CATERPILLER 16.2 (CLR16.2), a Novel NBD/LRR Family Member That Negatively Regulates T Cell Function*

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The newly discovered mammalian CATERPILLER (NOD,NALP,PAN) family of proteins share similarities with the NBD-LRR superfamily of plant disease resistance (R) proteins and are predicted to mediate important immune regulatory function. This report describes the first cloning and characterization of a novel CATERPILLER gene, CLR16.2 that is located on human chromosome 16. The protein encoded by this gene has a typical NBD-LRR configuration. Analysis of CLR16.2 suggests the highest expression among T lymphocytes. Cellular localization studies of CLR16.2 revealed that it is a cytoplasmic protein. Querying microarray studies in the public data base showed that CLR16.2 was significantly (>90%) down-regulated 6 h after anti-CD3 and anti-CD28 stimulation of primary T lymphocytes. Its reduction upon T cell stimulation is consistent with a potential negative regulatory role. Indeed CLR16.2 decreased NF-κB, NFAT, and AP-1 induction of reporter gene constructs in response to T cell activation by anti-CD3 and anti-CD28 antibodies or PMA and ionomycin. Following T cell stimulation, the presence of CLR16.2 reduced the levels of the endogenous transcripts for the IL-2 and CD25 proteins that are central in maintaining T cell activation and preventing T cell anergy. This reduction was accompanied by a delay of IκBα degradation. We propose that CLR16.2 serves to attenuate T cell activation via TCR and co-stimulatory molecules, and its reduction during T cell stimulation allows the ensuing cellular activation.

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¹ The abbreviations used are: NBD, nucleotide binding domain; LRR, leucine-rich repeat; MHCIITA, MHC Class II Transactivator; CLR, CATERPILLER; TCR, T-cell receptor; PBMC, peripheral blood mononuclear cell; ORF, open reading frame; PMA, phorbol 12-myristate 13-acetate; UTR, untranslated region; LMB, Leptomycin B; hCLR162, human CLR16.2; mCLR16.2, mouse CLR16.2; RACE, rapid amplification of cDNA ends; IL, interleukin; TNF, tumor necrosis factor; EGFP, enhanced green fluorescent protein; CMV, cytomegalovirus.

Recently, we discovered a family of human genes that contain a similar arrangement of NBD-LRR. We designated these as the CATERPILLER family (CARD, Transcription Enhancer, R (purine)-binding, Pyrin, Lots of Leucine Repeats) (3). Another group has found a similar gene family and designated it as NOD, christened after the founding member of the family (4,5). Others have found pyrin-containing subfamilies (5,6) that are designated here as the NALP and PAN families.

The CATERPILLER (CLR) family of genes emerged from our search of the human genome data base as genes related to MHC class II transactivator (MHCIITA, protein designation CIITA) via nucleotide homology and its members are predicted to have important roles in the immune system (3). MHCIITA, the founding member of the CATERPILLER gene family, has long been recognized as a transcriptional co-activator found in professional antigen-presenting cells required for the transcription of MHC class II surface glycoproteins and more recently plexin-A1 (7–11). Without the surface expression MHC class II proteins, individuals succumb to a severe immunodeficiency termed the type II Baireumyelocytes Disease. CATERPILLER 1.1, also named CIAS1, PYPAF1, and NALP3 has been linked genetically to the inflammatory disorders Muckle-Wells syndrome, familial cold urticaria (FCU), and neonatal-onset multisystem inflammatory disease (NOMID) (12–16). Mutations in CIAS1 associated with these disorders cause an increase in the basal secreted levels of IL-1β, a major inflammatory cytokine (12,17). Additionally, the protein product of CIAS1, cryopyrin, associates with an inflammasome complex consisting of ASC, Cardinal, and caspase-1, important for the conversion of pro-IL-1β to its secreted form (18–21). Other functional investigations have shown that cryopyrin can affect the NF-κB pathway as either an inhibitor or an activator depending on the experimental circumstances (22–24). The meaning of these latter results is unclear without further mechanistic studies, but is nevertheless consistent with a role for cryopyrin in inflammation. Another CATERPILLER protein, Monarch-1 (PYPAF7), has also been identified in a similar role. Overexpression of PYPAF7 along with ASC in COS-7L cells results in significant IL-1β secretion and activation of NF-κB (25). Our group has also implicated Monarch-1 in the regulation of classical and non-classical MHC class I genes (26) and in the down-regulation of an array of cytokines.²

The NOD1/CARD4 and NOD2/CARD15 proteins are yet an-
other example of the immunological significance of the CAT-ERPILLER genes. Reports have identified mutations in NOD2 as a contributing factor in Crohn’s disease and Blau syndrome (27–30). NOD1 and NOD2 have been described to bind diaminopimelic acid and muramyl dipeptide, respectively, common components of bacterial cell walls, and to activate NF-κB (31–34). Thus, multiple hypotheses have been put forth to explain how these proteins are intimately involved in the immune system and inflammatory diseases, including the intriguing possibility that NOD1 and NOD2 may serve as intracellular sensors (reviewed in Refs. 35–38).

