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Short Communication

Rapid translocation of intracellular toll-like receptors depends on endosomal NADPH oxidase

Nadine Müller-Calleja[1,2], Anne Hollerbach[1], Antje Canisius[1], Carolin Orning[1], Susanne Strand[3] and Karl J. Lackner[1]

[1] Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Mainz, Mainz, Germany
[2] Center for Thrombosis and Hemostasis, University Medical Center Mainz, Mainz, Germany
[3] Department of Internal Medicine I, University Medical Center Mainz, Mainz, Germany

Endosomal toll-like receptors (TLRs) must be translocated from the endoplasmic reticulum (ER) to the endosome and proteolytically cleaved within the endosome before they can induce cellular signals. As ligands for these TLRs are also liberated from apoptotic or necrotic cells, this process is controlled by several mechanisms which shall ensure that there is no inadvertent activation. We have shown previously that antiphospholipid antibodies induce endosomal NADPH-oxidase (NOX) followed by the translocation of TLR7/8 to the endosome. We show now that endosomal NOX is required for the rapid translocation of TLR3, TLR7/8, and TLR9. Deficiency of gp91phox, the catalytic subunit of NOX2, or inhibition of endosomal NOX by the chloride channel blocker niflumic acid both prevent immediate (i.e., within 30 min) translocation of these TLRs as shown by confocal laser scanning microscopy. Under these conditions, the induction of mRNA synthesis for TNF-α and secretion of TNF-α is delayed by approx. 6–9 h. However, maximal expression of TNF-α mRNA or secretion of TNF-α is not significantly reduced. In conclusion, these data add NOX2 as another component involved in the orchestration of cellular responses to ligands of endosomal TLRs.

Keywords: NADPH-oxidase · Rapid TLR translocation · TLRs

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Toll-like receptors (TLRs) are a family of type I transmembrane proteins that play a crucial role in the early detection of pathogen-associated molecular patterns (PAMPs) and the subsequent activation of the innate and adaptive immune response. The N-terminal ectodomain consists of multiple leucine-rich repeat domains involved in ligand binding, whereas the C-terminal cytosolic region mediates the recruitment of signaling components via a Toll/IL-1 receptor domain [1, 2]. Typical adapter molecules of TLRs are MyD88 (myeloid differentiation primary response gene 88) and/or TRIF (Toll/IL-1 receptor domain-containing adaptor, inducing IFN-β) [1].

There are 10 functional TLRs in human (TLR1–10) and 12 in mice (TLR1−9, 11−13). Each of these TLRs appears to recognize distinct PAMPs derived from various microorganisms, including bacteria, viruses, protozoa, and fungi [3]. The TLR family includes receptors residing both at the cell surface (TLR1, 2, 4, 5, 6, 10) and intracellularly (TLR3, 7–9, 11–13), highlighting the specialization of receptor subsets for particular tasks [4, 5].

Correspondence: Dr. Nadine Müller-Calleja
e-mail: nadine.mueller-calleja@unimedizin-mainz.de

In resting cells, the majority of the nucleic acid sensing TLRs 3, 7, 8, and 9 are located in the endoplasmic reticulum (ER). Upon stimulation, for example, by their specific ligands, translocation from the ER-pool of TLRs to the endolysosome and proteolytic activation rapidly enhances the response to nucleic acids [6, 7]. This sequestration is one mechanism to prevent cellular responses to host nucleic acids, because host DNA/RNA is usually (but not always) excluded from the intracellular space [6, 8]. Distinction between self and foreign nucleic acids is of great importance as uncontrolled activation and translocation of TLRs (particularly TLR7) can lead to substantial inflammation resulting in tissue damage and autoimmunity [9, 10].

