ORIGINAL ARTICLE
POSSIBLE FUNCTION OF NEURONAL LEUCINE-RICH REPEAT PROTEIN 3 (NLRR3) IN PRIMARY IMMUNE RESPONSE

Kyoko Ito\(^1\), Kazutaka Masuko\(^2\) and Koichi Ito\(^1,3\)

Abstract Contact between dendritic cells (DCs) and resting T cells is essential for initiation of the primary immune response. In this study, we examined whether neuronal leucine-rich repeat protein 3 (NLRR3), a receptor involved in nerve system development, participates in DC-T cell binding and T-cell activation. We confirmed that NLRR3 is expressed on naive T cells. NLRR3 contains an Arg-Gly-Asp (RGD) motif in its amino acid sequence, for which several integrins can be considered as potential ligands. Indeed, monocyte-derived DCs expressed several integrins that can bind to the RGD motif. Functionally, DC-induced resting allogeneic T-cell proliferation was partially inhibited by addition of integrin-specific antibodies and synthetic RGD peptides, indicating that RGD-containing molecules, including NLRR3 and several integrins, at least participate in the events of the initial primary immune response involving naive T cells and DCs. Furthermore, DCs were shown to bind directly to NLRR3-transfected Chinese hamster ovary cells in a NLRR3-dependent manner, and the binding was removed in the presence of integrin-specific antibodies. These data suggest that NLRR3 plays an important role in initiation of the primary immune response.


Key words: neuronal leucine-rich repeat protein; integrin; RGD motif; dendritic cells; adhesion molecule.

原著
一次免疫応答における Neuronal leucine-rich repeat protein 3 (NLRR3) の機能的役割

伊藤京子\(^1\) 増子和彦\(^2\) 伊藤巧一\(^1,3\)

抄録 一次免疫応答としてのT細胞活性化は、樹状細胞（DC）などの抗原提示細胞との接触により誘導される。本研究では、神経組織に発現しているNLRR3が接着分子として一次免疫応答に及ぼす影響を検証した。NLRR3はT細胞に特異的に発現していた。またNLRR3はDCに発現するインテグリンと結合可能なArg-Gly-Asp（RGD）モチーフを保有していた。そこで抗インテグリン抗体および合成RGDペプチドをDC刺激によるT細胞増殖系に加えたところ、その増殖活性が抑制された。またNLRR3を強制発現したチアニーズハムスター卵巣組織由来CHO細胞にDCを加えたところ、強制発していないCHO細胞よりも結合細胞数が増加した。さらにこの増加は、抗インテグリン抗体の添加により減少した。これらの結果は、T細胞上のNLRR3がDC上のインテグリンと直接結合して一次免疫応答の制御に関与していることを示唆する。

弘前医学 61: 46–57, 2010

キーワード：神経関連ロイシンリッチリピートタンパク質；インテグリン；RGD モチーフ；樹状細胞；接着分子。

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Received for publication, December 11, 2009
Accepted for publication, January 4, 2010
INTRODUCTION

In the immune system, T-cell activation requires interaction with antigen-presenting cells (APCs). In particular, dendritic cells (DCs), the major type of APC, have a unique ability to present antigen to naive T cells and thus initiate the primary immune response. During this antigen recognition, highly structured and localized adhesion complexes are constructed at the DC-T cell interface, forming a specialized junction known as the immunological synapse. This is the most important event for induction of T-cell proliferation and differentiation into effector T cells. Previous studies have indicated that both adhesion and costimulatory molecules with their corresponding ligands, such as LFA-3/CD2 and LFA-1/ICAM-1, -2 or -3, are potential candidate mediators of these DC-T cell interactions. However, DC-T cell interaction may reflect the numerous adhesion molecules involved in the stabilization of this initial contact.

Neuronal leucine-rich repeat protein (NLRR) genes were first isolated from a mouse brain cDNA library, and three distinct subfamilies (NLRR1, 2 and 3) have since been identified in mouse, rat and human. Recently, other NLRR subfamily members were also identified. NLRRs have been proposed to function as neuronal adhesion molecules. Essentially, NLRRs constitute a novel LRR family containing 11 LRRs, one immunoglobulin-like domain and one fibronectin type III-like domain. Mouse NLRR1 is expressed in the myotome of developing somites, but not in the presomitic mesoderm. NLRR2 expression appears to be prominent only in adults, while NLRR3 exhibits regulated expression in developing ganglia and motor neurons. In addition, NLRR3 expression has been shown to be upregulated during cortical injury, suggesting that this molecule plays an important role in regulation of synaptic reorganization.

