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Differential Expression of NLRP3 among Hematopoietic Cells

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Although the importance of the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in health and disease is well appreciated, a precise characterization of NLRP3 expression is yet undetermined. To this purpose, we generated a knock-in mouse in which the Nlrp3 coding sequence was substituted for the GFP (enhanced GFP [egfp]) gene. In this way, the expression of eGFP is driven by the endogenous regulatory elements of the Nlrp3 gene. In this study, we show that eGFP expression indeed mirrors that of NLRP3. Interestingly, splenic neutrophils, macrophages, and, in particular, monocytes and conventional dendritic cells showed robust eGFP fluorescence, whereas lymphoid subsets, eosinophils, and plasmacytoid dendritic cells showed negligible eGFP levels. NLRP3 expression was highly inducible in macrophages, both by MyD88- and Trif-dependent pathways. In vivo, when mice were challenged with diverse inflammatory stimuli, differences in both the number of eGFP-expressing cells and fluorescence intensity were observed in the draining lymph node. Thus, NLRP3 levels at the site of adaptive response initiation are controlled by recruitment of NLRP3-expressing cells and by NLRP3 induction. The Journal of Immunology, 2011, 186: 2529–2534.

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The online version of this article contains supplemental material.
Abbreviations used in this article: BMDM, bone marrow-derived macrophage; cDC, conventional dendritic cell; DC, dendritic cell; eGFP, enhanced GFP; Het, heterogeneous; HPRT, hypoxanthine phosphoribosyltransferase; KI, knock-in; LN, lymph node; MDP, muramyl dipeptide; NLR, NOD-like receptor; NLRP3, NOD-like receptor family, pyrin domain containing 3; pDC, plasmacytoid dendritic cell; PGN, peptidoglycan; poly(I:C), polyinosinic-polycytidylic acid; WT, wild-type.
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Materials and Methods

Mice

Six- to 12-wk-old (unless otherwise indicated) C57BL/6, eGFP KI (15), Trif<sup>−/−</sup> (16), and MyD88<sup>−/−</sup> (17) mice were housed at the animal facility of the University of Lausanne. All animal procedures were conducted in compliance with Swiss federal legislation for animal experimentation.

Cell preparations

Bone marrow-derived macrophages (BMDMs) were generated as previously described (18). Liver was harvested from PBS-perfused animals. Blood was collected from the tail vein. Spleen cell suspensions were prepared by grinding the organs through mesh filters. CD11b<sup>+</sup> splenocytes were isolated by MACS (Miltenyi Biotec), by using a two-step labeling procedure. First, splenocytes were incubated with allopurinol-labeled anti-CD11b (M1/70) Ab (eBioscience). Next, anti-allophycocyanin microbeads were used to magnetically label and select CD11b<sup>+</sup> cells (Miltenyi Biotec). For spleen dendritic cell (DC) analysis, cell suspensions were obtained after collagenase digestion as described in detail elsewhere (19).

Quantitative PCR

Murine tissue panel was purchased from Clontech. Total RNA extraction from macrophages was done using an RNeasy kit (Qiagen) with on-column DNase digestion according to the manufacturer’s instructions. RNA concentration was measured by a NanoDrop 1000 spectrophotometer (Thermo Scientific). cDNA synthesis was performed using SuperScript II reverse transcriptase on a Mastercycler gradient (Eppendorf). Gene expression was quantified using a LightCycler 480 (Roche) with SYBR Green (Roche). Expression was normalized relative to the control gene hypoxanthine phosphoribosyltransferase (Hprt) using the Roche LightCycler advanced relative quantification software. Primer sequences are available upon request.

In vitro stimulation experiments

The medium and the culture conditions used are described elsewhere (18). BMDM stimulations were done using 20 ng/ml ultrapure LPS, 2 g/ml muramyl dipeptide (MDP; Bachem), or 150 g/ml CpG 1826 (5′-CCATGGACTTCCGTACGTT-3′) (Microsynth). Inflammasome activation was performed with 3 μM nigericin (Sigma-Aldrich) or 300 g/ml alum (Pierce) for 150 min.

In vivo stimulations

Mice were injected s.c. in the footpad with PBS, 500 ng mouse TNF (Aлексис), 50 μg CpG 1826 (Microsynth), or 150 μg MDP (Bachem) in a volume of 30 μl. Sixteen hours later, mice were sacrificed and popliteal lymph node (LN) cells were analyzed by FACS.

