Figures and figure supplements

Inflammatory osteolysis is regulated by site-specific ISGylation of the scaffold protein NEMO

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Figure 1. NEMOK270A mutant expression in BMMs exacerbates RANKL-induced osteoclastogenesis. (A) Domain structure of NEMO. (B) Western blot showing expression of pMX-NEMO\textsuperscript{WT} (NM) and pMX-NEMO mutants (NM-K270A, NEMO-D304N and NM-K319A). (C) BMMs from WT and (LysM-cre-NEMO \textsuperscript{fl/fl}) NEMO-cKO mice were transduced with viral particles (generated by transfecting pMX- retroviral vectors in PLAT-E cells) expressing NEMO\textsuperscript{WT} (NM-WT) and NEMO\textsuperscript{K270A} (NM-KA) and cultured in the presence of MCSF (10 ng/ml) and RANKL (50 ng/ml). (D) Representative TRAP staining for osteoclast (n = 8) and quantification of TRAP positive OCs. qPCR analysis for OC marker genes (E) TRAP, (F) CTSK, (G) MMP9, (H) \(\beta\)3Integrin, (I) DC-STAMP and (J) NFATc1 (p=0.057). Representative data (n = 3 independent experiments). (K) BMMs from RelA\textsubscript{luc} reporter mice expressing NM-WT and NM-KA were cultured in the presence of MCSF (10 ng/ml) for 3 days followed by RANKL stimulation with RANKL (50 ng/ml) for 6 hr and RelA-luciferase activity measurement (n = 3). pMX-Flag-NEMO\textsuperscript{WT}-RFP (NM-WT), pMX-Flag-NEMO\textsuperscript{K270A}-RFP (NM-KA). (*p<0.05, **p<0.01 and ***p<0.001).
Figure 1—figure supplement 1. BMMs from wild type mice were transduced with viral particles (generated by transfecting pMX-retroviral vectors in PLAT-E cells) expressing NEMO<sup>WT</sup> (NM-WT), NEMO<sup>K270A</sup> (NM-KA), NEMO-D304N and NEMO-K319A constructs followed by culture in the presence of MCSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days. (A) Representative images for TRAP staining for osteoclast (n = 8). (B) Wild type BMMs were transduced with different dilution (1x, 0.5x, 0.25x, 0.1x and 0.05x) of retroviral particles expressing NEMO<sup>K270A</sup> (NM-KA). Wild type BMMs were also transduced with viral particles expressing NEMO-D304N and NEMO-K319N at 1x dilution. Western-blot using anti-flag antibody shows higher expression of NEMO<sup>K270A</sup> even at low viral dilution. pMX-Flag-NEMO<sup>WT</sup>-RFP (NM-WT), pMX-Flag-NEMO<sup>K270A</sup>-RFP (NM-KA).
Figure 2. Expression of NEMO<sup>K270A</sup> in vivo leads to inflammatory osteolysis and joint destruction. NEMO<sup>K270A</sup> was conditionally expressed in myeloid cells (NM-KA mice) by crossing NEMO<sup>K270A</sup> <sup>f/f</sup> mice with LysozymeM cre expressing mice. (A) Whole body images of NM-KA mice compared to littermate wild type control mice (6 weeks old). The arrows point to deformed joints and swelling. (B) Photomicrograph of spleen and bone from NM-WT and NM-KA mice. MicroCT analysis of bone from NM-WT and NM-KA mice showing (C) femur trabecular bone, (D) knee joint osteolysis (arrow) and quantification of (E) Bone volume/total volume (BV/TV), (F) Connectivity density, (G) Trabecular number (Tb.N), (H) Trabecular thickness (Tb.Th) and (I) Trabecular separation (Tb.Sp) in the femur trabecular region (n = 6). Long bones from 6 weeks old NM-WT and NM-KA mice were processed for Figure 2 continued on next page.
histology and stained for TRAP to visualize TRAP+ osteoclasts in (K) bone sections and (K) Articular surfaces of knee joint (arrow). Representative images (n = 6) Serum was collected from NM-WT and NM-KA to measure serum (L) TRAP and (M) CTX concentration as an indicator of increased osteoclast activity (n = 6–8). LysM-cre-NEMOWT-If/If (NM-WT), LysM-cre-NEMOK270A-If/If (NM-KA) mice. (*p<0.05, **p<0.01 and ***p<0.001).
