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The Transmembrane E3 Ligase GRAIL Ubiquitinates and Degrades CD83 on CD4 T Cells[1]

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Abstract

Ubiquitination of eukaryotic proteins regulates a broad range of cellular processes, including T cell activation and tolerance. We have previously demonstrated that GRAIL (gene related to anergy in lymphocytes), a transmembrane RING finger ubiquitin E3 ligase, initially described as induced during the induction of CD4 T cell anergy, is also expressed in resting CD4 T cells. In this study, we show that GRAIL can down-modulate the expression of CD83 (previously described as a cell surface marker for mature dendritic cells) on CD4 T cells. GRAIL-mediated down-modulation of CD83 is dependent on an intact GRAIL extracellular protease-associated domain and an enzymatically active cytosolic RING domain, and proceeds via the ubiquitin-dependent 26S proteosome pathway. Ubiquitin modification of lysine residues K168 and K183, but not K192, in the cytoplasmic domain of CD83 was shown to be necessary for GRAIL-mediated degradation of CD83. Reduced CD83 surface expression levels were seen both on anergized CD4 T cells and following GRAIL expression by retroviral transduction, whereas GRAIL knock-down by RNA interference in CD4 T cells resulted in elevated CD83 levels. Furthermore, CD83 expression on CD4 T cells contributes to T cell activation as a costimulatory molecule. This study supports the novel mechanism of ubiquitination by GRAIL, identifies CD83 as a substrate of GRAIL, and ascribes a role for CD83 in CD4 T cell activation.

Ubiquitination is an evolutionarily conserved process that covalently attaches polyubiquitin chains to target proteins. This modification can result in proteolytic degradation as well as nonproteolytic outcomes that regulate a broad range of critical cellular functions, including regulation of transcription and protein trafficking. Attachment of ubiquitin to target proteins occurs through a highly organized process involving the sequential actions of different classes of modifying enzymes (1). The first step in this process involves an ATP-dependent attachment of ubiquitin to the ubiquitin-activating enzyme (E1).[3] Next, the thiol ester-linked ubiquitin is transferred from the E1 enzyme to a cysteine residue in an ubiquitin-conjugating

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enzyme (E2). In the last step of the conjugation process, the E2 enzyme, together with ubiquitin-protein ligase (E3) transfers ubiquitin to target proteins, where a stable isopeptide bond is formed between the car-boxyl terminus of ubiquitin and the ε-amino group of a lysine residue on the target protein. The E3 ligase is the central determinant of specificity in the substrate conjugation process; however, mapping specific target lysine sites or consensus ubiquitination motifs on target proteins has been a challenge.

GRAIL (gene related to anergy in lymphocytes; rnf128) was initially identified in a differential display screen of cDNA obtained from anergized T cell clones (2), and subsequent studies demonstrated that GRAIL was a critical element in the induction of T cell anergy in Ag-specific murine $CD4^+$ T cell clones in vitro and in OVA-tolerized DO.11 mice in vivo (3). More recent studies have demonstrated that GRAIL is expressed in resting CD4 T cells and is degraded upon activation (4). Structure-function studies have characterized GRAIL as a type I transmembrane single sub-unit ubiquitin E3 ligase protein with a cytosolic zinc-binding RING finger domain and a luminal or extracellular protease-associated (PA) domain. In CD4 T cells, GRAIL has been shown to modulate expression of CD40L, a critical costimulatory molecule required for T cell activation (5), and to regulate the RhoA signaling pathway by ubiquitination of RhoGDI (6).

The ubiquitination process has been adopted by viruses to evade host antiviral immune responses. HSV-1 has been shown to infect human mature dendritic cells (DCs) by down-regulating the surface molecule CD83 via an ubiquitin-dependent 26S proteosome pathway (7). The degradation of CD83 was found to be dependent on the immediate-early gene product, ICP0 of HSV-1. ICP0 contains a RING finger domain highly homologous to that of GRAIL. Thus we asked whether CD83 might serve as a substrate for GRAIL.

Materials and Methods

Cell culture, plasmids, and transfections

HEK293 cells were cultured in DMEM supplemented with 10% FCS and antibiotics (Invitrogen). Wild-type murine CD83 cDNA, provided by A. Steinkasserer (University Hospital Erlangen, Erlangen, Germany), was subcloned into the pEF1-mycHis plasmid (Invitrogen). CD83 point mutants were generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Murine GRAIL (rnf128) and its mutants were cloned into a biscistronic internal ribosome entry site (IRES) enhanced GPF (eGFP) vector. All transfections were performed using Lipofectamine 2000 (Invitrogen).

Western blot and immunoprecipitation analysis

Whole cell extracts were prepared by washing the cells with ice-cold PBS, then resuspending the pellet in ice-cold lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% Brij96, 1 mM EDTA, 1 mM NaF, 1 mM β-glycerophosphate with freshly added protease inhibitor mixture, and PMSF). Lysates were clarified by centrifugation at

[3]Abbreviations used in this paper: E1, ubiquitin-activating enzyme; Ctrl, control; DC, dendritic cell; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; eGFP, enhanced GFP; GRAIL, gene related to anergy in lymphocytes; IRES, internal ribosome entry site; PA, protease-associated; RNAi, RNA interference; shRNA, short hairpin RNA.

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13,000 rpm for 15 min at 4°C. For denaturing immunoprecipitation, 1% SDS was added to lysates that were then boiled for 5 min. SDS was diluted to 0.1% final concentration and the lysates were subjected to immunoprecipitation with anti-myc conjugated agarose beads (Sigma-Aldrich). For Western blot analysis, proteins were separated by SDS-PAGE (Bio-Rad), transferred to polyvinylidene difluoride (Millipore), blocked with 5% nonfat dry milk, and probed with the following Abs: anti-myc-HRP, anti-V5-HRP (both purchased from Invitrogen), anti-Flag for ubiquitin detection (Sigma-Aldrich), monoclonal (BD Pharmingen) or polyclonal anti-GRAIL Ab. Secondary HRP Ab (Zymed Laboratories) was used where necessary. Membranes were reprobed with anti-cyclophilin-B (Abcam) or anti-β-actin (Millipore) to evaluate protein loading. Western blots were developed by ECL (GE Healthcare).

FACS analysis

Cell suspensions were prepared by washing the cells with ice-cold PBS and then staining with anti-CD83 PE conjugate (clone Michel-19; BD Pharmingen) at 1/200. Cells were then washed, resuspended in FACS buffer (1× PBS, 0.2% BSA), and analyzed on a FACS can analyzer (Stanford FACS Facility).

T lymphocyte isolation and CD4 T effector cell preparation

Single cell suspension were prepared from isolated spleens and lymph nodes. Lymphocytes were enriched by Lympholyte-M (Cedarlane Laboratories) gradient centrifugation, and $CD4^+$ T cells were isolated by depletion using pan T cell Macs Separation beads (Mltenyi Biotec). Cells were stimulated with 0.5 μg/ml plate-bound anti-CD3 (2C11) and 0.5 μg/ml soluble anti-CD28 (both purchased from BD Pharmingen) for 3 days, then rested in conditioned medium for 2 days, followed by an additional 2-day rest in fresh complete RPMI 1640 medium supplemented with 10% FCS. Live cells were then collected by Lympholyte-M centrifugation and subjected to T cell activation.