Because members of the same gene family often have similar but functionally distinct roles, we surmised that the predicted CLR16.2 (3) also has an important role in the adaptive or innate immune system. Our current report on the cloning and characterization of CLR16.2 provides evidence for this supposition. After cloning the full-length transcript of CLR16.2, we find evidence that CLR16.2 may be predominantly expressed in cells of the immune system, particularly in T lymphocytes. CLR16.2 expression decreases luciferase assay values for NF-κB, NFAT, and AP-1, major signal transduction pathways activated in innate and/or adaptive immunity, and decreases transcript levels of genes controlled by these transcription factors. This effect is observed in anti-CD3- and anti-CD28-stimulated T cells. Data mined from a microarray study indicates that CLR16.2 is strongly down-regulated in primary T cells following stimulation of the T-cell receptor (TCR) complex and CD28. Thus, CLR16.2 is the first protein within this family to potentially affect TCR signaling. With these results in mind, we postulate that CLR16.2 has a specific role in the attenuating the activation of T cells.

MATERIALS AND METHODS

Cell Culture—HeLa, HEK293, HEK293T, A549, K-562, MCF7, and B16-F10 cells were maintained in DMEM-H medium supplemented with 10% fetal calf serum and grown in a humidified, 37 °C incubator with 5% CO2. Raji, Jurkat E6–1, THP-1, U-937, HL-60, SB, HSB-2, and KU812 cell lines were cultured in RPMI media containing 10% fetal calf serum under the same incubation conditions. To create Jurkat stable cell lines, cells were electroporated, as described below, with 30 μg of pcDNA3 or pcDNA3-FLAGCLR16.2 and with 2000 μg/ml G418. The resulting polyclonal cultures were used in experiments. Stable 293 cells expressing EGFP or EGFCLR16.2 were created by transfection of cells with FuGENE 6 and treatment of cells with 500 μg/ml G418. PBMCs were isolated from buffy coats (American Red Cross) using technique separation medium (ICN Pharmaceuticals). For antibody stimulations, Jurkat E6.1 cells (1 × 10^6 cells/ml) were incubated with protein G-purified anti-CD3 antibody (mouse monoclonal OKT3×ATCC) at 2 μg/ml and anti-CD28 (mouse monoclonal CD28.2)(eBiosciences) at 5 μg/ml for 30 min, washed three times with phosphate-buffered saline, incubated with anti-mouse IgG (Sigma) at 10 μg/ml for 30 min, and plated in medium.

Cloning of Human CLR16.2 and Identification of mCLR16.2—Raji total mRNA was isolated with TRIzol reagent (Invitrogen). To perform race procedures, the FirstChoice™ RLM-RACE kit (Ambion) was used according to the manufacturer’s instructions with the following oligonucleotide sequences: 5’-ACCCGACATGATGCTGCGT, 5’-GCCAACAGTCTCTGAGTGTG, and 5’-GTCACCAGCCTCCTACTCT-GTCACATCTAC. To amplify the CLR16.2 open reading frame (ORF), cDNA was generated with Transcriptor Reverse Transcriptase (Roche Applied Science), and PCR was performed with Pfu DNA polymerase (Invitrogen). The following sense and antisense primers were used for 18 S real-time PCR: F, 5’-GCATTGACATTGG-TTGTCCCAGGACGAG; and R, 5’-ACCACACCAATGGCATTCCCTAAG; and CD25R, 5’-GCATTGACATTGG-TTGTCCCAGGACGAG. Real-time PCR experiments were performed three times using an AB Prism 7700 instrument (Applied Biosystems), and representative experiments were graphed. RNA levels of the gene of interest and 18 S rRNA were quantitated by comparing the experimental signal with a DNA plasmid standard curve. Values were normalized to 18 S rRNA levels. The following oligonucleotides were used for 18 S real-time PCR: F, 5’-GGCTGCTGGACACGATCT, R, 5’-CCGCTTACACATCCGAGAG; and 5’-CAAAATTCCAACCTCCCGACCC-GCG. For pcDNA3-FLAGCLR16.2-specific reverse transcription PCR, PCR was performed with Taq polymerase (Invitrogen) on randomly primed cDNA samples using the following primers: F, 5’-TCACACA-CGTGCTGCTCTCTCTATCT; and R, 5’-AGCCACACCAATGGCATTTACCTAGA.