The underlying signal transduction of TLR trafficking from the ER to the endosome which is a necessary requirement for a cellular response to nucleic acids or intracellular protozoan parasites is only partially understood. The chaperone UNC93b1 has been identified as important transport molecule. Very recently, the solute carrier SLC15A4 was identified as another component required for trafficking of TLRs from the ER to endosomes [11]. UNC93b1 deficient cells are unable to translocate the intracellular TLRs from the ER to the endosome [12–15]. However, it remains unknown that signals initiate translocation and UNC93b1 activation. We have previously shown that antiphospholipid antibodies (aPL) can induce the translocation of TLR7 from the ER to the endosome [16, 17]. This effect of aPL requires the activation of endosomal NADPH oxidase (NOX) and can be prevented by the inhibition of endosomal superoxide generation. Furthermore, in monocytes from NOX2 deficient mice, aPL are unable to induce translocation of TLR7. This observation led us to the hypothesis that the activation of endosomal NOX is required for translocation of intracellular TLRs in general which is needed for their activation by the respective ligands.

Results and discussion

Endosomal NOX2 activity is required for early TLR signaling

To better understand the exact mechanisms leading to the initialization of the translocation process, we compared the ability of the appropriate ligands for the three endosomal TLRs TLR3 (Poly I:C), TLR7/8 (R848), and TLR9 (CpG) to induce proinflammatory pathways in cultured mouse monocytes. The primary readout that we used was the induction of tumor necrosis factor (TNF)-α gene induction and TNF-α cytokine secretion, respectively. In monocytes isolated from C57/BL6 WT mice, all three TLR ligands rapidly induced TNF-α mRNA expression that peaked after 8–10 h (Fig. 1A–C). As expected, TNF-α secretion into the culture media followed a similar but slightly delayed time course (Fig. 1D–F). To study the role of UNC93B1 and NOX2 on early TLR activation, CD115+ monocytes were isolated from the spleens of the respective knock-out mice. As expected, the deficiency of UNC93B1 completely prevents TNF-α mRNA induction (Fig. 1A–C). None of the three specific TLR ligands could induce TNF-α mRNA upregulation or stimulate TNF-α secretion in UNC93B1$^{-/-}$ cells (Fig. 1D–F). Deficiency of gp91phox and the catalytic subunit of NOX2 prevented TNF-α mRNA induction in the first 6–9 h of incubation. However, different from UNC93B1 deficiency, TNF-α mRNA induction and subsequent TNF-α secretion were observed after this delay. TNF-α mRNA induction was slightly reduced (Fig. 1A–C), but TNF-α secreted into the culture media reached similar concentrations as observed in WT monocytes (Fig. 1D–F).

NOX2 deficiency prevents rapid TLR9 translocation

We wondered, if the delayed response to TLR stimulation was related to an effect of NOX2 deficiency on TLR translocation or on proteolytic processing of TLRs or disturbed signal transduction. To this end, TLR9 translocation induced by stimulation with CpG was analyzed by confocal microscopy. In unstimulated monocytes, TLR9 colocalized preferably with the ER-marker calnexin with much less colocalization with the endosomal marker EEA1. After stimulation with CpG for 30 min, TLR9 colocalization with the two markers was reversed, indicating translocation into the endosomal compartment (Fig. 1G). In gp91phox$^{-/-}$, monocytes incubation with CpG had no effect on the localization of TLR9 (Fig. 1H). This confirms that the delay in response to TLR stimulation is caused by delayed translocation of TLRs into the endosome.

Subcellular localization of superoxide production

Next we analyzed, if the subcellular localization of superoxide production could be better defined. The anion channel blocker niflumic acid (NFA) specifically inhibits superoxide production by endosomal NOX2 by the blockade of chloride channel 3 [18, 19]. When the human monocytic cell line MonoMac1 (MM1) is stimulated with the agonists for TLR3, TLR8, and TLR9, there is rapid ROS-production. This can be completely abolished by preincubation with NFA (Supporting Information Fig. 1). Over 6 h incubation, NFA completely prevented induction of TNF-α mRNA and TNF-α secretion by Poly I:C, R848, and CpG (Fig. 2), indistinguishable from the effects of NOX2 deficiency. This finding indicates that endosomal NOX2 is needed for early response of monocytes to ligands for endosomal TLRs.