Retroviral packaging and transduction of T cell

Retroviral constructs were transfected into 293 Phoenix packaging cells using standard calcium phosphate transfection to generate virus containing control vector or vector containing either wild-type or H2N2 GRAIL. Freshly isolated CD4 T cells were activated for 24 h before transduction by spin-fection at 32°C in the presence of polybrene. Cells were stained for CD83 and subjected to FACS analysis 24 h post-transduction.

Ionomycin-induced anergy

Resting CD4 T cells were treated with ionomycin (1 μM) for 18 h. Cells were then washed extensively in fresh RPMI 1640 medium before TCR stimulation.

GRAIL and CD83 RNA interference (RNAi) knockdown

Murine GRAIL and CD83 RNAi target sequences were obtained from Open Biosystems. Murine GRAIL target sequences: GRAIL RNAi no. 1: (5′CCATGTTGACAACCCAACCTT3′) and GRAIL RNAi no. 2: (5′CCACACAAGATTATAGACATA3′). Oligonucleotides were synthesized by Stanford

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PAN Core Facility, then annealed and cloned into pSiren RetroQ ZsGreen RNAi Ready vector (BD Clontech). GRAIL short hairpin RNA (shRNA) vectors were electroporated into CD4 T cells following the manufacture's recommended protocol (Amaxa). Post-transfection, cells were either left unstimulated or stimulated with plate-bound CD3 (0.5 μg/ml) and soluble CD28 (0.5 μg/ml) for 24 h, and CD83 surface expression level was evaluated by FACS. CD83 shRNA vectors were similarly introduced into DO11.10 CD4 T (Amaxa). Efficiency of knockdown was evaluated by FACS analysis 24 h post-transfection.

Cytokine and T cell proliferation assays

Sorted ZsGreen$^+$ DO11 T cells (5×10^4 per well) were cocultured with irradiated APC (1.5×10^5 per well) in the presence of peptide OVA $_{(323–339)}$ for 48 h. For cytokine assays, supernatants were collected and subjected to Luminex cytokine bead array assays (Stanford Immune Monitor Core Facility). To measure proliferation, cells were pulsed with [^{3}H]thymidine (1μCi/ well; Amersham Biosciences) for an additional 6 h, harvested, and the filter mat was counted (Wallac).

Results

GRAIL down-modulation of CD83 requires intact GRAIL PA and RING domains

The RING domain of ubiquitin E3 ligases is a specialized type of zinc finger of 40 to 60 residues known to bind two atoms of zinc. This domain is defined by the cross-brace motif of C-X_2-C-$X_{(9–39)}$-C-$X_{(1–3)}$-H-$X_{(2–3)}$-(N/C/H)-X_2-C-$X_{(4–48)}$C-X_2-C. The similarity in RING domain sequence of ICP0 and GRAIL is shown in Fig 1A, in which the conserved cysteine or histidine residues shared between ICP0 and GRAIL that form the zinc-binding sites are boxed. The RING domain of GRAIL forms a C3H2C3-type (RING-H2 finger), a slight variation of the C3HC4-type of RING found in ICP0.

GRAIL is a type I transmembrane protein that contains an extracellular or luminal PA domain that is separated from the cytosolic RING finger domain by a membrane-spanning sequence. Initially, to determine whether GRAIL could modulate CD83 expression, we transiently expressed CD83 with wild-type GRAIL in non-CD83-expressing HEK293 cells using a bicistronic vector with eGFP under control of an IRES. Transfection with the empty vector, encoding only eGFP, was used as a control. FACS analysis 36 h post-transfection showed a reduction in CD83 surface expression in GRAIL-eGFP positive cells compared with transfected vector control cells (Fig 1B). We found that the decrease in CD83 surface expression was dependent on a functional GRAIL-RING domain, as an enzymatically inactive mutant of GRAIL bearing two histidine to arginine substitutions (H2N2) in the RING domain abrogated the reduction in CD83 expression. Similar to the H2N2 RING mutant, a GRAIL mutant lacking the N terminus PA domain (ΔPA) was also unable to down-modulate CD83 levels in HEK293 cells.

We next asked if the down-modulation of CD83 surface expression was a consequence of decreased protein expression, and did not represent a defect in CD83 transport to the cell surface or retention of CD83 in cytoplasmic vesicles. In the presence of wild-type V5 epitope-tagged GRAIL, the protein level of Myc-tagged CD83 was reduced (Fig 1C, *lane 2*).

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Consistent with the FACS data, this decrease in CD83 protein level was not observed in the presence of either the ligase-defective H2N2 or the N-terminal PA deletion GRAIL mutant (Fig 1C, *lanes 3* and *4*, respectively). Though equivalent amounts of GRAIL vectors were used for transient expression, the auto-ubiquitinating property of GRAIL resulted in a smaller pool of GRAIL compared with H2N2 (Fig 1C, *lane 2*). Blots were reprobed for cyclophilin-B to ensure equal protein loading (Fig 1C, *bottom*).

To further explore the interaction of GRAIL with CD83, Myc/His-tagged CD83 was cotransfected with either full-length, H2N2, or ΔPA GRAIL. Coimmunoprecipitation studies demonstrated that while full-length and H2N2 GRAIL can both bind CD83, immunoprecipitation of ΔPA GRAIL via its V5 tag did not coprecipitate CD83 (Fig 2A, *lane 4*). Reciprocally, immunoprecipitation of CD83 via its Myc epitope tag coprecipitated full-length and H2N2 GRAIL (Fig 2A, *lanes 6* and *7*, respectively), but did not coprecipitate ΔPA GRAIL (Fig 2A, *lane 8*). These data show that the extracellular PA domain of GRAIL serves as a protein-protein interaction domain and is necessary to facilitate substrate binding to allow the cytosolic or luminal domain of GRAIL to transfer ubiquitin to its substrate. This represents a usual mechanism of E3 ligase substrate capture and recognition in which a single subunit E3 ligase contains a functional recognition domain separated from the catalytic E3 ligase domain by an intervening transmembrane sequence.

A pair of cysteine residues in the extracellular domain of CD83 is in position to permit disulfide bond formation that defines an Ig-like V-set domain, similar to those found in other costimulatory molecules, such as ICOS, CD28, and CTLA-4. We asked whether ICOS, a costimulatory molecule with a similar Ig-like domain, could serve as a target of GRAIL down-modulation. Though ICOS has structural framework similar to CD83, a significant difference between Myc epitope-tagged ICOS protein levels in the presence or absence of wild-type GRAIL (Fig 2B) was not observed. These data imply that regions in addition to the Ig-like domain may be necessary to confer specific GRAIL-sub-strate interaction. In support of this possibility, crystal structure analysis of CD28 family members (including ICOS) reveals a common ligand binding loop of MYPPPY (AA 99–104) (8) that is lacking in the extracellular region of CD83.

CD83 protein degradation proceeds via the 26S proteosome

We next asked whether CD83 is a substrate for GRAIL-mediated ubiquitination. Myc-tagged CD83, together with either vector control, wild-type GRAIL, or the H2N2 GRAIL mutant, were co-expressed in HEK293 cells along with Flag epitope-tagged ubiquitin. To observe the accumulation of ubiquitinated proteins, MG132, a specific 26S proteosome inhibitor, was added for 2 h before cell harvest. Following denaturing immunoprecipitation of epitope-tagged CD83 and probing with anti-Flag Ab, we observed distinctive m.w. laddering in the presence of wild-type GRAIL, indicative of polyubiquitin chain formation (Fig 3, *lane 5*). The laddering effect was markedly diminished in the presence of vector control (Fig 3, *lane 4*) and the H2N2 RING mutant (Fig 3, *lane 6*). From these results, we conclude that GRAIL down-modulates CD83 via the ubiquitin-dependent 26S proteosome degradation pathway.