Microarray Data—General expression data for hCLR16.2 and mCLR16.2 was obtained from Genomics Institute of the Novartis Research Foundation (GNF) (symatlas.gnf.org/SymAtlas) using NOD3 as the keyword identifier (40, 41). The mCLR16.2 gene identified by GNF corresponds to the cDNA Riken gene D230907K08. The mouse data set was downloaded from the gcmArray expression database source; www.biconductor.org software package is presented. In the human study, 2–4 samples for each tissue were examined. All tissues were taken from different individuals. In the mouse study, two arrays were performed for each tissue. The samples applied to each array were pooled RNA from seven mice of the same genetic strain. The Stanford Microarray Data Base (SMD) is found at the website, genome-www.stanford.edu/microarray (42). The unique clonal identifiers for the CLR16.2 sequence, IMAGE:713166 and IMAGE:712914, correspond to cDNA sequences contained within GenBank™. These sequences belong to the 3’-UTR of CLR16.2. The identifiers were used to query the microarray data produced by Diehn et al. (43). All data retrieved passed the exclusion software filters 1–7 set at default values. Data are reported as values normalized to the 0-min control and to the values obtained from Boldrick et al. (45) were retrieved and graphed in a similar manner.

Luciferase Assays—HEK293T cells were plated in 96-well plates and transfected with either 50 ng of AP-1 or NF-κB luciferase reporter construct and 100, 200, 300, or 400 ng of pcDNA3-FLAGCLR16.2 using FuGENE 6 (Roche Applied Science). Additional amounts of pcDNA3 were transfected to keep the total amount of DNA transfected per well constant. The AP-1 reporter constructs were activated by treatment with PMA (5 ng/ml) and the following oligonucleotides: F, 5’-CGGCTTACACATCCGAGAG; and R, 5’-AGCCACACCAATGGCATTTACCTAGA.

Jurkat luciferase assays were performed as follows: Cells in exponential growth were washed once with phosphate-buffered saline and resuspended in fresh, ice cold, phenol red-free RPMI media containing 2% fetal calf serum at 2 × 10^8 cells/ml. A total of 0.4 ml of cells were mixed with 3 μg of AP-1, NF-κB, or NFAT reporter constructs and 0, 5, 10, and 25 μg of pcDNA3-FLAGCLR16.2. Total amounts of transfected DNA were kept constant using additional amounts of pcDNA3. Cells were electroporated with the Gene pulser II instrument (Bio-Rad) at 960 microfarad and 250 V in a 0.4 cm gap cuvette. Cells were treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma) (10 ng/ml and 1 μM) or stimulated with anti-CD3 and anti-CD28 for 6 h to activate the AP-1, NF-κB, or NFAT reporter constructs. The control data with p53- and DR ε-luciferase constructs were generated in a
similar manner, using p53 and CIITA cotransfection to activate the reporter constructs and using 30 μg of pcDNA3-FLAGCLR16.2 where applicable. Average results of at least three experiments are graphed.

**Immunoprecipitation and Western Blots**—For immunoprecipitation of FLAGCLR16.2, Jurkat pcDNA3, or Jurkat pcDNA3-FLAGCLR16.2 stably transfected cells were lysed in phosphate-buffered saline plus 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM EDTA, and mammalian protease inhibitors mixture (Sigma) for 10 min. Lysates were cleared and mixed with M2 anti-FLAG-agarose beads (Sigma) for 2 h at 4°C. Beads were washed four times with fresh lysis buffer, and immunoprecipitates were eluted with SDS-PAGE loading buffer. Lysates or immunoprecipitates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Anti-IκBα (sc-371, Santa Cruz Biotechnology), M5 anti-FLAG (Sigma), anti-β-actin (Abeam), and anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon) antibodies were used to stain membranes, and secondary stains were performed with either goat anti-mouse-horseradish peroxidase or goat anti-rabbit-horseradish peroxidase antibodies (Santa Cruz Biotechnology). Supersignal Chemiluminescence Reagent (Pierce) was used for detection.

