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Leading Edge

Immunology Select

Cell

Inflammation is the body's defense against infection by pathogens, tissue injury, or cancer. The inflammatory response requires the eloquent orchestration of multiple networks of cells from both the innate and adaptive immune systems and their production of and response to a multitude of molecular mediators. This Immunology Select highlights a cluster of new studies that shed fresh light on the biology of some of the lymphoid and myeloid cell types involved in inflammation.

New Cells Hang Out In A Greasy Neighborhood

Cells of the innate immune system such as neutrophils and macrophages are the "first responders" during the initial steps of inflammation, rushing to the scene of pathogen invasion. All white blood cells of the myeloid lineage, including neutrophils and macrophages, are "innate-type" immune cells, but the only lymphoid cells that are also considered innate immune cells are natural killer (NK) cells. Moro et al. (2010) now identify a new comrade-in-arms for NK cells, a second "innate-type" lymphoid cell with a surprising location and with functional properties complementary to those of NK cells. The new kid on the innate immune system block is found in previously unrecognized cell clusters named "fat associated lymphoid clusters" (FALCs), present in adipose tissue located along the blood vessels of the mouse and human peritoneal cavity. Although this cell subpopulation has the morphological characteristics of lymphoid cells, they could not be persuaded to differentiate into T, B, or NK lymphoid lineages. They have been christened "natural helper (NH) cells." Unlike NK cells, which are innate lymphoid cells capable of secreting the cytokine IFN-γ, NH cells secrete IL-4, IL-5, and IL-13. Moro and colleagues identified three key functions for this new cell population. NH cells produce IL-5, which drives self-renewal of a subset of B lymphocytes in the peritoneal cavity. Secondly, they boost the secretion of IgA antibody by B cells in the spleen. Finally, by secreting IL-13, NH cells induce excess mucin secretion by goblet cells in the gut and, in so doing, facilitate the expulsion of a parasitic worm called *Nippostrongylus brasiliensis* in a mouse model of parasitic infection. Therefore, this new innate immune lymphocyte is able to join forces with its more celebrated NK cousin to fight invasion by pathogens. Yet why do these cells reside in fat tissue, a rather unusual neighborhood? Might fat tissue be the equivalent of potato chips for NH cells?

K. Moro et al. (2010). Nature ***463****, 540–544.*

Another Mercenary Molecule in the Innate Immune System

Natural killer (NK) cells may have acquired a new cousin, but they seem to have lost a sentinel inhibitory receptor, Ly49Q. In their new study, Sasawatari et al. (2010) identify Ly49Q, a member of the Ly49 family of inhibitory receptors expressed by NK cells, as a renegade. It turns out that Ly49Q is not expressed by NK cells at all but is expressed by neutrophils as well as other myeloid lineage cells expressing the marker Gr-1. The authors show that Ly49Q inhibits neutrophil pseudopod formation and hence migration under certain conditions while promoting it under others. More specifically, they demonstrate that when neutrophils are exposed to the chemotactic peptide fMLP, Ly49Q along with its MHC class I ligand (H-2 K^b) is internalized into endosomal compartments within the same neutrophil. Interestingly, upon stimulation of neutrophils, the phosphatase SHP-2 associates with H-2 K^b, and this association is dependent on Ly49Q. Ly49Q localizes to lipid rafts and is responsible for recruitment of Src and SHP-2 to the rafts. Subcellular redistribution of lipid raft components, which form platforms for chemokine signaling via Src-family kinases, is important for pseudopod formation and migration of neutrophils, and SHP-2 positively regulates Src kinase activity. When the investigators treated neutrophils with fMLP, those neutrophils expressing (but not those lacking) Ly49Q exhibited a time-dependent increase in Src activation and were found to adhere firmly to fibronectin substrates. Meanwhile, neutrophils cultured in the absence of fMLP were easily detached from their substrate. These results paint a picture whereby Ly49Q inhibits neutrophil adhesion and spreading in the absence of chemoattractant but promotes adhesion and pseudopod formation when the neutrophils are exposed to appropriate stimuli. It will be interesting to address the biological significance of the association of Ly49Q with H-2 K^b on the same neutrophil as well as the mode of transfer of signaling information from the fMLP receptor to Ly49Q. Nonetheless, Sasawatari and colleagues offer an interesting biological story of a renegade signaling molecule that can be persuaded to work either in favor of immune homeostasis (by blocking neutrophil migration) or in favor of inflammation (by promoting neutrophil migration) depending on the highest bidder.

S. Sasawatari et al. (2010). Immunity ***32****, 200–213.*

T Cell Receptors Send Their Flock For Indoctrination

T lymphocytes are critical elements of the cell-mediated immune response. Expression of the coreceptors $CD4^+$ and $CD8^+$ functionally distinguishes the two subpopulations of T lymphocytes responsible for supporting antibody-based and cell-mediated immunity, respectively. The bifurcation of these lineages in the thymus is thought to occur through differences in T cell receptor (TCR) signaling in thymocyte progenitor cells. The kinetic signaling model proposes that persistent signaling through the TCR in the thymus drives thymocytes to express the transcription factor ThPOK and to differentiate into $CD4^+$ helper T cells. In contrast, interruption of TCR signaling permits cytokine signals to drive differentiation of thymocytes into $CD8^+$ cytotoxic T cells, which in turn depends on induction of the transcription factor Runx3. In new work, Park et al. (2010) report that differentiation of thymocytes into $CD8^+$ T cells can occur in the absence of signaling through the TCR provided that immature thymocytes are rendered responsive to the cytokine IL-7 and that the local environment is enriched for IL-7. The authors tested the link between cytokine signaling and Runx3 expression by treating thymocytes with IL-7 at a critical stage of development in vitro. Most interestingly, mice genetically engineered to contain IL-7-responsive thymocytes that could not signal through their TCR had no $CD8^+$ T cells. However, these thymocytes could generate huge numbers of $CD8^+$ T cells in the thymus if the mouse expressed a transgene for IL-7 and started

Spatiotemporal regulation of intracellular trafficking of Toll-like receptor 9 by an inhibitory receptor, Ly49Q

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Toll-like receptor (TLR) 9 recognizes unmethylated microorganismal cytosine guanine dinucleotide (CpG) DNA and elicits innate immune responses. However, the regulatory mechanisms of the TLR signaling remain elusive. We recently reported that Ly49Q, an immunoreceptor tyrosine-based inhibitory motif–bearing inhibitory receptor belonging to the natural killer receptor family, is crucial for TLR9-mediated type I interferon production by plasmacytoid dendritic cells. Ly49Q is expressed in plasmacytoid dendritic cells, macrophages, and neutrophils, but not natural killer cells. In this study, we showed that Ly49Q regulates TLR9 signaling by affecting endosome/lysosome behavior. Ly49Q colocalized with CpG in endosome/lysosome compartments. Cells lacking Ly49Q showed a disturbed redistribution of TLR9 and CpG. In particular, CpG-induced tubular endolysosomal extension was impaired in the absence of Ly49Q. Consistent with these findings, cells lacking Ly49Q showed impaired cytokine production in response to CpG-oligodeoxynucleotide. Our data highlight a novel mechanism by which TLR9 signaling is controlled through the spatiotemporal regulation of membrane trafficking by the immunoreceptor tyrosine-based inhibitory motif–bearing receptor Ly49Q. (Blood. 2009;114:1518-1527)

Introduction

Toll-like receptors (TLRs) recognize molecular patterns unique to microorganisms, and then elicit host immune responses against the pathogens. Among the TLR family members, TLR3, 4, 7, and 9 can elicit type I interferon (IFN) production in response to microbial components.[1] TLR3, 7, and 9 reside in intracellular compartments such as endosomes and detect microbial nucleic acids. The intracellular localization of such TLRs might be necessary to prevent the recognition of self nucleotides while facilitating access to microbial ones.[2] TLR9 is a sensor of unmethylated cytosine guanine dinucleotide (CpG) DNA and resides in the endoplasmic reticulum.[2,3] TLR9's recognition of CpG DNA is accompanied by changes in membrane dynamics and trafficking, resulting in a strict spatiotemporal compartmentalization of the TLR9 and CpG DNA. The CpG oligonucleotide (CpG) moves into early endosomes and is subsequently transported to a tubular endolysosomal compartment, and in both of these structures it is colocalized with TLR9.[3,4] In endosomes, TLR9 forms a complex with myeloid differentiating factor 88 (MyD88) and IFN regulatory factor 7, and activates MyD88-IFR7–dependent type I IFN induction.[5] Interestingly, the manner of CpG internalization and the retention time of CpG in endosomes differ between CpG-A and CpG-B, and the retention of CpG/TLR9 complex in the endosomes is the primary determinant of TLR9 signaling.[6] After the TLR9 signaling complexes form in endosomes, they translocate to the juxtanuclear area, in tubular endolysosomal structures that extend toward the cell periphery and plasma membrane.[3,4] However, the molecular mechanisms underlying the intracellular trafficking of TLR9 are largely unknown.

Ly49Q is an immunoreceptor tyrosine-based inhibitory motif (ITIM)–bearing inhibitory receptor belonging to the lectin-type natural killer (NK) receptor family.[7,8] However, it possesses unique distinguishing features. Ly49Q is expressed neither on NK nor NKT cells, but on plasmacytoid dendritic cells (DCs), macrophages, and neutrophils.[7,9,10] The expression of Ly49Q appears to be regulated during the maturation of these cells and is significantly up-regulated by IFN-γ treatment.[7,10,11] Ly49Q can associate with both Src homology 2-containing protein tyrosine phosphatase (SHP)-1 and SHP-2 in a tyrosine phosphorylation-dependent manner.[7] We recently reported that Ly49Q is crucial for TLR9-mediated type I IFN production by pDCs.[12] pDCs in Ly49Q-deficient mice show impaired CpG-triggered IFN-α and interleukin (IL)-12 production; consequently, TLR9-dependent antiviral responses are diminished in Ly49Q-deficient mice. To gain insight into how Ly49Q regulates TLR9 signaling, we focused on the fact that Ly49Q localizes to endosome-like vesicular compartments. We found that Ly49Q was crucial for the efficient development of the tubular endolysosomes during the intracellular trafficking of CpG and TLR9. Our results reveal a novel mechanism by which TLR signaling is controlled through the spatiotemporal regulation of membrane trafficking by the receptor Ly49Q.

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This article is a continuation of a previous report.[9,12,14]

The online version of this article contains a data supplement.

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Methods

Mice

Mice (6-7 weeks old) were purchased from CLEA Japan. Experiments were performed according to the Guidelines for Animal Use and Experimentation as set by the International Medical Center of Japan, and were approved by the International Medical Center of Japan. Ly49Q knockout mice were described previously.

