript{38}.

In another experiment, the impact of two MHC haplotypes (H2\textsuperscript{a} and H2\textsuperscript{b}) in arthritis induction was investigated in B6N genetic background. B6N.Q (10N1i) mice heterozygous for both haplotypes (H2\textsuperscript{ab}) were studied by inducing CIA. Heterozygous mice developed high disease frequency and severity compared to the homozygous (H2\textsuperscript{a}, H2\textsuperscript{b}) littermate controls. But after day 52 disease severity in H2\textsuperscript{a} mice increased significantly compared to the H2\textsuperscript{ab} and H2\textsuperscript{b} mice. Analysis of total anti-CII antibody data shows that antibody levels are higher in the heterozygous mice compared to the homozygous mice during the primary and secondary immune response, which correlated with the disease phenotype observed. But the antibody response did not correlate with the chronic disease phenotype in the H2\textsuperscript{a} mice, which clearly emphasize the importance of cellular players in perpetuating chronicity in arthritis. This also infers that the H2\textsuperscript{a} haplotype even in B6N background increased the severity, which perpetuated the disease at later time points similar to B10 genetic background as reported earlier\textsuperscript{15}. Since the heterozygous mice (H2\textsuperscript{ab}) developed higher disease severity and frequency than the homozygous mice suggest MHC haplotype interactions as one of the major factors in contributing to arthritis disease development. Similar observations were found in an earlier experiment with another MHC heterozygous mice (H2\textsuperscript{qd}) using BALB/c and B10.Q mice\textsuperscript{59}.

The above studies on the effect of introducing Ncf1 gene mutation in B6N genetic background infers that, the mutation has only a minor effect on arthritis induction. The influence of B6N genetic background on arthritis induction with CII was earlier demonstrated by administering IL-12 cytokine. It was conferred that IL-12 can initiate T cell immunity but failed to trigger antibody response against CII in B6N genetic background\textsuperscript{60}. This infers that there exists yet to be explored influence of B6N genetic background on arthritis induction. Hence more substantial data is needed before making any valid conclusions, which can be obtained by doing experiments on T cell activation status, antigen presentation assays and oxidative burst assay using APCs.
6. Figures

**Figure 1.** J chain deficient mice develop collagen-induced arthritis (CIA)
CIA disease severity (A) and incidence (B) in J-/− and B10.Q mice. CIA was induced on day 0 with CII in CFA and mice were boosted on day 35 with CII in IFA. Male mice aged 8 to 12 weeks were used in the experiment. Number of mice used J-/− (n=15) and B10.Q (n=13). Error bars indicate the mean disease severity variance on the corresponding day in the mice groups.

**Figure 2.** Therapeutic vaccination approach with type II collagen in CIA
Intranasal vaccination effect on CIA disease activity in J-/− (A) and BQ mice (B), therapeutic approach. CIA was induced on day 0 and mice were boosted on day 35. Depending on arthritis development, mice were grouped into treated and non-treated groups. Mice in treated group were vaccinated on the day of disease onset as shown by arrow. Male mice of 8 to 12 weeks old were used in the experiment. Error bars indicate the variance in the mean arthritis severity on the corresponding day in the treated and non-treated J-/− and B10.Q mice groups. Number of mice in treated (J-/−=3, B10.Q= 4) and non-treated (J-/−=2, B10.Q=4) groups.
Figure 3. Preventive vaccination approach with type II collagen in CIA
Intranasal vaccination effect on CIA disease activity in J/- (A) and BQ mice (B), preventive approach. In preventive vaccination mice were vaccinated on day -7 before primary immunization. Primary immunization was done on day 0 and secondary immunization on day 35. Male mice aged 8 to 12 weeks were used and number of mice in treated J/- (n=8) and B10.Q (n=9). Number of mice in non-treated group J/- (n=12) and B10.Q (n=13). Error bars indicate the variance in mean arthritis severity on the corresponding day in the groups.

