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CD155 (PVR/Necl5) Mediates a Costimulatory Signal in CD4$^+$ T Cells and Regulates Allergic Inflammation

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Although Th1 and Th2 cells are known to be involved in allergic inflammatory diseases, the molecular mechanisms underlying their differentiation are incompletely understood. In this study, we identified CD155 as a costimulatory molecule on CD4$^+$ T cells. Importantly, CD155-mediated signaling induced Th1 development in both humans and mice, as evidenced by production of IFN-$\gamma$ and upregulation of $\text{Tbx21}$ transcription; these effects were independent of IL-12 but dependent on NF-$\kappa$B–induced autocrine IFN-$\gamma$ that triggered positive feedback via STAT1 activation. Mice genetically deficient in CD155 or treated with anti-CD155 Ab exhibited attenuated Th1-type contact hypersensitivity. Thus, CD155 plays an important regulatory role in helper T cell differentiation and allergic diseases. The Journal of Immunology, 2015, 194: 5644–5653.

Allergic diseases are caused by hypersensitivity reaction of immune responses and are commonly classified according to the type of immune responses. The Th2 immune response is involved in IgE production and mediates type I allergic responses (so-called immediate hypersensitivity), which are due to mast cell activation induced by the crosslinking of Fc$\varepsilon$RI with an IgE immune complex (1, 2). In contrast, the Th1 immune response leads to delayed-type hypersensitivity through the production of proinflammatory cytokines from Th1 and CD8$^+$ T cells. Imbalance in the numbers of Th1 and Th2 cells is responsible for many allergic inflammatory disorders and correction of the imbalance may ameliorate allergic inflammation. For example, the administration of recombinant IFN-$\gamma$ or IL-12, which induce the polarization of CD4$^+$ naive Th cells into Th1 cells, attenuates allergic airway inflammation (3, 4).

Allergic contact dermatitis (ACD) is one of the most common skin diseases, affecting 15–20% of the general population worldwide. Contact hypersensitivity (CHS) is the most frequently used murine model of human ACD (5). The pathogenesis of CHS is based on delayed-type hypersensitivity, which is mediated mainly by Th1 immune responses. The CHS reaction is divided into the sensitization and elicitation phases. Ag-specific Th1 cell clones differentiate from naive CD4$^+$ Th cells during the sensitization phase; later on, re-exposure to Ag activates Th1 and CD8$^+$ T cells, thus inducing cytokine-mediated inflammation during the elicitation phase. Recent evidence has demonstrated that regulatory T cells, Th17 cells, and dermal dendritic cells (DC) also play important roles in the pathogenesis of CHS (5). Moreover, CHS reactions are attenuated in mice deficient in CD28, OX40L, and LFA-1 (6–8), suggesting that these costimulatory molecules are involved in the development of CHS.

Th1 cells produce IFN-$\gamma$, IL-2, and TNF-$\alpha$ and are involved in cellular immune responses against intracellular pathogens. The differentiation of Th1 cells from naive CD4$^+$ T cells is driven mainly by IL-12 produced by APCs. IL-12–mediated signaling in CD4$^+$ T cells activates STAT4, which induces IFN-$\gamma$ production (9). IFN-$\gamma$ promotes the phosphorylation of STAT1 by binding to the receptor for IFN-$\gamma$ that is expressed on CD4$^+$ T cells, resulting in upregulation of T-bet, a master regulator of Th1 development. Thus, Th1 development is reinforced by autocrine IFN-$\gamma$ via the STAT1 and T-bet signaling pathways in a positive-feedback manner (10, 11). However, although IL-12–deficient mice show severely impaired Th1 responses, they still carry low levels of Th1 cells, suggesting that these cells might alternatively be developed through an IL-12–independent mechanism (12).

CD155, initially identified as a receptor for poliovirus in humans (13), is a member of poliovirus receptor–related (PRR) family of adhesion molecules, which consists of CD111 (nectin-1/PRR-1), CD112 (nectin-2/PRR-2), nectin-3 (PRR-3), and nectin-4 (PRR-4). The CD155 transcript is ubiquitously expressed in most organs in both human and mice (14–15) and on various hematopoietic cells in mice, including T cells, macrophages, and DC (16). CD155 binds to another PRR family member, nectin-3, as well as the matrix protein vitronectin, thereby mediating cell–cell or cell–matrix adhesion, respectively, and cell migration (17). Additionally, CD155 is a ligand for the Ig-like receptors DNAM-1 (CD226), Tactile (CD96), and T cell immunoreceptor with Ig domain (TIGIT) on T cells and NK cells (18–22). Mice deficient in CD155 show diminished humoral immune responses and thymic retention of mature CD8$^+$ cells (16, 23–25). Although several studies have demonstrated various functions of CD155, particularly on nonhematopoietic cells, the role of CD155 in lymphocyte function remains undefined.