To further analyze, if the inhibition of endosomal superoxide production by NFA also inhibited the translocation of TLRs into the endosome, we visualized the appropriate TLR molecules via confocal microscopy. For this purpose, we stained MM1 cells with fluorescently labeled antibodies against TLR3, TLR8, TLR9, and a marker for early endosomes (EEA1) or for the ER (calnexin), respectively. In resting cells, there was significant overlap between TLR3, TLR8, or TLR9 and calnexin, whereas there was no colocalization of TLRs and EEA1 (Fig. 3A–C, left panels). When cells were incubated with the appropriate TLR ligands, there was no longer colocalization of TLRs and calnexin. Instead, the colocalization of TLR3, TLR8, or TLR9 and EEA1 could be detected (Fig. 3,

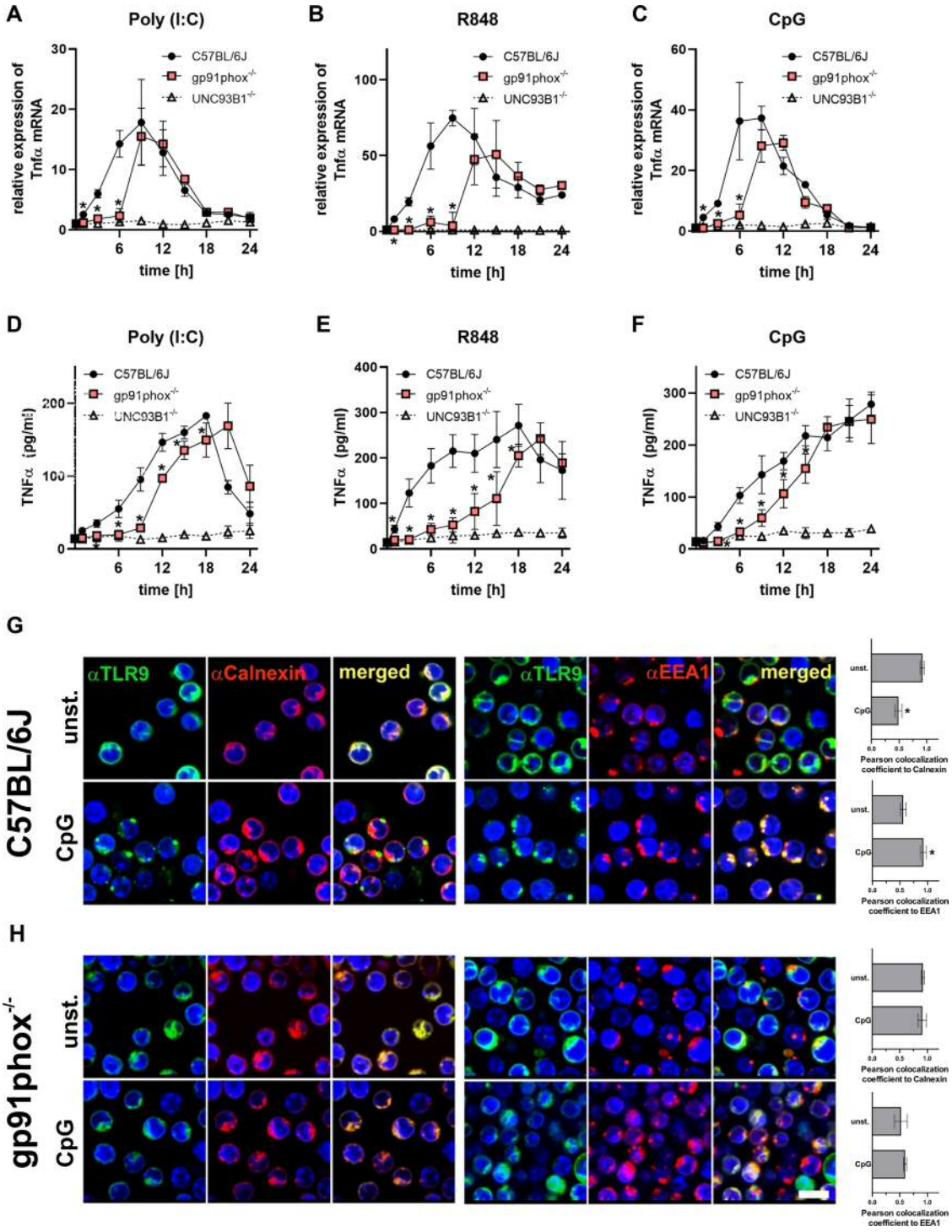

Figure 1. Role of endosomal NADPH-oxidase 2 (NOX2) and UNC93b1 in endosomal TLR signaling. CD115$^+$ splenocytes were isolated from indicated mouse strains and stimulated with TLR3 (A + D), TLR7/8 (B + E), and TLR9 ligands (C + F) for up to 24 h. (A–C) Expression of TNF-α mRNA by TLR ligand-stimulated CD115$^+$ splenocytes relative to unstimulated (A + B) or control ODN-stimulated cells (C). TNF-α secretion was measured by ELISA in the cell culture supernatants at indicated time points (D and E). $n = 6$ biological replicates, $^*p < 0.005$; two-way ANOVA, Sidak's multiple comparisons test. Gp91phox$^{-/-}$ versus WT. (G + H) Representative confocal microscopy images (single confocal plane) showing TLR9 translocation: Monocytes isolated from the spleen of WT C57BL/6J mice (G) or gp91phox$^{-/-}$ mice (H) were stimulated with CpG for 30 min. After fixation, cells were stained with anti-(α)-TLR9 antibodies (green), anti-(α)-calnexin, or anti-(α)-EEA1 (red) and 4,6-diamidino-2-phenylindole (DAPI) (blue) and visualized by confocal laser scanning microscopy. In WT monocytes, stimulation with CpG leads to the colocalization of TLR9 and the endosomal marker EEA1, whereas in monocytes from gp91phox−/− mice, no translocation is observed. Bar = 100 μm. Quantification of colocalization; $n = 3$ regions of interest (ROIs) consisting of at least three cells, generated by three independent experiments. $^*p < 0.0001$; two-tailed *t*-test.