Antibodies and reagents

The preparation of the anti-Ly49Q antibody was described previously.[7] The following monoclonal antibodies were purchased from BD Pharmingen: phycoerythrin (PE)-conjugated anti–mouse Mac-1 (M1/70); streptavidin-conjugated allophycocyanin and PE; control rat immunoglobulin (Ig)G2a and IgG2b; and anti–mouse CD18 (M18/2). Biotin-conjugated and purified anti-FLAG M2 antibodies were purchased from Upstate Biotechnology. PE-conjugated H-2K^b tetramer was purchased from MBL. Alexa Fluor 594-conjugated anti–rat IgG, Alexa Fluor 594-conjugated anti–mouse IgG, and Alexa Fluor 488-conjugated anti–rat IgG were purchased from Molecular Probes. Antibodies for phospho-c-Jun N-terminal kinase (JNK) and JNK were purchased from Santa Cruz Biotechnology. Antibodies for phospho-p38 and p38 were purchased from Dai-ichi Pure Chemicals. Antibody for Glu-tubulin was purchased from Chemicon International. Recombinant IFN-γ was purchased from PeproTech. CpG-oligodeoxynucleotide1668 and rhodamine-conjugated CpG-oligodeoxynucleotide1668 were purchased from Hokkaido System Science.

Cell culture

The murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection. Cells were cultured in complete RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 1% (vol/vol) nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. Macrophages and pDCs were treated with CpG-N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylanmonium methylsulfate (DOTAP; Roche Diagnostics), prepared following the manufacturer's instructions. Peritoneal exudation macrophages were prepared by injecting 2 mL of 4% thioglycolate medium intraperitoneally into mice. Cells infiltrating the peritoneal cavity were collected 4 days after the injection.

Flow cytometric analysis

Acid treatment and immunofluorescence analysis were performed, as previously described.[13] Stained cells were analyzed with a FACSCalibur (BD Biosciences).

Vectors and cDNA transfection

The expression vector for TLR9-green fluorescent protein (GFP; pCAGGS-TLR9-GFP) was a gift of Dr K. Miyake (Tokyo University).[14,15] Expression vector for Rab5-DsRed was a gift of Dr C. R. Roy (Yale University). WEHI3 transfectants expressing wild-type Ly49Q (Ly49Q-WT), the ITIM-less mutant, or containing the mock vector were previously described.[7] The vectors for the Ly49Q knockdown were a gift of Dr Takayanagi (Tokyo Medical and Dental University). RAW264.7 cells were transfected by electroporation using a Microporator MP-100 (Digital Bio; NanoEnTek), following the manufacturer's instructions.

Immunohistochemical staining

Cells adhering to glass coverslips were fixed with 3.7% formalin in phosphate-buffered saline (PBS) at room temperature for 15 minutes, and then treated with 0.1% Triton X-100 in PBS for 20 minutes. After being washed with PBS containing 0.05% bovine serum albumin, the cells were treated with 3% bovine serum albumin in PBS to prevent nonspecific protein binding. The cells were then stained with the indicated antibodies or reagents, mounted, and analyzed by confocal (Zeiss) or fluorescence (Olympus) microscopy.

Western blot analyses

The cells were lysed in radioimmunoprecipitation assay buffer consisting of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA (ethyleneglycoltetraacetic acid), 1.5 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, and complete EDTA (ethylenediaminetetraacetic acid)-free protease inhibitor mixture (Roche Diagnostics). The lysates were resolved by 5% to 20% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The proteins were detected by incubating the membranes with the indicated antibodies and visualized using SuperSignal West Dura Extended Duration Substrate (Pierce). In some experiments, SuperSignal West Femto Extended Duration Substrate was used for detection (Pierce). For quantification of the precipitated proteins, a LAS-3000 (Fuji Photo Film) was used.

Reverse transcription–polymerase chain reaction and quantification of mRNA

For RNA preparations, Isogen was used according to the manufacturer's instructions (Wako Pure Chemical). cDNA synthesis was performed according to standard protocols using oligo(dT) and random hexamer oligonucleotides. For semiquantitative reverse transcription–polymerase chain reaction (RT-PCR), gene-specific fragments were obtained by linear phase PCR amplification, and standardized using the β-actin or hypoxanthine guanine phosphoribosyl transferase 1 level. Quantitative differences in mRNA levels were determined by real-time RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems) and a thermal cycler controlled by the 7900HT Fast Real Time PCR system (Applied Biosystems). Primers used for PCR analyses are shown in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

RNA interference

Double-stranded RNA for Ly49Q interference (UGAGGACAAUCAAGGGUCAAGAGAA) was purchased from Hokkaido System Science. To introduce the small interfering RNA (siRNA) into RAW264 cells, a Microporator MP-100 (Digital Bio; NanoEnTek) was used according to the manufacturer's instructions. Transfection efficiency was estimated by using a fluorescein isothiocyanate–conjugated control siRNA-A (Santa Cruz Biotechnology).

Statistical analysis

The statistical significance of differences in values for the migration, invasion, adhesion, and spreading of neutrophils was determined with the 2-tailed Student t test. Differences with a P value of less than .05 were considered significant.

Results

Colocalization of Ly49Q with CpG in lysosomal compartments

We recently reported that, in Ly49Q-deficient mice, the TLR9-triggered production of cytokines such as IFN-α and IL-12 is severely impaired.[12] To investigate how Ly49Q affects TLR9 signaling, we first examined whether Ly49Q colocalizes with CpG-containing endosomes. Because Ly49Q is internalized and localizes to endocytic compartments, we hypothesized that Ly49Q colocalizes with CpG/TLR9 in endosomal compartments and affects TLR9 signaling. To facilitate the immunohistochemical analyses, peritoneal macrophages expressing FLAG-tagged Ly49Q were obtained from Ly49Q-expressing transgenic (Tg) mice (supplemental Figure 1). Ly49Q was detected not only at the cell surface,

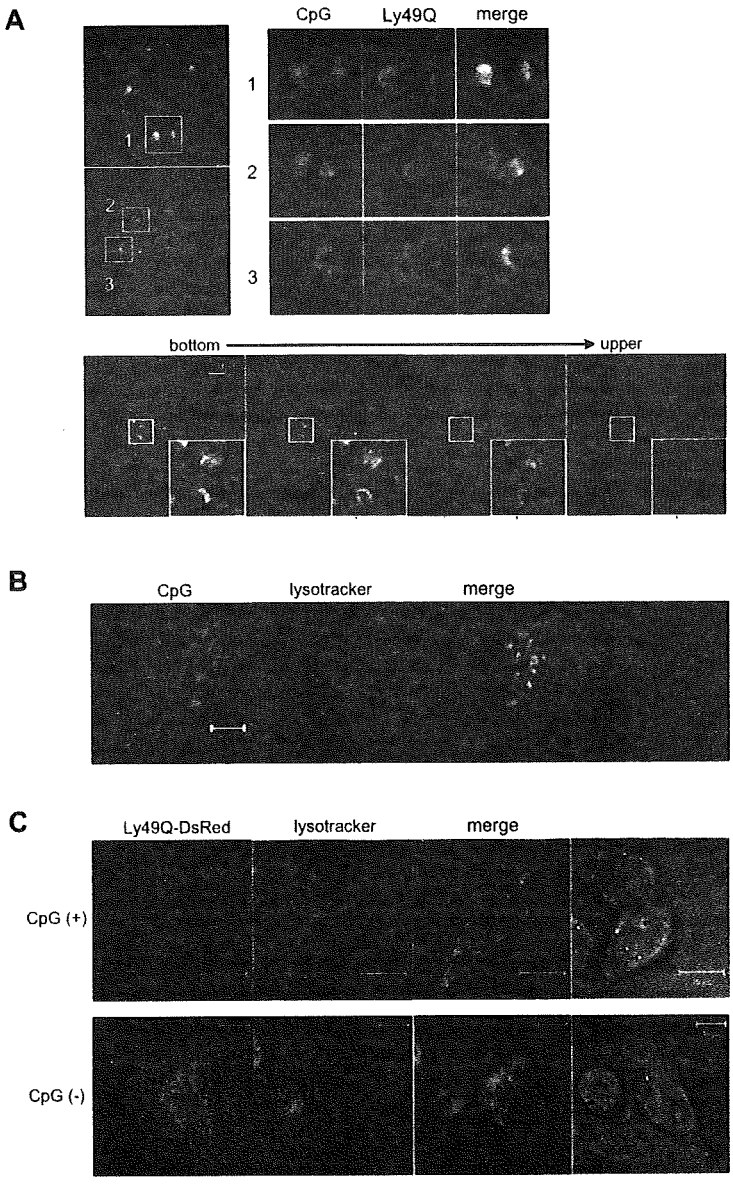

Figure 1. Ly49Q colocalized with CpG in endosome/lysosome compartments. (A) Colocalization of Ly49Q with CpG in endosomes. Peritoneal exudate macrophages were prepared from Ly49Q Tg mice. The cells were incubated with rhodamine-conjugated CpG (0.3 μM) for 15 minutes, fixed with 4% formalin in PBS, then stained with an anti-FLAG antibody and analyzed by confocal microscopy. The bottom 4 photographs show serial *Z*-axis–sectioned patterns after 60-minute incubation with rhodamine-conjugated CpG. Squares indicate the region shown in higher magnification. (B) Localization of CpG to late endosomes/lysosomes. RAW264 cells were incubated with rhodamine-conjugated CpG for 60 minutes. To visualize the late endosomes/lysosomes, the cells were incubated with lysotracker for the last 30 minutes. (C) Localization of Ly49Q in the late endosomes/lysosomes. A Ly49Q-DsRed fusion construct was introduced into RAW264 cells. Twenty-four hours after transfection, the cells were cultured with lysotracker at 37°C for 30 minutes, and the intracellular localization of Ly49Q was examined by confocal microscopy. Ly49Q localized to the late endosomes/lysosomes. In the presence of CpG stimulation, Ly49Q-containing compartments showed a tubular structure

but also in annular-shaped vesicular compartments (Figure 1A). The CpG fluorescence partially overlapped with the Ly49Q-associated vesicles, and some of the CpG-containing endosomes were closely encircled by Ly49Q vesicular structures, suggesting that the Ly49Q-associated vesicles had fused with the CpG-containing endosomes. *Z*-axis sections revealed that the Ly49Q-containing compartments were intricately intertwined with the CpG-containing endosomes. Similar results were obtained using the RAW264 macrophage cell line (data not shown). As previously demonstrated, the CpG-containing endosomes subsequently acquired lysosome-like features along with acidification, which was detectable with an acidic organelle-targeting fluorescent dye (Figure 1B).[16,17] To clarify whether Ly49Q also localized to the lysosome-like compartments, a Ly49Q-DsRed fusion protein was expressed in RAW264 cells. The Ly49Q localized to lysotracker-targeted compartments in the steady state (Figure 1C). CpG stimulation caused the Ly49Q to be redistributed to tubular vesicular compartments, which were previously described as tubular endolysosomal structures.