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Abbreviations used in this article: ACD, allergic contact dermatitis; alum, aluminum hydroxide; CHS, contact hypersensitivity; cIg, control Ig; DC, dendritic cell; DNCB, 1-chloro-2,4-dinitrobenzene; IFN-$\gamma$R, IFN-$\gamma$ receptor $\alpha$-chain; PRR, poliovirus receptor-related; Tg, transgenic; WT, wild-type.
In this study, we investigated the role of CD155 in lymphocyte function and immune responses. We show that CD155-mediated signaling plays an important role in the pathogenesis of contact hypersensitivity.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Clea Japan (Tokyo, Japan). I-Fg-γ−/− mice and Rag1−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CD155-deficient (Per−/−) mice were generated as described (16) and were crossed with C57BL/6 (Tg) mice (26). DNAM-1−/− mice were generated as described (27) and crossed with Rag1−/− mice. All mice were housed and bred under specific pathogen-free conditions at the Laboratory Animal Resource Center (University of Tsukuba, Ibaraki, Japan). All animal experiments were performed according to the guidelines of the Animal Ethics Committee of the Laboratory Animal Resource Center, University of Tsukuba.

Human samples

Human peripheral blood was obtained from healthy volunteers after obtaining informed consent. Human cord blood was purchased from the RIKEN BioResource Center (Ibaraki, Japan). All experiments with samples of human blood were approved by the Ethics Review Board of the University of Tsukuba.

Abs and reagents

Anti-mouse CD1 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD28 (37.51), CD62L (MEL-14), CD44 (J67, B220 (RA3-6B2), CD49b (DX5), CD11c (HL3), CD11b (M1/70), Ly6G (1A8), S IgG-F (ES0-2400), IFN-γ receptor α-chain (IFN-γR-α), CD4 (R4-6A2), IFN-γ (XM-2-12), IL-2 (11B11), IL-17A (TC11-18H10.1), and IL-12 (C17.8) mAbs; anti-human CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD19 (B19), CD56 (B159), CD28 (CD28.2), CD45RA (HI100), CD45RO (UCHL1), and CD44 (44-26)-mAbs; isotype-matched control Abs; and secondary Abs and fluorescein-conjugated streptavidin were purchased from BD Biosciences (San Jose, CA). Anti-human CD14 (TUK4) mAb was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-mouse IFN-γRα (2E2) was purchased by BioLegend (San Diego, CA). Anti-mouse α-chain (IFN-γ) was purchased from eBioscience (San Diego, CA). Anti-mouse p-STAT1 (42H3) and STAT1, K-B (TX42, rIgG2a), and anti-CD155 mAbs; isotype-matched control Abs; and secondary Abs and fluorescein-conjugated streptavidin were purchased from BD Biosciences (San Jose, CA). Anti-human CD14 (TUK4) mAb was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-mouse iNOS (iNOS-1) was purchased from Abcam (Cambridge, MA, USA). Anti-mouse NF-κB translocation-dependent Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse β-actin (AC515) mAb was purchased from Sigma-Aldrich (St. Louis, MO). For ELISA, anti-mouse IFN-γ (AN-18), IL-2 (JES6-1A2), and IgE (R35-118) mAbs were used as capture Abs, and biotinylated anti-mouse IFN-γ, IL-2, and IgE were used as detection Abs; these Abs were purchased from BD Biosciences. Streptavidin-conjugated HRP was purchased from GE Healthcare Biosciences. OVA323-339 peptide (1 μg/ml) was used as a high-affinity anti-OVA Abs. OVA-specific Abs were purchased from BioLegend (San Diego, CA). Anti-CD3, anti-CD4, and anti-CD8 mAbs were purchased from eBioscience (San Diego, CA). Anti-CD155 Abs were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-mouse CD45RA (53-6.7), CD45RO (53-1), and CD44 (44-26)-mAbs were purchased from BD Biosciences (San Jose, CA). Anti-mouse β-actin (AC515) mAb was purchased from Sigma-Aldrich (St. Louis, MO). For ELISA, anti-mouse IFN-γ (AN-18), IL-2 (JES6-1A2), and IgE (R35-118) mAbs were used as capture Abs, and biotinylated anti-mouse IFN-γ, IL-2, and IgE were used as detection Abs; these Abs were purchased from BD Biosciences. Streptavidin-conjugated HRP was purchased from GE Healthcare Biosciences. OVA323-339 peptide (1 μg/ml) was used as a high-affinity anti-OVA Abs. Anti-CD155 mAbs were generated in hybridoma cultures and purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Flow cytometry

Cell suspensions were analyzed using FACSAria flow cytometers (BD Biosciences). FlowJo software (Tree Star, Ashland, OR) was used for data analysis.

Isolation of CD4+ T cells

CD4+ T cells were isolated from the spleens of wild-type (WT) mice by using anti-CD4 MACS technology (Miltenyi Biotec) according to the manufacturer’s instructions. Naïve CD4+ T cells were sorted from mouse spleens or human peripheral blood, respectively, by using FACSARia (BD Biosciences).