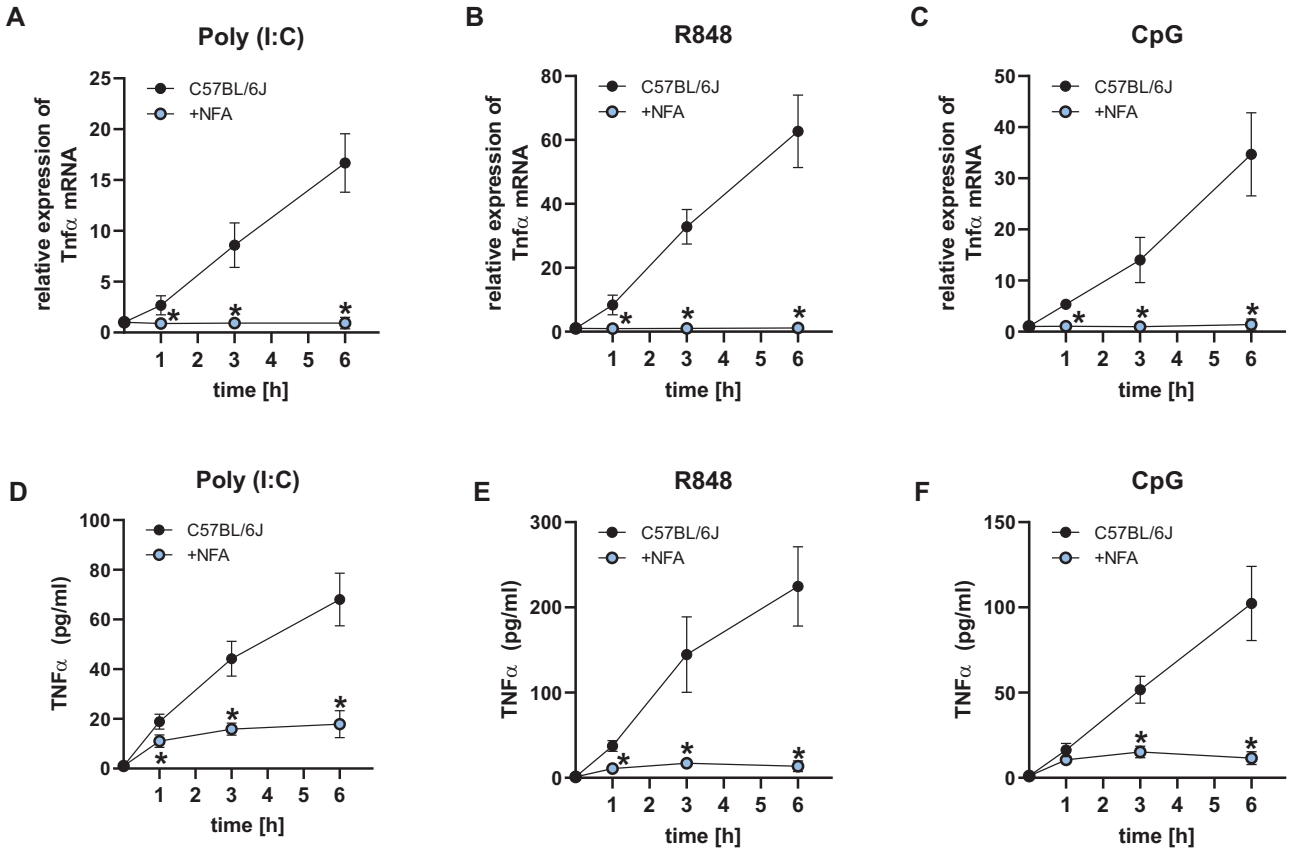

Figure 2. The pharmacological NADPH-oxidase 2 (NOX2) inhibitor niflumic acid (NFA) can block early translocation of intracellular TLRs. CD115$^+$ splenocytes from WT mice were stimulated with TLR3 (A + D), TLR7/8 (B + E), and TLR9 ligands (C + F) for up to 6 h either alone or in the presence of NFA. Thereby, NFA (0.1 mM) was added 15 min before stimulation. (A–C) Expression of TNF-α mRNA by TLR ligand-stimulated CD115$^+$ splenocytes relative to unstimulated (A + B) or control ODN-stimulated cells (C). (D–F) TNF-α secretion was measured by ELISA in the cell culture supernatants at indicated time points. $n = 6$ biological replicates, $^*p < 0.005$; two-way ANOVA, Sidak's multiple comparisons test. NFA versus untreated WT animals.

middle panels). When cells were pretreated with the endosomal NOX inhibitor NFA, translocation from the ER into the endosome was prevented in all three cases (Fig. 3A–C, right panels). This experiment provides further evidence that the rapid translocation of TLR3, TLR7/8, and TLR9 from the ER to the endosome was triggered by endosomal NOX2 activation.