**IkBa Degradation Assay**—For the IkBa degradation assay, HEK293T cells plated in 6-well plates were transfected with 1 μg of pcDNA3 or pcDNA3-FLAGCLR16.2 using FuGENE 6 (Roche Applied Science). After 18 h, cells were stimulated with 20 ng/ml TNF for the indicated amounts of time, and cells were lysed for 2 min in radioimmunoprecipitation assay lysis buffer with 1 mM EDTA, 1 mM dithiothreitol, and mammalian protease inhibitor mixture (Sigma). The protein concentrations of cleared lysates were determined by Bradford assay (Bio-Rad), and equal amounts of protein were analyzed by immunoblot as described above. The IkBa degradation assay performed with EGFPCLR16.2 was accomplished in a similar manner, with the exception that stably transfected 293 cells were electroporated with 30 μg of pcDNA3 or pcDNA3-FgCLR16.2, and stimulated after 12–18 h with PMA and ionomycin for the indicated amounts of time.

**RESULTS**

**Cloning and Description of the CLR16.2 Gene**—We previously predicted the partial sequence of human CLR16.2 (3). Based on this prediction, primers were designed and used to amplify 98% of the CLR16.2 coding region (Fig. 1, A and B, ii). Additionally, 5′-RACE and 3′-RACE procedures were performed, and the resulting bands (Fig. 1, A and B, i and iii) sequenced to reveal the full-length mRNA sequence of the CLR16.2 gene (NCBI accession AT601811). The cloned ORF of CLR16.2 gene is similar to a predicted sequence within GenBankTM, NOD3 (NCBI accession NM_178544), with the differences resulting in an extra 47 residues in the N terminus and a Q41H point mutation in the NOD3 protein. Another cDNA reported and cloned by the NEDO human cDNA sequencing project (www.nedo.go.jp/bio-e/index.html) (NCBI accession AK090431) is also similar to CLR16.2. The mRNA sequence of the NEDO clone contains two insertions, one in the 5′-UTR and the other within the ORF of CLR16.2, resulting in a frameshift at the nucleotide corresponding to residue 727 of CLR16.2 and the addition of 54 out-of-frame residues to the CLR16.2 protein (schematically represented in Fig. 1A, iv).

Consistent with CATERPILLER proteins, CLR16.2 contains an N terminus dominated by one large exon and 14 exons coding for LRRs (schematically represented in Fig. 1C). Where many of the CATERPILLER proteins contain an N-terminal pyrin domain, the N terminus of CLR16.2 does not adhere to any current domain definition. CLR16.2 was aligned with several other prominent CATERPILLER family members, CIITA, CIAS1, Monarch-1, NOD1/CARD4, and NOD2/CARD15, to demonstrate their homology (Fig. 2A). Additionally, the mouse CLR16.2 (mCLR16.2) gene was identified and deposited in GenBankTM. A protein alignment between human CLR16.2 (hCLR16.2) and mCLR16.2 is shown (Fig. 2B).

**Expression of CLR16.2**—Real-time PCR has been used to quantify the amount of hCLR16.2 mRNA expressed in various human cell lines. CLR16.2 was detected in Raji, an EBV-transformed B cell line, and U937, a myeloid-monocytic cell line. We have used the mouse cell line B16-F10 as a negative control. The real-time PCR signal in HeLa, HEK293, A549, and MCF7 cells lines was comparable to the signal from the B16-F10 cell line (Fig. 3A), indicating that CLR16.2 is not expressed significantly in transformed epithelial cells within the same experiment. CLR16.2 was also expressed in the following cell lines: SB (a B lymphoblastoid cell line), HS6-2 (a T lymphoblastoid line autologous to SB), and Jurkat E6.1 (T cell line), THP-1 and HL-60 (monocytic lines), and KU812 cells (basophilic line) (Fig. 3B). CLR16.2 levels have been examined in Raji cells in both sets of experiments to demonstrate the large difference in expression observed between the cell lines surveyed (Fig. 3, A and B).

Additional expression data from primary tissues have been obtained by mining microarray data produced by the Genomics Institute of the Novartis Research Foundation (GNF) (40, 41). Two sets of data, derived from probes sets 09358 and 09041, are available for hCLR16.2 but reveal different results. Thus, we have compared the data to mCLR16.2 expression to determine which probe accurately represented the expression of CLR16.2. GNF has analyzed the mCLR16.2 data with both MAS v5 and gcRMA software packages. The two analyses were consistent with one another, but we have presented the results from the gcRMA analysis, because studies have concluded that gcRMA is superior to MAS v5 (46–48). Fig. 3C compares the expression data for mCLR16.2 (right panel) and hCLR16.2 (left panel). The expression values for mCLR16.2 were highest in CD4+ and CD8+ T cells and moderate in the thymus and lymph node, tissues of the immune system. In the other tissues and cells, mCLR16.2 expression values were low, although there appeared to be some mCLR16.2 expressed in B cells. Values for the expression of hCLR16.2 were also highest in CD4+ and CD8+ T cells as well as NK cells. Moderate expression values