Stimulation of CD4+ T cells

To examine tyrosine phosphorylation of CD155, CD4+ T cells were stimulated with anti-CD155 mAb or control Ig (clg; 10 μg/ml) and then cross-linked with anti-rat IgG secondary Ab (10 μg/ml). For proliferation assays, CD4+ T cells were stimulated for 48 h with plate-bound anti-CD3 mAb and either plate-bound rat IgG2a isotype control or anti-CD155 mAb (20 μg/ml). CFSE-labeled CD4+ T cells were stimulated for 48 h with plate-bound anti-CD3 mAb and either plate-bound rat IgG2a isotype control or anti-CD155 mAb (20 μg/ml). CFSE dilution was measured by flow cytometry. For analysis of 4iAb, CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD155 mAbs. In the case of Erk and NF-κB, CD4+ T cells were stimulated with soluble anti-CD3 and anti-CD155 mAbs, followed by cross-linking with secondary Abs. Mouse and human CD4+ naïve T cells were stimulated for 6 d with plate-bound anti-CD3 mAb (1 μg/ml) and anti-CD155 mAb (20 μg/ml) in the presence of IL-2 (20 ng/ml). For coculture assays, splenocytes were treated with 50 μg/ml mitomycin C. Naïve CD4+ T cells (105) and the splenocytes (2×106) were cocultured for 6 d with OVA323-339 peptide (1 μM) in the presence or absence of neutralizing anti-IL-12 mAb or anti-IFN-γ mAb (40 μg/ml).

Analysis of intracellular cytokines

Cells (105–106/ml) were stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 2 h, after which brefeldin A (10 μg/ml; Sigma-Aldrich) was added and cells were cultured for another 2 h. Cells then were harvested, resuspended, stained for intracellular cytokines (Fix and Perm Kit, Invitrogen) according to the manufacturer’s instructions, and analyzed by flow cytometry.

ELISA

The concentration of mouse IFN-γ and IL-2 in culture supernatants and IgE, IgG1, and IgG2a in sera were measured by ELISA using capture and detection mAbs against mouse IFN-γ, IL-2, and IgE. Mouse IL-4 and human IFN-γ and IL-4 concentrations were measured by using ELISA kits from BD Biosciences, and mouse and human IL-17A levels were measured by using ELISA kits from R&D Systems; all assays were performed according to the manufacturer’s instructions.

Biochemistry

After respective stimulations as described in Materials and Methods, cells were lysed by using a buffer containing 1% Nonidet P-40, protease inhibitors, and phosphatase inhibitors, as described (30). Total cell lysates were immunoprecipitated by using control Ab or anti-CD155 mAb. Proteins were separated by SDS-PAGE under nonreducing conditions, immunoblotted by using anti-phosphotyrosine (4G10) (0.3 μg/ml) Ab, and rebotted with anti-CD155 mAb (2 μg/ml). For analysis, 4iAb and NF-κB–stimulated cells were lysed by using 1% Nonidet P-40 lysis buffer as described above, or by a cytoplasmic and nuclear extraction kit (Invent, Eden Prairie, MN), respectively, and immunoblotted with appropriate Abs, according to the manufacturer’s instructions.

Quantitative RT-PCR

Total RNA was isolated from cells pellets by using Isogen reagent (Nippon Gene, Tokyo, Japan). For reverse transcription, we used 2 μg total RNA and a final volume of 20 μl with a high-capacity CDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Real-time PCR analysis of Tbx21, Gata3, Rorc, Foxp3, and Ifng was performed by using an ABI 7500 sequence detector (Applied Biosystems), the Power SYBR Green PCR master mix (Applied Biosystems), and appropriate primers. Primer sequences of the target genes are: Tbx21, forward, 5′-AGC AAC GAC GGC GAA GAT TGT T-3′; reverse, 5′-GGG TGG TAC ACA TAT AGG CGG TTC-3′; Gata3, forward, 5′-TTC ACA GGC ACC GAA GAC GG-3′; reverse, 5′-CAT CGT CCT TCC TCC TTC AGA G-3′; Row, forward, 5′-GGG GAC GAG GCA ACC CAT TTC-3′; reverse, 5′-CAT CGT CCT TCC TCC TTC AGA G-3′; Foxp3, forward, 5′-ACC ATG ACT AGG GGC ACT GTA-3′; Ifng, forward, 5′-ACA GGA AGC CCA AAA AGT ATG-3′; reverse, 5′-GGG TGG TAC ACT GGG ATG A-3′; Actb, forward, 5′-ACC ATG ACT GAG CGG CTG G-3′; reverse, 5′-GGG TGG TAC ACT GGG ATG A-3′; β-actin level was measured as an internal control to normalize data. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The mRNA level was determined relative to that in mouse spleen. All values were determined in triplicate.

Induction of CHS

Aliquots (200 μl) of 5% 1-chloro-2,4-dinitrobenzene (DNCB; Sigma-Aldrich) in ethanol were applied to the shaved abdomens of mice. Five days later, mice were challenged by the application of 1% DNCB (40 μl)
total) to the inner and outer sides of the left ear and of ethanol only to the right ear. Response was assessed by using a digital thickness gauge (G-1A; Ozaki, Tokyo, Japan) to measure ear thickness. Net ear swelling was calculated as the difference between the thickness of the right and left ears of each mouse. For the transfer model, either WT or Pvr−/− CD4+ T cells (10^7) and WT CD8+ T cells (10^6) were injected i.v. into Rag1−/− or Rag1−/− Cd226−/− mice. One week after transfer, CHS was induced as described above. Five days after sensitization, draining lymph nodes were harvested, and cytokine production was analyzed by flow cytometry. For treatment of Ab, mice were injected i.v. with anti-CD155 mAb (TX56) or control mAb (MP Biomedicals, Solon, OH) at 2 h prior to sensitization (anti-CD155 dose, 1.0 mg) or challenge (0.5 mg).