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CD83 degradation is dependent on specific lysine residues in its cytoplasmic tail

The highly ordered process of ubiquitination ultimately results in the attachment of mono-or polyubiquitin chains onto the ε-amino group of specific lysine residues on the E3 substrate. However, definition of consensus ubiquitin target motifs and identification of acceptor lysine residues on target proteins has been a growing challenge confronting the field of ubiquitination research. In an attempt to determine the specific lysine residue(s) on CD83 targeted by GRAIL ubiquitination, we synthesized point mutants of the CD83 cytosolic lysine residues. Shown in Fig 4A is a partial protein sequence of the intracellular domain of CD83, in which the locations of the lysine residues subjected to site-directed mutagenesis are highlighted.

When coexpressed in HEK293 cells, wild-type GRAIL was able to degrade each of the single lysine to arginine (K → R) CD83 point mutants (Fig 4B), suggesting that there is either no discrimination between the lysine used for GRAIL-mediated ubiquitination, or alternatively, the availability of any two of the three lysines is sufficient for polyubiquitination. To distinguish between these two possibilities, we generated CD83 point mutants bearing double lysine to arginine substitutions. When these mutants were transiently expressed with wild-type GRAIL, we observed degradation of the CD83 mutants bearing the K168/192R or K183/192R point mutations (Fig 4C, *lanes 2* and *4*, respectively). However, the K168/183R CD83 point mutant was resistant to GRAIL-mediated degradation (Fig 4C, *lane 6*), indicating a preference for either the K168 or K183 lysine for ubiquitin attachment by GRAIL, while the transmembrane distal lysine residue (K192) is dispensable for ubiquitin-mediated degradation. Finally, we generated the triple lysine to arginine CD83 mutant to confirm that no other residues were involved in GRAIL-mediated degradation of CD83. Consistent with the model, wild-type GRAIL was unable to degrade the K168/183/192R CD83 triple mutant (Fig 4C, *lane 8*).

GRAIL down-modulation of CD83 in CD4 T cells

GRAIL originally was identified to be up-regulated in a differential display of anergic T cell clones; however, endogenous GRAIL has recently been reported to be expressed in resting $CD4^+$ effector T cells (4). In this study, we demonstrate that expression of this endogenous pool of GRAIL protein diminishes upon in vitro TCR activation (anti-CD3/anti-CD28), presumably via auto-ubiquitination and degradation (Fig 5A).

Although CD83 is best studied as a cell surface marker for mature DCs, CD83 has been reportedly expressed on a subset of activated T and B cells (9). To confirm this report, resting CD4 T cells were subjected to TCR activation for the indicated times and stained for inducible CD83 surface expression (Fig 5B). Because CD83 expression inversely correlated with GRAIL protein, we asked whether ectopic expression of GRAIL in CD4 T cells could modulate the expression of CD83. Using a myeloproliferative sarcoma virus-based retroviral vector (pMP71) encoding either wild-type GRAIL or H2N2 GRAIL together with eGFP under control of an IRES, CD4 T cells were transduced. CD83 levels on GFP+ cells were evaluated by FACS analysis 24 h post-transduction. Following GRAIL transduction, 20% of the CD4 T cells expressed high levels of CD83, compared with 25% and 26% of the vector and H2N2-transduced T cells, respectively (Fig 5C). The percentage of retrovirally

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transduced CD83-expressing cells, normalized to the vector control, is shown in Fig 5D. Hence, the presence of GRAIL reduced the surface levels of CD83 by degrading CD83 in a RING domain-dependent manner. GRAIL and H2N2 transduction of CD4 T cells was verified by immunoblot (data not shown).

Next, we wished to determine whether modulating endogenous levels of GRAIL can affect the levels of CD83 expression on T cells. Sustained calcium mobilization has been shown to induce anergy and increase GRAIL levels in CD4 T cells (10, 11). Increased GRAIL expression in ionomycin (1 μM) treated cells was verified by immunoblot (Fig 5E). To determine whether this method of induced T cell unresponsiveness influences CD83 expression levels, CD4 T cells were treated with ionomycin (1 μM) for 18 h before TCR stimulation. Cells treated with ionomycin displayed reduced CD83 surface expression compared with TCR-stimulated non-ionomycin-treated cells (Fig 5F). To further investigate the regulation of CD83 by GRAIL, CD4 T cells were subjected to GRAIL knockdown by RNAi. CD83 levels dramatically increased on GRAIL RNAi-treated ZsGreen$^+$ cells (RNAi no.1: 36%; RNAi no. 2: 31%) compared with ZsGreen-containing control (Ctrl) vector electroporated cells (Ctrl: 15%) (Fig 5G, *left*). This difference in CD83 levels is also observed upon TCR stimulation (Ctrl: 41%; RNAi no. 1: 65%; RNAi no. 2: 55%) (Fig 5G, *right*). These data indicate that CD83 does indeed serve as a target of GRAIL degradation in CD4 T cells.

CD83 expression on CD4 T cells is important for T cell proliferation and function

We used RNAi technology to assess the functional importance of CD83 expression in peripheral CD4 T cells. The mRNA CD83 sequences targeted by the designed sense/antisense RNAi oligonucleotides are listed in Fig. 6A. CD4 T cells were isolated from DO11.10 OVA-specific TCR transgenic mice and electroporated with either a Luciferase ZsGreen-containing control vector or a mix of CD83-targeted ZsGreen-expressing shRNA vectors. To evaluate the efficiency of the shRNA-mediated knockdown of CD83, the CD4 T cells were stimulated with irradiated APCs plus peptide OVA 24 h post-electroporation. Gating on ZsGreen$^+$ cells, cells electroporated with the CD83-targeted shRNA vector displayed a reduced level of CD83 (49%), compared with cells treated with the control vector (64.4%) after 24-h OVA stimulation (Fig 6B, *top*). No marked difference in up-regulated expression of CD25 was observed in the control and CD83 shRNA electroporated cells, supporting the specificity of the shRNA-mediated gene silencing (Fig 6B, *bottom*).

Next, we tested whether CD83 knockdown on CD4$^+$ T cells would result in impaired T cell function by measuring cytokine production and T cell proliferation. DO11 OVA-specific CD4 T cells were electroporated with either control or CD83 shRNA vectors. ZsGreen+ cells were isolated by FACS 24 h post-electroporation and cocultured with irradiated APCs in the presence of OVA peptide. After 48-h coculture, the supernatants were collected and cytokine levels were determined by cytokine bead array assay. Significantly lower amounts of IL-2 and IL-17 were detected in the supernatants of CD83 siRNA-electroporated CD4 T cells, compared with vector control-treated cells (Fig 6C, *left*). Although previous reports have noted a difference in IFN-γ and TNF-α expression in T cells cocultured with CD83 siRNA-treated DCs, IFN-γ and TNF-α levels were not significantly different in the CD83

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RNAi-electroporated T cells, compared with vector control-treated cells (Fig 6C, *right*). The proliferative capacity of the T cells were similarly evaluated by coculture with irradiated APCs together with OVA peptide, followed by a 6-h pulse with [^{3}H]thymidine. CD4 T cells electroporated with CD83 shRNA vectors showed a reduced capacity to proliferate when compared with cells electroporated with control vector (Fig. 6D). Even stimulation with increased OVA peptide concentration (500 ng/ml) was unable to overcome the deficiency in CD83 expression. These observations provide evidence that up-regulated CD83 expression on T cells serves as a positive costimulatory signal for full T cell proliferation and function.