In cellular interaction, cell-cell contacts may be accomplished through similar molecular events in different cell types. In particular, the nervous system as a regulator of synapse formation, also plays a role in regulating the formation of the immunological synapse. Therefore, in this study, we examined whether NLRR molecules participate in the initial exploratory events involved in adhesion between naive T cells and DCs to elicit the primary immune response. Here we describe the expression of NLRR3 on naive T cells and their potential ligand, an integrin, on DCs.

MATERIALS and METHODS

Blood samples

Peripheral blood samples were taken from three healthy volunteers after informed consent had been obtained. The mononuclear cell fraction was collected by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare). This study was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine, Hirosaki, Japan.

Magnetic separation

For RT-PCR experiments, human T cells, B cells, and monocytes were positively isolated from the mononuclear cell fraction by the magnetic method with anti-CD3, anti-CD19 and anti-CD14 monoclonal antibodies (Beckman Coulter), respectively, followed by Dynabeads M-450 goat anti-mouse IgG (Life Technologies Inc.). In addition, T cells and monocytes were also negatively isolated from the mononuclear cell fraction using a Pan-T isolation kit II and a Monocyte isolation kit II, respectively, with a MACS column system (Miltenyi Biotec) for cell culture experiments.

Cell line

The Chinese hamster ovary (CHO) cell line was provided by Dr. Nobukata Shohara (Kitasato University).
Reagents for cell culture

RPMI1640 containing 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 μM L-glutamine, and 10 mM HEPES was used as complete medium for cell culture experiments. For generation of monocyte-derived DCs, recombinant human GM-CSF, IL-4, and TGF-β, purchased from PeproTech Inc., were added to the complete medium.

DC induction

Monocytes that had been isolated by negative selection were used for DC generation. Immature DCs (imDCs) were cultured from monocytes in the presence of GM-CSF and IL-4 (50 ng/ml each) at 3×10^5 cells/well in 6-well tissue culture plates in complete medium for 6 days in a 5% CO₂ incubator, and continuous culture was performed to generate mature DCs (mDCs) with 50 ng/ml TGF-β for 3 days \(^{[41]}\). To confirm maturation into DCs, cultured cells were analyzed by staining with FITC-labeled anti-CD14, FITC-labeled anti-HLA-DR, and PE-labeled anti-DC-SIGN, and then subjected to flow cytometric analysis (data not shown).

RT-PCR

Positively selected T cells, B cells, and monocytes were used for detection of NLRRs and integrin mRNA expression by RT-PCR. Total mRNAs extracted from these cells were transcribed into cDNAs using M-MLV reverse transcriptase (Takara Bio Inc.) and oligo-dT primers (Life Technologies Inc.). A similar procedure for cDNA synthesis was also performed on monocyte-derived immature and mature DCs. PCR was performed for 35 cycles using specific oligonucleotide primers (denaturation at 95°C for 15 s; annealing at 58°C for 15 s; extension at 72°C for 1 min for NLRR1, 2, 3\(^{[3]}\) and GAPDH, and denaturation at 94°C for 30 s; annealing at 58°C for 1 min; extension at 72°C for 2 min for the integrin α chains (αIIb, αV, α5, and α8) and β chains (β1, β3\(^{[46]}\), β5, β6, and β8\(^{[27]}\) ). The primer sequences are shown in Table 1. PCR products were visualized on 1.5% agarose gels with ethidium bromide staining. Expression of mRNAs for NLRR1, 2, and 3 was also examined using a Human Blood Fraction MTC Panel (Clontech Laboratories, Inc.).