Defective CpG redistribution and endolysosome extension in Ly49Q^{-} pDCs and macrophages

Previous studies demonstrated that the intracellular trafficking of CpG is spatiotemporally regulated, and that the behavior of CpG endosomes affects the quality of TLR9 signaling.[5,6] Therefore, we next investigated whether Ly49Q is involved in CpG trafficking using Ly49Q$^{-/-}$ pDCs and macrophages. The internalization of CpG and its subsequent redistribution to tubular endolysosomes were observed in bone marrow-derived pDCs prepared from a Ly49Q$^{-/-}$ mouse (Figure 2A). The tubular endolysosomal structures radiated from the perinuclear region, extending toward the cell periphery. Lysosomal-associated membrane protein-1 (LAMP-1)$^{+}$ late endosomes were localized to the perinuclear region, and some of the CpG colocalized with LAMP-1. These results are consistent with a previous report showing that internalized CpG is transported to the perinuclear region through late endosomes and subsequently distributed into tubular endolysosomes.[5]

In contrast, Ly49Q$^{-/-}$ pDCs showed fractionated and undirected CpG-containing compartments. Only a small portion of the

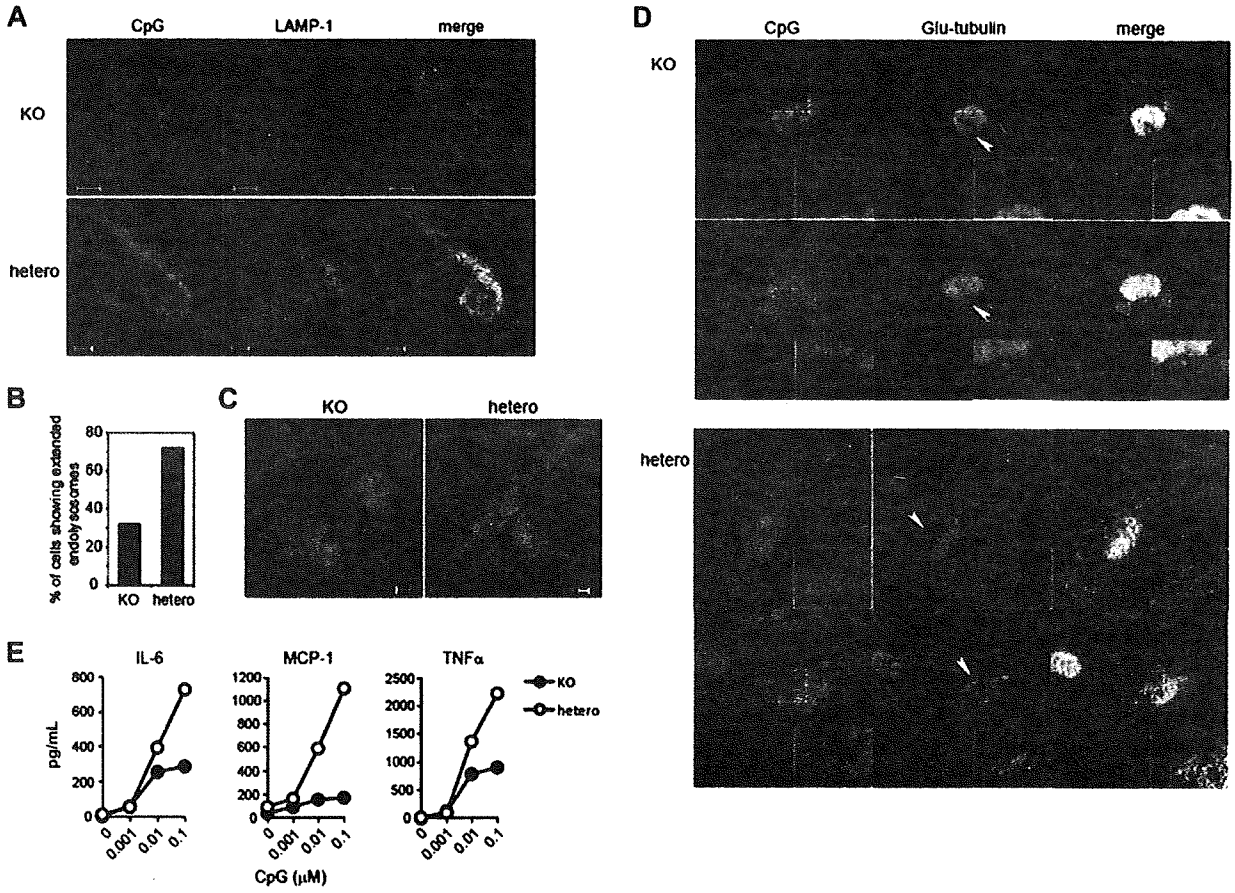

Figure 2. Formation of tubular endolysosomal structures was impaired in pDCs and macrophages derived from Ly49Q knockout mice. (A) Bone marrow–derived pDCs were prepared by culturing bone marrow cells with Flt3L, and they were then incubated with rhodamine-conjugated CpG (0.3 μM) for 60 minutes. The cells were fixed and then stained with an anti–LAMP-1 antibody. Confocal images of representative cells are shown. (B) The number of cells showing the directionally extending CpG-including tubular endolysosomal structures was counted, and the proportion of total cells was calculated. (C) Peritoneal exudate macrophages were prepared from Ly49Q knockout and control littermate mice and incubated with rhodamine-conjugated CpG (0.3 μM) for 24 hours. The intracellular distribution of CpG was examined by confocal microscopy. (D) The peritoneal exudate macrophages were treated with rhodamine-conjugated CpG (0.3 μM) for 24 hours. The cells were fixed and then stained with anti–detyrosinated tubulin (Glu-tubulin) antibody. Arrowheads indicate MTOC. (E) Cytokine production by peritoneal exudate macrophages was compared between Ly49Q-deficient and control littermate mice. Macrophages were stimulated with CpG, and the amount of cytokine in the culture supernatant was estimated by cytokine bead array 24 hours later.

CpG compartments colocalized with the late endosomes, and the perinuclear localization of the late endosomes was not obvious in the Ly49Q$^{-/-}$ pDCs. The frequency of cells possessing radiating and elongating endolysosome structures was decreased in the Ly49Q$^{-/-}$ pDCs compared with the Ly49Q$^{+/-}$ pDCs (Figure 2B). A similar defect in the redistribution of CpG-containing endolysosomes was observed in Ly49Q$^{-/-}$ peritoneal exudate macrophages (Figure 2C). The directional extension of CpG-containing endolysosomes was not observed in the Ly49Q$^{+/-}$ macrophages, whereas numerous directional CpG-containing endolysosomes elongating from the perinuclear region were observed in the Ly49Q$^{+/-}$ macrophages. The tubular endolysosome elongation has been shown to be MyD88 independent, but microtubule dependent.[3,18] Stabilized microtubules are enriched with posttranslationally modified tubulins such as detyrosinated tubulin (Glu-tubulin), in which the C-terminal tyrosine of α-tubulin is removed by tubulin carboxypeptidase.[19] Immunohistochemical staining of Glu-tubulin clearly demonstrated that, in Ly49Q$^{+/-}$ macrophages, Glu microtubules colocalized with the tubular endolysosomes, indicating that stabilized microtubules exist along the endolysosomes (Figure 2D). In addition, the Glu-tubulin at the microtubule organizing center (MTOC) appeared to be connected to the tubular endolysosomes in Ly49Q$^{-/-}$ macrophages. In contrast, in Ly49Q$^{-/-}$ macrophages, Glu-tubulin did not colocalize with the endolysosomes, and no connection between Glu-tubulin and the endosomes at the MTOC was observed. Thus, the impairment of endolysosome elongation in the Ly49Q$^{-/-}$ macrophages was associated with decreased stability of the microtubules. Consistent with these observations, in Ly49Q$^{-/-}$ macrophages, the CpG-triggered production of cytokines, including IL-6, tumor necrosis factor α, and monocyte chemoattractant protein-1, was severely diminished (Figure 2E). Thus, the absence of Ly49Q upset the CpG trafficking in both pDCs and macrophages, in close correlation with the failure of cytokine production in both pDCs and macrophages.

Importance of Ly49Q in TLR9-mediated IFN-β and IL-6 production in RAW264 cells

Next, we tried to investigate the intracellular trafficking of CpG and TLR9 in the presence or absence of Ly49Q using the mouse macrophage cell line RAW264, which expresses TLR9 and readily permits the introduction of various expression constructs. To do this, we established Ly49Q^{hi} and Ly49Q^{lo} RAW264 clones to examine the relevance of Ly49Q in CpG/TLR9 trafficking, because the expression level of Ly49Q in RAW264 cells was heterogeneous in the steady state (Figure 3A). Four clones each of Ly49Q^{hi} or

