

Activation of the Inflammasome in Gout

Background

Gout is an auto-inflammatory arthritis that can be triggered by the sedimentation of monosodium urate crystals in articular joints and is characterized by severe pain, swelling, redness and stiffness in the affected joints. The overall prevalence of gout in the U.S. was determined to be 41 in 1000 (1). Men are at a higher risk than women for gout, and the risk increases with age for both males and females (1). The initiation and development of gout is not completely understood. Diagnostically, serum uric acid content is measured in symptomatic patients and lowering uric acid levels is one of the therapeutic goals in the management of this disease. Although hyperuricemia can promote the development of gout by increasing the likelihood of urate crystal deposition, hyperuricemia *per se* does not automatically lead to gout. This is highly suggestive of a variable ability among humans to react to crystal load. Resident macrophages are responsible for the production of potent pro-inflammatory cytokines, IL-1 β and IL-6, as well as chemokines that promote the infiltration of neutrophils during crystal-induced inflammation (2). The molecular mechanism by which macrophages are induced to produce IL-1 β is not completely understood. Recent studies have described the role of inflammasomes in mediating crystal-induced inflammatory responses (3,7).

The inflammasomes are multimolecular platforms that promote the activation of pro-caspase-1, which in turn proteolytically processes pro-IL-1 β into mature IL-1 β . Active inflammasomes are assemblies of NLR (nucleotide-binding oligomerization domain-like receptors) family members, adaptor proteins (TUCAN/CARD-8 or ASC) and pro-caspase-1 (4,5). The NALP3/NLRP3/cryopyrin inflammasome has been reported to be the particular assembly that mediates crystal-induced IL-1 β production (3). Activation of the NALP3 inflammasome is depicted in Figure 1. The NALP3 inflammasome is comprised of NALP3 and adaptor proteins TUCAN/CARD8 and ASC. When activated, the macromolecular complex recruits and activates pro-caspase-1, which then proteolytically cleaves pro-IL β thereby promoting the release of mature IL- β (5).

In 2008, Verma et al. found that a patient with familial periodic fever syndrome and with a high level of serum IL-1 β carry compound SNPs in the NALP3 (Q750K) and the CARD8 (C10X) genes (6). In their population-based study, the frequencies of these alleles are 6.5% and 34%, respectively, and 4% of the population carry both mutations (6). The NALP3 (Q750K) mutation occurs in the LRR (Leucine-rich repeat) domain, the 'sensing' region of NALP3, while the CARD8 (C10X) mutation results in a premature stop codon. It is unclear how the NALP3 mutation leads to a heightened response. Does this mutation lead to a disengagement of an inhibitory component? Does his mutation lead to increased sensitivity to intracellular danger signals? Investigating this mutation using *in vitro* and *in vivo* model systems would give insight on how the NALP3 component of the NALP3 inflammasome reacts to intracellular danger signals. CARD8 has been shown to inhibit caspase-1 (8). Therefore, the loss of CARD8 as a result of the C10X mutation could lead to a heightened caspase-1 activity. Since crystal-induced IL-1 β production utilizes the NALP3 inflammasome, it is possible that these activating mutations also play a role not only in familial periodic fever syndrome but also in the development of gout. The elevated IL-1 β associated with these mutations suggest a hyperactive inflammasome state, therefore, understanding these mutations would eventually provide insight as to how the NALP3 inflammasome is activated by uric acid crystals.

It has been suggested that lysosomal instability leads to the leakage of the lysosomal exopeptidase, cathepsin B, into the cytosol and that cathepsin B is 'sensed' by the components of the inflammasome leading to its immediate assembly (7). The mechanistic role of cathepsin B in the activation of the NALP3 inflammasome is not known. In the NALP3 mutation, does the need for cathepsin B no longer exist? Revealing the direct or indirect action of cathepsin B on the

inflammasome assembly or components thereof is important in elucidating how the danger signals of crystals are communicated to the inflammasome and in understanding inflammasomal dysfunction, which could influence the pathogenesis of gout.

Questions

This proposal centers on the following specific questions: 1) Would the Q750K mutation in NALP3 result in crystal-induced hyper-inflammatory responses *in vitro* and *in vivo*? 2) Would the loss of CARD8 result in elevated IL-1 β secretion and 3) Does cathepsin B directly cleave components of the inflammasome leading to inflammasome activation?

Hypotheses

This proposal presents the following hypotheses: 1) The Q750K mutation in NALP3 would lead to hyper-inflammatory responses, 2) The loss of CARD8 would lead to hyper-inflammatory responses and 3) Cathepsin B would cleave the inhibitory component of the inflammasome.

