Cutting Edge: Processing of Oxidized Peptides in Macrophages Regulates T Cell Activation and Development of Autoimmune Arthritis

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APCs are known to produce NADPH oxidase (NOX) 2–derived reactive oxygen species; however, whether and how NOX2-mediated oxidation affects redox-sensitive immunogenic peptides remains elusive. In this study, we investigated a major immunogenic peptide in glucose-6-phosphate isomerase (G6PI), a potential autoantigen in rheumatoid arthritis, which can form internal disulfide bonds. Ag presentation assays showed that presentation of this G6PI peptide was more efficient in NOX2-deficient (Ncf1mut/hmut) mutant mice, compared with wild-type controls. IFN-γ–inducible lysosomal thiol reductase (GILT), which facilitates disulfide bond–containing Ag processing, was found to be upregulated in macrophages from Ncf1 mutant mice. Ncf1 mutant mice exhibited more severe G6PI peptide-induced arthritis, which was accompanied by the increased GILT expression in macrophages and enhanced Ag-specific T cell responses. Our results show that NOX2-dependent processing of the redox-sensitive autoantigens by APCs modify T cell activity and development of autoimmune arthritis. *The Journal of Immunology, 2017, 199: 3937–3942.

The central interaction of cell-mediated adaptive immunity is between CD8 T cells and MHC molecule loaded with Ag peptides. To produce immunogenic peptides in APCs, the Ag processing is one of the key steps, which normally involves proteolysis and disulfide bond reduction. Of interest is the induced burst of reactive oxygen species (ROS) mediated by the NADPH oxidase (NOX) 2 complex expressed in APCs, which regulate Ag processing with quite divergent observations, ranging from a change in phagosomal pH to effects on cysteine oxidation (1, 2). In dendritic cells (DCs), NOX2 mediates sustained production of low levels of ROS and controls phagosomes pH to maintain alkalization of the phagosomal lumen, which facilitates cross-presentation (3). In contrast, in macrophages, NOX2 has been suggested to inhibit phagosomal proteolysis through reversible oxidative inactivation of local cysteine cathepsins to modulate the local redox environment (4). Moreover, it has been shown that NOX2 activity significantly compromises the ability of the phagosomes to reduce disulfides both in macrophages and in DCs (4, 5). Reduction of disulfide bonds in Ag is an important step in MHC class II–restricted processing, and multiple epitopes require disulfide bond reduction for efficient stimulation of T cells (6, 7).

In addition to NOX2, IFN-γ–inducible lysosomal thiol reductase (GILT) has been identified as the only reductase known to be localized in lysosomes (7). Accumulating data have indicated that GILT is crucial for processing Ags with disulfide bond(s), including the model Ag hen egg lysosome, viral glycoprotein, and melanoma Ag tyrosinase-related protein 1 (7–9). Moreover, GILT increases the expression and stability of a mitochondrial enzyme, superoxide dismutase 2, to regulate the cellular redox state, and maintains the activity of cysteine proteases in phagosomes of IL-4–activated macrophages, particularly in the absence of high NOX2 activity (10, 11).

However, it remains unclear whether NOX2-mediated oxidation can affect the processing of redox-sensitive antigenic peptides. To clarify this question, we used a peptide named as hGPlc-c from glucose-6-phosphate isomerase (G6PI) protein, a ubiquitously expressed glycolytic enzyme and a potential autoantigen in rheumatoid arthritis (12). It has been shown that this peptide can induce arthritis, and the disease severity is largely affected by NOX2 activity (13).

In this study, we found that hGPlc-c peptide can easily form intradisulfide and interdisulfide bonds, which need to be processed before being presented, and that NOX2 deficiency...
facilitated this peptide processing at least partially because of GILT expression. Moreover, the arthritis severity correlated with elevated GILT expression in macrophages together with enhanced Ag-specific T cell response. Collectively, our findings clearly show that NOX2-dependent processing of the redox-sensitive Ag in macrophages modifies T cell response and arthritis development.

Materials and Methods

Mice

B10.Q.Ncf1+/+ mice with a point mutation m1J in the Ncf1 gene and MN. Ncf1-/- mice carrying MN transgene encode functional Ncf1 under the control of the hCD68 promoter in B10.Q.Ncf1+/+ mice, and age-sex-matched B10.Q mice were used (14). All animal experiments were approved by the Stockholm ethical committee, Sweden (license no. N49012 and N35/16).