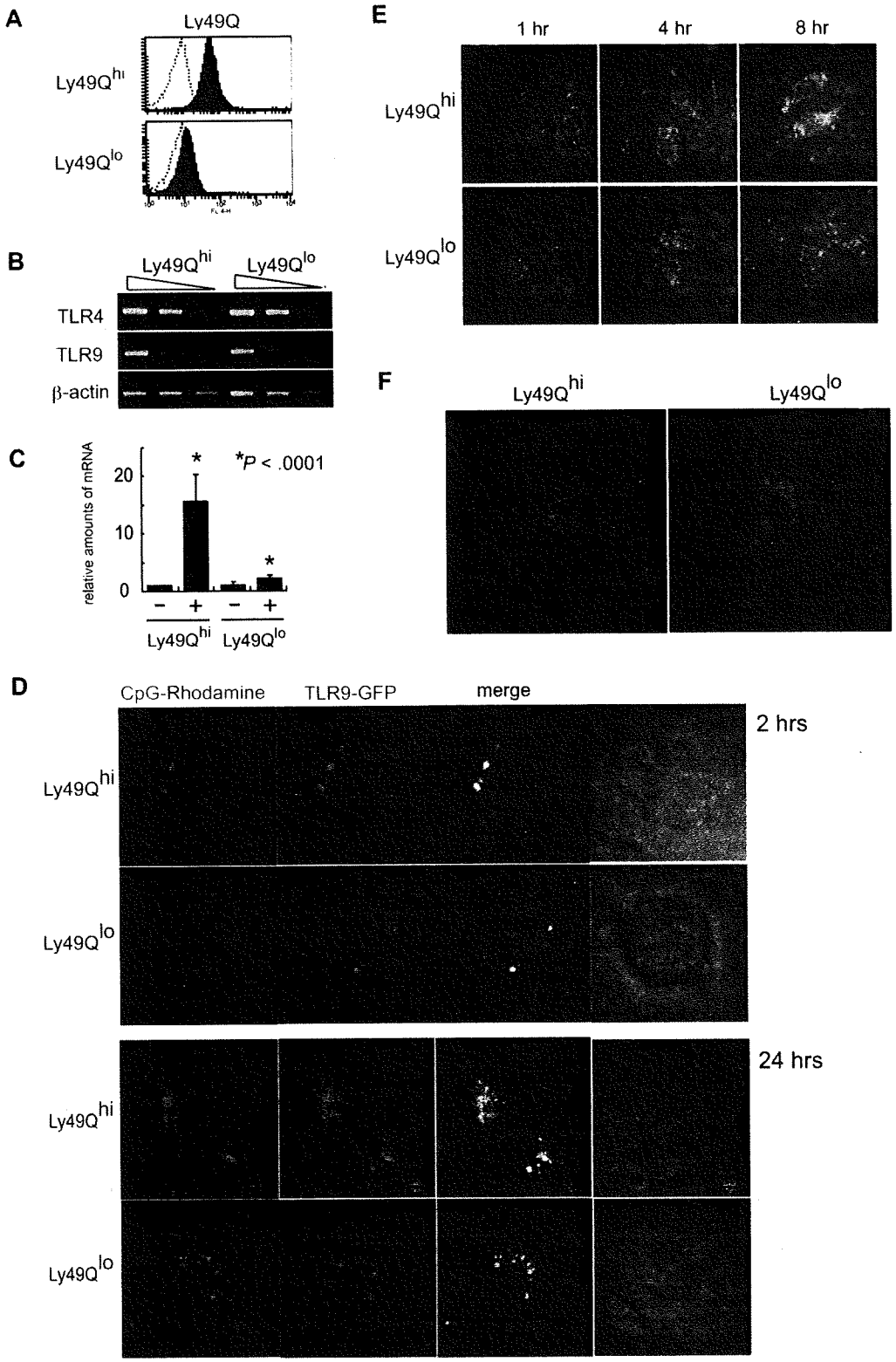

Figure 3. Inefficient uptake of CpG and retarded redistribution of TLR9 in Ly49Q^{lo} cells. (A) Expression of Ly49Q in RAW264 clones. Ly49Q^{hi} or Ly49Q^{lo} RAW264 clones were established from bulk RAW264 cells by limiting dilution. Four clones of each type were analyzed, and similar results were obtained. Data from representative clones are shown. (B) No difference in TLR9 and TLR4 expression in RAW264 cells. Semiquantitative RT-PCR was carried out using RNA prepared from the indicated cells. The presence or absence of Ly49Q had no effect on the transcription of these TLRs. In the photographs, the PCR templates were sequentially diluted by 5-fold. (C) Quantitative analysis of IFN-β transcription in RAW264 cells in response to CpG. Ly49Q^{hi} or Ly49Q^{lo} RAW264 cells were stimulated with CpG1668 (0.3 μM) for 4 hours, and quantitative RT-PCR analyses were performed. The histograms show the relative amounts of IFN-β mRNA evaluated by real-time PCR. (D) Intracellular redistribution of CpG and TLR9. TLR9-GFP expression plasmids were introduced into RAW264 cells. Twenty-four hours after transfection, the cells were incubated with rhodamine-conjugated CpG (0.3 μM) for the indicated periods. Impaired CpG/TLR9 redistribution was observed in the Ly49Q^{lo} RAW264 cells. (E) Internalization and distribution of CpG in RAW264 cells. RAW264 cells were incubated with rhodamine-conjugated CpG (0.3 μM) and fixed at the indicated time points. (F) Intracellular distribution of rhodamine-conjugated CpG. After 24 hours of incubation with CpG, tubular endolysosomal structures were observed in Ly49Q^{hi} RAW264, but not in Ly49Q^{lo} RAW264 cells.

Ly49Q^{lo} RAW264 cells were analyzed, and the same results were obtained (data not shown). Ly49Q^{hi} and Ly49Q^{lo} RAW264 clones showed comparable expression levels of CD11b, F4/80, TLR4, and TLR9 (supplemental Figures 2A, 3B). To confirm the importance of Ly49Q in TLR9 signaling in RAW264 cells, Ly49Q^{hi} or Ly49Q^{lo} RAW264 cells were treated with CpG, and the TLR9-triggered cytokine production was examined. When the cells were stimulated with CpG, the Ly49Q^{hi} RAW264 cells produced IFN-β and IL-6, and the Ly49Q^{lo} RAW264 cells produced little, if any (Figure 3C and supplemental Figure 2B). These results were consistent with our findings that pDCs in Ly49Q$^{-/-}$ mice show impaired TLR9-mediated type I IFN production and that Ly49Q^{-} pDCs from bone marrow are less potent producers of IFN-β and IL-6 (Figure 2E).[10] In addition, the Ly49Q^{hi} and Ly49Q^{lo} RAW264 cells were morphologically different in their spreading and adhesion properties and in their formation of cytoplasmic vacuolar structures in the presence of CpG (supplemental Figure 2C). When exogenous Ly49Q-WT was expressed in Ly49Q^{lo} RAW264 cells, the CpG-induced IL-6 production recovered (supplemental Figure 2D-E). However, the ITIM-less mutant (Ly49Q-YF) did not rescue the IL-6 production efficiently, indicating that the ITIM is important for TLR9 signaling.

To further confirm that the effects in the Ly49Q^{lo} RAW264 cells were due to the decreased Ly49Q and not to some other difference, we performed siRNA knockdown experiments. Ly49Q expression in the Ly49Q^{hi} RAW264 cells was down-modulated by introducing Ly49Q antisense RNA, and the CpG-induced IL-6 production was examined. The expression of Ly49Q short hairpin RNA in the Ly49Q^{hi} RAW264 cells resulted in diminished IL-6 production in response to CpG (supplemental Figure 3A-B). The down-modulation of Ly49Q expression also caused a decrease in lysosome-like vesicular structures after CpG stimulation, implying a functional correlation between Ly49Q and lysosome and/or vesicle trafficking (supplemental Figure 3C). In addition, Ly49Q expression was induced in Ly49Q^{lo} RAW264 cells by IFN-γ treatment, as we reported previously (supplemental Figure 3D). In association with the increased Ly49Q expression, the IL-6 production and vacuolar formation by CpG stimulation were recovered in these originally Ly49Q^{lo} RAW cells (supplemental Figure 3E-F). Furthermore, the inhibition of Ly49Q expression using Ly49Q-specific short hairpin RNA in IFN-γ-treated RAW264 cells diminished the CpG-induced IL-6 production and vacuolar formation, indicating that Ly49Q is important for the IL-6 production triggered by TLR9 (supplemental Figure 3G-H). Therefore, TLR9-mediated cytokine production in RAW264 cells was also dependent on Ly49Q.

Defective TLR9 trafficking in the absence of Ly49Q

Therefore, we next investigated the intracellular trafficking of a TLR9-GFP fusion protein using the Ly49Q^{hi} and Ly49Q^{lo} RAW264 cells. Two hours after CpG stimulation, internalized CpG was colocalized with TLR9 in endosomes in both the Ly49Q^{hi} and Ly49Q^{lo} RAW264 cells (Figure 3D). However, there were great differences in the diameter, number, and cytoplasmic localizations of the CpG/TLR9 endosomes between the Ly49Q^{hi} and Ly49Q^{lo} RAW264 cells. In the Ly49Q^{lo} RAW264 cells, the diameters of the TLR9^{+} vesicles appeared smaller than in the Ly49Q^{hi} RAW264 cells (Figure 3D). In addition, several TLR9^{+} vesicles were observed scattered throughout the cytoplasm in the Ly49Q^{lo} RAW264 cells. After 24 hours of stimulation, the difference in TLR9 distribution between the Ly49Q^{lo} and Ly49Q^{hi} RAW264 cells was remarkable. TLR9 in the Ly49Q^{hi} RAW264 cells was localized along tubular endosomal structures and distributed at the edges of protrusions that were focal adhesion-like attachment sites. In contrast, in the Ly49Q^{lo} RAW264 cells, even though the TLR9^{+} vesicles colocalized with the CpG-containing endosomes, they remained scattered in the cytoplasm, and no vesicular fusion or elongation was observed. These tubular structures did not colocalize with either Rab11 or the transferrin receptor (data not shown).

Defects in tubular endolysosome extension, as observed in the Ly49Q knockout pDCs and macrophages, were also observed in Ly49Q^{lo} RAW264 cells (Figure 3E-F). Kinetic analyses of CpG trafficking demonstrated that both Ly49Q^{hi} and Ly49Q^{lo} RAW264 cells internalized CpG, although the amount of internalized CpG in Ly49Q^{lo} RAW264 cells seemed slightly lower than in Ly49Q^{hi} RAW264 cells at the early time point (1 hour; Figure 3E). After 8 hours of CpG stimulation, in the Ly49Q^{hi} RAW264 cells, CpG-containing endosomes appeared to spread or diffuse through the cytoplasm, in contrast to Ly49Q^{lo} RAW264 cells, which showed no diffuse distribution in CpG fluorescence in the cytoplasm. In addition, no obvious change of distribution pattern was observed in Ly49Q^{lo} RAW264 cells from 4 to 8 hours. A remarkable difference was also observed after 24 hours of CpG stimulation in the extension of tubular endolysosomal structures (Figure 3F). These results strongly suggest that the CpG trafficking in RAW264 cells was also regulated by Ly49Q.

Mitogen-activated protein kinase activation, but not NF-κB-related transcription factor expression, was affected by Ly49Q

Next, we examined which signaling pathways could be affected by Ly49Q. RT-PCR analyses revealed no great differences in the expression of NF-κB–related transcription factors between the Ly49Q^{hi} and Ly49Q^{lo} RAW264 cells (supplemental Figure 4; data not shown). However, the TLR9-triggered activation of p38 was severely impaired in the Ly49Q^{lo} RAW264 cells (Figure 4A). In addition, CpG-induced JNK activation was dysregulated in the Ly49Q^{lo} RAW264 cells. The activation of JNK in Ly49Q^{hi} RAW264 cells was sustained between 4 and 7 hours after CpG stimulation, but in the Ly49Q^{lo} RAW264 cells, the level of phospho-JNK decreased during this time (Figure 4B). In addition, immunohistochemical analyses clearly showed that Ly49Q colocalized with phosphorylated p38 in late endosome/lysosome compartments after CpG stimulation. A portion of the phosphorylated JNK also colocalized with LAMP-1^{+} late endosome compartments (Figure 4C). These results strongly suggest that Ly49Q influences TLR9-mediated p38 and JNK activation in the late endosome/lysosome compartments.