Northern blot analysis

Complementary T-cell DNA synthesized as

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described above was amplified by PCR with NLRR3-specific oligonucleotide primers, and the PCR products were labeled with [\(\alpha^{32}\text{P}\)]dCTP using a Rediprime II DNA labeling system (GE Healthcare) in accordance with the manufacturer's instruction. The radioactive probes were hybridized onto premade nylon membrane filters of Human Fetal MTN Blot II and Human Immune System MTN Blot II (both purchased from Clontech Laboratories, Inc.), and the signals were detected on GE Hyperfilm MP (GE Healthcare) after overnight exposure.

**Flow cytometry**

Negatively isolated T cells and monocytes, and monocyte-derived immature and mature DCs were stained with anti-\(\alpha5\beta1\) (IIA1, BD Biosciences), anti-\(\alpha\V{3}\) (LM609, Millipore), and anti-\(\alpha\V{5}\) (P1F6, Millipore) integrin-specific monoclonal antibodies, followed by phycoerythrin (PE)-labeled goat anti-mouse IgG (Beckman Coulter). The stained cells were then washed and analyzed on an EPICS XL flow cytometer using EXPO32 software (Beckman Coulter). These antibodies were also used as blockers in mDC-induced alloimmune T-cell proliferation.

**T-cell proliferation inhibition assay**

An assay was performed to examine the inhibition of mDC-induced resting alloimmune T-cell proliferation. Alloimmune resting responder T cells (1x10^5 cells) were added to monocyte-derived mDCs (5x10^5 cells) in each well of a U-bottomed 96-well plate with each (10 \(\mu\)g/ml) of the blocking antibodies against integrins \(\alpha5\beta1\) (IIA1), \(\alpha\V{3}\) (LM609), and \(\alpha\V{5}\) (P1F6). Purified IgGl was used as a negative control. In addition, RGD synthetic peptides (Hokkaido System Science) were used for this assay as blocking reagents at various concentrations (0 \(\mu\)g, 5 \(\mu\)g, 25 \(\mu\)g, 50 \(\mu\)g/ml). The cells were cultured for 5 days and pulsed with 37-kBq \([\text{3}^\text{H}]\)-thymidine for 16 h, then harvested onto glass fibers. \(\text{3}^\text{H}\) incorporation was determined using a liquid scintillation counter (Aloka, LSC-5100).

**Binding assay with NLRR3 transfectant**

Direct interaction of NLRR3 and integrin was demonstrated by binding between mDCs and wild-type CHO cells or CHO cells that had been transfected with NLRR3. Full-length cDNA for NLRR3 was obtained from positively isolated human T cells by 30 cycles of RT-PCR with primeSTAR DNA polymerase (Takara Bio Inc.) utilizing a forward primer for the BamHI site: 5’-CCCCGATTCGCAATGAAGACATGCCACTCCGA-3’ and reverse primer for the NotI site: 5’-CCCGCGGCGCTTACGATATTGTTG T-3’ (denaturation at 98°C for 10 s; annealing at 55°C for 5 s; extension at 72°C for 2 min). The amplified NLRR3 full-length cDNA was inserted into the corresponding BamHI-NotI site of the pIRESneo3 expression vector, and the construct was then transfected into CHO cells using Lipofectamine 2000 (Life Technologies Inc.). Stable NLRR3-expressing CHO transfectants were obtained by continuous culture with G418 (1.5 mg/ml) for 4 weeks.

mDCs were labeled with PKH2-GL green fluorescent dye (Sigma-Aldrich Chemical Co.) in accordance with the manufacturer's instructions. The labeled mDCs were added to a non-transfected or transfected CHO monolayer grown to confluence in a 24-well plate at a mDC/CHO or transfected CHO ratio of 3:5 and cultured for 1 h at 37°C in a 5% CO_2 incubator in serum-free RPMI1640 medium containing 3% bovine serum albumin. After culture, the non-binding mDCs were gently removed and then washed twice with Ca\(^{2+}\), Mg\(^{2+}\)-free PBS buffer. The remaining cells in the plate were detached and suspended as single cells by treatment with trypsin-EDTA, and the number of fluorescent mDCs in the suspension that had bound CHO cells with or without NLRR3 transfection was measured by flow cytometry described previously\(^{48}\). Furthermore, the binding
Effect between mDCs and NLRR-transfected CHO cells was examined in the presence of blocking antibody (10 μg/ml) against each integrin used, as shown in Figure 5A.