**Induction of eosinophilic airway inflammation**

WT and Pvr−/− BALB/c mice were injected i.p. with OVA (100 μg) plus aluminum hydroxide (alum) on days 0 and 7. On days 14, 15, and 16, these mice were challenged intranasally with OVA (10 μg in PBS) or PBS alone. On day 18, the numbers of total cells and eosinophils in bronchoalveolar lavage fluid were determined by using flow cytometry. Serum IgE, IgG1, and IgG2a levels on days 0 and 14 were measured by using ELISA.

**Histology**

Mouse ears and lungs were fixed with formalin and then stained with H&E for histopathologic examination by light microscopy (×200 objective).

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**FIGURE 1.** CD155 mediates a costimulatory signal in CD4+ T cells. (A) Immunoblot analysis of phosphorylated CD155 (blotted with anti-phosphotyrosine [pY]) and CD155 (blotted with anti-CD155 mAb [CD155]) in mouse CD4+ T cells that were stimulated with anti-CD155 mAb or isotype control (clg) and immunoprecipitated (IP) with anti-CD155 mAb or clg. (B and C) Immunoblot analysis of phosphorylated Erk and Erk (B) or p65 NF-κB of nuclear (Nuc) and cytoplasmic (Cyt) extracts (C) of mouse CD4+ T cells at indicated time points after stimulation with anti-CD3 plus either anti-CD155 mAb or clg. Bar graphs show the relative amount of phosphorylated Erk to total Erk at 2 min (B) or p65 NF-κB at 30 min (C). (D–G) Frequencies of CD25+ and CD69+ CD4+ T cells analyzed by flow cytometry (D), proliferation assay by CFSE dilution (E), and IL-2 production according to ELISA (F) after stimulation of WT CD4+ T cells with plate-bound anti-CD3 plus anti-CD155 mAbs or clg. (G) Proliferation assay by CFSE dilution after stimulation of CD155-deficient (Pvr−/−) CD4+ T cells with plate-bound anti-CD3 plus anti-CD155 mAbs or clg. The mean ± 1 SD is shown. Data are representative of three or four (B–F) or two (A and G) independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005.
Statistical analysis

Statistical analyses were performed by using the unpaired or paired Student t test (GraphPad Prism 5, GraphPad Software, La Jolla, CA). A p value <0.05 was considered statistically significant.

Results

**CD155 mediates a costimulatory signal in CD4+ T cells**

To analyze the function of CD155 in immune responses, we first investigated the expression profile of CD155 on spleen cells. CD155 was broadly expressed on both myeloid and lymphoid lineage cells. Of note, CD155 was expressed most strongly on CD4+ T cells (Supplemental Fig. 1A). Stimulation of CD4+ T cells with plate-bound anti-CD3 plus anti-CD28 mAbs further augmented the expression of CD155 (Supplemental Fig. 1B). Therefore, we focused our present study on the role of CD155 in immune responses by CD4+ T cells. CD4+ T cells purified from the spleen were stimulated with anti-CD155 mAb. Immunoblot analysis demonstrated that CD155 became tyrosine phosphorylated after stimulation (Fig. 1A), suggesting that CD155 is a signal-transducing molecule in CD4+ T cells. We next examined whether CD155 mediates a costimulatory signal in CD4+ T cells. Erk is phosphorylated after stimulation of the TCR with a cognate Ag or anti-CD3 mAb (31). Stimulation of CD4+ T cells with anti-CD155 together with anti-CD3 mAb augmented Erk phosphorylation as early as 2 min after stimulation, compared with stimulation with control mAb and anti-CD3 mAb (Fig. 1B). Furthermore, stimulation of CD4+ T cells with anti-CD155 mAb markedly increased activation of NF-κB, as determined by translocation of p65 NF-κB from the cytoplasm into the nuclei (Fig. 1C), increased number of activated CD4+ T cells (Fig. 1D), augmented proliferation of CD4+ T cells (Fig. 1E), and increased IL-2 production by CD4+ T cells (Fig. 1F), compared with stimulation with control mAb plus anti-CD3 mAb. In contrast, stimulation with anti-CD155 mAb plus anti-CD3 mAb did not augment the proliferation of CD155-deficient (Pvr-/-) CD4+ T cells (Fig. 1G). Taken together, these results suggest that CD155 is a costimulatory molecule in CD4+ T cells.

**CD155 mediates IL-12–independent Th1 development from naive CD4+ T cells**

We next investigated whether CD155-mediated signal is involved in development of Th cell subsets. Naive CD4+ T cells, prepared by sorting CD3+CD4+CD62LhiCD44dull cells from the spleen by using flow cytometry, were stimulated for 6 d with anti-CD3 mAb and either plate-bound anti-CD155 or control mAb in the presence of IL-2 and analyzed for cytokine production. Stimulation with anti-CD155 and anti-CD3 mAbs significantly increased the production of IFN-γ but not IL-4 or IL-17 by CD4+ T cells, compared with stimulation with control mAb and anti-CD3 mAb (Fig. 2A, 2B). These results suggest that CD155 is involved in the development of Th1 cells. Because the in vitro Th1 differentiation assay was free from IL-12, these results suggest that CD155 is involved in IL-12–independent Th1 development from naive CD4+ T cells.