**FIG. 1. Cloning of CLR16.2 cDNA.** A, schematic representation of cloned CLR16.2 cDNA products. The 5′-RACE product we isolated is illustrated in i, the CLR16.2 ORF in ii, and the 3′-RACE product in iii. Black regions depict untranslated segments and white regions depict ORF. The NEDO clone (accession AK090431) is shown in iv and contains a frameshift mutation resulting in a truncated product (indicated with arrow). B, agarose gels of original PCR products respectively representing i to iii are also shown with approximate nucleotide length. C, CLR16.2 protein domain organization, schematically represented, is characteristic of a CATERPILLER gene. One large exon encodes the nucleotide binding domain and individual exons encode 14 leucine-rich repeats. The N terminus does not conform to any current domain definition.
FIG. 2. The homology of CLR16.2 protein to the CATERPILLER family members and mCLR16.2. A, protein alignment of the CLR16.2 and other well-known CATERPILLER family members, Monarch-1, CIAS1, NOD1/CARD4, NOD2/CARD15, and CIITA, demonstrates significant homology between these proteins. Identity is denoted by white letters in shaded boxes, and similarity is shown by black letters in shaded boxes. B, protein sequence of experimentally verified hCLR16.2 is aligned with predicted mCLR16.2.
for hCLR16.2 were found in B cells. However, it is difficult, if not impossible, to definitively compare hCLR16.2 expression in the remaining tissues and cells as their errors overlap. It is also difficult to compare the pattern of expression in these human tissues to that of the mouse. It is not surprising that the hCLR16.2 expression data were less stratified as only 2–4 human samples were taken for each human tissue, and every sample was from a different individual. Accordingly, the mouse expression data may be more accurate because the two arrays run for each mouse tissue were comprised of pooled RNA samples from seven mice with identical genetic background. We conclude from these studies that CLR16.2 is likely expressed highest in T cells. The mouse data suggest that expression in most other organs, except in the thymus and lymph node, is low.

Next, we performed immunofluorescent microscopy to determine the localization of hCLR16.2 within the cell. Although this assay does not always yield specific functional information regarding the activity of a protein, it provides basic insight as to where in the cell the protein may function. For example, CIITA was found both in the cytoplasm and nucleus, consistent with its function as a transcriptional co-activator. In contrast, examination of FLAGCLR16.2 overexpressed in HeLa cells localized CLR16.2 to the cytoplasm (Fig. 3D). The nuclear stain, DAPI, was used to delineate the nucleus, and CLR16.2 staining did not overlap with DAPI. Cellular localization has been examined under conditions of TNFα stimulation and also showed cytoplasmic staining. Finally, cells were treated with LMB and the localization of CLR16.2 determined. Treatment of
cells with LMB, an inhibitor of CRM1-mediated nuclear export, allows the visualization of protein that is rapidly exported from the nucleus by causing its accumulation in the nucleus, and hence its visualization (39, 49–52). LMB did not cause nuclear staining of CLR16.2. In contrast, the positive control, CIITA, was enriched in the nucleus upon LMB treatment. These experiments indicate that CLR16.2 is not likely a nuclear protein.

Knowing the full-length mRNA sequence of CLR16.2 and finding substantial expression in T cell lines, we sought to acquire additional expression data through the use of the Stan-

**FIG. 3.** **Expression patterns of CLR16.2.** A and B, real-time PCR has been used to assess CLR16.2 mRNA expression in standard cell lines and PBMCs. The signal values for CLR16.2 in K-562, HeLa, 293, A549, and MCF7 cells are similar to that of the negative control, cDNA from the mouse cell line B16-F10, and are interpreted as none detected (ND). The levels of CLR16.2 in Raji cDNA have been assayed in both sample sets to illustrate the large range of CLR16.2 present across cell lines. C, publicly available microarray data indicate that human and mouse CLR16.2 is enriched in T cells. D, FgCLR16.2 localizes to the cytoplasm in transfected HeLa cells using DAPI to outline the nucleus (panels i). TNF stimulation or LMB treatment does not alter the cytoplasmic localization of CLR16.2, whereas LMB treatment does result in nuclear accumulation of the transcriptional co-activator CIITA (panels ii).
CLR 16.2 Decreased NF-κB, AP-1, and NFAT Function