Figure 2—figure supplement 1. Generation of NEMO transgenic mice. Schematic diagram showing generation of conditional (A) NEMO<sup>K270A</sup>-KI (NM-KA) and (B) NEMO<sup>WT</sup>-Tg (NM-WT-Tg) mice (6 weeks old). (C) MicroCT images of paw and ankle joint showing osteolysis in NM-KA mice (arrow). X-ray images of NM-WT and NM-KA mice showing significant bone loss and deformities in (D) whole body (E) Knee joint (F) ankle and paw (arrow pointing towards osteolysis and deformed bones). (G) Whole body images of NM-WT and NM-WT-Tg mice (6 weeks old). (H) Photomicrograph of spleen and bone from NM-WT and NM-WT-Tg mice. MicroCT analysis showing quantification of (I) Bone volume/total volume (BV/TV), (J) Trabecular number (Tb.N), (K) Trabecular thickness (Tb.Th) and (L) Trabecular separation, between NM-WT and NM-WT-Tg mice (n = 3).
Figure 3. NEMO<sup>K270A</sup> mutation instigates systemic inflammation. Serum was collected from NM-WT and NM-KA mice (n = 8–10) to measure concentration of inflammatory cytokines (A) Interleukin (IL) 1β, (B) IL-4, (C) IL-6, (D) IL-10, (E) IL-13, (F) IL-17, (G) Monocyte chemoattractant protein 1 or CCL2, (H) Tumor necrosis factor alpha, (I) Macrophage colony stimulating factor, (J) Macrophage Inflammatory protein (MCP)-1 or CCL3, (K) Keratinocyte chemoattractant or neutrophil activating protein 3 or CXCL1 and (L) Granulocyte colony stimulating factor (GCSF). (M) BMMs from NM-WT and NM-KA mice were isolated and cultured in the presence of MCSF (10 ng/ml) and RANKL (10 ng/ml). Representative TRAP staining for osteoclast (n = 8) is shown. (N–R) Representative qPCR analysis for OC marker genes TRAP, CTSK, β3integrin, DC-STAMP and NFATC1 (n = 3). (S) BMMs from NM-WT and NM-KA mice were isolated and cultured in the presence of MCSF (10 ng/ml) four days followed by serum starvation and stimulation with RANKL (50 ng/ml) for different time points (n = 8). Representative western-blot showing activation of p65 (phos-p65/p65 ratio) post RANKL stimulation in BMMs from NM-WT and NM-KA mice. LysM-cre-NEMO-WT-f/f (NM-WT), LysM-cre-NEMO-K270A-f/f (NM-KA) mice. (*p<0.05, **p<0.01 and ***p<0.001).
Figure 3—figure supplement 1. BrdU was injected to NM-WT and NM-KA mice. 1 day after injection single cell suspensions from bone marrow were prepared by flushing the marrow out of femur and tibia. Following RBC lysis, cells were stained with different antibody cocktails. Flow analysis showing percentage of (A) Lin-Sca1+Kit+ (LSK) cells in haemopoietic stem cells (HSC), (B) percentage of common myeloid progenitor (CMP), granulocyte-monocyte progenitors (GMP) cells; proliferation of (C) LSK, (D) CMP and (E) GMP cells, (F) Myeloid cells and (G–J) proliferation of neutrophils populations. LysM-cre-NEMO<sup>WT</sup>-f/f (NM-WT), LysM-cre-NEMO<sup>K270A</sup>-f/f (NM-KA) mice.