Peptides

All peptides were synthesized from Biomatik (Wilmington, DE), including hGPIc-c (NH2-IWYNCFCGETHAML-OH), mGPlc-c (NH2-IWYNCGETHALL-OH), and hGPls-s (NH2-IWYNFGSFETHAML-OH), as well as hGPlc-c with different conjugations.

Arthritis model and evaluation

Arthritis was induced by hGPIc-c peptide, and disease development was monitored using a macroscopic scoring system (13). Serum Abs against hGPIc-c or hGPls-s were detected by ELISA. At the end of the experiment, mice were sacrificed, and hind paws were collected for the section and followed by H&E and Safranin O staining.

HPLC-MS

hGPlc-c or hGPls-s peptide was incubated at 37°C for 24 h with or without immobilized TCEP disulfide reducing gel (Thermo Scientific). Then 30 µl of each peptide solution was subjected to analysis by analytical reversed-phase HPLC using a GraceVydac, MS C18-column with a gradient of ACN/H2O from 10 to 90% ACN in 30 min. The identities of the peptides were confirmed with MALDI-TOF-MS (Voyager PRO; Applied Biosystems) using α-cyano-4-hydroxycinnamic acid as a matrix with detection in the positive mode.

Molecular modeling

The homology model of MHC class II Aβ in complex with hGPIc-c or hGPls-s was constructed using a previously reported model (15), and the potential core binding residues (INCFGCETH) of hGPIc-c were predicted using Consensus algorithm (16). For modeling the hGPIc-c in the binding groove of Aβ2, several residues were modeled by the Swrl4 and R. Holmdahl, unpublished observations), several residues were modeled by different conjugations.

Flow cytometry analysis

Unless otherwise indicated, all Abs for staining were obtained from BioLegend (San Diego, CA). Live/dead cells were distinguished by near-IR dead cell stain (Molecular Probes: Life Technologies) before surface staining. Following cell fixation and permeabilization (Cytofix/Cytoperm; BD Biosciences), CD68 and GILT (PE, clone Map.mGILT6; BD Pharmingen) were detected intracellularly. Data were collected by LSRII flow cytometer and analyzed by FlowJo software (Tree Star).

Results and Discussion

Disulfide bond formation is critical for immune responses

The hGPlc-c peptide derived from 325–339 of human G6P1 is, so far, the only known single peptide that can induce polyarthritis (13, 21). The induction of arthritis is dramatically enhanced in Ncf1-mutated mice, that is, lacking an inducible ROS response. Because there are two cysteines in hGPIc-c, a contributing explanation could be that the ROS modify the formation of disulfides. To determine the disulfide formation, we performed HPLC followed by MALDI-MS analysis. As shown in Fig. 1A, hGPlc-c was oxidized, where 70% of the resulting peptide mixture contained an intramolecular disulfide and 30% contained two intermolecular disulfides between two peptides with molecular masses of 1800 and 3598 Da, respectively, whereas after 24-h TCEP treatment, hGPlc-c peptide was fully reduced with a molecular mass of 1802 Da. In contrast, the hGPls-s peptide, where the two cysteines are changed to two serines, shows no difference with or without TCEP treatment with molecular mass of 1769 Da (Fig. 1B). Thus, our results indicate that the two cysteines in hGPlc-c are prone to form disulfides upon oxidation.

Next, we sought to explore whether disulfides are critical for immune response and arthritis development. As shown in Fig. 1C and 1D, hGPlc-c could induce arthritis in both B10. Q and B10Q.Ncf1+/+ mice, whereas hGPls-s immunization did not induce disease at all. Consistently, hGPlc-c immunization could provoke higher T cell response (corresponding to immunized peptide) based on IFN-γ–secreting T cells in lymphoid organs, as well as higher serum Ab production on both B10.Q (Fig. 1E, 1G) and B10Q.Ncf1+/+ mice (Fig. 1F, 1H). As expected, there was almost no T and B cell response after hGPls-s immunization. Notably, serum Ab response from hGPlc-c immunization was higher with hGPls-s coating, suggesting that the drop in the arthritogenic capacity with hGPls-s immunization is not due to B cell recognition of a disulfide bridge.