**FIG. 4.** CLR16.2 is down-regulated in primary T lymphocytes upon TCR activation and CD28 co-stimulation. CLR16.2 mRNA levels sharply decline in primary T cells upon T cell activation with anti-CD3 and anti-CD28 beads or with PMA and ionomycin treatment compared with a mock control, anti-CD3 beads alone, or anti-CD28 beads alone (upper left panel) (43). The transcripts for IL-2 and CD25 are strongly up-regulated in response to anti-CD3 and anti-CD28 stimulation or PMA and ionomycin treatment (lower panels). Treatment of PBMCs with bacteria (upper right panel) causes a modest decrease in CLR16.2 mRNA levels, however, PMA and ionomycin treatment of PBMCs leads to a stronger reduction (80%) in CLR16.2 message levels and of longer duration (45). A logarithmic scale is used for CLR16.2 mRNA expression and a linear scale is used for CD65 and IL-2 mRNA levels.

CLR16.2 expression was not altered by anti-CD28 alone, was modestly reduced by anti-CD3, and was significantly down-regulated by greater than 90% after stimulation with both anti-CD3 and anti-CD28 or with PMA and ionomycin treatment (Fig. 4, upper left panel). Treatment of cells with PMA, which act as a protein kinase C pathway activator, and ionomycin, which non-specifically permits Ca²⁺ influx, are typically used to mimic the activation of lymphocytes (53). In contrast to CLR16.2 mRNA levels, the transcripts for well studied proteins such as IL-2 and CD25 (IL-2Rα) were, as expected, up-regulated in response to stimulation (Fig. 4, lower panels) (39). This indicates that the expression pattern of CLR16.2 is opposite that of two T cell activation markers. A separate study that examined the transcriptional response of PBMCs to various types of bacteria and to PMA and ionomycin treatment has also been queried (42, 44, 45). As shown in Fig. 4 (upper right panel), CLR16.2 mRNA levels were modestly reduced with bacterial stimulation (~40%) with the exception of *Escherichia coli*, which caused a more substantial reduction (~60%). Similar to the T cell study, PMA, and ionomycin treatment resulted in strong down-regulation of CLR16.2 (~80%). Implicit with these types of studies, a large change in mRNA levels of a gene is indicative that the respective protein plays a function in mediating the cellular response to the stimulation. Thus, these data demonstrate that CLR16.2 likely has a specific function in the T lymphocytes following their activation through the TCR when accompanied by a second co-stimulatory signal.

CLR16.2 Decreased NF-κB, AP-1, and AP-1 Reporter Gene Activation—In an effort to understand how the down-regulation of CLR16.2 in response to T cell activation may yield functional consequences, we assessed the ability of CLR16.2 to affect signal transduction pathways triggered in activated T cells. NF-κB and AP-1 are two major transcription factors activated upon T cell stimulation. We tested the effect of exogenous CLR16.2 on these two pathways in HEK293T cells, which do not express CLR16.2 (Fig. 3A). Luciferase assays in HEK293T cells show that overexpressed FLAG-tagged CLR16.2 decreased NF-κB reporter gene activation elicited by TNFα (Fig. 5A, top left panel). Changing the tag to EGFP did not alter the outcome indicating that the epitope tag did not have a functional effect (Fig. 5A, top right panel). AP-1 reporter gene activation by PMA was also decreased significantly by CLR16.2 (Fig. 5A, lower left panel). As a control, we examined the effect of CLR16.2 on a p53 luciferase. The highest quantity of CLR16.2 used to show the reduction in NF-κB and AP-1 reporter values had no effect on the p53 control luciferase assay (Fig. 5A, lower right panel).

Because CLR16.2 is likely to be highly expressed in T cells, we tested the effect of CLR16.2 expression on NF-κB, NFAT, and AP-1 luciferase constructs in Jurkat T lymphoid T cell line. Using PMA and ionomycin treatment as a stimulus, CLR16.2 decreased 70% of the NF-κB and AP-1 reporter gene activity, and 60% of NFAT activity in Jurkat T cells (Fig. 5B, top left panel). CLR16.2 caused a similar decrease in NF-κB and NFAT activity induced by anti-CD3 and anti-CD28 (Fig. 5B, top right panel). Data for AP-1 luciferase assays using anti-CD3 and anti-CD28 stimulation were not included because we consistently achieved only a 2-fold activation, not allowing us to determine with confidence whether a decrease in activity occurred. As controls, we performed these assays with DRα- and p53-responsive promoter luciferase constructs. CLR16.2 expression did not decrease the activation of these constructs by their respective transactivator, CIITA and p53 (Fig. 5B, bottom panels). CIITA had been chosen as a control because it is structurally similar to CLR16.2. Not only does p53 serve as a control for the potential nonspecific effects of CLR16.2, but it is also an important indicator of cell survival because p53 regulates this process.