Immunoblot analysis

Rabbit polyclonal Ab to β-actin was purchased from Abcam, and the monoclonal anti-NLRP3 (NALP3) Ab was from AdipoGen (Cryo-2).

Abs and flow cytometry

Cells were preincubated with anti-mouse CD16/32 (2.4G2) culture supernatant to block FcRs, then washed and surface-stained using combinations of the following mAbs: anti-CD8 (Ly-2), anti-CD3e (145-2C11), anti-CD4 (L3T4), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (N20), anti-F4/80 (BM8), anti–PDCA-1 (eBio927), and anti-NK1.1 (PK136) were purchased from eBioscience; and anti–Ly-6G (AL-21), and anti–Ly-6C (AL-26) were purchased from BD Biosciences. Allopurinol-Cy7–labeled streptavidin was purchased from eBioscience. All of the Abs were labeled with an appropriate combination of fluorophore. Propidium iodide (Sigma-Aldrich) was used to exclude dead cells from in vitro cultures. Samples were analyzed on either FACSCalibur or FACS-Canto flow cytometers (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

For in vitro experiments, statistical analyses were calculated with an unpaired Student t test (GraphPad Prism version 5.0; GraphPad Software). For in vivo experiments, differences between stimulated groups and reference group were calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism version 5.0).

Results

eGFP fluorescence correlates with NLRP3 expression

To reliably use eGFP fluorescence as a measure of NLRP3 expression in the eGFP KI cells (Supplemental Fig. 1), we first confirmed that the level of the reporter mirrors that of NLRP3 protein expression. Because it is known that NLRP3 expression is strongly induced upon LPS treatment (9), we stimulated BMDMs from eGFP KI, heterozygous (Het), and wild-type (WT) mice with LPS. Over time, the levels of Nlrp3 mRNA expression in WT and Het BMDMs were coherent with those of egfp mRNA in KI and Het BMDMs, showing a peak at 6 h and decreasing overnight (Fig. 1A). Note, however, that the decrease in egfp mRNA had a slightly delayed kinetics than the one of Nlrp3. A clear gene dose effect was observed for Nlrp3 and egfp gene transcripts in Het BMDMs compared with WT or KI BMDMs, respectively.

We next examined whether the expression of egfp would be consistent with NLRP3 also at the protein level. When eGFP fluorescence was determined by FACS, we observed higher basal

**FIGURE 1.** eGFP is a reporter for NLRP3 expression at both mRNA and protein levels. WT, eGFP Het, and KI BMDMs were treated 1.5, 3, and 6 h, and overnight with LPS or left untreated. A, egfp and Nlrp3 mRNA expression, relative to Hprt, was determined by quantitative PCR (qPCR). Means and SDs of technical triplicates are depicted. B, eGFP fluorescence as determined by FACS. Values shown on the right side of the graph refer to the respective geometric mean fluorescence intensities (MFIs). C, NLRP3 protein levels (upper) compared with β-actin loading control (lower) were assessed by Western blot. One representative experiment of three is shown (A–C).
levels of eGFP fluorescence in eGFP KI and Het BMDMs as compared with the WT BMDM controls, with Het BMDMs displaying an intermediate level between WT and KI BMDMs (Fig. 1B). The strongest increase in fluorescence was observed when stimulating with LPS overnight. As expected, WT BMDMs showed no significant change in fluorescence following LPS treatment. We then determined NLRP3 protein expression by Western blot. A basal level of NLRP3 protein could be observed in unstimulated WT and Het cells, but not in eGFP KI control cells (Fig. 1C). This was consistent with the basal eGFP fluorescence detected in unstimulated Het and eGFP KI cells. The strongest expression of NLRP3 was observed after a minimum of 6 h stimulation. Het BMDMs showed again an intermediate level of NLRP3 expression, thus confirming a clear gene dose effect of the Nlrp3 and the egfp alleles. In agreement with observations at the mRNA level, eGFP protein also showed slightly delayed kinetics as compared with NLRP3.

Taken together, these results indicate that eGFP fluorescence can be used as a reliable reporter for NLRP3 expression.