Endosomal ROS determines the timing of TLR signaling

Thus, we describe here that the immediate translocation of endosomal TLRs from the ER to the endosome/lysosome after incubation with the appropriate ligands is delayed for several hours in gp91phox−/− monocytes, that is, in cells deficient in the catalytic subunit of NOX2. A similar effect is observed when monocytes are pretreated with the inhibitor of endosomal NOX NFA. We observed previously that the translocation of TLR7/8 is induced by incubation with antiphospholipid antibodies that activate endosomal NOX after binding to the complex of EPCR/LBPA on the cell surface [16, 17]. This could be also blocked by pretreatment with NFA. These two observations together provide evidence that endosomal NOX is able to effectuate the first step of activation of endosomal TLRs, that is, translocation to the endosome, where they are activated by proteases present in the acidic milieu of these organelles. However, the deficiency of NOX does not lead to complete inability to respond to the respective ligands as is the case for the deficiency of UNC93B1. In the absence of NOX TLR-signaling is observed with a delay of 6–9 h. However, the extent of induction of TNF-α transcription and secretion is similar or only slightly reduced.

Conclusions and perspectives

The observation that endosomal NOX2 is involved in the compartmentalization of nucleic acid sensing TLRs adds another facet to the understanding of this complex process. Decreased NOX-activity has been associated with increased autoimmunity in mice and humans [20–25]. However, NOX2 has many cellular effects besides endosomal signaling [26]. Without in vivo data in appropriate animal models, the overall effect of delayed activation of TLR signaling described here cannot be predicted. This is even more so because the sequestration of TLRs to different