Statistical analysis

Statistical significance of differences was determined using Student’s t-test. Differences at \( p<0.05 \) were considered significant.

RESULTS

Expression of NLRR3 by human T cells

NLRR3 expression has been studied predominantly in the developing nervous system. Because synapse formation in the immune system and the nervous system has common features, we analyzed whether immune cells expressed NLRRs using RT-PCR. Expression of mRNA for NLRR1 and 2 was marginal in all fractions of blood total mononuclear cells (MNCs), CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes, and this was the case in both the resting group and the mitogen-stimulated group (Figure 1A).

Interestingly, NLRR3 mRNA was specifically expressed on CD4+ T cells and CD8+ T cells in both groups. Total MNCs including T cells were also amplified. GAPDH amplification was similar in all blood fractions. These results indicate that, among immune cells, NLRR3 is expressed specifically on T cells. Also, the expression levels of mRNAs for NLRR1, 2 and 3 were measured in CD3+ T cells, CD19+ B cells, CD14+ monocytes, and monocyte-derived immature and mature DCs from the mononuclear cell fraction in our laboratory (Figure 1B). Results essentially similar to those shown in Figure 1A were obtained, indicating T-cell-specific expression of NLRR3 mRNA. NLRR3 expression on monocyte-derived DCs was not detectable in immature and mature DCs. No difference of GAPDH amplification was observed among the blood fractions.

Accumulation of NLRR3-expressing cells in lymph nodes

Expression of NLRR3 mRNA was also examined by Northern blot analysis. In fetal tissues, a
high level of NLRR3 expression was detected in brain, in accordance with previous reports\(^6,12,15\) (Figure 2, upper panel). In contrast, other fetal tissues, including lung, liver and kidney, showed no detectable expression of NLRR3. Among immune tissues, expression of NLRR3 was predominant in lymph nodes, where T cells and DCs accumulate to initiate a primary immune response (Figure 2, lower panel). Although spleen, thymus and PBL also contain many T cells, none showed detectable NLRR3 expression. A similar expression level of β-actin, used as an endogenous control, was observed in all tissues (data not shown).

**Monocyte-derived DCs express integrins as potential ligands for NLRR3**

The above studies showed that NLRR3 was specifically expressed on T cells. We next searched for potential ligands of NLRR3 on DCs. Among the three NLRR family members, only NLRR3 contains an Arg-Gly-Asp (RGD) motif in its amino acid sequence\(^5,6\). As potential ligands for this RGD motif, integrin molecules have already been identified. Each integrin molecule is a heterodimer of α and β chains, and half of the more than 20 known integrin dimers recognize the RGD motif\(^19,20\). In this study, expression of the integrin α and β chains, known to be ligands for the RGD on DCs, was examined by RT-PCR (Figure 3). After PCR, integrin chains αV, α5 and β1 were commonly amplified not only in immature and mature DCs but also in T cells. In contrast, the integrin β3 and β5 chains were amplified in immature and mature DCs, but not in T cells. Therefore, potential integrins capable of binding to the RGD motif in NLRR3, expressed on DC, were αVβ1, αVβ3, αVβ5 and α5β1.

**Integrins αVβ3, αVβ5 and α5β1 are expressed on monocyte-derived DCs**

Of the 4 integrins expected to act as ligands for the RGD in NLRR3, cell surface expression of αVβ3, αVβ5 and α5β1 on DCs was detected by flow cytometry using commercially available
Specific monoclonal antibodies against them (Figure 4). Integrin α5β1 was commonly expressed at a high level in T cells, monocytes, and monocyte-derived immature and mature DCs. On the other hand, expression of αVβ3 and αVβ5 increased with the course of maturation of monocytes to DCs. An especially high level of αVβ5 expression was detected in both immature and mature DCs. In contrast, integrins αVβ3 and αVβ5 were not expressed on T cells, indicating that their expression on DCs was specific.