To examine the costimulatory function of CD155 under more physiological conditions, we cocultured OVA-specific naive CD4+ T cells derived from Pvr+/- or Pvr-/- OT-II Tg mice with WT splenocytes loaded with OVA peptide for 6 d. Pvr+/- CD4+ T cells produced significantly more IFN-γ than did Pvr-/- CD4+ T cells, (Fig. 2C).
indicating that CD155 plays an important role in Th1 development when naive CD4+ T cells are stimulated by using a cognate Ag and APCs that express DNAM-1, a natural ligand for CD155 (Fig. 2C, Supplemental Fig. 1C). In contrast, IL-4 and IL-17 production was comparable between \( Pvr^{+/+} \) and \( Pvr^{-/-} \) CD4+ T cells. The addition of a neutralizing anti–IL-12 mAb into the assay still permitted significantly greater IFN-\( \gamma \) production from \( Pvr^{+/+} \) CD4+ T cells than from \( Pvr^{-/-} \) CD4+ T cells (Fig. 2C), indicating that CD155 does not necessarily require IL-12 to induce Th1 development. Alternatively, the generation of Th1 cells from naive CD4+ T cells after stimulation with anti-CD3 and anti-CD155 was comparable to that after stimulation with anti-CD3
plus control Ab in the presence of exogenous IL-12 and anti–IL-4 (Th1 condition) (Supplemental Fig. 2A). Moreover, the coculture of Pvr−/− naive CD4+ T cells and APCs in the presence of a cognate Ag generated Th1 cells at a comparable level to that of Pvr+/− CD4+ T cells and APCs under the Th1 condition (Supplemental Fig. 2B), indicating that CD155 is not necessarily required for Th1 differentiation from naive CD4+ T cells when in the presence of adequate exogenous IL-12.

The transcription of Tbx21, which encodes T-bet, was upregulated in CD4+ T cells after stimulation with anti-CD155 and anti-CD3 mAbs (Fig. 2D). In contrast, these cells showed downregulation of Gata3, a master regulator of Th2 development (Fig. 2D). There was no difference in expression of Rorc, which encodes retinoic acid–related orphan receptor γt, and Foxp3, master regulators of Th17 and regulatory T cells, respectively, between CD4+ T cells stimulated with anti-CD155 mAb plus anti-CD3 mAb and those stimulated with control mAb and anti-CD3 mAb (Fig. 2D). Taken together, CD155-mediated signal is involved in IL-12–independent Th1 development.

CD155 mediates IFN-γ–dependent Th1 development from naive CD4+ T cells

We next investigated the molecular mechanism of Th1 development induced by CD155-mediated signaling. Because STAT1 activation upregulates T-bet expression and Th1 development (10), we examined whether CD155 mediates STAT1 phosphorylation. Although stimulation of CD4+ T cells with anti-CD3 mAb plus control mAb moderately induced STAT1 phosphorylation, co-stimulation with plate-bound anti-CD155 together with anti-CD3 mAbs markedly increased both the expression and phosphorylation of STAT1 (Fig. 3A), suggesting that CD155-mediated Th1 development is dependent on the STAT1 pathway. Because IFN-γ also activates STAT1, we investigated whether CD155-mediated activation of STAT1 required IFN-γ. STAT1 expression and phosphorylation were negligible in Ifng−/− CD4+ T cells after stimulation with anti-CD3, regardless of whether anti-CD155 mAb was included (Fig. 3A), indicating that the CD155–associated induction of STAT1 expression and phosphorylation is dependent on IFN-γ. Moreover, CD155-mediated upregulation of T-bet expression was undetectable in Ifng−/− CD4+ T cells (Fig. 3B). A neutralizing anti–IFN-γ mAb decreased IFN-γ production from both Pvr−/− and Pvr−/− OT-II CD4+ T cells at the same level after coculture with WT splenocytes loaded with OVA peptide (Fig. 3C). These results suggest that CD155-mediated STAT1 phosphorylation and subsequent Th1 development were dependent on IFN-γ.

We therefore investigated whether CD4+ T cells produced IFN-γ before STAT1 phosphorylation after stimulation with anti-CD155 mAb. We found that Ifng transcript was significantly upregulated in naive CD4+ T cells just 10 h after stimulation with plate-bound anti-CD155 mAb and anti-CD3 mAb compared with stimulation with control mAb and anti-CD3 mAb (Fig. 3D). We also found that the expression of IFN-γRα on CD4+ T cells was comparable regardless of stimulation with anti-CD155 mAb, as analyzed by using an anti–IFN-γRα mAb 2E2 (Fig. 3E). However, when we used GR20 anti–IFN-γRα mAb, which, unlike 2E2 mAb, competes with IFN-γ for binding to IFN-γRα, GR20 mAb binding was significantly suppressed on CD4+ T cells after stimulation with anti-CD155 mAb and anti-CD3 mAb, compared with stimulation with control mAb and anti-CD3 mAb (Fig. 3E). Interestingly, we further found that autocrine IFN-γ bound to CD4+ T cells stimulated with anti-CD155 mAb and anti-CD3 mAb significantly more than on CD4+ T cells stimulated with control mAb and anti-CD3 mAb before STAT1 phosphorylation. Taken together, these results indicated that CD155-mediated signal induced STAT1–independent production of IFN-γ, triggering the positive feed-