Discussion

Ubiquitination has been demonstrated to play a major role in modulating immune responses. Several E3 ligases, including GRAIL (2), Cbl-b (12), c-Cbl (13), and Itch (10) have been identified as T cell intrinsic anergy factors. GRAIL ligase substrates represent a diverse array of signaling nodes that are critical in T cell activation. For example, GRAIL activity has been implicated in alterations of actin cytoskeleton by modulating the RhoA pathway by ubiquitination of RhoGDI (6). Tetraspanins, a family of four-pass transmembrane molecules that contribute to cell adhesion binding complexes, also have been shown to be ubiquitinated by GRAIL (14). Costimulatory pathways are another key node of regulation targeted by GRAIL. Down-modulation of CD40L by GRAIL in bone marrow-reconstituted mice was associated with diminished lymphoid follicle formation (5). In studies reported in this paper, the surface molecule CD83 was identified as a novel substrate of GRAIL. The ubiquitin-dependent down-modulation of CD83 requires not only an intact RING domain, but also the extracellular or luminal PA domain of GRAIL to recruit and retain the substrate for ubiquitination. This transmembrane capture-ubiquitination mechanism was recently described in GRAIL-mediated degradation of CD40L, a type 2 transmembrane molecule with an extracellular TNF-R-like domain (5), and of CD151, a member of the tetraspanin family with two extracellular loop domains (14). Primary protein sequence analysis does not identify similarities between the Ig-like domain of CD83 with that of the TNF-R domain of CD40L and the loop domains of CD151; however, topology predictions have revealed possible secondary structural domain shared between the these molecules. Crystallography experiments will be required to investigate the protein interactions between the PA domain of GRAIL and the extracellular domain of these GRAIL substrate molecules to reveal essential structural domains or motifs required for GRAIL-specific substrate recognition and binding.

CD83 ubiquitination by GRAIL was shown to require a functional cytosolic RING domain, as the RING-defective H2N2 GRAIL mutant was unable to degrade CD83. Polyubiquitin transfer to specific lysine residues of substrate proteins results in diverse biological outcomes, but prediction of these ubiquitin acceptor sites has been difficult. GRAIL has demonstrated preferential sites or residue positions for ubiquitin transfer, but these preferences differ among the identified GRAIL substrates. For example, a cytosolic domain lysine-less CD40L mutant was shown to be ubiquitinated by GRAIL, suggesting either non-lysine or amino-terminal ubiquitination (5). Site-directed mutagenesis of CD81 and CD151, members of the tetraspanin family, suggested localized regional specificity, as GRAIL-mediated ubiquitin transfers to lysine residues of only one of two cytoplasmic regions in the

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tetraspanin molecule (14). In this study, we demonstrated that lysine ubiquitination at either K168 or K183 on the cytoplasmic region of CD83 results in proteosomal degradation, while the membrane distal K192 residue was not demonstratively involved. How GRAIL directs transfer of ubiquitin moieties from the E2 transferase to preferential target substrate sites is still unresolved, but may depend on orientation or topology of substrate binding or association of other accessory molecules, such as OTU-1 and USP8 (15).

CD83 was originally defined as a specific marker for activated DCs, and has been described as mediating intercellular interaction between DCs and T cells. Subsequent studies have shown CD83 up-regulation in subsets of hematopoietic cells, including naturally occurring $CD4^+CD25^+$ regulatory T cells (16), activated B cells, a subset of activated NK cells, and activated macrophages (9, 16– 18). In this study, we show robust up-regulated expression of CD83, correlating inversely with GRAIL levels, on murine CD4 T cells following TCR engagement. Forced ectopic GRAIL expression, as well as induced GRAIL expression by ionomycin treatment, resulted in diminished CD83 levels, whereas GRAIL knockdown in CD4 T cells resulted in increased CD83 surface expression. These data conclusively demonstrate GRAIL-mediated regulation of CD83 expression on CD4 T cells.

In vivo mouse models have provided evidence of a vital role for CD83 function in regulating T cell development and modulating immune responses. $CD83^{-/-}$ mice displayed impaired T cell development and reduced numbers of peripheral $CD4^+$ T cells (19). The requirement for CD83 on thymic epithelial cells for proper thymic selection and egress from the thymus may explain these observations. Alternatively, T cells from the $CD83^{-/-}$ mice may harbor T cell-intrinsic defects that affect development, as these cells exhibit decreased cell survival in an adoptive transfer model (9). Our data corroborate the notion that CD83 is functionally relevant on peripheral CD4 T cells. By specifically knocking down CD83 in peripheral CD4 T cells, we show in an Ag-specific co-culture system that impaired CD83 expression results in decreased IL-2 production and reduced proliferative capacity of these cells. These data are consistent with previous reports that T cells isolated from CD83 transgenic mice exhibited an activated phenotype, producing increased amounts of IL-2 (20). Interestingly, in our studies, reduction of CD83 expression on T cells also resulted in a significant reduction of IL-17 production. A soluble form of CD83 has been shown to inhibit Ag-specific T cell activation and prevent murine experimental autoimmune encephalomyelitis (21), presumably by interfering with the CD83:CD83 ligand interaction. Because IL-17 has been found to drive differentiation of pathogenic Th-17 cells in experimental autoimmune encephalomyelitis, CD83 may serve as an attractive target for development of disease intervention therapy. In conclusion, these observations illustrate the importance of CD83 in APC:T interactions, and suggest that CD83 may represent a novel molecular target for GRAIL function in peripheral CD4 T cells.