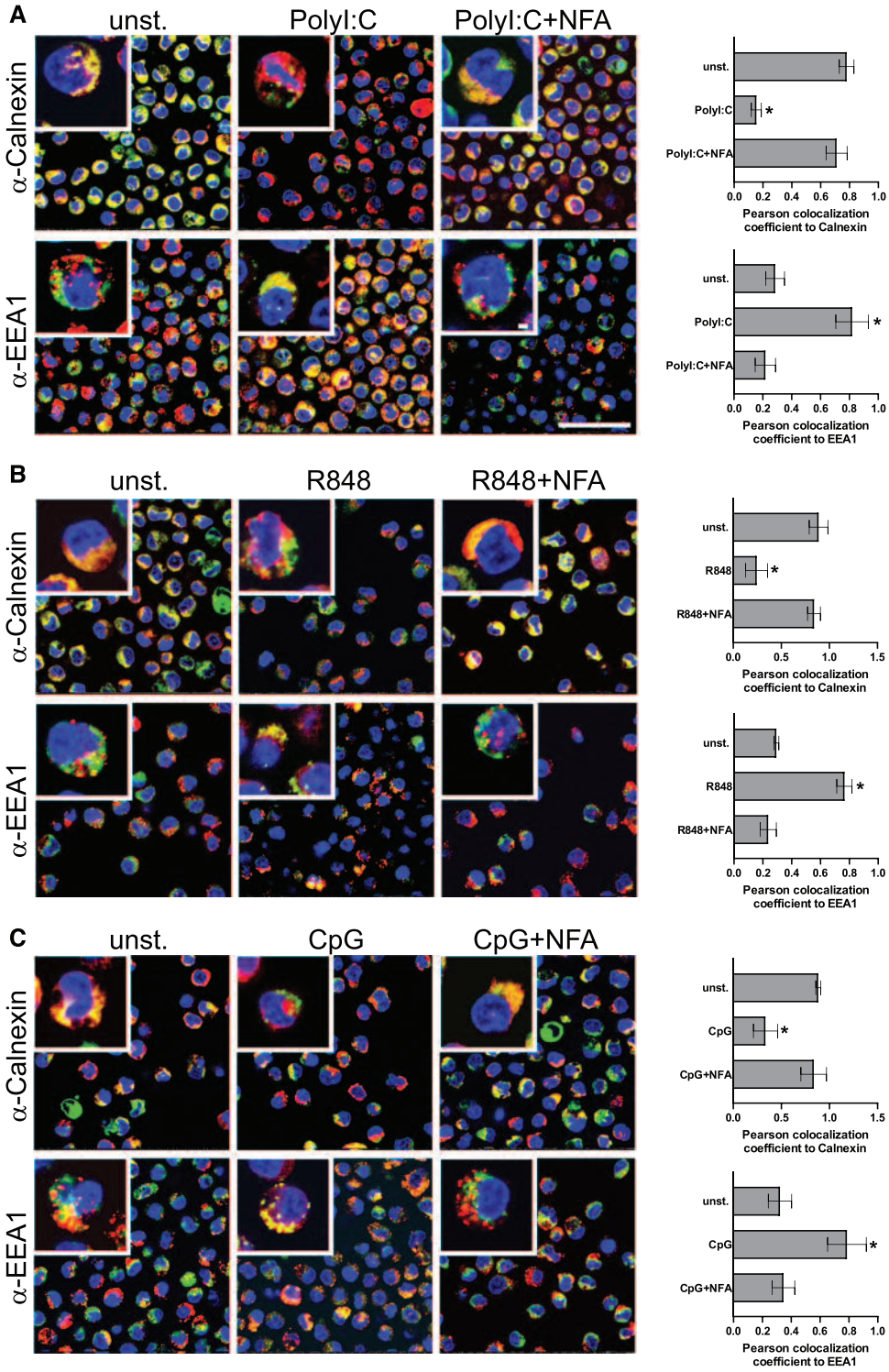

Figure 3. Niflumic acid (NFA) blocks TLR translocation from the ER into the endosome. MonoMac1 cells (MM1) were stimulated for 30 min with (A) Poly I:C, (B) R848, or (C) CpG. NFA (0.1 mM) was added 15 min before stimulation. After fixation, cells were stained with anti-(α)-TLR antibodies (green), anti-(α)-calnexin, or anti-(α)-EEA1 (red) and 4,6-diamidino-2-phenylindole (DAPI) (blue) and visualized by confocal laser scanning microscopy (single confocal plane). In unstimulated cells, intracellular TLRs colocalized with calnexin in the ER. Incubation with Poly I:C, R848, or CpG leads to the translocation of TLR3, TLR8, or TLR9 to the endosome as shown by colocalization with EEA1. Rapid translocation was prevented by the addition of NFA. Bar = 50 μm. Quantification of colocalization; $n = 3$ regions of interest (ROIs) consisting of at least three cells, generated by three independent experiments. $^*p < 0.003$; one-way ANOVA.

cellular compartments is different between cell types, for example, macrophages and B-cells [27]. As we did not analyze B-cells that apparently have more TLRs in the endosome under resting conditions, we do not know, if NOX2 deficiency would have similar effects in this cell type. Our data also suggest that TLRs may be affected differently by NOX2 deficiency, if one compares the delay of peak secretion of TNF-α, it is conceivable that the balance between TLR responses is shifted. If these differences are sufficient to have biological, relevance remains to be determined.