CLR16.2 Decreased Gene Expression of CD25 and IL-2—Next, we examined whether CLR16.2 could decrease the transcription of genes normally induced after T cell stimulation. We have chosen to look at the transcript levels of IL-2 and CD25 because their gene promoters have been shown to be heavily regulated by NF-κB, NFAT, and AP-1 transcription factors, and their pattern of expression is opposite that of CLR16.2 (see Fig. 4) (54–56). Jurkat T cells stably transfected with pcDNA3 or pcDNA3-FLAGCLR16.2 have been established. To verify the expression of FLAGCLR16.2, reverse transcriptase PCR was performed with primers specific for FLAGCLR16.2 transcript, yielding a PCR product of the correct length only in the pcDNA3-FLAGCLR16.2-transfected cell line (Fig. 6A). Addi-
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FIG. 5. CLR16.2 reduces activation of NF-κB, NFAT, and AP-1 in Jurkat T cells. A, increasing amounts of pcDNA-FLAGCLR16.2 (FgCLR16.2) or pEGFP-CLR16.2 have been transfected into 293T cells along with either NF-κB or AP-1 reporter constructs. TNFα (A, top panel) has been used to activate NF-κB. The AP-1 luciferase construct has been activated by treatment of 293T cells with PMA (bottom left panel). A decrease in relative luciferase units is observed in all cases when FgCLR16.2 was present. In contrast, FgCLR16.2 expression does not decrease the luciferase activity of a p53-luciferase construct (bottom right panel). B, FgCLR16.2 strongly reduces NF-κB, NFAT, and AP-1 activity in Jurkat T cells upon PMA and ionomycin treatment, also judged by luciferase activity (top left panel), and reduces NF-κB and NFAT reporter gene values following activation with anti-CD3 and anti-CD28 co-stimulation (top right panel). CLR16.2 does not reduce DRα and p53 luciferase activity (bottom panels). All data are graphed as percent activity such that stimulated samples without FgCLR16.2 are normalized to 100% activity. A minimum of three replicates was performed for each panel.

FIG. 6. CLR16.2 decreases mRNA induction of IL-2 and CD25 in activated Jurkat T cells. Jurkat T cells have been stably integrated with pcDNA3 or pcDNA3-FLAGCLR16.2. A and B, expression of FgCLR16.2 has been verified by reverse transcriptase PCR and by anti-FLAG immunoblot analysis of anti-FLAG immunoprecipitates. C, cells have been stimulated with PMA and ionomycin for 1 h or 6 h and mRNA levels of IL-2 and CD25 have been measured using real-time PCR. The normalized expression of CD25 and IL-2 mRNA is less in cells expressing FgCLR16.2 after 6 h of stimulation. A representative experiment from three replicates is shown. Above each time point the average percent reduction is shown from the three separate experiments along with their standard deviation in parenthesis. At time 0, no IL-2 message is detected (ND). At time 0 and 1 h, no repression (NR) is detected for CD25.

mRNA levels for IL-2 and CD25 were reduced, respectively, by 58 and 70% in FLAGCLR16.2-expressing cells compared with cells transfected with the mock vector control (Fig. 6C).

CLR16.2 Altered the Degradation of IκBα—One explanation for the decrease in NF-κB luciferase readings upon overexpression of CLR16.2 is that CLR16.2 directly or indirectly acts to inhibit the transduction pathway. If CLR16.2 acts as an inhibitor, it should negatively regulate signaling molecules. The degradation of IκBα is a well established step needed to activate the canonical p50:p65 NF-κB pathway. Prevention or mitigation of IκBα degradation may mean that CLR16.2 acts on IκBα directly or upstream of this point in the pathway, possibly even before the signal in T cells splits into separate NF-κB, NFAT, and AP-1 signaling modules. When TNFα was used to stimulate the degradation of IκBα in HEK293T cells, there was a reproducible difference in the kinetics of IκBα protein degradation between cells transiently transfected with FLAGCLR16.2 and with vector control (Fig. 7A, middle panel). The presence of FLAGCLR16.2 delayed IκBα degradation. This pattern was reproducible in four independent experiments using the FLAG-tagged protein. To further verify that this was not caused by the specific epitope tag, an EGFP-tagged CLR16.2 protein was utilized in the same experiment. EGFPCLR16.2 delayed the degradation of IκBα compared with EGFP alone (Fig. 7B). To assess whether these results could be recapitulated in Jurkat T cells, PMA and ionomycin were used to stimulate the degradation of IκBα in cells that were transiently transfected with FLAGCLR16.2 or with vector control. There was a reproducible difference in the IκBα protein degradation between cells transiently expressing FLAGCLR16.2 compared with control (Fig. 7C). The typical transfection efficiency of Jurkat T cells was ~15% (data not shown). The difference observed in the latter experiment may be an underestimation.
of the actual difference in specific cells overexpressing FLAG-CLR16.2. These experiments indicate that overexpression of CLR16.2 leads to the altered degradation of IκBα, which is expected to reduce NF-κB activation.