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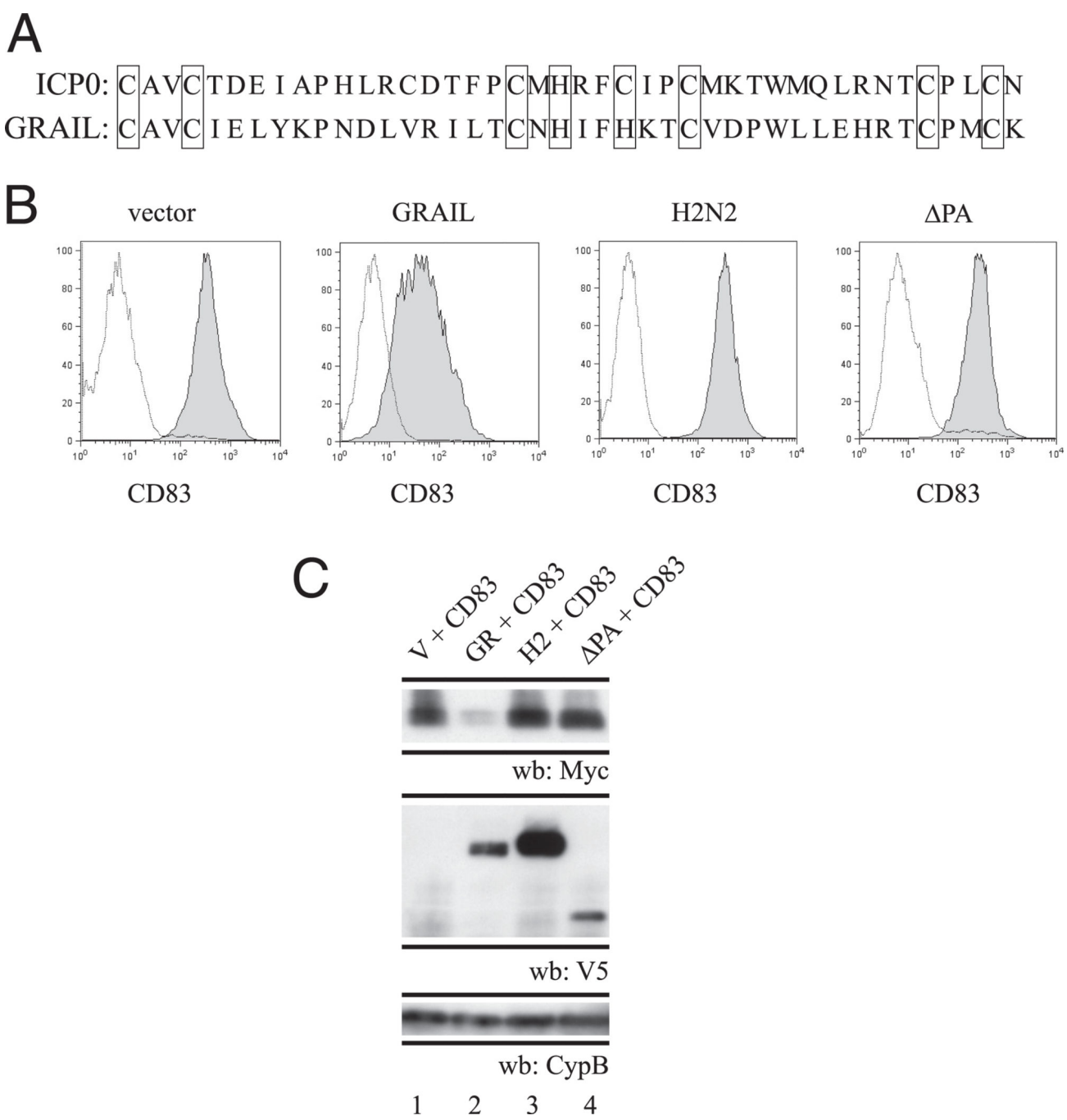

FIGURE 1.

GRAIL down-regulation of CD83 requires an intact RING domain and N-terminal PA domain. *A*, Sequence alignment of HSV-1 ICP0 and murine GRAIL RING domains. Conserved residues required for zinc binding are boxed. *B*, HEK293 cells were transfected with murine CD83 plasmid (0.5 μg) along with either control vector, wild-type GRAIL, H2N2, or APA GRAIL IRES eGFP plasmid (1.0 μg each). CD83 surface expression levels were evaluated on $eGFP^+$ cells by FACS analysis 36 h post-transfection. Rat IgG1 isotype control included for each sample. (mean fluorescence intensities: vector, 359; GRAIL, 43.8;

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H2N2, 351; ΔPA, 253.) *C*, HEK293 cells were transfected as described in *B*. Cell lysates were prepared and subjected to SDS-PAGE. The levels of CD83-myc and GRAIL-V5 were detected by Western blot (wb) using anti-myc-HRP (*top*) and anti-V5-HRP (*middle*) Ab, respectively. Blots were reprobed for cyclophilin-B (CypB) as a protein loading control (*bottom*). V, Vector; GR, GRAIL; H2, H2N2 GRAIL; ΔPA, N-terminal PA deletion.

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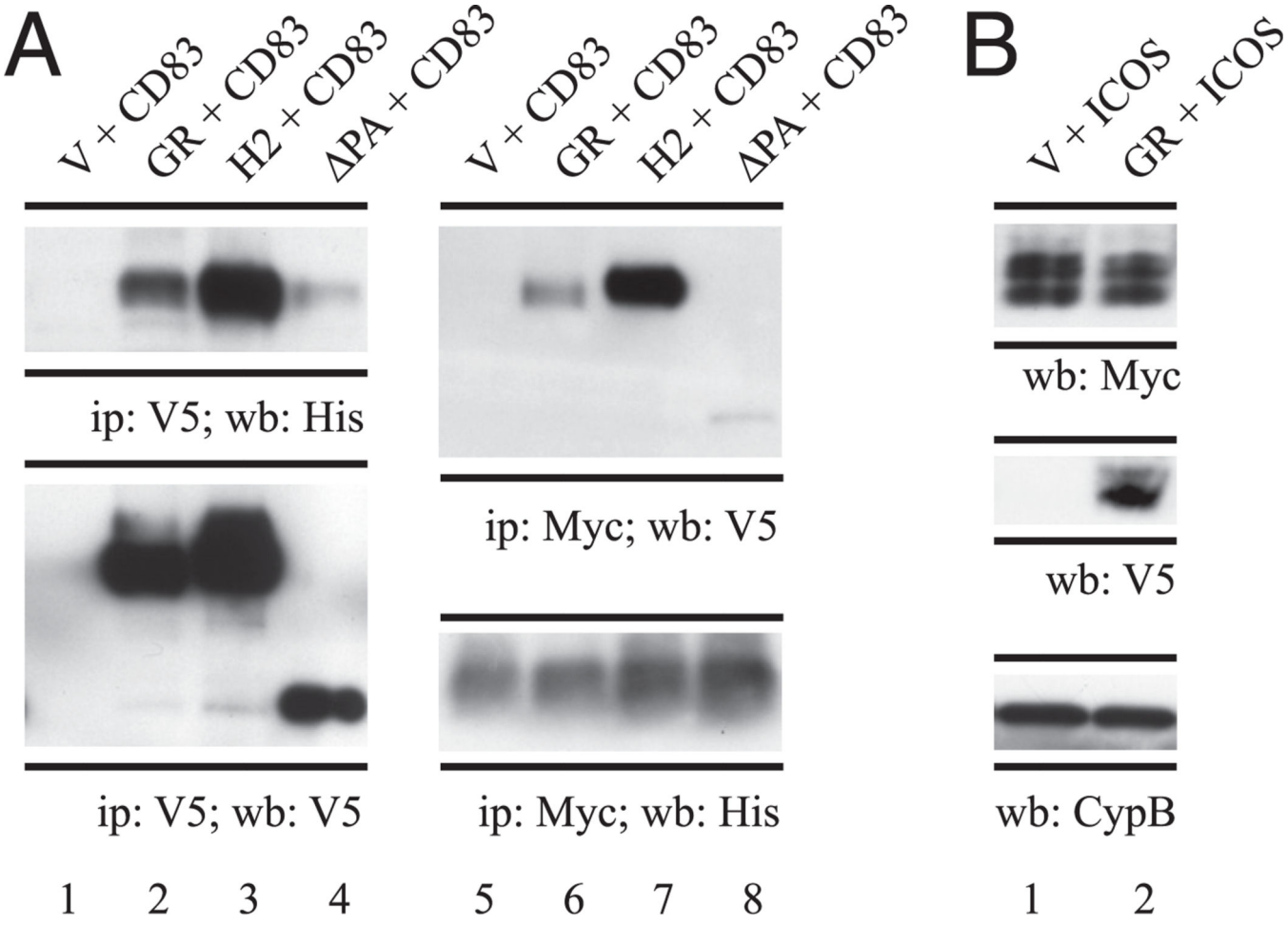

FIGURE 2.