Basal NLRP3 levels in BMDMs suffice for inflammasome activation

Next, we sought to understand whether the induction of NLRP3 upon LPS treatment was dependent on MyD88 or Trif activation. For this purpose, we stimulated WT, MyD88<sup>−/−</sup>, or Trif<sup>−/−</sup> BMDMs with LPS (Fig. 2A). Although at reduced potency, cells deficient for either MyD88 or Trif were still able to upregulate NLRP3, suggesting that both TLR 4 signaling pathways triggered by LPS can induce NLRP3 expression. We therefore reasoned that also the Trif-dependent TLR3 agonist poly(I:C) as the MyD88-dependent TLR9 agonist CpG DNA would upregulate NLRP3 expression. Indeed, CpG and poly(I:C) both caused an increase in NLRP3 levels (Fig. 2A). We also stimulated BMDMs with the inflammatory cytokine TNF, with the NOD2 ligand MDP, and with the TLR2 agonist PGN. All of these treatments increased the levels of NLRP3 protein, as shown by Western blot, as mirrored by a raise in the eGFP fluorescence values in KI cells (Fig. 2B, 2C). Taken together, proinflammatory stimuli of diverse nature can induce NLRP3 expression in BMDMs, and in the case of TLR agonists both MyD88- and Trif-dependent signaling pathways can be alternatively used.

Because we observed detectable, albeit weak, NLRP3 expression prior to LPS stimulation, we hypothesized that the NLRP3 inflammasome would be competent for activation without prior stimulation with one of the above-mentioned proinflammatory molecules. To this purpose, we treated cells with two potent NLRP3 activators, nigericin and alum, in the presence or absence of 5 h prior LPS priming. As shown in Fig. 2D, although the extent of caspase-1 activation was strongly enhanced by prior priming with LPS, caspase-1 cleavage was detectable also in LPS unprimed cells. Not surprisingly, pro–IL-1β induction and mature IL-1β secretion were observed exclusively when cells were LPS treated, as pro–IL-1β synthesis is known to be dependent on the priming step.

**NLRP3 is primarily expressed by myeloid cells such as conventional DCs and monocytes**

To determine NLRP3-expressing organs, Nlrp3 mRNA expression was tested in various murine tissues. Nlrp3 was most strongly transcribed in secondary lymphoid organs, namely the spleen and the LNs, and in organs densely populated by immune cells, such as the lung and the liver (Fig. 3A). A few reports also suggested that IL-1 secretion by keratinocytes is NLRP3-dependent (20–22). However, we could detect neither a significant shift in eGFP fluorescence by the eGFP KI keratinocytes nor NLRP3 expression in WT keratinocytes by Western blot (Supplemental Fig. 2). Taken together, our data suggest that NLRP3 is strongly expressed in immune cells. This prompted us to more closely examine eGFP expression within cells of the hematopoietic system.

First, we examined expression of the NLRP3 reporter in splenocytes; eGFP expression was almost exclusively found in cells expressing the myeloid marker CD11b (Fig. 3B). This indicates that within the spleen, the myeloid compartment predominates over the lymphoid compartment for NLRP3 expression. Similar results were obtained when bone marrow, blood, and liver were analyzed (Supplemental Fig. 3). In s.c. LNs and thymus the eGFP reporter was expressed by a small percentage of the few CD11b<sup>+</sup> cells, suggesting that NLRP3 is poorly expressed in these lymphoid organs under resting conditions (Supplemental Fig. 3). To
corroborate the eGFP reporter pattern with endogenous NLRP3 expression, we separated CD11b+ from CD11b− cells of WT spleen and assessed NLRP3 expression by Western blot (Fig. 3C). This supported a tight correlation between eGFP fluorescence and NLRP3 protein expression also ex vivo.

To more precisely define NLRP3 expression in cell types of the hematopoietic system, we analyzed the eGFP fluorescence in specific splenic subpopulations (Fig. 3D). T cells, B cells, and NK cells of eGFP KI mice showed no or a minimal increase of eGFP fluorescence compared with WT control mice, further corroborating that NLRP3 is barely expressed by lymphoid cells under resting conditions. Plasmacytoid DCs (pDCs) also showed negligible eGFP fluorescence. In contrast, splenic conventional DCs (cDCs), Ly6Chigh monocytes, and macrophages all displayed a very clear shift between WT and eGFP KI. Interestingly, in the granulocyte compartment, eosinophils did virtually not express eGFP whereas neutrophils did. These results thus indicate that NLRP3 is expressed, among hematopoietic cells and under resting conditions, by myeloid cells and most strongly by cDCs, monocytes, and, to a lesser extent, by macrophages and neutrophils.