**FIGURE 4.** CHS is attenuated in CD155-deficient mice. (A and B) Net swelling of the left ear at 24 h after challenge of WT (n = 7) or Pvr−/− (n = 10) mice, which had been immunized with DNBC onto the abdomen on day 0 and challenged with DNBC onto the left ear or with medium alone on the right ear on day 5. (B) H&E-stained mouse ear tissues at 24 h after challenge. Scale bars, 100 μm. (C) Net ear swelling on day 6 in Rag1−/− (n = 4 per group) or Rag1−/− Cd226−/− (n = 3 per group) mice, into which CD4+ T cells from WT or Pvr−/− mice had been transferred i.v. together with WT CD8+ T cells 1 wk before immunization with DNBC, followed by challenge on day 5 with DNBC (left ear) or medium alone (right ear). (D and E) Intracellular analysis of cytokines in CD4+ T cells obtained from axillary lymph nodes of WT (n = 3) or Pvr−/− (n = 3) mice on day 5 after immunization with DNBC. Numbers indicate the percentage of cells in each region. *p < 0.05, **p < 0.01, ***p < 0.005.
back of IFN-γ-dependent STAT1 phosphorylation and Th1 development. It remains undetermined how CD155 upregulates STAT1-independent IFN-γ expression. However, we found that stimulation with anti-CD155 and anti-CD3 mAbs induced degradation of IkBα significantly more than that with control mAb and anti-CD3 mAbs even in the absence as well as in the presence of IFN-γ (Fig. 3F). Moreover, the expression of Ifng gene was significantly decreased in the presence of NF-κB inhibitor (JSH-23) regardless of the stimulation of CD155 and CD3 (Fig 3G). Taken together, these results suggest that CD155 triggers Ifng gene transcription via NF-κB.

CHS is attenuated in CD155-deficient mice

Th1 cells play an important role in CHS (32). Because CD155-mediated costimulatory signaling is involved in Th1 development, we investigated whether CD155 deficiency affects the development of CHS. WT and Pvr−/− mice were sensitized by using epicutaneous application of DNCB and then were challenged with DNCB on the left ear and medium alone on the right ear. The net swelling in the left ear, as determined by the difference in the thicknesses of the left and right ears, of both WT and Pvr−/− mice was increased at 24 h after antigenic challenge. However, swelling was significantly greater in WT than Pvr−/− mice (Fig. 4A). Supporting these results, histological analysis demonstrated that the ear swelling at 24 h after application of DNCB was considerably greater in WT mice than in Pvr−/− mice (Fig. 4B).

Because CD155 is expressed on various immune cells, we next examined whether CD155 on CD4+ T cells alone was responsible for the difference in the development of CHS between WT and Pvr−/− mice. WT or Pvr−/− CD4+ T cells were transferred with WT CD8+ T cells into Rag1−/− mice, which were immunized i.p. with OVA plus alum on days 0 and 7. Flow cytometric analysis of total cells, eosinophils (CD11b+Siglec-F+), macrophages, CD3+/B220+ T cells, CD3+ Macs, CD3− Macs, and lymphocytes (T cells, CD3+/B220−, B cells, CD3−/B220−) in bronchoalveolar lavage fluid (BALF) and histological analyses of H&E-stained sections of lung tissue (D) from WT (n = 6) or Pvr−/− (n = 6) mice immunized i.p. with OVA plus alum on days 0 and 7, challenged intranasally with OVA or PBS on days 14, 15, and 16, and analyzed on day 18. Scale bars, 100 μm. (E) Intracellular analysis of cytokines in CD4+ T cells obtained from spleen of WT (n = 5) or Pvr−/− (n = 6) BALB/c mice on day 11 after immunization i.p. with OVA plus alum on days 0 and 7, followed by restimulation with PMA plus ionomycin for 4 h. The mean ± 1 SD is shown. Data are representative of two independent experiments. *p < 0.05, ***p < 0.005.
and then challenge intranasally with OVA. Pvr$^{-/-}$ mice showed significantly higher concentration of serum IgE than did WT mice on day 14 after the immunization. However, there were no differences in IgG1 and IgG2a levels between WT and Pvr$^{-/-}$ mice after the immunization (Fig. 5B). Pvr$^{-/-}$ mice exhibited higher numbers of eosinophils in bronchoalveolar lavage fluids (Fig. 5C) and infiltrated inflammatory cells in the lung (Fig. 5D), compared with WT mice, after challenge of OVA. Moreover, the frequency of IL-4– and IL-13–producing cells among total CD4$^+$ T cells in the spleen from Pvr$^{-/-}$ mice was significantly increased, compared with that from WT mice (Fig. 5E). Taken together, these results suggest that CD155-mediated signal stimulates Th1 pathway and reciprocally suppresses Th2 development both in vitro and in vivo.