Experimental Approaches and Alternatives

Aim 1. Preparation of mutant constructs and siRNA. Prepare a NALP3 (Q750K) mutation construct by site directed mutagenesis and introduce mutant DNA or wildtype DNA into a GFP-encoding plasmid. This reagent will be used in Aim 2. Design or commercially obtain siRNA to CARD8. (This will mimic the loss-of-function mutation of CARD8 C10X). This reagent will be used in Aim 3. Prepare the mouse equivalent construct of the NALP3 Q750K mutation and introduce this mutant gene in a retroviral vector encoding a Thy1.1 marker. This reagent will be used in Aim 4.

Aim 2. Determination of the functional consequences of the NALP3 (Q750K) mutation in a monocytic cell line. Introduce GFP-NALP3 mutant construct, GFP-NALP3 wildtype construct or empty vector into the human monocytic cell line, THP1 by transfection. Sort GFP positive monocytes by flow cytometry, culture and induce differentiation into macrophages. Analyze the THP1 transfectants. Confirm GFP-NALP3 protein expression by Western blot of lysates of THP1 cells transfected with GFP-NALP3 mutant or wildtype GFP-NALP3. Stimulate transfected cells with increasing concentration of monosodium urate (MSU) crystals, similar to what Martinon et al. described (3). Stimulate cells for 6 hours. Analyze supernatants for the presence of mature IL-1 β and IL-18 by ELISA. Analyze cell extracts for the presence of pro-IL-1 β and pro-IL-18. Confirm the observed levels of IL-1 β by performing a transwell migration assay in which neutrophils are placed in the upper wells and pre-differentiated and MSU pre-treated THP1 cells in the bottom wells. Measure the degree of neutrophil migration towards the bottom wells. Assess cell extracts of transfected and treated cells for pro-caspase 1 and mature caspase 1 levels by Western blot. By immunoprecipitation, assess the ability of each transfectant to assemble the inflammasome upon stimulation. Using anti-GFP antibodies, pull down GFP and blot the immunoprecipitates with anti-NALP3, anti-CARD8, anti-ASC and anti-pro-caspase-1.

Expectations and Alternatives

The expectation is that cells transfected with NALP3 mutant will show enhanced inflammatory responses compared to wildtype GFP-NALP3 and empty GFP plasmid due to an alteration in the sensing (LRR) region of the molecule making it more sensitive to cytoplasmic signals. If this is

not the case, then perhaps the elevated IL-1 β in a patient with NALP3 (Q750K) plus CARD8 (C10X) mutations is largely due to the loss of CARD8.

If THP1 cells prove to be difficult to transfect, the use of HEK cells would be considered for transfections.

Aim 3. Determination of the functional consequences of the loss of CARD8 in a monocytic cell line. Transfect THP1 cells with siRNA targeting the CARD8 domain or with control siRNA. Confirm the loss of CARD8 expression by Western blot. Stimulate differentiated THP1 cells with increasing concentrations of MSU and assess transfectants as described in AIM 2, except for the immunoprecipitation assay to assess assembly of the inflammasome. For Aim 3, anti-NALP3 antibodies will be used to pull down NALP3 and immunoprecipitates will be blotted with anti-ASC and anti-pro-caspase 1.

Expectations and Alternatives

The expectation is that cells transfected with CARD8 siRNA will show enhanced inflammatory responses compared to cells transfected with control siRNA because CARD8 regulates the self-activation of pro-caspase-1 and without this regulator in the macromolecular complex, pro-caspase-1 autoactivation is uninhibited leading to an elevated pro-IL-1 β processing. If this is not the case, then perhaps another adaptor molecule keeps pro-caspase-1 in check.

If THP1 cells prove to be difficult to transfect, the use of HEK cells would be considered for transfections.

Aim 4. Determination of the functional consequences the NALP3 mutation *in vivo*. Obtain donor NALP3 knockout mice (9) and treat these animals with 5-fluorouracil. Isolate bone marrow (enriched with HSCs) and culture. Transduce stem cells with a NALP3 mutant-encoding retroviral vector or control retroviral vector with a Thy1.1 marker prepared in Aim 1. After 2-3 days in culture, purify Thy1.1 expressing HSCs and propagate for 2-3 days. Intravenously transfer HSCs to lethally irradiated mice. After 6-8 weeks, induce peritonitis by injecting animals with crystals as described by Martinon et al. (3). Measure serum IL-1 β and IL-18 by ELISA. Perform a peritoneal lavage and analyze fluids for PMN recruitment using a neutrophil marker. Derive macrophages from these animals and analyze macrophages for pro-caspase-1 activation by Western blot.

Expectations and Alternatives

Reconstitution of lethally irradiated mice with bone marrow from NALP3 null mice transduced with mutant NALP3 retrovirus will reconstitute the monocytic lineage bearing the mutation in the LRR domain. The expectation is that cells transfected with NALP3 mutant will show enhanced inflammatory responses. It is possible that reconstituted animals will appear sick.