**Inflammatory cells with increased NLRP3 expression are recruited to inflamed LNs**

Finally, to address the question of whether NLRP3 expression is also increased by proinflammatory stimuli in vivo, we s.c. injected MDP, TNF, CpG, or saline only in the footpad of eGFP KI and WT mice. Popliteal LNs were harvested 16 h postinjection and eGFP-expressing cells were examined. We identified by FACS the fluorescent cells in the eGFP KI LNs as cDCs, monocytes, and neutrophils (Fig. 4). Although MDP, TNF, and CpG all shared the ability to increase LN cellularity (Supplemental Fig. 4A), eGFP-expressing cDCs, monocytes, and neutrophils were most strongly recruited to the LN draining the site of TNF and CpG injection. Additionally, at a single cell level, monocytes and neutrophils strongly augmented their eGFP emission in response to these two stimuli, suggesting Nlrp3 promoter activation. In contrast, the eGFP fluorescence in cDCs recovered from untreated or treated mice was similar, suggesting that cDCs do not further upregulate...
NLRP3 expression in an inflammatory environment. Importantly, for all three cell types, the intensity of eGFP was higher compared with the emission by WT cells, which remained constant upon the different inflammatory conditions, indicating the specificity of the fluorescence detected (Supplemental Fig. 4B).

In conclusion, these data indicate that augmented expression of NLRP3 in draining LNs is achieved by two mechanisms; that is, NLRP3 reporter-expressing cells are recruited to the LN, and also individual cells upregulate expression of the NLRP3 reporter.

Discussion

The NLRP3 inflammasome is a pivotal host platform for the sensing of endogenous and exogenous danger and for the subsequent orchestration of inflammatory responses. Despite this central role, its expression is still poorly characterized. We thus generated a reporter mouse by substituting the coding sequence for Nlrp3 with the egfp coding sequence. By using BMDMs, we first demonstrated that eGFP regulation indeed mirrored that of NLRP3. This was further confirmed in splenocytes, where both eGFP of Ki mice and NLRP3 of WT mice were coherently present almost exclusively in myeloid CD11b+ cells, and thus we validated the use of our reporter mouse for interrogating the expression of NLRP3 in specific cellular subsets.

As already described, NLRP3 expression is highly inducible by various TLRs (13). TLRs can signal via two pathways that depend on two different adaptor proteins, MyD88 and Trif. Consistent with what has been suggested by a previous report (13), our results indicate that each pathway can individually upregulate NLRP3. In line with that, TLR4, which triggers both pathways in parallel, can even more strongly augment NLRP3 expression (9, 13, 14). Inducers of NLRP3 expression were, however, not limited to TLRs. TNF and the NOD2 agonist MDP were equally effective, suggesting that NLRP3 is inducible by any pathway that activates the proinflammatory transcription factor NF-kB (9, 13, 14). In agreement with published data, we detected basal levels of NLRP3 in BMDMs also without LPS priming (13). However, in contrast to what was observed for ATP (13), this NLRP3 basal expression supported weak, but detectable, caspase-1 activation upon exposure to nigericin or alum. This difference might be explained by the longer duration of alumn and nigericin stimulation compared with the usual very short stimulations used for ATP.

Longer time courses might allow positive feedback loops to take place and amplify caspase-1 cleavage over time.

Nlrp3 mRNA is predominantly expressed in lymphoid organs and organs highly populated by immune cells (10). Consistent with previous publications and with the widespread use of bone marrow-derived DCs for studying the NLRP3 inflammasome, we found that splenic cDCs show very strong NLRP3 expression (3, 10, 14). In contrast to cDCs, pDCs presented negligible NLRP3 reporter levels. This compartmentalization of NLRP3 expression among DCs might favor the local secretion of IL-1β by activated tissue-resident cDCs, while avoiding more systemic production of this cytokine by pDCs, which are mainly circulating cells. Splenic monocytes also showed very high eGFP expression, consistent with the common use of monocytes as a model for NLRP3-expressing cells (9, 15), whereas macrophages presented modest reporter expression. This observation might suggest that monocytes recently recruited to the site of inflammation could activate the inflammasome more efficiently than do fully differentiated macrophages, which may need an additional stimulation provided, for example, by TLRs, to re-express high NLRP3 levels. Eosinophils did not express NLRP3 reporter, while in agreement with previous observations, neutrophils showed a clear expression (10, 11). Our results also indicate that NLRP3 reporter is barely expressed by splenic lymphoid B, T, and NK cells, an observation that diverges with some previous publications (10, 11), but which may be due to differences in expression profiles between mice and humans or differences in T cell activation status.

Primary murine keratinocytes appear not to express NLRP3; we could not detect NLRP3 by Western blot in WT or eGFP fluorescence in KI cells. Importantly, previous literature showing basal NLRP3 protein expression was based on studies with human keratinocytes. It is therefore possible that expression of NLRP3 diverges in keratinocytes from humans and mice, or that NLRP3 expression requires to be induced in these cells.