N-terminal PA domain of GRAIL facilitates CD83 binding. *A*, HEK293 cells were transfected with CD83 myc/His-tagged vector (1.0 μg) along with wild-type and mutant GRAIL-V5-tagged vectors (1.0 μg each). Thirty-six hours post-transfection, interaction between GRAIL and CD83 was determined by immunoprecipitation (ip) with anti-V5 Ab, followed by Western blot (wb) probed with anti-His Ab (*top left*), or immunoprecipitation with anti-myc Ab, followed by Western blot probed with anti-V5 Ab (*top right*). Blots were reprobed with appropriate Ab to show presence of immunoprecipitated protein (*bottom*). *B*, HEK293 cells were transfected with 0.5 μg of ICOS-myc-tagged plasmid, along with vector or GRAIL-V5 tagged plasmid (1.0 μg each). Protein levels were analyzed as described in Fig. 1C. V, Vector; GR, GRAIL; H2, H2N2 GRAIL; ΔPA, N-terminal PA deletion.

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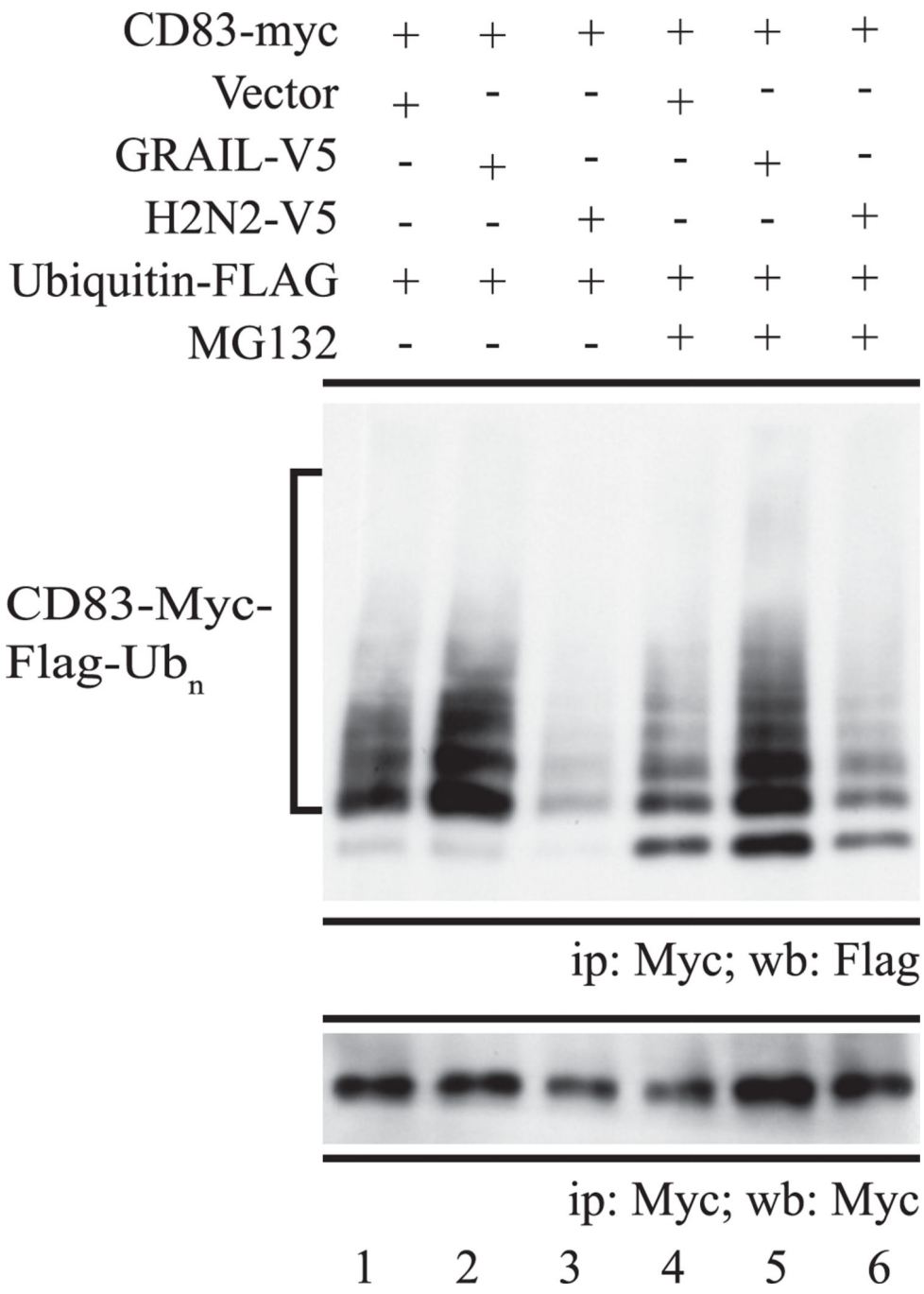

FIGURE 3.

GRAIL-mediated degradation of CD83 proceeds via an ubiquitin-mediated process. HEK293 cells were transfected with 0.5 μg of CD83-myc, 0.5 μg of Flag-Ubiquitin, and either 1.0 μg of vector, GRAIL, or H2N2 plasmid, as indicated. Before lysate preparation, cells were either left untreated or treated with MG132 (25 μM_f) for 2 h. To eliminate the possibility of protein coprecipitation in the samples, lysates were boiled in lysis buffer containing 1% SDS for 5 min before a 10-fold dilution and subsequent immunoprecipitation (ip) using anti-myc Abs. The blot was probed with anti-Flag-HRP to detect the

polyubiquitinated CD83 species (*top*). The blot was reprobed with anti-myc to show CD83 immunoprecipitation levels (*bottom*). wb, Western blot; ip, immunoprecipitation.

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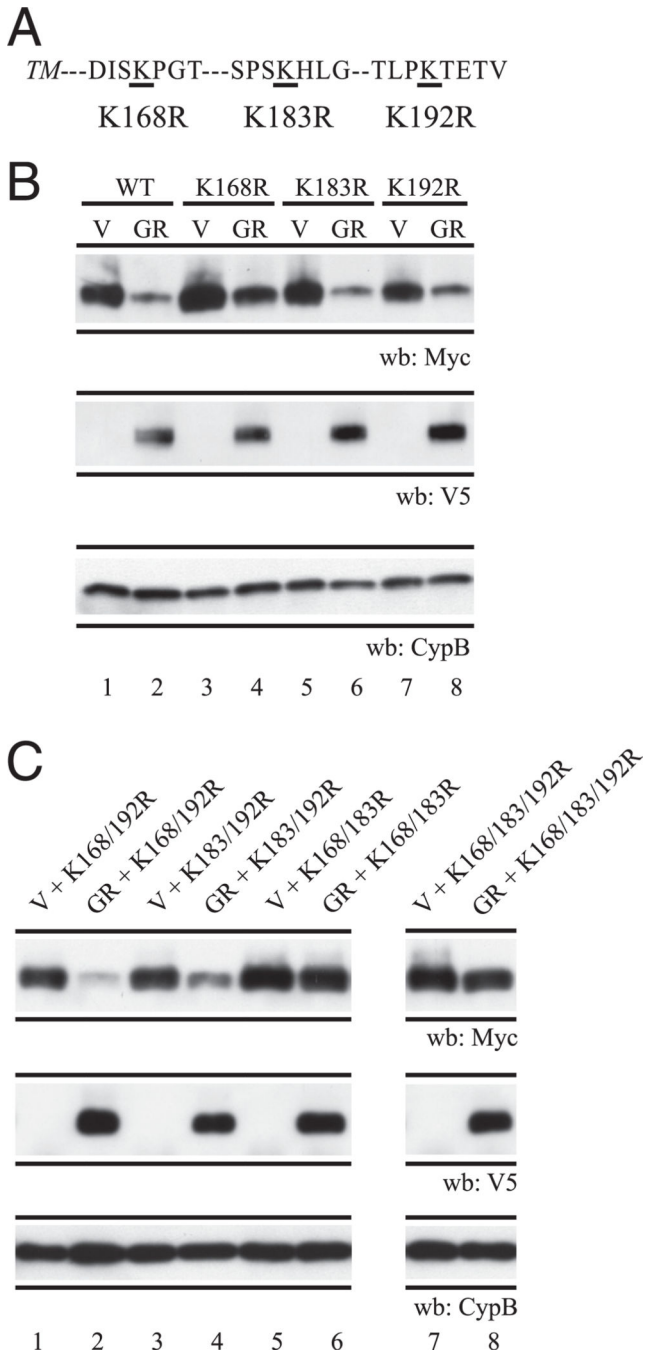