Cysteine is not critical for TCR recognition

Then we sought to investigate whether the deficit T cell response of hGPIs-s is due to MHC II processing and presentation or TCR recognition. By aligning with the major collagen II–derived peptide from 259 to 273 (CII259–273) associated with the development of collagen-induced arthritis, we found that the hGPlc-c peptide has identical anchoring residues with Aβ in P1, P4, and P7. By molecular modeling, we found that the cysteine at position 330 is in the P3 position, which is likely not critical for either TCR or Aβ binding, whereas cysteine at position 333 is in the P6 position, which could to a minor extent interact with Aβ (Fig. 2A). We produced a T cell
A disulfide bond–containing peptide processing is regulated by NOX

Although we have ruled out that both cysteines in hGPlc-c peptide are needed for T and BCR recognition, disulfide formation as such in hGPlc-c is critical for immune response and arthritis development. Notably, Ncf1 deficiency allows more severe disease and enhanced T cell response than wild-type mice, as indicated in Fig. 1. Thus, it is of importance to understand the influence of redox regulation on peptide processing. Live splenocytes, containing B cells, DCs, and macrophages from both B10.Q and B10Q.Ncf1*/* mice, were used to present hGPlc-c and hGPls-s peptides. There was no major difference between the strains when presenting hGPls-s, whereas an increase of IL-2 secretion was observed in cells from B10Q.Ncf1*/* mice, upon stimulation with hGPlc-c (Fig. 3A). To further address whether these two peptides need to be processed before being presented, fixed splenocytes were used instead of live splenocytes. As shown in Fig. 3B, the presentation of hGPlc-c by fixed splenocytes was completely abolished, whereas the hGPls-s could still be presented, albeit with less efficiency as compared with live APCs. Therefore, our results indicate that hGPlc-c needs to be processed before being presented, and lack of intracellular ROS due to the Ncf1 mutation enhances its Ag processing.

To confirm the effect of redox regulation on hGPlc-c peptide processing, we performed in vivo Ag presentation assay to avoid too much manipulation in vitro. We detected an increase of IL-2 level from B10Q.Ncf1*/* mice comparing with B10.Q mice when presenting hGPlc-c peptide, but surprisingly, the IL-2 level was nondetectable when presenting hGPls-s peptide, probably because of low stability in circulation (Fig. 3C). In addition, we compared Ag uptake ability of different APCs, including B220+, CD11b+, and CD11c+ macrophages from both B10.Q and B10Q.Ncf1*/* mice regarding Ag uptake, but no major difference between the strains when presenting hGPls-s, whereas an increase of IL-2 secretion was observed in cells from B10Q.Ncf1*/* mice, upon stimulation with hGPlc-c (Fig. 3D). The reasonable explanation could be because of the static acidic environment in macrophages, which favors the reduction of disulfides (22).

**GILT enhances hGPlc-c peptide processing in Ncf1-deficient macrophages**

We have already demonstrated that Ncf1-dependent ROS production by macrophages limits their capacity to activate T cells (14, 23). GILT is the only reductase known to be localized in lysosome and exerting intracellular reducing activity (7), which could provide an explanation for the ROS-mediated effect on processing of cysteine-containing peptides. As shown in Fig. 4A and 4B, ROS production by BMMs was

**hybridoma named G5 and by stimulating G5 with various peptides, including both oxidized and reduced form of hGPlc-c/hGPls-s, we found that there was no significant difference of activation strength between these peptides, whereas there was only around one-tenth of a response toward hGPlc-c/hGPls-s, we found that there was no significant difference of activation strength between these peptides, whereas there was only around one-tenth of a response toward hGPlc-c**
FIGURE 3. Disulfide bond–containing peptide processing is regulated by NOX. The activation of G5 cells with live splenocytes (A) \( (n = 11 \text{ for each}) \) or fixed splenocytes (B) \( (n = 8 \text{ for each}) \) loaded with hGPlc-c or hGPls-s (10 \( \mu \text{M} \)) in vitro or with splenocytes preloaded with peptides in vivo (C) \( (n = 9 \text{ and } 6 \text{ from left}) \) was determined by IL-2 secretion in both B10.Q (empty circles) and B10Q.Ncf1*/* mice (filled squares). Bars are shown as mean \( \pm \text{SEM} \), and significance was determined by unpaired \( t \)-test. Uptake of FAM-labeled hGPlc-c by different cell populations was measured by flow cytometry analysis; bars are shown as mean \( \pm \text{SEM} \) of mean fluorescence intensity (D) \( (n = 3 \text{ for each}) \). **p < 0.0001.