Figure 4. NEMO<sup>K270A</sup> mutation hampers autophagy. PLAT-E cells were transfected with retroviral pMX-Flag-NEMO<sup>-WT</sup>-RFP (NM-WT) and pMX-Flag-NEMO<sup>-K270A</sup>-RFP (NM-KA) expression vector. (A) Fluorescence images showing distribution of NM-WT-RFP in cytoplasm compared to puncta (yellow arrows) juxtaposed to nuclei- DAPI stained) formation in case of NM-KA-RFP in PLAT-E cells. (B) Western blot for LC3 using WES (protein simple). BMMs were cultured for 2 days with RANKL (preOC) followed by 6 hr of serum starvation and western blotting. Fold change of LC3 relative to actin is indicated on top. (C) Quantification of LC3+ cells per high magnification field. (D) For flow cytometry, BMMs were transduced with pMX-GFP-LC3-RFP retrovirus generated in PLAT-E packing cells, and flow analysis was done to detect GFP signal or LC3 flux. Contour plots showing LC3-GFP+ expressing cells in NM-WT and NM-KA preOC (Blue: NM-WT without serum starvation, Red: NM-KA without serum starvation, and Black: after 6 hr of serum starvation). (E) Histograms representing shift in LC3-GFP+ cells following induction of autophagy (Red histogram: background signal in uninfected cells, Blue histogram: No serum starvation or 10% FBS control, yellow: 6 hr serum starvation, and pink: chloroquine), (F) Change in Mean fluorescent intensity (MFI) showing LC3-GFP signal in NM-WT and NM-KA preOC cells post autophagy induction. LysM-cre-NEMO-WT-f/f (NM-WT), LysM-cre-NEMO-K270A-f/f (NM-KA) mice. (*p<0.05). (*p<0.05, **p<0.01 and ***p<0.001).
Figure 4—figure supplement 1. Autophagy is negatively impacted in NEMO \textsuperscript{K270A} cells. (A) Pre-OC (RANKL-treated BMMs) from NM-WT and NM-KA mice were pelleted and processed for electron microscopic and Immunofluorescence (IF) analysis after 6 hr of serum starvation. Representative Electron microscopic images (x7500) showing nucleus, Cell membrane (CM) lysosome (L) in NM-WT preOC and cytoplasmic aggregates (yellow arrow) in NM-KA preOC. (B) Representative IF images for NEMO (red), LC3 (green) and NEMO-LC3 colocalization (yellow). Arrows indicate accumulation of NEMO in LC3 positive vacuole-like structures. (C) Representative western blot showing expression of LC3 from BMMs starved and stimulated with RANKL as shown. (D) Representative Western blot for mTOR expression in BMMs from NM-WT and NM-KA mice treated as shown. (E) Pre-osteoclasts were treated with chloroquine as indicated and number of TRAP+ multi nucleated osteoclasts (MNC) per well were counted in triplicate wells from three independent experiments (*p<0.05).
Figure 5. NEMO<sup>K270A</sup> is restricted to autophagosomes whereas NEMO<sup>WT</sup> is delivered to lysosomes. preOC from NM-WT and NM-KA mice were pelleted and processed for Immunofluorescence (IF) and electron microscopic analyses after 6 hr of serum starvation. (A) Representative IF images showing NEMO (red) and LAMP1 (green). Arrows indicate colocalization of NEMO in LAMP1 positive vacuole-like structures in NM-WT, which is decreased in NM-KA preOC. (B) Representative electron microscopic images (x7500) lysosome (L), Autophagosome (AP) and APL (Autophagolysosome). (C) Representative IF images showing changes in cellular NEMO organization in response to autophagy induction by serum starvation in NM-WT and NM-KA preOC cells. NEMO-puncta (white) and nucleus (blue). (D) NEMO-puncta quantification. LysM-cre-NEMO-WT-f/f (NM-WT), LysM-cre-NEMO-K270A-f/f (NM-KA) mice. (*p<0.05, **p<0.01 and ***p<0.001).
Figure S5—figure supplement 1. preOC from NM-WT and NM-KA mice were processed for Immunofluorescence (IF) analysis after 6 hr of serum starvation. (A) NEMO-puncta determination in IgG control and (B) chloroquinone (CQ) treated pOC and quantification. (C) NEMO puncta quantification in presence of chloroquinone (CQ).