**Treatment with anti-CD155 mAb suppresses CHS responses**

To investigate whether CD155 is a potential molecular target for preventive and therapeutic applications, we treated mice with a neutralizing anti-CD155 mAb, which blocks the interaction between CD155 and DNAM-1 (28), at 2 h before sensitization or challenge with DNCB (Fig. 6A). Suppression of ear swelling was greater in mice that received anti-CD155 mAb before sensitization than in mice that received control mAb (Fig. 6B, 6C). Similarly, administration of anti-CD155 mAb before challenge significantly suppressed CHS responses (Fig. 6D, 6E), suggesting that CD155 may be a candidate target for therapeutic approaches for ACD.

**CD155 is involved in Th1 development in humans**

We next examined the expression of CD155 on human PBMCs. Whereas CD155 was expressed abundantly on monocytes, it was scarcely detectable on lymphoid cells, including CD4$^+$ and CD8$^+$ T cells and NK cells (Supplemental Fig. 3A). However, activation of CD4$^+$ T cells through stimulation with anti-CD3 plus anti-CD28 mAbs markedly upregulated the expression of CD155 (Supplemental Fig. 3B). To investigate whether CD155 is involved in Th1 development in human, we purified CD4$^+$CD45RA$^{hi}$CD45RO$^2$ naive T cells from the cord blood, stimulated them with anti-CD3 mAb and either anti-CD155 or control Ig, and analyzed subsequent cytokine production. The concentration of IFN-$\gamma$, but not IL-4 or IL-17, in the culture supernatants of naive CD4$^+$ T cells that had been stimulated with anti-CD155 plus anti-CD3 was significantly higher than that in supernatants from cells

![FIGURE 6. Treatment with anti-CD155 mAb suppresses CHS responses. (A–E) Schedule of treatment with anti-CD155 mAb to prevent CHS development (A): mice were injected i.v. with anti-CD155 mAb or cIg 2 h before either sensitization (B and C) or challenge (D and E) with anti-CD155 mAb ($n = 6$ or $5$) or cIg ($n = 4$ or $5$). Net ear swelling and H&E-stained sections of ear tissue from mice treated with anti-CD155 mAb or cIg before either sensitization (B and C) or challenge (D and E). The mean ± 1 SD is shown. Data are representative of two independent experiments. Scale bars, 100 μm. *$p < 0.05$, **$p < 0.01$.]

![FIGURE 7. CD155 is involved in Th1 development in humans. Cytokine analyses of IFN-$\gamma$, IL-4, and IL-17 in the culture supernatant after stimulation of human CD4$^+$ naive T cells with plate-bound anti-CD3 plus anti-CD155 mAbs or cIg for 6 d, followed by restimulation with PMA plus ionomycin for or 48 h. The mean ± 1 SD is shown. Data are representative of three experiments. ***$p < 0.005$.]
CD155 initially was identified as a receptor for poliovirus that was expressed on human epithelial cells (13). Although mice are not infected with poliovirus, we previously identified the mouse counterpart of CD155 (33). CD155 is expressed on various hematopoietic and nonhematopoietic cells in both humans and mice. However, the physiological function of CD155, particularly in hematopoietic cells, is incompletely understood. We previously demonstrated that DNAM-1, which is a counterreceptor for CD155 and is expressed also on CD4 T cells, mediates an activating signal for Th1 development in naive CD4 T cells in human (30). In agreement with this context, Lozano et al. (34) suggested that DNAM-1 on CD4+ naive T cells mediates an activating signal for Th1/Th17 differentiation as a result of ligation with CD155. In sharp contrast, the present study demonstrated that CD155 itself mediates a costimulatory signal in CD4+ T cells that promotes Th1 development. These results were further supported by the in vivo study, which showed that whereas CHS was significantly greater in Rag1-/- mice that received WT CD4+ T cells than in those that received Pvr-/- CD4+ T cells, it was comparable between Rag1-/- Cd226-/- mice that received WT CD4+ T cells and those mice that received Pvr-/- CD4+ T cells. These results suggest that CD155-mediated signal in CD4+ T cells triggered by the trans interaction with DNAM-1 on APC, rather than DNAM-1-mediated signal in CD4+ T cells triggered by the cis interaction with CD155 on CD4+ T cells, was involved in the development of CHS.

Th1 development is induced through both IL-12–dependent and –independent mechanisms (12). In the present study, we showed that CD155-mediated Th1 development does not require IL-12. Previous reports demonstrated that the costimulatory molecule LFA-1 is involved in Th1 development in an IL-12–independent manner (30, 35), in which Erk phosphorylation is sustained for >30 min. The sustained phosphorylation of Erk downregulates GATA-3 but upregulates T-bet, resulting in Th1 development from naive CD4+ T cells (30, 35). CD27, a member of the TNF receptor family, mediates another costimulatory signal that induces Th1 differentiation in an IL-12–independent manner (36, 37), although the molecular mechanisms underlying this effect remain underdetermined as yet. Although we showed that the CD155-mediated costimulatory signal also enhances Erk phosphorylation, Erk was dephosphorylated in several minutes in our experiments (Fig. 1B). Therefore, CD155 is involved in Th1 development by a mechanism distinct from LFA-1. Instead, we showed that CD155 induced Th1 development in an autocrine IFN-γ–dependent manner. CD155 also induced NF-κB activation, which was independent of IFN-γ. Evidence demonstrated that p50 and p60 NF-κB subunits bind to the IFN-γ promoter and upregulate IFN-γ expression (38). Therefore, CD155 signaling induces IFN-γ secretion via NF-κB, leading to autocrine IFN-γ–dependent STAT1 activation and the subsequent upregulation of T-bet expression (Supplemental Fig. 4). Previous reports also demonstrated that CD155 recruits the Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP-2), which activates MAPK (39, 40). How CD155 activates NF-κB is unknown currently but SHP-2 might also be involved in NF-κB activation in CD4+ T cells. A recent study has demonstrated that the CD155 promoter region contains an NF-κB binding site (41), suggesting a positive feedback loop in which CD155 and NF-κB stimulate each other.