FIGURE 4. GILT enhances hGPlc-c peptide processing. Total ROS production response to PMA by BMMs was measured by chemiluminescence assay. Data are shown (mean \( \pm \text{SEM} \)) from B10.Q (open circles), B10Q.Ncf1*/* (filled squares), and MN.Ncf1*/* (filled triangles, \( n = 6 \text{ for each} \)) (A and B). Lysates of BMMs were subjected to SDS-PAGE analysis, and STAT1, GILT, and \( \beta \)-actin as loading control were blotted on the same membrane (C). The Ag presentation capability of BMMs from naive B10.Q, B10Q.Ncf1*/*, and MN.Ncf1*/* mice \( (n = 8 \text{ for each}) \) were determined by Ag presentation assay, and corresponding IL-2 level (mean \( \pm \text{SEM} \)) was shown (D). BMMs from naive B10Q.Ncf1*/* mice were silenced with GILT siRNA (S82440, filled squares, \( n = 6 \text{ or negative control (Neg; open circles, } n = 6 \text{) and subjected to Ag presentation assay, and IL-2 secretion (mean \( \pm \text{SEM} \)) was detected by ELISA (E). Significance was determined either by one-way ANOVA or unpaired Student \( t \)-test. *p < 0.05, **p < 0.01, ****p < 0.0001.

Ncf1-sufficient macrophages ameliorate peptide-induced arthritis

To investigate whether Ncf1-sufficient macrophages can revert arthritis development in hGPlc-c–induced arthritis (GIA), we included MN.Ncf1*/* mice. As shown in Fig. 5A and 5B, all B10Q.Ncf1*/* mice experienced severe arthritis, whereas only 40% of the MN.Ncf1*/* mice experienced development of disease but much milder. In addition, the disease development was not fully resolved in B10Q.Ncf1*/* mice until termination of the experiment, whereas the disease development in both B10.Q and MN.Ncf1*/* mice was naturally resolved around 30 d. Moreover, arthritic joints from B10Q.Ncf1*/* mice were marked by synovial infiltration, hyperplasia, cartilage destruction, and bone erosion, whereas joints of both B10.Q and MN.Ncf1*/* mice were largely intact with few infiltrates on day 60 following immunization (Fig. 5C). Because GIA is a T cell–dependent arthritis model, T cell recall assay was performed to detect Ag-specific or autoreactive T cells in lymphoid organs following day 10 immunization. As shown in Fig. 5D, upon restimulation with hGPlc-c and mGPlc-c peptide, numbers of spots of both IFN-\( \gamma \)–producing T cells and IL-17–producing T cells were dramatically increased in splenocytes from B10Q.Ncf1*/* mice as compared with B10.Q and MN. Ncf1*/* mice. Taken together, Ncf1-sufficient macrophages alleviate the Ag-specific autoreactive T cell response and development of arthritis.

GILT-expressing macrophages are increased in Ncf1-deficient mice in the GIA model

Next, we addressed the role of macrophages in the GIA model. We found that splenic macrophages (CD11b+CD68+) were 2- to 3-fold increased in B10Q.Ncf1*/* as compared with both B10.Q and MN.Ncf1*/* mice, whereas similar frequencies of both DCs (CD11b+CD11c+) and B cells (B220+) were observed in all three strains (Fig. 6A).
Consequence, provide an explanation why lack of ROS could promote certain autoimmune diseases. It will certainly have an importance for the regulation of the immune response to all proteins containing potential internal disulfide bonds like in CXCC motifs. As such, it could have a fundamental importance in how the immune system is regulated. One obvious consequence is a break of tolerance to autoantigenic peptides. Because the GIA is induced mainly by such a peptide from the G6PI protein, it is possible that this is one of the major explanations why this model is more severe in Ncf1-deficient mice. Human diseases are at least as complex as the murine models, and it remains to be shown whether specific redox-regulated peptides could provide an explanation on how redox could regulate both Ag-specific tolerance but also the regulation of the immune response to Ag-specific T cells, which may additionally contribute to the observed enhancement of autoreactive T cell responses and the severe development of arthritis in Ncf1-deficient mice.

In summary, in this article, we have proposed a novel explanation how ROS could regulate T cell activation and, as a consequence, provide an explanation why lack of ROS could promote certain autoimmune diseases. It will certainly have an importance for the regulation of the immune response to all proteins containing potential internal disulfide bonds like in CXCC motifs. As such, it could have a fundamental importance in how the immune system is regulated. One obvious consequence is a break of tolerance to autoantigenic peptides. Because the GIA is induced mainly by such a peptide from the G6PI protein, it is possible that this is one of the major explanations why this model is more severe in Ncf1-deficient mice. Human diseases are at least as complex as the murine models, and it remains to be shown whether specific redox-regulated peptides could provide an explanation on how redox could regulate both Ag-specific tolerance but also the regulation of the immune system.

**Disclosures**

The authors have no financial conflicts of interest.

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