Ly49Q was internalized and recycled through an ITIM-mediated mechanism

To obtain insights into a mechanism of the Ly49Q-mediated TLR-containing vesicular trafficking, we analyzed trafficking of Ly49Q and its ligand, major histocompatibility complex (MHC) class I. We previously demonstrated that Ly49Q associates with MHC class I in *cis* at the cell surface.[20] By confocal microscopic analyses, we found that Ly49Q was colocalized with H-2K^{b} in peritoneal exudate macrophages (Figure 5A). This colocalization occurred not only at the cell surface, but also in cytoplasmic vesicles, suggesting that Ly49Q was internalized together with H-2K^{b}. We next tested whether the removal of β_2-microglobulin (β_2m) from the cell surface by acid treatment would elicit binding of the H-2K^{b} tetramer to Ly49Q, as shown for Ly49A.[13] As we previously reported, Ly49Q on the pDCs in C57BL/6 mice showed

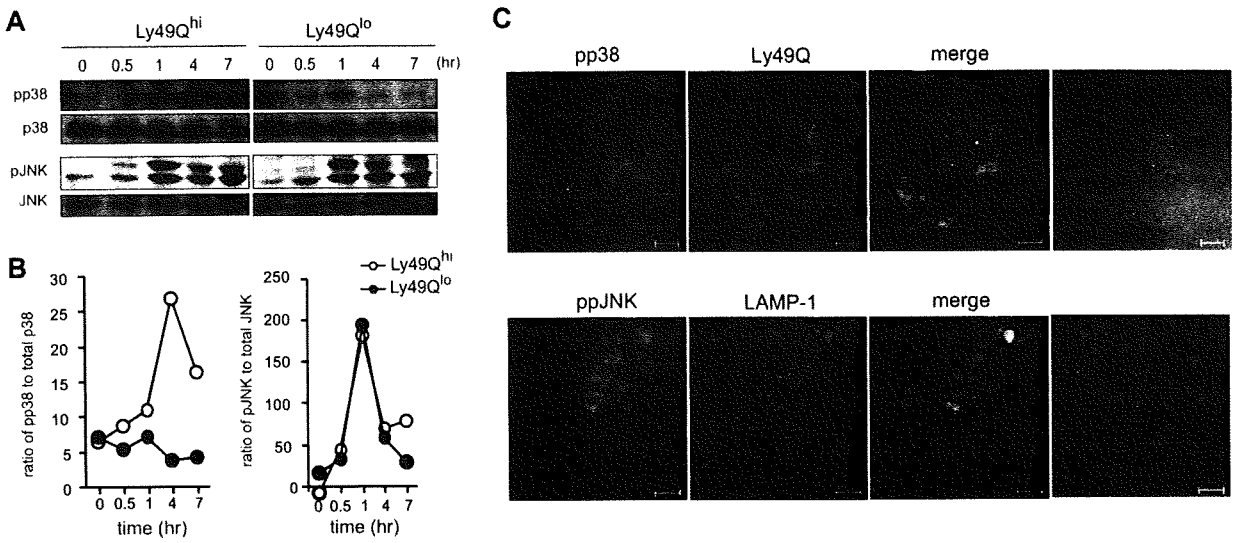

Figure 4. Ly49Q is involved in the regulation of MAP kinase activation after CpG stimulation. (A) Activation kinetics of p38 and JNK. RAW264 cells were treated with CpG (0.3 μM) for the indicated periods, and the total cell lysates were prepared by directly adding SDS sample buffer. The total lysates were separated by SDS–polyacrylamide gel electrophoresis, and the activation of p38 and JNK was examined by Western blotting. (B) The signal intensity (pixel numbers) of phosphorylated MAP kinases was normalized to that of each total MAP kinase, and semiquantitative values for the MAP kinase activation are shown. (C) Intracellular distribution of phosphorylated p38 and JNK in the presence of CpG stimulation. FLAG-tagged Ly49Q expression plasmids were introduced into Ly49Q^{hi} RAW264 cells, and 24 hours after transfection, the cells were incubated with unlabeled CpG (0.3 μM) for 2 hours. The cells were then fixed and stained with antibodies against FLAG and phosphorylated p38. To analyze the distribution of phosphorylated JNK, Ly49Q^{hi} RAW264 cells were treated with CpG for 2 hours and stained with antibodies against phospho-JNK and LAMP-1

low levels of H-2K^b binding (Figure 5B).[20] C57BL/6 $\beta_2m^{-/-}$ pDCs showed increased H-2K^b binding, which was completely inhibited by an anti-Ly49Q antibody. These results indicate that Ly49Q associates with H-2K^b in a *cis* configuration. The acid treatment of pDCs did not affect the H-2K^b binding, even though β_2m was successfully removed from the cell surface (Figure 5C-D; data not shown). Furthermore, neither the cell viability nor the Ly49Q expression levels on the cell surface were affected by acid treatment (data not shown). These results strongly suggested that Ly49Q's association with H-2K^b is β_2m independent and stable under acidic conditions; therefore, this interaction should be sustained in intracellular acidic compartments, such as endosomes/lysosomes.

Next, we asked whether the ITIM of Ly49Q was involved in the internalization of Ly49Q, because the tyrosine motifs (Yxxϕ) in ITIMs have been suggested to function as an internalization signal.[21] We first investigated Ly49Q endocytosis in the presence of various inhibitors of membrane trafficking, using Ly49Q-null myeloid lineage WEHI3 cells transduced with Ly49Q-WT or an ITIM-less mutant (Ly49Q-YF). In the absence of inhibitors, Ly49Q-WT was largely observed at the cell surface, but Ly49Q-YF inhabited perinuclear intracellular granules (Figure 5E). Methyl-β-cyclodextrin, which inhibits raft-dependent endocytosis by depleting cholesterol from the plasma membrane,[22] abrogated both the perinuclear distribution of Ly49Q-YF and the juxtamembranous endosomal distribution of Ly49Q-WT. Chlorpromazine, an inhibitor of clathrin-dependent endocytosis,[23] did not inhibit the internalization of either Ly49Q-YF or Ly49-WT. These results strongly suggest that Ly49Q is internalized via lipid raft-mediated endocytosis, and that the ITIM is not necessary for endocytosis itself, but important for the retention of Ly49Q at the cell surface.

We also found that the internalized endosomes were transported along microtubules, because nocodazole treatment strikingly diminished the accumulation of Ly49Q-YF in the perinuclear regions.[24] Importantly, treatment with a phosphatase inhibitor, sodium vanadate,[25] caused Ly49Q-WT to be redistributed to the perinuclear region in the same pattern as Ly49Q-YF. Given that Ly49Q-WT can associate with tyrosine phosphatases via its ITIM, these results suggest that the ITIM-associated phosphatase is important for regulating the intracellular distribution of Ly49Q. Because MHC class I recycles between the cell surface and endosomes, this finding also suggests that Ly49Q recycles together with MHC class I along microtubules in the steady state.

Discussion

In this study, we demonstrated that an inhibitory receptor, Ly49Q, plays an important role in the signaling of TLR9 by controlling the intracellular trafficking of TLR9 and CpG. The spatiotemporal regulation of the vesicular compartments containing TLR9 and CpG and their associated adaptor proteins is crucial for TLR9 signaling.[5,6] Ly49Q itself was internalized and appeared to move along microtubules in the steady state. The observation that Ly49Q associated with MHC class I in a *cis* configuration, even in an acidic environment, strongly suggests that Ly49Q recycles together with MHC class I. Importantly, the Ly49Q movement was regulated by an ITIM- and tyrosine phosphatase-dependent mechanism. Because Ly49Q itself can recruit tyrosine phosphatases such as SHP-1 and SHP-2 to the ITIM,[7] it is possible that Ly49Q-associated phosphatases play a role in the trafficking of Ly49Q itself.

How Ly49Q influences TLR9/CpG trafficking in addition to its own recycling process is an important question. Not all the CpG-containing endosomes included Ly49Q, and some endosomes contained only Ly49Q. This observation suggests that Ly49Q-containing and CpG-containing endosomes fused after these molecules were internalized separately. In endosomes/lysosomes, where the pH ranges from 5.0 to 6.5 depending on the type of compartment, some receptor-ligand interactions are disrupted due to the acidic environment. However, internalized Ly49Q might maintain an association with MHC class I in