FIGURE 4.

GRAIL-specific degradation of CD83 requires membrane proximal lysine residues. *A*, Protein sequence of carboxyl-terminal domain of CD83-myc, with lysine residues underlined. Position of lysine to arginine (K→R) substitutions noted. *B*, HEK293 cells were transfected with either wild-type CD83 or CD83 bearing single K→R mutations (0.5 μg each) in the absence or presence of 1.0 μg vector encoding wild-type (wt) GRAIL. Lysates were prepared and levels of CD83 were detected by Western blot (wb) with anti-myc Ab 36 h post-transfection (*top*). All blots were reprobed with anti-V5 Ab to assess GRAIL

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expression. The expression of cyclophilin-B (CypB) was used as an internal loading control. *C*, HEK293 cells were transfected with either CD83 bearing double (*left*), or triple (*right*) K→R mutations (0.5 μg each) in the absence or presence of wild-type GRAIL plasmid (1.0 μg). Protein levels of CD83 were determined as described in *B*. TM, Transmembrane; V, vector; GR, GRAIL.

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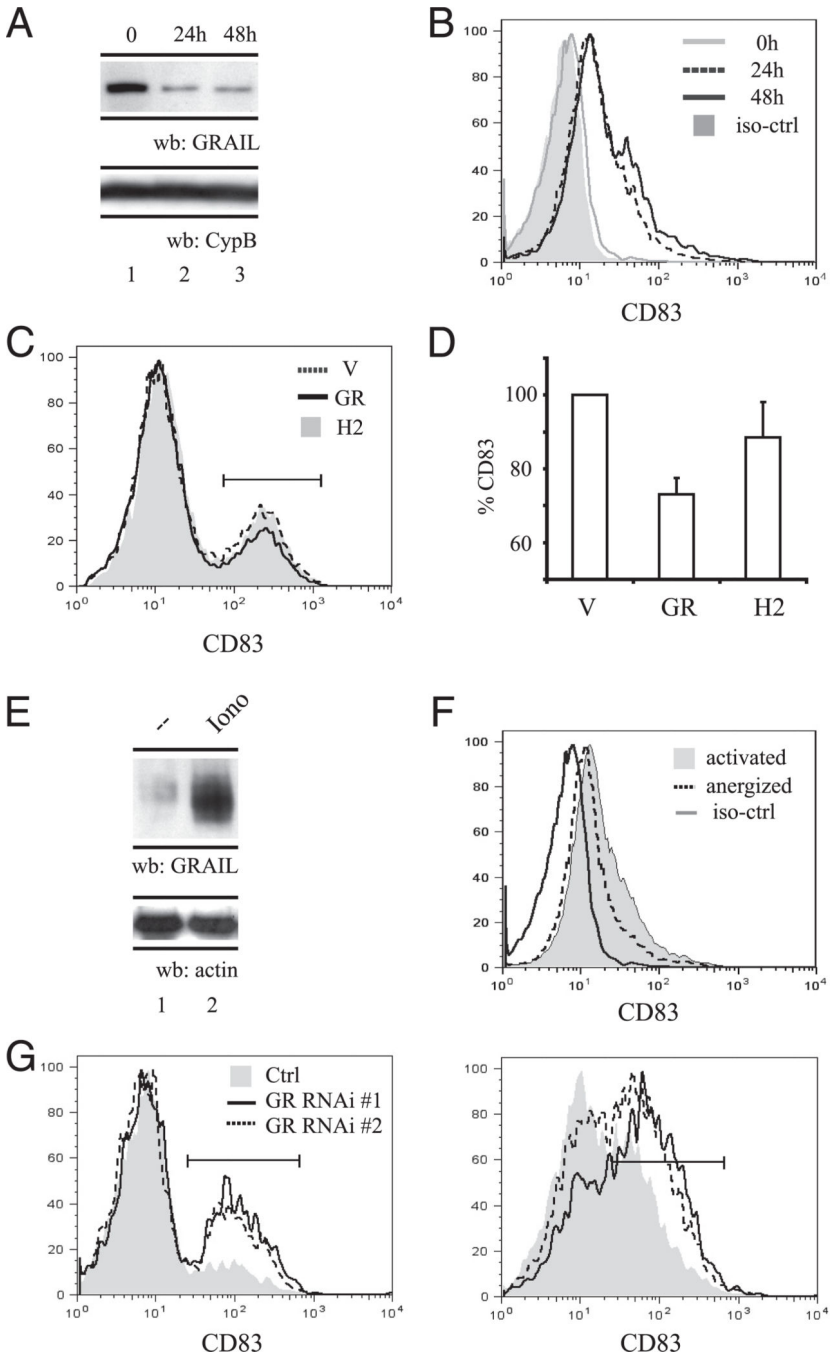

FIGURE 5.

GRAIL expression results in decreased CD83 expression in CD4 T cells. *A*, GRAIL levels in CD4 T cells decrease upon TCR engagement. $CD4^+$ T effector cells were prepared by CD4 T cell MACS depletion and 3-day anti-CD3/CD28 stimulation, followed by 4-day rest in medium. Cells were then subjected to plate-bound anti-CD3 (0.5 μg) and soluble anti-CD28 (0.5 μg) stimulation for times indicated. Cell lysates were prepared and subjected to SDS-PAGE. GRAIL protein level was detected using a monoclonal GRAIL Ab (*top*). Blot was reprobed with anti-cyclophilin-B as protein loading control (*bottom*). *B* TCR