DISCUSSION

Immunity represents a double-edged sword in that proper immune activation is necessary to sense foreign antigens and to contain invading pathogens, whereas an overzealous response is detrimental to the host, as witnessed by a variety of autoimmune and autoinflammatory disorders and by sepsis. Although an array of positive regulators of immune activation has been discovered, relatively few negative feedback modulators have been reported. Here we identify a novel CATERPILLER protein and provide evidence that it may attenuate T cell activation. This study is the first description of a CLR protein that may be involved in the negative regulation of T cell signaling produced through TCR and CD28 co-stimulation.

We describe the isolation and characterization of a predicted gene CLR16.2. The full-length mRNA sequence of CLR16.2 was obtained by assembling the sequence of the cloned ORF with the sequences from its 5′-RACE and 3′-RACE products. The protein sequence of CLR16.2 conforms to the definition of the CATERPILLER protein. There is a central, putative nucleotide binding domain as defined by the presence of Walker A and Walker B motifs (3, 57), which is followed by a series of LRRs. Structures of LRR-containing proteins have been solved previously (58, 59). The LRRs of CLR16.2 are expected to have the same general fold as these previous structures; however, it is in the arrangement of the specific side chain residues that will determine the function of this region. Similar to our hypothesis with CIITA, we postulate the LRRs may act in conjunction with the NBD to regulate the binding of other proteins or itself in such a way as to mediate the specific function of CLR16.2 (60).

We found that the expression of CLR16.2 is highest in T cells. Our conclusion is based upon the CLR16.2 array expression values being highest in T cells in both the mouse and human data in comparison to the other tissues examined in the GNF tissue profiling studies. There is still a possibility, however, that there is higher expression in tissues or cell types not assayed. It seems likely that the human data contains higher error and has less stratification than the mouse data because all the human samples were derived from different individuals. Therefore, comparison of the patterns of expression in many of the tissues between human and mouse is impossible. Since the RNA samples for the mouse data were prepared from mice with identical genetic backgrounds, the resulting data may be much more accurate. The mouse data interpreted by itself without comparison to the human data suggest that CLR16.2 is expressed highest in the immune tissues and cells. The low signals detected in other mouse tissues may indicate a total lack of expression of CLR16.2 except in immune tissues.

The expression of CLR16.2 contrasts with other studied CATERPILLER genes in that its expression is greatest among T lymphocytes. NOD2/CARD15, Monarch-1/Pypaf7 and cryopyrin/CIAS/Pypaf1 have restricted expression among the myeloid-monocytic lineage, although some non-immune expression has also been found (14, 22, 25, 26, 61, 62). Others such as NOD1/CARD4 have a broad tissue distribution (61). An interesting pattern of CLR16.2 expression that piqued our interest is its reduction upon T cell stimulation, suggestive of a potential negative regulatory function.

Other reports examining CATERPILLER proteins have found modulating effects on the NF-κB signal transduction pathway. In the canonical pathway, the IKK complex receives an activating signal and triggers the proteosomal-dependent degradation of IκBα. The heterodimeric complex of p50:p65 is then free from IκBα and can migrate into the nucleus to activate gene specific transcription (reviewed in Refs. 63–65). Our experiments indicated that CLR16.2 may modulate this pathway because the overexpression of CLR16.2 caused a delay in the kinetics of IκBα degradation and decreased the activity of our NF-κB reporter construct upon lymphocyte activation. Although we only see a modest effect on the degradation of IκBα, it has clearly been demonstrated that T cells have a complex mechanism to recognize the magnitude of signaling and even slight alterations in signal strength result in different cellular...
The activation of this family of transcription factors and T cell survival.

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