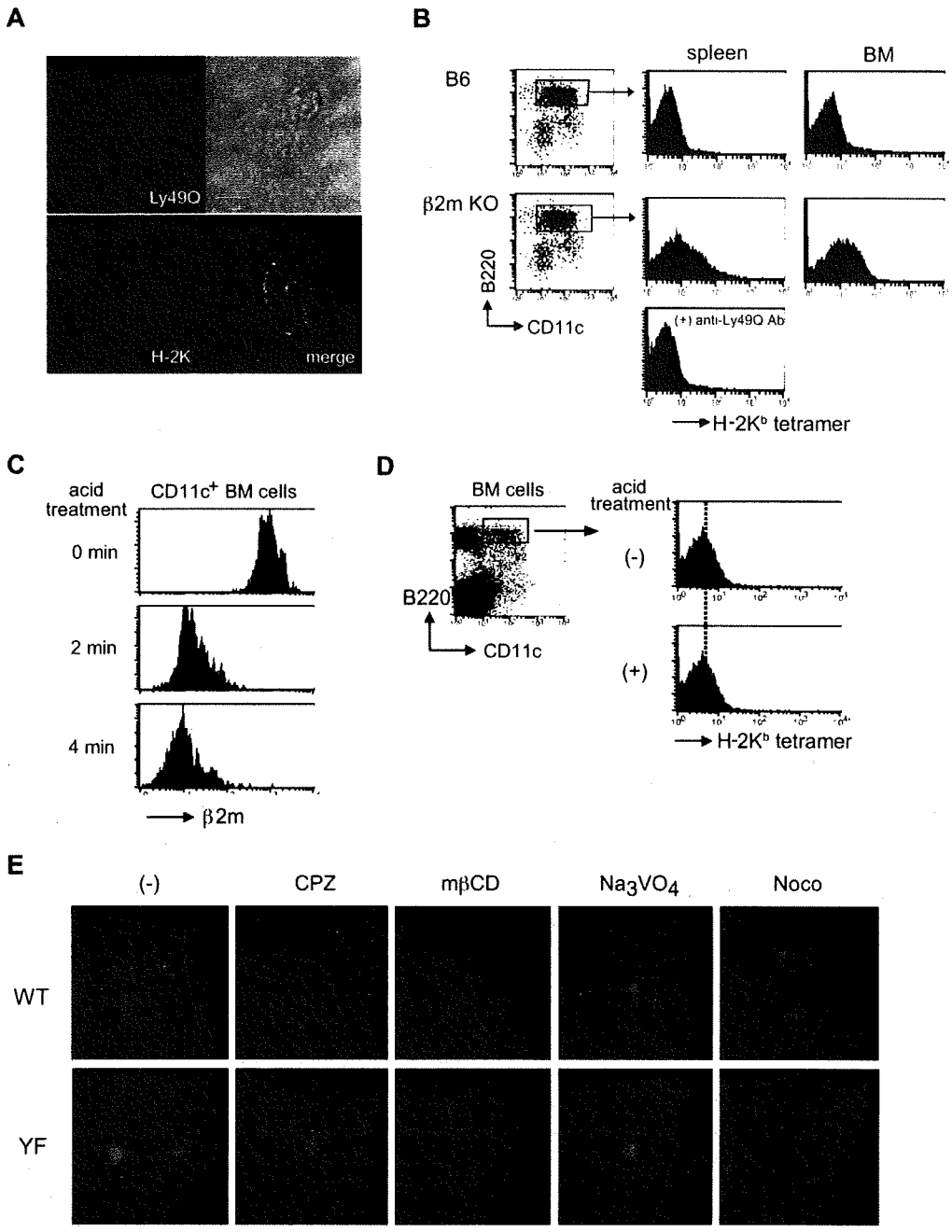

Figure 5. Ly49Q was internalized with MHC class I, and its localization was regulated in an ITIM- and tyrosine phosphatase-dependent manner. (A) Colocalization of Ly49Q with H-2K^b in intracellular vesicular compartments. Peritoneal exudation macrophages were prepared from Ly49Q Tg mice and examined for Ly49Q (red) and MHC class I (green) by immunohistochemical analyses with a confocal microscope. FLAG-tagged Ly49Q in Tg mice was detected with an anti-FLAG antibody. (B) Binding of H-2K^b tetramer to pDCs. Cells enriched in bone marrow pDCs were obtained by AutoMACS using an anti–plasmacyloid DC Ag-1 antibody, and the binding of PE-conjugated H-2K^b tetramer was examined by flow cytometry. In the absence of β_2m, binding of the H-2K^b tetramer in *trans* was detectable due to loss of the *cis* interaction. The tetramer binding was abrogated by an anti-Ly49Q antibody. (C) Removal of β_2m from the cell surface by acid treatment. Bone marrow cells were treated with citrate buffer (0.133 M citric acid and 0.066 M Na_2HPO_4, pH 3.3) at 20°C for the indicated periods. Removal of β_2m from the cell surface was confirmed by a decreased fluorescence intensity of anti-β_2m antibody staining. CD11c$^+$ cells were gated and analyzed. (D) Binding of H-2K^b tetramer before and after acid treatment. H-2K^b tetramer bound in *trans* to Ly49Q after the removal of β_2m from the cell surface, indicating that the *cis* interaction between Ly49Q and H-2K^b was still maintained, and the interaction was β_2m independent and acid resistant. (E) ITIM and tyrosine phosphatase dependence of Ly49Q redistribution. WEHI3 transfectants expressing Ly49Q-WT or Ly49Q-YF were incubated at 37°C in the presence or absence of the indicated inhibitors of membrane trafficking. The cells were then fixed and stained with an anti-FLAG antibody to visualize Ly49Q.

endosomes, because the Ly49Q-MHC class I association was β_2m independent and acid resistant. In fact, Ly49Q and MHC class I were still colocalized in the endosomes/lysosomes after being internalized. Therefore, Ly49Q may influence endosome behavior and TLR9 signaling events in such vesicular compartments through an interaction with MHC class I, which may contribute to the sustained activation of mitogen-activated protein (MAP) kinases that are colocalized with Ly49Q at the endosomes. Further investigations focusing on the roles of this inhibitory receptor not only at the plasma membrane, but also at

intracellular vesicular compartments, may help us in understanding the fine-tuning of immune responses.

In this study, we found that the TLR9-triggered extension of tubular endolysosome structures was severely impaired in both Ly49Q^{lo} RAW264 cells and Ly49Q knockout pDCs and macrophages. Tubular endolysosomal structures have attracted attention for their functional associations not only with TLR signaling, but also with various cellular activities, including cytokine secretion and phagocytosis, and antigen presentation.[3,18,26-30] Although neither the regulatory mechanisms that govern the formation and trafficking of these compartments nor their nature are fully understood, it has been established that the dynamic movement and reconstitution of the TLR9-associated endoplasmic reticulum and endolysosomes provide a site where TLR9-associated signal complexes containing the receptor, the ligand, MyD88, and IFN regulatory factor 7 can be assembled.[3,5] In addition, the location and retention of these molecules in endosomes are the key factors controlling the nature of TLR9 responses. These observations together lead to a novel concept that the trafficking of TLR signaling complexes to the correct membrane compartment with appropriate timing may be important for eliciting effective and controlled immune responses. In light of our present findings, we propose that Ly49Q is involved in the optimization of TLR9 signaling by controlling the redistribution of endolysosome compartments spatiotemporally.

In addition to the impaired formation of tubular endolysosomal structures, Ly49Q^{lo} RAW264 cells contained smaller TLR9/CpG endosomes than Ly49Q^{hi} cells. This observation suggests that Ly49Q plays a role not only in the intracellular movement of endosomes/lysosomes, but also in the endocytic process itself. Given that the CpG-containing endosomes were smaller in Ly49Q^{lo} than in Ly49Q^{hi} RAW264 cells, Ly49Q may affect the dimension of the endocytic cups. It has been proposed that the Yxxϕ (where ϕ is a hydrophobic amino acid) of the ITIM sequence functions as the internalization signal for membrane protein trafficking.[21] Tyrosine-mediated internalization has been demonstrated for inhibitory receptors such as cytotoxic T lymphocyte-associated antigen-1 and CD33.[31,32] In these receptors, tyrosine phosphorylation of the ITIM is essential for the recruitment of the internalization machinery, including the μ2 or suppressor of cytokine signaling 3-E3 ligase complex, and the subsequent internalization and degradation of the receptors. However, Ly49Q internalization seems to be mediated by a different mechanism from that reported for cytotoxic T lymphocyte-associated antigen-1 and CD33, because the ITIM is essential for the maintenance of Ly49Q at the cell surface, and Ly49Q-YF, whose mutant ITIM lacked tyrosine, was internalized. Alternatively, Ly49Q may be involved in the directional transport of recycling endosomes from the perinuclear region to the plasma membrane along microtubules. The accumulation of Ly49Q-YF at the perinuclear region may reflect stagnating endolysosomes that ought to have been added to the tubular endolysosomal structures to extend them. Importantly, the improper trafficking of TLR9/CpG vesicular compartments in Ly49Q^{-} and Ly49Q^{lo} cells was closely related to the impaired production of cytokines such as IFN-β and IL-6. Although the precise molecular basis for Ly49Q's regulation of endosome/lysosome trafficking is still largely unknown, our data indicate that Ly49Q is important for the physical positioning of TLR9 signaling within a cell.

The mechanistic linkages among the tubular endolysosome extension, MAP kinase activation, and cytokine production still need to be elucidated. We showed that p38 activation was impaired, and JNK activation was temporally dysregulated in Ly49Q^{lo} cells during CpG stimulation. We also demonstrated that phospho-p38 and phospho-JNK distributed to Ly49Q-containing endosome/lysosome compartments. Therefore, TLR9, CpG, pp38, pJNK, and Ly49Q might be colocalized in the same endosomes/lysosomes and functionally associated. The JNK pathway is required for the formation of the immunologic synapse between NK and their target cells: the expression of dominant-negative JNK or its siRNA knockdown blocks the cytotoxic granule movement along microtubules to the interface between the NK and its target cells, preventing NK cell polarization.[33] The cytotoxic granules have been suggested to share a common biogenesis with lysosomes.[34] Therefore, it is possible that an impairment of sustained JNK activation disrupts the directional movement of TLR9/CpG-riding endolysosomes along microtubules. In addition to JNK, p38 modulates endocytosis, by regulating a guanosine diphosphate dissociation inhibitor in the cytosolic cycle of Rab5, a key regulator of endocytic membrane traffic.[35,36] Further detailed analyses will be necessary to examine these possibilities.

Our present study established that Ly49Q is important for the regulation of TLR trafficking, which is associated with temporally regulated MAP kinase activation and cytokine production. The inappropriate positioning and timing of TLR9 signaling complexes may be caused by the lack of Ly49Q, resulting in impaired cytokine responses. Therefore, the fine-tuning of the intracellular trafficking of TLR9/CpG compartments by such an inhibitory receptor might be crucial for optimizing the responses to various infectious microbes. It is intriguing that the inflammatory cell-specific inhibitory receptor, Ly49Q, is a key regulator for the correct positioning of the TLR9 signaling complex. Understanding the multiple functions of this inhibitory receptor will help reveal the molecular bases of TLR9 receptor functioning as well as that of the NK receptors, and will shed light on the origin and role of inhibitory receptors recognizing MHC class I in the regulation of immune cells ranging from macrophages to NK cells.

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Authorship

Contribution: M.Y., A.T., E.K., S.S., and K.I. performed experiments; T.D., K.I., T.S., and A.P.M. analyzed results; and N.T.-S. designed the research and wrote the paper.

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Ly49Q, an ITIM-bearing NK receptor, positively regulates osteoclast differentiation

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ABSTRACT

Osteoclasts, multinucleated cells that resorb bone, play a key role in bone remodeling. Although immunoreceptor tyrosine-based activation motif (ITAM)-mediated signaling is critical for osteoclast differentiation, the significance of immunoreceptor tyrosine-based inhibitory motif (ITIM) has not been well understood. Here we report the function of Ly49Q, an Ly49 family member possessing an ITIM motif, in osteoclastogenesis. Ly49Q is selectively induced by receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) stimulation in bone marrow-derived monocyte/macrophage precursor cells (BMMs) among the Ly49 family of NK receptors. The knockdown of Ly49Q resulted in a significant reduction in the RANKL-induced formation of tartrate-resistance acid phosphatase (TRAP)-positive multinucleated cells, accompanied by a decreased expression of osteoclast-specific genes such as *Nfatc1*, *Tm7sf4*, *Oscar*, *Ctsk*, and *Acp5*. Osteoclastogenesis was also significantly impaired in Ly49Q-deficient cells *in vitro*. The inhibitory effect of Ly49Q-deficiency may be explained by the finding that Ly49Q competed for the association of Src-homology domain-2 phosphatase-1 (SHP-1) with paired immunoglobulin-like receptor-B (PIR-B), an ITIM-bearing receptor which negatively regulates osteoclast differentiation. Unexpectedly, Ly49Q deficiency did not lead to impaired osteoclast formation *in vivo*, suggesting the existence of a compensatory mechanism. This study provides an example in which an ITIM-bearing receptor functions as a positive regulator of osteoclast differentiation.