Figure 4. J/- mice have compromised IgM and total antibody response to self-antigen during CIA disease course. Mice in preventive vaccination experiment were bled on days 35 and 90, to assess the anti-CII antibody levels in the serum. Serum anti-CII total Igs response in J/- and B10.Q mice at day 35 (A) and day 90 (B). Anti-CII IgM antibody
levels in J-/ and B10.Q mice at day 35 (C) and day 90 (D). Error bars indicate the variance in the absorbance values for IgM and total Ig antibody in the mice groups.

Figure 5. Modified Therapeutic vaccination experiment. Therapeutic vaccination in disease induced J-/ and Cia9i mice. Mice in treated group were vaccinated with 100 µg of CII on day of disease onset day 0, day 2 and day 4. (A) Disease severity increased in vaccinated J-/ group compared to non- treated J-/ group. (B) Mice in both the groups have same disease incidence on day of disease onset. Arrows indicate the day of intranasal vaccination. (C) Serum anti-CII IgM antibody levels were significantly decreased in joining chain deficient (J-/ ) mice than joining chain sufficient wild type mice. (Male mice, n=5)
mice in each group). Error bars indicate the variance in mean arthritic scores on the corresponding day in the treated and non–treated J/- mice groups. In IgM ELISA error bars indicate the variance in absorbance values in the J/- and Cia9i mice groups at 1:100 and 1:1000 serum dilutions.

Figure 6. In vivo modified preventive vaccination experiment in J/- and B10.Q mice Effect of intranasal vaccination in CIA induced J/- and B10.Q mice. (A) disease severity among different groups of J/- and B10.Q mice with mean arthritis score on Y-axis and days on X-axis. (B) disease incidence of vaccinated and non-vaccinated mice groups. 8 to 12 weeks old, male mice were used in the experiment. Number of mice used J/- (treated =10, non-treated = 9) and B10.Q (treated=10, non-treated = 9). Error bars in the disease severity indicate the mean score variance inside the group on the corresponding day.
Figure 7. Biotinylation quality and determination of CII concentrations by ELISA. Antigens are coated directly as shown in (A) and loaded onto avidin-coated wells (B). Numbers on the X-axis indicate the type of antigen coated on X-axis and their corresponding absorbance values at 405 nm are shown on Y-axis. 1, 2 are the wells coated with BioCII and detected with CB20 and Gb8 primary antibodies respectively. 3, 4 are the wells loaded with NCII and DN CII as a positive controls for the primary antibodies CB20 and GB8 respectively. 5, is the well loaded with biotinylated antibody as a positive control for the assay and 6, 7 wells are negative controls loaded with NCII and DN CII without any primary antibody detection. (C) Standard curve for determining concentration of collagen was obtained by using known concentration of denatured CII (1 μg/ml) and diluting down five times. Error bars indicate the variance in mean absorbance values in the group.
Figure 8. *In vivo* study of collagen uptake in nasal mucosa. B10.Q mice vaccinated intranasally with 100 µg of biotinylated and normal CII. After 24 hours NALT tissue obtained, fixed and stained with streptavidin peroxidase to visualize uptake of Biotinylated CII (A) and native CII (B) as negative control. Four B10.Q mice of 8 to 12 weeks old were used. Biotinylated and Normal CII of two concentrations 100 µg and 50 µg were used for vaccination. NALT sections from 100 µg concentration of biotinylated CII and normal CII are shown here. 60 x magnification.
Figure 9. CIA induction in B6N.Ncf1 and B6N mice with chicken collagen in normal CFA. Tolerance break down experiment in B6N.Ncf1 mice. Disease severity (A) and incidence (B) of the mice are shown in graphs. B6N.Ncf1 mice developed very mild disease after LPS injection on day 57. (C) both the mice groups have same anti-CII antibody levels in serum collected at three time points. Male and female mice of 8 to 12 weeks old, were used. B6N (n=23) and B6N.Ncf1 (n=26). Mean score B6N vs B6N.Ncf1 significance p < 0.0001(***). Error bars indicate the mean arthritis score variance on the corresponding day in the group and variance in mean absorbance values for total Ig in serum in the groups.
B6N vs. B6N.Ncf1 p=0.0002(***)