In summary, our data presented here show that endosomal NOX is involved in the timely and efficient orchestration of endosomal TLRs when cells are exposed to their respective ligands. If and how this might affect the efficacy of innate immune responses and the development of autoimmunity remains to be investigated.

Material and methods

Reagents

Reagents were purchased as follows: TLR3 ligand Poly I:C, TLR7/8 ligand R848, and TLR9 ligand CpG C (ODN 2395 and respective ODN control) (all Invivogen, San Diego, CA, USA); NFA (Sigma-Aldrich, St. Louis, MO, USA); mouse TNF-α ELISA kit (R&D Systems, Wiesbaden, Germany); rabbit anti-mouse TLR9 (Acris/Origene, Rockville, MD, USA); rabbit antihuman TLR3 and rabbit antihuman TLR9 (both ThermoFisher, Dreieich, Germany); biotin-labeled monoclonal antihuman TLR8 (Origene, Rockville, MD, USA), anti-Calnexin-Biotin (Novus Biologicals, Centennial, CO, USA); goat anti-EEA1 (Abcam, Cambridge, UK); Streptavidin DyLight 549 and DyLight 488 (both Biozol, Eching, Germany); donkey anti-Goat IgG DyLight 550 and anti-rabbit Alexa Fluor 488 (both ThermoFisher, Dreieich, Germany); H_2DCFDA ROS indicator (ThermoFisher, Dreieich, Germany); and CD115 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Mice