The costimulatory signal mediated by CD155 induced Th1 development from naive CD4+ T cells in both humans and mice and exacerbated Th1-mediated CHS and ameliorated Th2-mediated airway inflammation in mice. We showed that treatment with anti-CD155 mAb dampened CHS. Treatment with anti-LFA-1 mAb has been reported to similarly inhibit the development of CHS (42). However, although LFA-1 also mediates a costimulatory signal that leads to Th1 development from naive CD4+ T cells, mice deficient in LFA-1 or treatment of mice with anti–LFA-1 mAb show impaired migration of T cells to draining lymph nodes (43), leading to overall T cell dysfunction. In contrast, we observed that cell migration to draining lymph nodes was not impaired in CD155-deficient mice, suggesting that CD155 is not involved in T cell migration into lymph nodes. Notably, treatment with anti-CD155 mAb was effective when injected after as well as before sensitization with DNCB Ag. These characteristics of anti-CD155 treatment are advantageous for its clinical use in the prevention of ACD, because most patients have been already sensitized to a specific allergen.

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Disclosures

The authors have no financial conflicts of interest.

References

13. 1536.


Supplemental Figure 1. CD155 is expressed most strongly on CD4+ T cells and DNAM-1, a ligand for CD155, is expressed on APCs.

(A and B) Flow cytometric analysis of mouse CD4+ T cells, CD8+ T cells, NK1.1+ CD3− NK cells, B220+ B cells, CD11b+ Ly6G− macrophages/monocytes (Mφ/Mo), and CD11c+ dendritic cells (DCs) in the spleen stained with biotinylated anti-CD155 mAb (open histogram) or isotype control (clg; shaded histogram), followed by allophycocyanin (APC)-conjugated streptavidin (A). CD4+ T cells were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 mAbs (1 μg/ml), and CD155 expression was analyzed. Numbers in histograms indicate mean fluorescence intensities (B).

(C) Flow cytometric analysis of the expression of DNAM-1, (open histogram) on dendritic cells (CD11c+ cells), macrophages (Mφ) (CD11b+ Ly6G− cells), and B cells (B220+ cells) from the spleen of mice. Shaded histograms indicate staining with isotype control.

Data are representative of two independent experiments.
Supplemental Figure 2. CD155 is not necessarily required for Th1 differentiation when in the presence of adequate exogenous IL-12.

(A) Cytokine analyses of IFN-γ in the cytoplasm after stimulation of CD4+ naïve T cells from C57BL/6 mice with plate-bound anti-CD3 plus anti-CD155 mAbs or clg in the presence of IL-2 for 6 d, followed by restimulation with phorbol myristate acetate (PMA) plus ionomycin for 4 h. Th1 condition; 20 ng/ml IL-12 plus 2.5 μg/ml anti-IL-4, Th2 condition; 50 ng/ml IL-4 plus 10 μg/ml anti-IFN-γ, Th17 condition; 10 ng/ml IL-6 plus 5 ng/ml TGF-β plus 10 μg/ml anti-IFN-γ plus 2.5 μg/ml anti-IL-4. Statistical data are indicated.

(B) Cytokine analyses of IFN-γ in the cytoplasm after stimulation of CD4+ naïve T cells from Pvr+/+ or Pvr−/− OT-II Tg mice after coculture for 6 d with WT splenocytes loaded with OVA peptide, followed by restimulation as in (A).

The mean ± 1SD is shown. Data are representative of two independent experiments.
Supplemental Figure 3. Expression of CD155 on human peripheral blood.

(A and B) Flow cytometric analysis of human CD4+ T cells, CD8+ T cells, CD56+ CD3− NK cells, CD19+ B cells, and CD14+ monocytes in the peripheral blood that were stained with PE-conjugated anti-CD155 mAb (open histogram) or isotype control (shaded histogram) (A). Peripheral blood mononuclear cells were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) mAbs for 24 or 48 h in the presence of IL-2 (10 ng/ml), after which CD155 expression was analyzed (B). Data are representative of three experiments.
Supplemental Figure 4. CD155 triggers *Ifng* gene transcription via NFκB.

CD155 cooperates the TCR signaling and promotes NFκB activation (1), which then induces *Ifng* transcription (2). IFN-γ binds to IFN-γ receptor (3) and induces STAT1 activation (4), followed by transcription of *Tbx21* (5), which encodes T-bet, the master regulator of Th1 development.