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stimulation induces CD83 expression on CD4 T cells. CD4 T cells were prepared as described in *A*. Cells were stained for CD83 surface expression at 0-, 24-, and 48-h TCR stimulation. Isotype control for 24-h stimulation sample included. (MFIs: 0 h 8.1; 24 h 31.4; 48 h 48.5) *C* Ectopic GRAIL expression reduces CD83 expression levels. CD4 T cells were isolated by AutoMACS separation stimulated for 24 h then transduced with retrovirus containing vector control (V) GRAIL (GR) or H2N2 (H2). Levels of CD83 surface expression on transduced GFP+ cells were evaluated by FACS analysis at 24 h post-transduction. Percentage of CD83 surface expression on GFP^+ cells denoted by gated region: V 25.0%; GR 20.2%; H2 26.0%. *D* Percentage of CD83 surface expression on GRAIL and H2N2-transduced cells normalized to vector control. Average of three independent experiments. *E* Ionomycin-anergized CD4 T cells display increased GRAIL expression. CD4 T cells were prepared as described in *A* then either left untreated or treated with ionomycin (1 μM) for 18 h. Lysates were prepared and subjected to SDS-PAGE. Blots were probed with polyclonal GRAIL anti-serum (*top*) and reprobed with anti-actin (*bottom*) to ensure equal loading. *F* Anergized CD4 T cells display reduced surface expression of CD83. Ionomycin-anergized CD4 T cells were prepared as described in *E* then subjected to plate-bound anti-CD3 (0.5 μg) and soluble anti-CD28 (0.5 μg) stimulation for 24 h and CD83 levels were evaluated by FACS. (Stimulated non-ionomycin treated = activated MFI 31.5; stimulated ionomycin treated = anergized MFI 22.3; isotype control MFI 8.8). *G* GRAIL RNAi results in increased CD83 expression levels. CD4 T cells were electroporated with GRAIL shRNA vectors (GR RNAi no.1 GR RNAi no.2) or pSiren Luciferase control. Twenty-four hours post-electroporation cells were either left unstimulated (*left*) or stimulated with plate-bound anti-CD3 (0.5 μg/ml) and soluble anti-CD28 (0.5 μg/ml) for 24 h (*right*) then CD83 expression on $ZsGreen^+$ cells were determined by FACS. Percentage of CD83 surface expression on $ZsGreen^+$ cells denoted by gated region: unstimulated (*left*): Ctrl 15.4%;RNAi no.1 36.0%; RNAi no. 2 31.1%; stimulated (*right*): Ctrl 41.1%; RNAi no. 1 65.5%; RNAi no. 2 55.6%.

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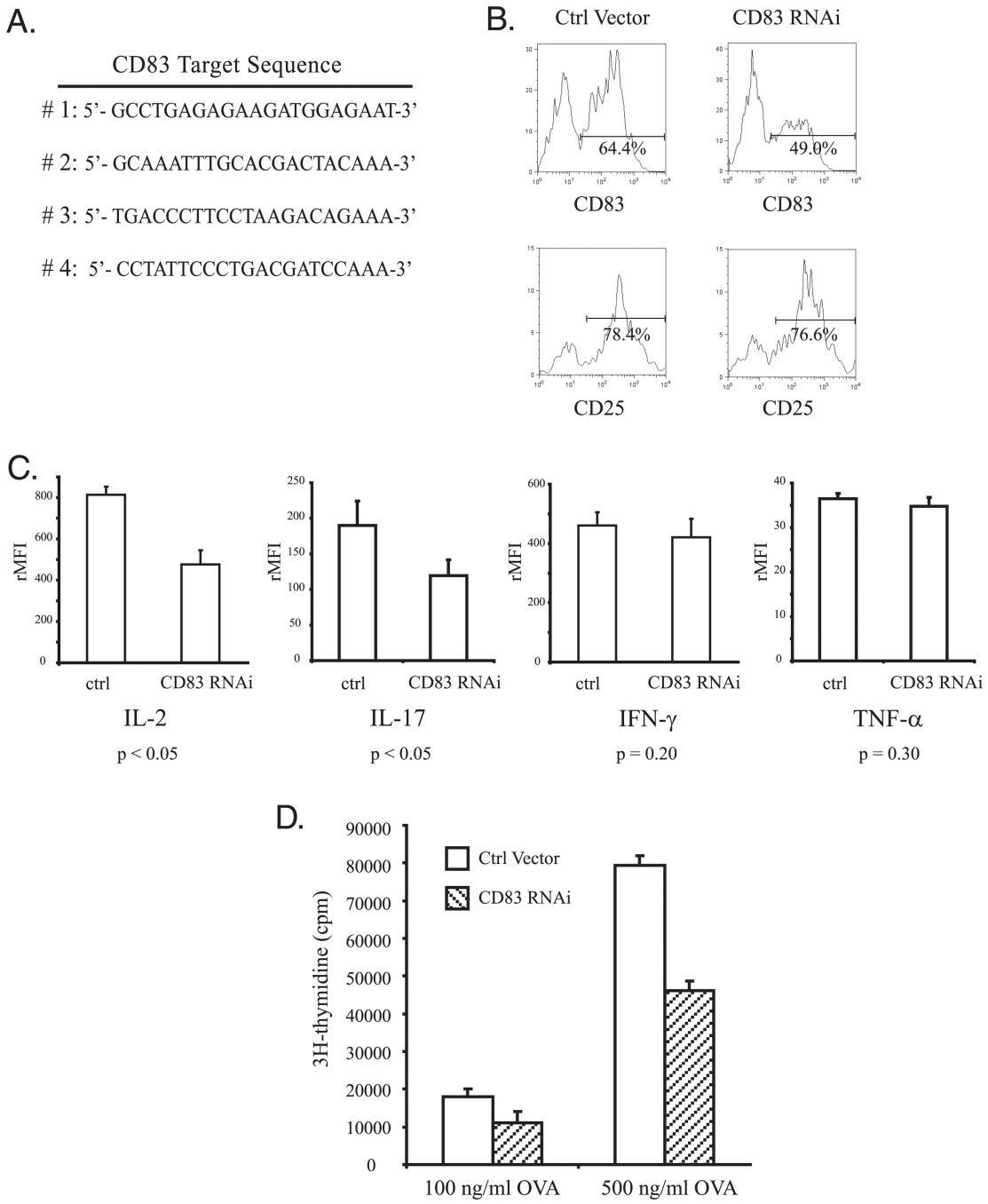

FIGURE 6.

CD83 expression on murine CD4$^+$ T cells is important in TCR-mediated T cell proliferation. *A*, Target sequence of murine CD83 for RNAi oligo design. Four different hairpin loop-containing sense and antisense oligonucleotides were synthesized, annealed, and cloned into the pSiren-Ret-roQ-ZsGreen RNAi vector. *B*, Specific CD83 knockdown in CD4$^+$ T cells. A pSiren Luciferase RNAi control, or a mixture of the CD83 pSiren RNAi vectors (4 μg total) were electroporated into DO11 OVA-specific CD4$^+$ T cells. Cells were stimulated with pOVA (100 ng/ml) presented by irradiated APC 24 h post-electroporation. Surface

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expression of CD83 (*top*) and CD25 (*bottom*) on ZsGreen$^+$ CD4 cells were determined by FACS analysis (Gated regions). *C*, Cytokine expression profile of CD83 RNAi-electroporated CD4$^+$ T cells. DO11 OVA-specific CD4 T cells were electroporated with either control or CD38 shRNA vectors (6 μg total). Zs-Green$^+$ cells were FACS sorted 24 h post-electroporation and subsequently stimulated with irradiated APCs in the presence of 500 ng/ml OVA peptide for 48 h. Supernatants were collected and subjected to Luminex cytokine bead assay. The *p* values were determined using paired *t* test. *D*, CD83 knockdown on CD4$^+$ T cells diminished TCR-mediated T cell proliferation. DO11 OVA-specific CD4 T cells were treated with CD83 RNAi and stimulated with irradiated APCs as described in *C*. Forty-eight hours post-pOVA peptide stimulation, cells were pulsed with [^{3}H]thymidine for 6 h, harvested, and filter was counted. Results are an average of four individual wells.