Introduction

Bone homeostasis is controlled by the coordinated balance maintained between bone formation by osteoblasts and bone resorption by osteoclasts [1]. Osteoclasts, multinucleated cells that uniquely have the ability to resorb bone, play a central role in calcium homeostasis as well as bone remodeling [2]. Increased osteoclast differentiation and function have been implicated in the pathogenesis of various osteopenic conditions, including postmenopausal osteoporosis and bone loss in inflammatory arthritis [3,4]. Therefore, understanding the regulatory mechanisms of osteoclast differentiation and function is important for the development of novel therapeutic strategies for these disorders.

Osteoclasts differentiate from monocyte/macrophage lineage cells in the presence of macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) [1,2]. This process is tightly regulated by mesenchymal lineage cells such as osteoblasts and bone marrow stromal cells, which provide M-CSF and RANKL [1,2]. M-CSF signaling through its receptor, c-Fms, is required for the survival and proliferation of osteoclast precursor cells [5]. RANKL binding to its receptor, RANK, results in the activation of tumor necrosis factor receptor-associated factor 6 (TRAF6), c-Fos, and calcium signaling pathways, each of which is essential for the induction and activation of nuclear factor of activated T cells (NFAT) c1, a critical transcription factor for osteoclastogenesis [1,6]. In fact, mice deficient in RANKL, RANK, TRAF6, c-Fos, and NFATc1 exhibit severe osteopetrosis due to impaired osteoclastogenesis [1,3,7].

In addition to RANKL and M-CSF, costimulatory signals mediated by immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptors, DNAX-activating protein 12 (DAP12), and Fc

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Lnk regulates integrin αIIbβ3 outside-in signaling in mouse platelets, leading to stabilization of thrombus development in vivo

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The nature of the in vivo cellular events underlying thrombus formation mediated by platelet activation remains unclear because of the absence of a modality for analysis. Lymphocyte adaptor protein (Lnk; also known as Sh2b3) is an adaptor protein that inhibits thrombopoietin-mediated signaling, and as a result, megakaryocyte and platelet counts are elevated in *Lnk*$^{-/-}$ mice. Here we describe an unanticipated role for Lnk in stabilizing thrombus formation and clarify the activities of Lnk in platelets transduced through integrin αIIbβ3–mediated outside-in signaling. We equalized platelet counts in wild-type and *Lnk*$^{-/-}$ mice by using genetic depletion of Lnk and BM transplantation. Using $FeCl_3$- or laser-induced injury and in vivo imaging that enabled observation of single platelet behavior and the multiple steps in thrombus formation, we determined that Lnk is an essential contributor to the stabilization of developing thrombi within vessels. *Lnk*$^{-/-}$ platelets exhibited a reduced ability to fully spread on fibrinogen and mediate clot retraction, reduced tyrosine phosphorylation of the β3 integrin subunit, and reduced binding of Fyn to integrin αIIbβ3. These results provide new insight into the mechanism of αIIbβ3-based outside-in signaling, which appears to be coordinated in platelets by Lnk, Fyn, and integrins. Outside-in signaling modulators could represent new therapeutic targets for the prevention of cardiovascular events.

Introduction

Platelet activation is controlled through a series of highly regulated processes and is critical for maintaining normal homeostasis (1). The nature of hemostasis and thrombosis mediated in vivo by activated platelets and its contribution to cardiovascular events remains unclear, however. Particularly challenging has been the characterization of the multicellular network of interactions among platelets, endothelial cells, leukocytes, and erythrocytes that occur during thrombus formation in pathological settings and analysis of the kinetics of platelet activity. Injury to vascular endothelial cells exposes matrix proteins, which induce platelets to adhere to the vessel wall, where they subsequently spread and become activated. At the high shear rates found within the circulation, vWF immobilized on the vessel wall binds to the platelet receptor glycoprotein Ib-V-IX complex (GPIb-V-IX), facilitating platelet adhesion to injured sites, where collagen and/or laminin are exposed (2, 3). Once activated, the adhering platelets secrete soluble mediators to recruit additional circulating platelets, and, through their aggregation, bleeding is stopped. Platelet activation is mediated via several signaling pathways, including the integrin αIIbβ3 pathway (1). Other receptor-ligand interactions, including the binding of GPVI-collagen, $P2Y_1$/$P2Y_{12}$-ADP, and protease-activated G protein–coupled receptor–thrombin (PAR-thrombin), synergistically promote integrin αIIbβ3 activation (inside-out signaling) and the subsequent binding of fibrinogen or vWF to integrin αIIbβ3. This binding triggers signaling that promotes cytoskeletal changes that lead to the spread and stabilization of platelet thrombi through a process termed outside-in signaling (1, 2).

It is also known that αIIbβ3 physically interacts with non-receptor tyrosine kinases such as Src and Syk (4, 5) and that activation of these kinases upon engagement of integrin with fibrinogen contributes to the stability of thrombi in vivo (6). The kinases and adaptors involved in the assembly of the αIIbβ3-based signaling complex are believed to include Syk, lymphocyte cytosolic protein 2 (Lcp2, also known as SH2 domain–containing leukocyte protein of 76 kDa [SLP-76]), Vav, and Fyn-binding protein (Fyb, also known as adhesion and degranulation promoting adaptor protein [ADAP]) (4, 5). Tyrosine phosphorylation of the cytoplasmic domain of the integrin β3 subunit, at least on Tyr747, is required for stable platelet aggregation and the interaction of myosin with the β3 subunit in platelets (7), which is believed to be necessary for full clot retraction (8–10).

Lnk (SH2B adaptor protein 3 [Sh2b3]) belongs to the Src-homology 2 (SH2) adaptor family, which also includes SH2-B (Sh2b1) and adaptor protein with PH and SH2 domains (APS; Sh2b2) (11).

Authorship note: Hitoshi Takizawa and Satoshi Nishimura contributed equally to this work.

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TRANSLATIONAL AND CLINICAL RESEARCH

Lnk Deletion Reinforces The Function of Bone Marrow Progenitors in Promoting Neovascularization and Astrogliosis Following Spinal Cord Injury

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Key words. Lnk • bone marrow • angiogenesis • astrogliogenesis • spinal cord injury • regeneration

ABSTRACT

Lnk is an intracellular adaptor protein reported as a negative regulator of proliferation in c-Kit positive, Sca-1 positive, lineage marker-negative (KSL) bone marrow cells. The KSL fraction in mouse bone

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ORIGINAL ARTICLE

Time of initial appearance of renal symptoms in the course of systemic lupus erythematosus as a prognostic factor for lupus nephritis

Yuko Takahashi · Tetsuya Mizoue · Akitake Suzuki · Hiroyuki Yamashita · Junwa Kunimatsu · Kenji Itoh · Akio Mimori

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Abstract The prognosis of lupus nephritis (LN) was studied retrospectively in two LN categories, LN manifested initially at systemic lupus erythematosus (SLE) onset (I-LN) and LN of delayed manifestation after SLE onset (D-LN), based on a chart review (C) of 154 SLE (85 LN) patients with a mean observation of 20.8 ± 9.3 years and a questionnaire study (Q) of 125 LN patients outside our hospital with mean observation of 17.6 ± 9.2 years. In both study groups, half of I-LN patients were relapse-free by Kaplan–Meier analysis after initial therapy, and the relapsed I-LN patients responded to retherapy at higher 5-year relapse-free rates than those of patients receiving initial therapies for D-LN. At last observation, a higher frequency of prolonged remission was shown in I-LN compared with D-LN patients (C: 22/31, 71% versus 14/49, 29%, $P < 0.01$; Q: 65/89, 73% versus 11/33, 33% $P < 0.01$) and also a higher frequency of irreversible renal damage in D-LN compared with I-LN patients (C: 25/49, 51% versus 2/31, 6%, $P < 0.001$; Q: 14/33, 42% versus 6/89, 7%, $P < 0.001$), although class IV pathology was common in patients (C) in both LN categories. Onset time of lupus nephritis in the course of SLE may affect renal prognosis.

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Introduction

Systemic lupus erythematosus (SLE) is a multiple-organ disease, and lupus nephritis (LN) is a major clinical problem because of its high morbidity and mortality rates. In accordance with the chronic nature of SLE, renal symptoms can manifest at various times in the disease course, and a physician cannot predict the future development of LN at the time of SLE onset. Furthermore, it is unclear whether there is a difference in prognosis between LN manifested at the onset of SLE and LN developed later in the course of SLE, because the clinical significance of time of LN onset in the disease course of SLE has not been clearly described in the literature, to our knowledge. Accordingly, in recent clinical trials on therapies for LN including cyclophosphamide and mycophenolate mofetil, mixtures of cases having various time intervals between SLE onset and LN onset have been studied [1, 2]. Although a prognostic impact of renal pathology and a poor prognosis of class IV disease have been established in LN based on the World Health Organization (WHO) classification [3–5] and more recent criteria [3, 6, 7], a later progression or transformation of the pathology cannot necessarily be predicted at the time of the initial biopsy [8, 9].

In our preliminary chart review, we found that numerous cases of remitted SLE had class IV LN at SLE onset. On the other hand, irreversible renal damage was precipitated in patients who initially showed no renal symptoms but later developed LN with various renal pathologies, and the LN of later development was never a case of senile-onset LN. Thus we undertook to study the possible relationship

Pivotal Role of Lnk Adaptor Protein in Endothelial Progenitor Cell Biology for Vascular Regeneration

Sang-Mo Kwon,* Takahiro Suzuki,* Atsuhiko Kawamoto, Masaaki Ii, Masamichi Eguchi, Hiroshi Akimaru, Mika Wada, Tomoyuki Matsumoto, Haruchika Masuda, Yoshihiro Nakagawa, Hiromi Nishimura, Kenji Kawai, Satoshi Takaki, Takayuki Asahara

Abstract—Despite the fact that endothelial progenitor cells (EPCs) are important for postnatal neovascularization, their origins, differentiation, and modulators are not clear. Here, we demonstrate that Lnk, a negative regulator of hematopoietic stem cell proliferation, controls endothelial commitment of c-kit$^+$/Sca-1$^+$/Lineage$^-$ (KSL) subpopulations of bone marrow cells. The results of EPC colony–forming assays reveal that small (primitive) EPC colony formation by CD34$^-$ KSLs and large (definitive) EPC colony formation by CD34$^{(dim)}$ KSLs are more robust in *lnk*$^{-/-}$ mice. In hindlimb ischemia, perfusion recovery is augmented in *lnk*$^{-/-}$ mice through enhanced proliferation and mobilization of EPCs via c-Kit/stem cell factor. We found that Lnk-deficient EPCs are more potent actors than resident cells in hindlimb perfusion recovery and ischemic neovascularization, mainly via the activity of bone marrow-EPCs. Similarly, *lnk*$^{-/-}$ mice show augmented retinal neovascularization and astrocyte network maturation without an increase in indicators of pathogenic angiogenesis in an in vivo model of retinopathy. Taken together, our results provide strong evidence that Lnk regulates bone marrow-EPC kinetics in vascular regeneration. Selective targeting of Lnk may be a safe and effective strategy to augment therapeutic neovascularization by EPC transplantation. (***Circ Res.* 2009;104:969-977.**)

Key Words: endothelial progenitor cell ■ lnk ■ vascular regeneration

Stem cell–related, postnatal neovascularization requires several activities of putative stem cells and their progeny, endothelial progenitor cells (EPCs), including the ability to self-renew in bone marrow (BM), commitment and differentiation into mature endothelial cells (ECs), mobilization from BM into the circulatory system, and recruitment to sites of neovascularization.[1,2] Many cytokines augment mobilization and/or recruitment of BM-derived EPCs,[3,4] including granulocyte colony-stimulating factor and granulocyte/macrophage colony-stimulating factor; angiogenic growth factors such as vascular endothelial growth factor (VEGF) and stromal cell–derived factor (SDF)-1; estrogen; and pharmaceutical drugs such as statins. However, these factors act not only on immature stem/progenitor cells but also on hematopoietic cells and mature ECs. Thus, the identification of a novel molecule that specifically regulates immature populations involved in EPC kinetics in BM is warranted.