Age- and sex-matched (6–12 weeks) mice were used for these studies. C57BL/6J and gp91phox−/− mice were obtained from The Jackson Laboratory. UNC93B1 mutant mice were a gift from Prof. Dr. Stefan Bauer, Institute of Immunology Marburg, Germany. Mice were killed by cervical dislocation, and spleens were excised for cell isolation. All animal procedures were performed in accordance with the legal and institutional guidelines.

Cell isolation and culture

All cells were maintained in RPMI with 10% FCS, L-glutamine, and sodium pyruvate at 37°C with 5% CO2. The MM1 cell line was routinely passaged twice a week. CD115+ monocytes were isolated from spleens by collecting CD115 Beads on Miltenyi Biotec's LS columns and MidiMacs magnet, followed by elution. Monocytic cells were cultured at a density of 0.5 × 106 cells/mL overnight on 24-well cell culture dishes (Nunc). Inhibitors were added 15 min before stimulation, and cells were stimulated at the following concentrations: 5 μg/mL Poly(I:C) (high-molecular weight); 2μg/mL R848 or 2,5 μM CpG C (ODN2395).

mRNA expression analysis

Relative quantification of gene expression was performed by real-time PCR on the iCycler iQ thermal cycler (Bio-Rad) normalized to GAPDH levels. Relative expression was normalized to GAPDH levels. Primer sequences were as follows:

Gene	Sense Mouse	Antisense
Gapdh	GGCAAATTCAACGGCACAGT	GTCTCGCTCCTGGAAGATGG
TNF-α	CCAGACCCTCACACTCAGATC	CACTTGGTGGTTTGCTACGAC

ELISA

Mouse TNF-α was measured in the cell culture supernatant by the use of the DuoSet ELISA Development System from R&D Systems (Wiesbaden, Germany) according to the manufacturer's instructions.

Confocal laser scan microscopy

To show translocation of intracellular TLRs on stimulation, cells were fixed and stained with the respective anti-TLR antibodies and anti-Calnexin as marker for the ER or anti-EEA1 (both Abcam) as marker for early endosomes as described by Latz *et al.* [28]. Imaging was performed with a Zeiss LSM 710 NLO confocal laser scanning microscope and a 1.4 Oil Dic M27 63× plan apochromat objective (Zeiss). Colocalization of TLRs with calnexin or EEA1 was measured in three regions of interest consisting of at least three cells. Analyzation was performed by calculating the Pearson correlation coefficient using the plugin Coloc2 in Fiji. Significance was determined by two-tailed *t*-test.

Flow cytometric determination of ROS-production

Endosomal ROS was detected in cells loaded with the fluorescent probe H_2DCFDA (10 mM) in Krebs-Ringer phosphate buffer (pH 7.4) after defined stimulation by flow cytometry on an FACS Canto.

Statistics

Data shown are mean and standard deviation (SD), unless otherwise indicated. To compare two groups, GraphPad Prism 8 software was used for unpaired *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) and Tukey's/Dunnett multiple comparison test or two-way ANOVA and Sidak's multiple comparison test were used. Normal distribution was confirmed using Shapiro–Wilk test.

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Ethics statement: Experiments in mice received approval of the Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany (23177-07/G18-1-003).

Data availability statement: The data that supports the findings of this study are available in the supplementary material of this article.

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Abbreviations: **aPL:** antiphospholipid antibodies · **MM1:** Mono-Mac1 · **NFA:** niflumic acid · **NOX:** NADPH-oxidase

Full correspondence: Dr. Nadine Müller-Calleja, Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstr. 1, D-55131 Mainz, Germany
e-mail: nadine.mueller-calleja@unimedizin-mainz.de

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