Figure 6. Intact NEMO K270 residue is essential for post-translational modification (PTM) by ISG15. (A) Volcano plot showing changes in autophagy and PTM related proteins in immunoprecipitated lysates from NM-WT compared with NM-KA BMMs using anti-NEMO antibody. PreOC from NM-WT and NM-KA mice were processed for Immunofluorescence (IF) and Immuno-electron microscopy (EM) analysis after 6 hr of serum starvation. (B) Representative IF images of NEMO (red) and ISG15 (green) co-localization in preOC. White arrows indicate foci of expression of ISG15 (enlarged inset at bottom of panel B). Yellow arrows indicating NEMO-ISG15 co-localization. (C) ISGylated proteins (upper panel) and free ISG15 in response to RANKL treatment. (D) BMMs from WT and ISG15-KO mice were isolated and cultured in the presence of MCSF (10 ng/ml) and RANKL (50 ng/ml) for four days. Representative TRAP staining for osteoclast (D) and quantification (E). (**p<0.01).
**Figure 6—figure supplement 1.** Representative Immuno-EM images (x7,500) showing localization of NEMO (black arrows) and ISG15 (blue arrow) in NM-WT and NM-KA cells; lysosomes (L), autophagosome (AP). Large black dot: NEMO (18 nm gold particle), small black dot: ISG15 (12 nm gold particle). BMMs from NM-WT and NM-KA mice cells treated with RANKL for different time points followed by western blot.
Figure 7. ISGylation of NEMO is essential to restrain osteoclastogenesis. BMMs from NM-WT and NM-KA mice were transduced with viral particles (generated by transfecting pMX-retroviral vectors in PLAT-E cells) expressing ISG15 and cultured in the presence of MCSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days. (A) Representative TRAP staining for osteoclast (n = 6) and (B) quantification of TRAP positive OCs. (C) BMMs from NM-WT and NM-KA mice were transduced with ISG15 and pMRX-GFP-LC3-RFP retrovirus generated in PLAT-E packing cells. The cells were cultured for 2 days (preOC) followed by 6 hr of serum starvation and flow analysis to detect GFP signal or LC3 flux. (C) Histograms representing shift in LC3-GFP+ cells following induction of autophagy. Blue histogram: serum starvation, yellow histogram: serum starvation + ISG15 expression (D) Change in Mean Fluorescent Intensity (MFI) showing LC3-GFP signal. (E) Wild type BMMs transduced with viral particles (generated by transfecting pMX-retroviral vectors in PLAT-E cells) expressing NEMO+/+ISG15, NEMO-K270A+/+ISG15 NEMO-WT::ISG15 (fused) and NEMO-K270A::ISG15 (fused) protein and cultured in the presence of MCSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days. (E) Representative TRAP staining for osteoclast (n = 3) and (F) quantification of TRAP positive OCs. (G) NEMO puncta regulation by ISG15: Live images of preOC expressing RFP-NEMO<sub>WT</sub>/+ISG15, RFP-NEMO<sub>K270A</sub>/+ISG15, GFP-NEMO<sub>WT</sub>::ISG15 and GFP-NEMO<sub>K270A</sub>::ISG15 fusion protein. Yellow arrows indicate NEMO<sub>K270A</sub> puncta. ISG15 panel which is not tagged serves as Figure 7 continued on next page.
background control. (H) Quantification of LC3 puncta+ preOC cells shown in Figure 7—figure supplement 1. (I) WB for LC3 in preOC expressing NEMOWT+/−ISG15, NEMO^{K270A}+/−ISG15, NEMOWT::ISG15 and NEMO^{K270A}::ISG15 fusion protein. (*p<0.05, **p<0.01 and ***p<0.001). (·) denotes fusion. (J) Representative IF images (NEMO (Red); LAMP1(green)). NEMO localization in preOC expressing NEMOWT+/−ISG15, NEMO^{K270A}+/−ISG15, NEMOWT::ISG15 and NEMO^{K270A}::ISG15 fusion protein. Green arrow- Lysosome and Yellow arrow-localization of NEMO in Lysosome.
Figure 7—figure supplement 1. LC3 puncta accumulation of NEMO^{K270A} is reduced by forced fusion with ISG15. (A) Representative IF images for LC3 puncta+ cells (arrow) in preOC expressing NEMO^{WT}+/−ISG15, NEMO^{K270A}+/−ISG15, NEMO^{WT}::ISG15 and NEMO^{K270A}::ISG15 fusion protein (quantified in Figure 7H).