B6N vs. B6N.Ncf1 p=0.0002(***)

B6N vs. B6N.Ncf1 p=0.0002(***)

C

ant-Cl Ig conc (µg/ml)

B6N
B6N.Ncf1

d-1  d20  d57
Figure 10. Strong CFA is needed to induce disease in B6N mice with chicken CII
(A) Disease severity was high in B6N mice and low in B6N.Ncf1 mice. (B) Disease incidence was also high in B6N mice compared to B6N.Ncf1 mice. (C) Serum anti-CII antibody levels were same on day 20, but on day 57 antibody levels were significantly high in B6N mice which correlate with disease severity. (D) anti-CII subtypes IgM and IgG subclasses IgG1, IgG2c, IgG2b were same between the groups on day 20. Both male and female mice aged 8 to 12 weeks were used. B6N.Ncf1 (n=13) and B6N (n=14). Mean score and incidence significance B6N vs. B6N.Ncf1 P=0.0002 (**). Error bars indicate the mean arthritis variance on the corresponding day in the mice groups and in ELISA they indicate the variance in mean absorbance values in the mice groups for total Ig, IgM and IgG subclasses.
Figure 11. *Ncf1* mutated B6N mice didn’t develop CAIA, but the control mice developed disease. Four monoclonal antibody induced arthritis in B6N and B6N. *Ncf1* mice. (A) monoclonal antibodies against the major CII epitopes induced disease in B6N mice, but not in B6N. *Ncf1* mice. Mice were given 40 µg of Lipomannan intraperitoneally after 3 hours of antibody transfer. (B) disease incidence was in B6N mice, but not in B6N. *Ncf1* mice as they developed disease. Only male mice of 8 to 12 weeks old were used. B6N (n=9) and B6N. *Ncf1* (n=10). Error bars indicate the mean arthritis score variance on the corresponding day in the mice groups.
Figure 12. CIA experiment in B6N.Q 10N1i (+/-, +/-, +/-) mice with chicken CII
Influence of H2q haplotype on disease induction in B6 background. (A) Disease severity was initially high in qb mice, but qq mice developed more arthritis score after day 52. (B) Disease incidence was very much high in qb mice, compared to the qq and bb mice. (C) Total anti-CII antibody concentration was high in qb mice, but qq and bb mice had similar antibody concentration. Both male and female mice of 8 to 12 weeks old were used. Number of mice used qq (n=8), bb (n=9) and qb (n=17). Mean score bb vs. qb p=0.0244(*) and incidence qq vs. qb p=0.0315(*). But after day 52 there is significant difference in disease severity between qq vs. bb (p=0.0020, **) and qq vs. qb (p=0.0095, **). Error bars indicate the mean arthritis score variance on the day in the mice groups.
<table>
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<th>S.No</th>
<th>Microsatellite markers</th>
<th>Sequence</th>
<th>Peak range (qq-bb)</th>
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<td>5’-TTC CTG TGG CGG CCT TAT CAG-3’</td>
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<td>D17 Mit 24 R</td>
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Table 1. Microsatellite markers used and their forward and reverse sequences are shown in the table. B6N.Q 10N1i mice were genotyped to find their MHC haplotype (qq, bb or qb). The value of the peak range is used to identify the haplotype of the mice. In Nds3, 47 and 24 markers the peaks at 113, 220 and 124 base pairs corresponds to the qq haplotype respectively. Similarly, peaks at 135, 235 and 135 base pairs correspond to bb haplotype. If the DNA sample contains both the peaks in any of the marker, then the sample is considered to be heterozygous having both q and b haplotypes.

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8. References

38. Nandakumar, K. S. & Holmdahl, R. Efficient promotion of collagen antibody induced arthritis (CAIA) using four monoclonal antibodies specific for the major epitopes