Effect of NLRR3 and its potential ligand integrin on T-cell proliferation

To determine whether NLRR3 and integrin are required for initiation of the primary immune response, we studied the involvement of these receptors in DC-induced allogeneic T-cell proliferation. The T-cell proliferation was inhibited when T cells and mDCs were cultured with blocking antibodies against integrins αVβ3, αVβ5, and α5β1 (Figure 5A) or synthetic RGD peptides (Figure 5B), indicating that NLRR3 and integrins αVβ3, αVβ5, and α5β1 at least participate in the events of the initial primary immune response involving naïve T cells and DCs. In Figure 5B, the minimal dose (5 μg/ml) of RGD peptide may already have represented the saturation level for inhibition of the primary immune response. In these assays, the inhibitory effect of RGD peptide did not represent the result for NLRR3 alone in the primary immune response, because numerous RGD-containing molecules contribute simultaneously to these events. Furthermore, evidence of direct binding of NLRR3 to integrin would be necessary to confirm their interaction.

NLRR3 can bind αVβ3 integrin expressed on mDCs

There is still no direct evidence that NLRR3 is a ligand for integrin(s). For this purpose, binding of NLRR3 and integrin(s) was examined using mDCs and NLRR3-transfected or non-transfected CHO cells. The number of green fluorescence-labeled-mDCs bound to transfectedants or non-transfectants was measured using flow cytometry. CHO transfectedants expressed NLRR3 at a high level (Figure 6A). We observed an increased
number of mDCs bound to NLRR3-transfected CHO cells in comparison with non-transfected CHO cells (Figure 6B), and the increased binding of mDCs to NLRR3-transfected CHO cells was significantly inhibited to a level similar to that of non-transfected CHO cells in the presence of antibody against αVβ3, but not antibody against αVβ5 (double-headed arrow in Figure 6C). In contrast, addition of antibody against α5β1 enhanced the binding of DCs to NLRR3-expressing CHO cells (data not shown). These results suggest that integrin αVβ3 expressed on DCs is a potential ligand for NLRR3 expressed on T cells.

**DISCUSSION**

T cells are activated by interaction with APCs. During an immune response, this happens first when naive T cells meet DCs in the secondary lymphoid organs, which triggers T-cell proliferation and differentiation into effector T cells. The effector T cells then interact with APCs a second time, this time in the periphery, which contributes to antigen elimination. During each of these encounters, the T cells must establish stable contact with the APCs and maintain this contact in order to allow sufficient signaling at the T cell-APC interface. Essentially, the same scenario applies to pre- and post-synaptic neurons after their initial contact. Both processes result in the formation of highly structured and localized adhesion complexes that are necessary for T-cell activation and neurotransmission, respectively.

In this study, we focused on NLRR3, a well known adhesion molecule that functions in early embryonic patterning, brain development and nervous system regeneration. Using RT-PCR, we found that NLRR3 was expressed on T cells, but not on other immune cells (Figure 1A.
and B). Other NLRR family members, NLRR1 and 2, were not essential for immune function. In addition, northern blot analysis revealed a high level of NLRR3 expression in lymph nodes, where many T cells accumulate to obtain antigenic information from DCs to initiate the primary immune response (Figure 2). However, expression of NLRR3 was not detectable in other T-cell-containing tissues, i.e., the spleen, thymus and PBL, indicating that detection of NLRR3 by this technique may require gene amplification.

Next, we searched for potential ligands of NLRR3 on DCs. Since the amino acid sequence of NLRR3 contains the RDG motif\(^{10}\), integrins are considered to be potential ligands for NLRR3 on DCs, as described previously\(^{10}\). Integrins are important for T-cell interactions with APCs\(^{22}\) and for migration of leukocytes from blood into tissues. The ligand-binding affinity of integrins can be regulated by various stimuli, and the cytoplasmic domains of integrins bind to the cytoskeleton\(^{22}\). We examined integrins that can bind to NLRR3 expressed on DCs, and selected integrins αβ1, αβ3, αβ5 and α5β1 as potential candidates by RT-PCR (Figure 3). Of these 4 integrins, flow cytometric analysis demonstrated expression of integrins αβ3, αβ5 and α5β1 on DCs (Figure 4). Especially, the expression of αβ3 and αβ5 increased with the course of maturation from monocytes to DCs. Although previous reports have suggested that integrins αβ3 and αβ5 are expressed on DCs\(^{10}\), their immunological function has been unclear. Harui et al. have suggested that DCs expressing a high level of integrin αβ3 are preferentially transduced by adenoviral vectors and activate the CD8\(^+\) T-cell response against adenovirus-encoded antigens\(^{23}\).