Finally, we compared the in vivo effects of different inflammatory stimuli on NLRP3 expression in the draining LNs. Whereas the dose of MDP used was a poor stimulator, TNF and CpG were potent inducers of eGFP expression among cells of the popliteal LNs. These two stimuli not only recruited higher numbers of cDCs, monocytes, and neutrophils to the draining LN, but also efficiently upregulated NLRP3 reporter expression in neutrophils and monocytes. Interestingly, eGFP fluorescence in cDCs remained unchanged by challenge, suggesting that this cell type already expresses NLRP3 at a maximal level. Given the importance of the NLRP3 concentration in the efficacy of inflammasome activation (13), it seems likely that NLRP3 levels are tightly regulated to avoid spontaneous activation leading to chronic inflammation.

Mounting evidence supports a crucial role for the NLRP3 inflammasome in infectious disease control and in the priming of T cell responses, as exemplified by the discovery that the vaccine adjuvant alum activates the inflammasome and the function of the NLRP3 inflammasome in generating antimonial immunity (3–7, 23). Thus, a detailed knowledge of the regulation of NLRP3 expression is crucial to better understand immune system function and regulation. We anticipate that the NLRP3-eGFP KI mouse we have characterized in this study will enable such future studies.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. *egfp* fluorescent reporter gene replaces *Nlrp3* and is controlled by its promoter. An eGFP cassette was inserted in frame with the ATG of exon 2, the first coding exon of *Nlrp3* gene, completely replacing *Nlrp3* coding sequence downstream of the ATG with the *egfp* gene. In this way, the KI strategy as much as possible retains the endogenous regulatory elements for the NLRP3 gene. In fact, both the proximal promoter and the first intron, which in many genes contain enhancer activity, are left untouched. The eGFP cassette is followed by the SV40 poly(A) tail, resulting in the disruption of the endogenous *Nlrp3* transcript. A selection cassette PGK-neo (a neomycin resistance gene driven by the mouse phosphoglycerate kinase promoter) flanked by two loxP sites was inserted in the intron 2. The neo cassette was deleted by backcrossing the mice with a Cre-expressing deleter strain (C57BL/6).
Supplementary Figure 2. Primary mouse keratinocytes were tested for NLRP3 expression.
Primary adult murine tail epidermal keratinocytes were isolated from WT and KI homozygous adult mice according to standard procedure (Redvers, R. and Kaur. P. 2005. *Epidermal Cells: Methods and Protocols, Methods in Molecular Biology*, Humana, Totowa, NJ). Tail skin was digested overnight in 10mg/ml Dispase II (Roche). After removal of the epidermal sheets and trypsin digestion (0.05% Trypsin/EDTA), the epidermal suspension was filtered through a 100 μm mesh. The cells were cultured in Cnt-57 basal keratinocytes medium (Cellntec) on collagen-coated dishes (Purecol, Nutacon) for 5 days. A, Graph shows eGFP fluorescence in WT and KI homozygous keratinocytes. Values shown on right side of graph refer to the respective eGFP geometric mean fluorescence intensities. B, NLRP3 expression in WT keratinocytes was analyzed by western blot. As a control, the levels of NLRP3 were measured also in BMDMs unprimed or treated 8 hours with LPS.
Supplementary Figure 3. NLRP3 expression correlates with CD11b positivity in different organs.
Organs were harvested from WT, eGFP KI heterozygous and homozygous mice. Cell populations from a pool of subcutaneous lymph nodes, the bone marrow, the thymus, the blood and the liver were FACS-analyzed for eGFP fluorescence based on their CD11b expression (as CD11b low or CD11b high). Graphs show eGFP fluorescence in WT, KI heterozygous and homozygous cell subpopulations. Values shown on right side of graph refer to the respective eGFP geometric mean fluorescence intensities.
Supplementary Figure 4. MDP, TNF and CpG increase the cellularity of the draining LN.

WT and eGFP KI mice were subcutaneously injected in the footpad either with MDP, TNF, CpG, saline only or left untreated, as control. 16 hours post-injection, popliteal LNs were harvested. 

A, The cellularity of the draining LNs in the WT animals as determined by cell counts. B, cDC, monocyte and neutrophil fluorescence in the eGFP channel in the WT animals as measured by FACS. A,B, Data represent mean and s.e.m. of three individual experimental points.