Aim 5. Determination of potential inflammasomal targets of cathepsin B in a monocytic cell line treated with MSU. Incubate THP1 cell cultures (differentiated cells) in media containing MSU plus the cathepsin B inhibitor, CA074, or control vehicle. Lyse the treated cells and subject cell lysates to 2D gel electrophoresis. Perform spot analysis and compare spots between inhibitor treated and untreated samples. Purify differentially displayed spots and analyze by mass spectrometry. Determine which of these protein or protein fragments are derived from components of the NALP3 inflammasome.

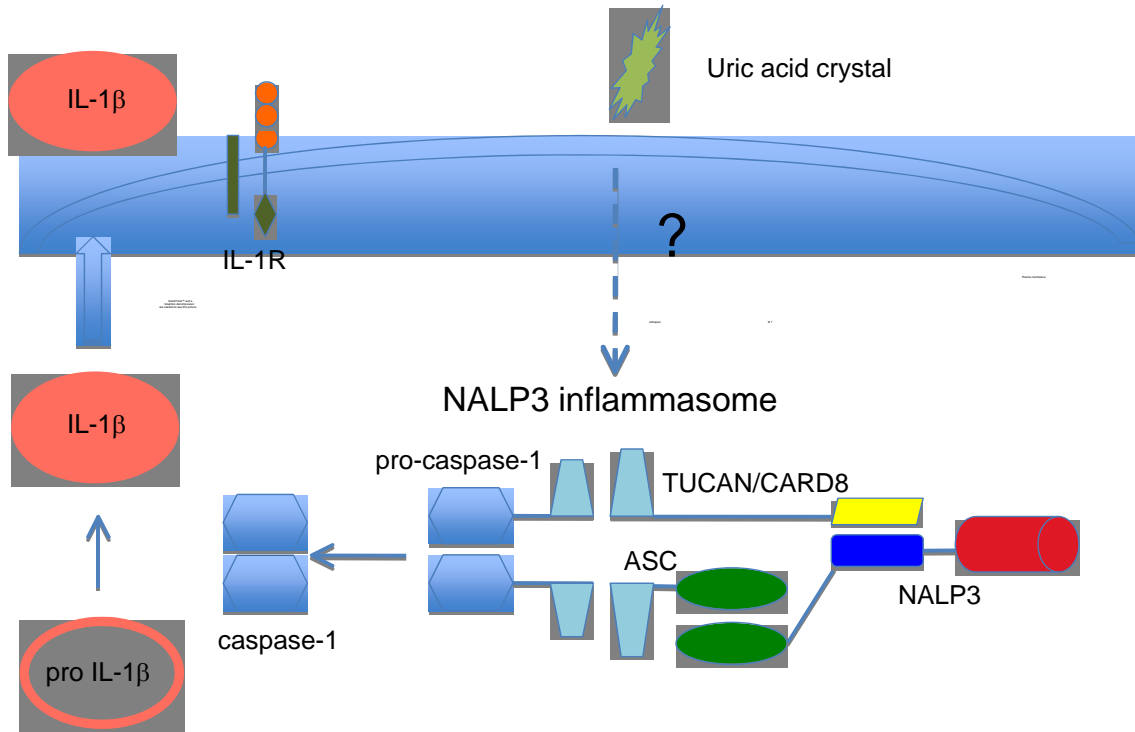
Expectations and Alternatives

Alternatively, if a large number of spots is obtained and proves to be difficult to analyze, an isolated system can be designed. Purified NALP3, ASC and CARD8 can be directly subjected to cathepsin B activity in the presence or absence of cathepsin B inhibitor and the resulting protein fragments can be analyzed by mass spectrometry.

Collaboration

The HHMI-sponsored Med-Into-Grad program is a unique and excellent program that aims to narrow the gap between basic science research and medicine. This program opened the doors for me to an arena of rheumatologic diseases and their clinical management. This program also introduced me to faculty members who may be interested in the outcome of this proposal. My graduate research experience thus far has allowed me to learn and master some of the laboratory techniques that this proposal calls for. Nevertheless, feedback from Rheumatology faculty members at UCSD School of Medicine will be extremely important for this proposal.

Figure 1



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Figure 1. Schematic representation of the assembly of the NALP3 inflammasome. The NALP3 inflammasome is comprised of NALP3, a nucleotide-binding oligomerization domain-like receptor and adaptor proteins TUCAN/CARD-8 and ASC. Upon activation, the macromolecular complex recruits and activates pro-caspase-1, which then proteolytically cleaves pro-IL β thereby promoting the release of mature IL- β .

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