T-cell proliferation induced by allogeneic mDCs was interrupted in the presence of antibody

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**Figure 5** Effect of NLRR3 and integrin on allogeneic DC-induced T-cell proliferation. The requirement of NLRR3 and integrin for DC-induced allogeneic T-cell proliferation was investigated. mDCs and T cells were cultured with blocking antibody against αβ3, αβ5 or α5β1 (10 μg/ml) (A) or the indicated dose of synthetic RGD peptide (B) for 4 days. T cells were then pulsed for 16 h with [³H]-thymidine, and their proliferation was measured by [³H] incorporation. Bar graphs show the ratio of the inhibitory effect on T-cell proliferation. T-cell proliferation in the presence of purified IgG1 (A) or in the absence of synthetic RGD peptide (B) is shown as 1. Results are expressed as mean ± SD of two independent experiments. A significant inhibitory effect was observed in the presence of antibodies against αβ3 and α5β1 (A) and in the presence of all doses of RGD peptides (B), indicating that their corresponding molecules at least participate in initiation of the primary immune response. (Student’s t-test; *p < 0.05, **p<0.01)
Figure 6  Evidence of direct binding between NLRR3 and integrin. Direct binding between NLRR3 and integrin was examined using mDCs and NLRR3 transfectants. (A) NLRR3 was expressed in CHO transfectants but not in non-transfectants. (B) Green fluorescence-labeled mDCs were added to NLRR3-transfected and non-transfected CHO cells grown to confluence in culture plates. After gentle washing, the remaining cells on the plate were harvested with trypsin-EDTA, and green fluorescence-labeled mDCs that had bound CHO cells were counted using flow cytometry. Bar graphs show the ratio when the number of mDCs bound to transfected CHO is shown as 1. (C) Involvement of integrin in the direct binding of mDCs to NLRR3-transfected CHO cells was examined. Bar graphs show the percentage binding inhibition when mDCs were cultured with NLRR3-transfected CHO cells in the presence of antibody against αVβ3 or αVβ5. Mouse purified IgG1 was used as a negative control. The inhibition assay suggested that NLRR3 can bind directly to integrin αVβ3. Results are expressed as mean ± SD of two independent experiments in (B) and (C).
(Students t-test; **p<0.01)

against integrin αVβ3, αVβ5 or α5β1 (Figure 5A). Such an inhibitory effect has also been observed in previous studies upon addition of synthetic RGD peptides (Figure 5B). These results indicate that integrins αVβ3, αVβ5 and α5β1, and RGD-containing molecules (including NLRR3) at least contribute to the events responsible for inducing T-cell proliferation upon stimulation with allogeneic DCs. However, the evidence from this analysis dose not allow confirmation of direct interaction of integrins with DCs and NLRR3 on T cells, because other possible ligands for NLRR3 and integrins also contribute simultaneously to DC-T interaction. To clarify this issue, NLRR3-overexpressing CHO cells were used as a binding partner for mDCs. Their increased
binding activity, shown in Fig. 6B, was lost in the presence of antibody against integrin αVβ3, indicating that NLRR3 binds directly to integrin αVβ3 (Figure 6C).

Previous reports have suggested that immunological and neurological synapses use a common molecule, agrin[24], and that the neuronal repellent Slit[25] regulates leukocyte migration. In addition, Tordjman et al. have reported that a neuronal receptor, neuropilin-1, mediates interactions between DCs and T cells that are essential for initiation of the primary immune response[26]. Our present findings have revealed that NLRR3 is another feature common to both the nervous and immune system. We conclude that NLRR3 is expressed on T cells, and that its potential ligand, integrin αVβ3, is expressed on DCs, their association apparently influencing the primary immune response. Future studies to evaluate the direct interaction between integrin αVβ3 and NLRR3 at the DC-T cell interface are warranted.

ACKNOWLEDGEMENTS

This work was supported by a Grant for Hirosaki University Institutional Research.

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