Differentiation of progenitor cells into hematopoietic and endothelial lineage cells has been intensively investigated. During development, hemangioblastic aggregates originate from the mesodermal yolk sac, migrate to the fetal liver, and finally establish themselves in the BM. The results of a number of gene-targeting studies contribute to our understanding of functional molecules such as Scl/Tal,[5] c-kit, CD34, Runx-1,[6] and Flk-1,[7] which regulate the developmental kinetics of hemangioblasts and are also expressed in the common precursors of hematopoietic cells and ECs. Postnatal hematopoietic stem cells (HSCs) and EPCs also share common markers; however, the precise characteristics of hemangioblasts, mechanisms regulating cell growth in adults, and endothelial commitment of putative stem cells and/or common precursors for hematopoietic cells and ECs for postnatal vasculogenesis have not previously been reported.

The Lnk protein shares a pleckstrin homology domain, a Src homology 2 domain, and potential tyrosine phosphorylation sites with APS and SH-2B. It belongs to a family of adaptor proteins implicated in integration and regulation of multiple signaling events.[8] Lnk has been studied in the immune system, where Lnk regulates B cell production via negative regulation of pro–B-cell expansion.[8] Recently, Lnk was reported to play a critical role in maintaining the ability of HSCs to self-renew, in a study that based on BM c-Kit$^+$/Sca-1$^+$/Lineage (Lin)$^-$ (KSL) CD34$^-$ cells, which are

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CONCISE COMMUNICATION

Reciprocal recognition of an HLA-Cw4-restricted HIV-1 gp120 epitope by CD8$^+$ T cells and NK cells

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Objectives: The HIV-1 Nef protein selectively downregulates human leukocyte antigen (HLA)-A and HLA-B but not HLA-C molecules on the surface of infected cells. This allows HIV-infected cells to evade recognition by most cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. We investigated the recognition of an HLA-Cw4-restricted HIV-1 gp120 epitope SFNCGGEFF (SF9) and its variant SFNCGGEFL (SL9) by T cells and NK receptors.

Design and method: Recognition of HIV-1 gp120 peptides (SF9 and SL9) by T-cell clones was measured by staining with HLA-Cw4-peptide tetrameric complexes and cytolytic assays using target cell pulsed with either peptides. KIR2DL1 binding to these two peptides was measured using surface plasmon resonance and tetramer staining of an NK cell line.

Result: CTLs could recognize SF9 better than the variant SL9, as shown by both tetramer staining and cytolytic assays. Intriguingly, an HLA-Cw4 tetramer folded with the 'escape' variant SL9 could bind to KIR2DL1 on NK cell lines with higher affinity than HLA-Cw4-SF9. The binding of KIR2DL1 to its ligand results in inhibition of NK cell function. Our results indicate that the HIV-1 gp120 variant peptide SL9 could potentially escape both from NK cell and CTL recognition by increasing its affinity for KIR2DL1 binding.

Conclusion: These data suggest that HIV-1 can acquire mutations that are capable of escaping from both CTL and NK cell recognition, a phenomenon we have termed 'double escape'.

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Keywords: AIDS, cytotoxic T cells, human, natural killer cells

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Efficient silkworm expression of human GPCR (nociceptin receptor) by a *Bombyx mori* bacmid DNA system

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BmNPV

ABSTRACT

Guanine nucleotide-binding protein (G protein) coupled receptors (GPCRs) are frequently expressed by a baculovirus expression vector system (BEVS). We recently established a novel BEVS using the bacmid system of *Bombyx mori* nucleopolyhedrovirus (BmNPV), which is directly applicable for protein expression in silkworms. Here, we report the first example of GPCR expression in silkworms by the simple injection of BmNPV bacmid DNA. Human nociceptin receptor, an inhibitory GPCR, and its fusion protein with inhibitory G protein alpha subunit ($G_i\alpha$) were both successfully expressed in the fat bodies of silkworm larvae as well as in the BmNPV viral fraction. Its yield was much higher than that from Sf9 cells. The microsomal fractions including the nociceptin receptor fusion, which are easily prepared by only centrifugation steps, exhibited [^{35}S]GTPγS-binding activity upon specific stimulation by nociceptin. Therefore, this rapid method is easy-to-use and has a high expression level, and thus will be an important tool for human GPCR production.

Introduction

Guanine nucleotide-binding protein (G protein) coupled receptors (GPCR) are cell surface membrane proteins that regulate a broad range of physiological functions related to numerous diseases [1]. Thus, many widely used drugs directly target GPCR-mediated signaling. GPCRs have seven transmembrane regions, and associate with trimeric G protein, composed of alpha (Gα), beta (Gβ), and gamma (Gγ) subunits, at the cytoplasmic region [2]. The binding of specific ligands (peptides, lipids, steroids, etc.) to the extracellular region of GPCRs induces the enhancement of the GTPase activity of trimeric G protein, to exchange Gα-bound GTP to GDP. This hydrolysis leads to the dissociation of trimeric G protein subunits from GPCRs, and this triggers the following G protein-mediated signal transduction [3].

To date, several hundred GPCR family genes have been identified in the human genome [4]. However, the physiological ligands of many GPCRs remain unknown, and thus they are designated as "orphan receptors" [5]. Many researchers are searching for the physiological GPCR ligands, to understand their functions and to develop agonists or antagonists as effective, new drugs. In order to perform ligand screening and biochemical studies, large amounts of GPCR proteins are required. However, the expression of GPCRs is still difficult, thus poses a major obstacle for GPCR studies.

Escherichia coli (*E. coli*) expression systems are frequently used for the large-scale expression of recombinant proteins. However, *E. coli* systems have critical disadvantages for the expression of eukaryotic proteins, such as the lack of intramolecular disulfide bond formation and post-translational modifications. In the case of GPCRs, a small number of groups reported the successful expression of recombinant GPCRs using *E. coli*. [6–8]. Many researchers prefer eukaryotic expression systems to produce GPCRs. The baculovirus expression vector system (BEVS) is one of the attractive tools for the large-scale expression of recombinant GPCRs. BEVS has many advantages for the expression of eukaryotic proteins, including (1) high level expression by strong promoters (Polyhedrin and P10), and (2) post-translational modifications similar to those generated by mammalian cell expression. BEVS was employed in the successful crystallization of human adrenergic receptor protein for structure determination [9–12]. However, the commercially available BEVS system, which utilizes *Autographa californica* nucleopolyhedrovirus (AcNPV) and insect cell lines (High five™, Sf9 and Sf21), requires large-scale cultivation and virus handling techniques.

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Silkworm expression and sugar profiling of human immune cell surface receptor, KIR2DL1

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ABSTRACT

Immune cell surface receptors are directly involved in human diseases, and thus represent major drug targets. However, it is generally difficult to obtain sufficient amounts of these receptors for biochemical and structural studies because they often require posttranslational modifications, especially sugar modification. Recently, we have established a bacmid expression system for the baculovirus BmNPV, which directly infects silkworms, an attractive host for the large-scale production of recombinant sugar-modified proteins. Here we produced the human immune cell surface receptor, killer cell Ig-like receptor 2DL1 (KIR2DL1), by using the BmNPV bacmid expression system, in silkworms. By the direct injection of the bacmid DNA, the recombinant KIR2DL1 protein was efficiently expressed, secreted into body fluids, and purified by Ni^{2+} affinity column chromatography. We further optimized the expression conditions, and the final yield was 0.2 mg/larva. The sugar profiling revealed that the *N*-linked sugars of the purified protein comprised very few components, two paucimannose-type oligosaccharides, Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc and Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc. This revealed that the protein product was much more homogeneous than the complex-sugar type product obtained by mammalian cell expression. The surface plasmon resonance analysis demonstrated that the purified KIR2DL1 protein exhibited specific binding to the HLA-Cw4 ligand. Moreover, the CD spectrum showed the proper secondary structure. These results clearly suggested that the silkworm expression system is quite useful for the expression of cell surface receptors that require posttranslational modifications, as well as for their structural and binding studies, due to the relatively homogeneous *N*-linked sugar modifications.

Introduction

The cell surface receptors of immune cells regulate various biological functions via highly specific binding to their cognate ligands. Immune cell surface receptors tend to be directly involved in immune-related diseases, and thus they are one of the main targets for drug design. Moreover, the soluble forms of such receptors and their specific antibodies could become biopharmaceutical drugs for immune regulation. However, it is presently difficult to obtain sufficient amounts of the immune cell surface receptors for biochemical and structural studies, because they generally require sugar modifications. The baculovirus expression vector system (BEVS) is quite useful for the preparation of such post-translationally-modified recombinant proteins.

There are two types of baculoviruses currently utilized in BEVS. One of them is *Autographa californica* nuclear polyhedrosis virus (AcNPV), which can infect only *Spodoptera frugiperda* and *Tricoplusia ni*. The AcNPV BEVS is widely used and commercially available. The other is the *Bombyx mori* nuclear polyhedrosis virus (BmNPV), which can only infect the silkworm and its cell lines. The BmNPV BEVS using silkworms is one of the most suitable systems, because silkworms, which produce extreme amounts of silk fibers containing fibroin, are very attractive as a protein-production factory. Indeed, the protein expression using silkworms is 10- to 100-fold higher than that using *B. mori* cells. The conventional BmNPV BEVS, which requires a difficult, time-consuming plaque assay technique to obtain the recombinant viruses, was successfully utilized for the expression of interleukin receptors and interferon-α [1,2].

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