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MERTK as negative regulator of human T cell activation

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ABSTRACT

The aim of this study was to test the hypothesis whether MERTK, which is up-regulated in human DCs treated with immunosuppressive agents, is directly involved in modulating T cell activation. MERTK is a member of the TAM family and contributes to regulating innate immune response to ACs by inhibiting DC activation in animal models. However, whether MERTK interacts directly with T cells has not been addressed. Here, we show that MERTK is highly expressed on dex-induced human tol-DCs and participates in their tolerogenic effect. Neutralization of MERTK in allogenic MLR, as well as autologous DC–T cell cultures, leads to increased T cell proliferation and IFN-γ production. Additionally, we identify a previously unrecognized noncell-autonomous regulatory function of MERTK expressed on DCs. Mer-Fc protein, used to mimic MERTK on DCs, suppresses naïve and antigen-specific memory T cell activation. This mechanism is mediated by the neutralization of the MERTK ligand PROS1. We find that MERTK and PROS1 are expressed in human T cells upon TCR activation and drive an autocrine proproliferative mechanism. Collectively, these results suggest that MERTK on DCs controls T cell activation and expansion through the competition for PROS1 interaction with MERTK in the T cells. In conclusion, this report identified MERTK as a potent suppressor of T cell response. *J. Leukoc. Biol.* 97: 000–000; 2015.

Introduction

DCs are the most potent APCs connecting innate to adaptive immune responses. DCs are crucial in promoting proinflammatory responses against pathogenic microbes and tumors, in addition to establishing and maintaining tolerance to self- or harmless antigens [1]. Cellular therapies based on DCs have been used to treat different pathologic conditions with the aim of inducing a specific immune response in cancer patients or infectious diseases [2]. Immunogenic DCs are phenotypically well characterized, and their cytokine secretion profile and functional responses are firmly described and established. Up-regulated, costimulatory molecules or maturation-associated receptors are currently used as standard biomarkers to determine DC activation status. Recently, the interest in developing tol-DCs and their potential role in ameliorating autoimmune or immune-based diseases and transplantation have paved the way to apply these cells in clinical protocols [3, 4]. Although great efforts are being made to understand fully tol-DC physiology, as well as to identify specific molecules that mediate their tolerogenic function, no appropriate biomarker for these cells has been identified so far. Furthermore, the signaling pathways that program human DCs into a tolerogenic state are poorly understood. As a result of their attractive and potential role to be applied in clinical trials in human diseases [5], identification of tol-DC markers and characterization of the mechanisms involved in mediating tolerance are of utmost importance.

DNA microarray technology has been used to study the maturation, as well as the effect, of immunosuppressive agents on mouse and human DCs [6, 7]. Here, after gene-expression profile characterization of human tol-DCs cultured with dex and a MC of cytokines, as described previously [8], we identified MERTK as one of the most up-regulated molecules in tol-DCs.

Receptor tyrosine kinases of the TAM family [9, 10] are molecules involved in tempering the immune response in murine macrophages and DCs [11]. Specifically, MERTK have

Abbreviations: AC = apoptotic cell, DC = dendritic cell, dex = dexamethasone, Fla2 = flagellin 2, GAS6 = growth arrest-specific 6, GR = glucocorticoid receptor, iDC = immature dendritic cell, MC = maturation cocktail, mDC = mature dendritic cell, Mer-Fc = rMer tyrosine kinase Fc, MERTK = Mer tyrosine kinase, MFI = mean fluorescence intensity, PROS1 = protein S, qPCR = quantitative PCR, TAM = Tyro-3, Axl, and Mer, tol-DC = tolerogenic dendritic cell

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been reported as a major macrophage AC receptor. Activation of MERTK by their soluble ligands—GAS6 or PROS1—bound to AC restricts the intensity of inflammatory cytokine production and immune responses mainly by inhibiting DC activation, thereby maintaining self-tolerance [12–14]. It has been described that MERTK regulates murine DC production of BAFF [15]. In humans, MERTK is expressed on DCs, NK cells, B cells, M2c macrophages, and platelets [16–18]. Although the role of MERTK and its ligands has become more apparent in the last few years, most of the studies have been performed based on engineered loss-of-function mutants in mice [11, 13]; hence, MERTK regulation in humans still remains unclear. Recently, it has been described that activated human T lymphocytes are able to produce PROS1, which in turn, regulates DC activation and the subsequent immune response [19]. However, whether MERTK regulates T cell activation directly has not been investigated.

In this study, we sought to identify the mechanisms underlying the tolerogenic properties of human tol-DCs and identified MERTK to be highly up-regulated in these cells, contributing to their immunosuppressive function. In addition, although MERTK is up-regulated under tolerogenic conditions, non-tol-DCs also expressed MERTK. Our results revealed that MERTK represents a novel immune-regulatory receptor within the Ig superfamily by directly inhibiting T lymphocyte activation in humans. Thus, MERTK regulates human T cell activation and expansion by sequestering the TAM ligand PROS1 and limiting its proproliferative effect on T cells. Furthermore, the modulation of MERTK activity could be a promising therapeutic approach when control of the immune response is required.

MATERIALS AND METHODS

Generation of human DCs

The present study was approved by the Ethics Committee at the Hospital Clinic of Barcelona, and the authors declare no violation of the Helsinki Doctrine on human experimentation. Buffy coats were obtained from Banc de Sang i Teixits (Barcelona, Spain), and written informed consent was obtained from all blood donors. Monocyte-derived DCs were generated from the peripheral blood samples of healthy volunteers, as reported previously [8]. In summary, PBMCs were allowed to adhere for 2 h at 37°C. Nonadherent cells—PBLs—were gently removed, washed, and used for $CD4^+$ naïve T cell isolation. The adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker, Lonza, Belgium), supplemented with 2% AB human serum (Sigma-Aldrich, Madrid, Spain) and IL-4 (300 U/ml) and GM-CSF (450 U/ml; Miltenyi Biotec, Madrid, Spain) for 6 days to obtain iDCs. The MC consisted of IL-1β and IL-6 (both at 1000 IU/ml) and TNF-α (500 IU/ml; CellGenix, Freiburg, Germany) and PGE_2 (10 μg/ml; Dinoprostona; Pfizer, New York, NY, USA) and was added on day 6 for 24 h. mDCs were harvested and analyzed on day 7. dex (10^{-6} M; Fortecortin; Merck, Madrid, Spain) was added on day 3. We did not observe differences in viability and yield among iDC, dex-iDC, mDC, and tol-DC generation. RU-486 was used in some experiments and added 2 h before dex addition at different concentrations (10, 50, 150, 300 ng/ml; RU-486; Mifepristone). Ethanol was used as a vehicle control, and it did not affect viability or DC phenotype.

RNA isolation

Total RNA was isolated by use of RNeasy Mini Kit columns with on-column DNase I treatment (Qiagen, Hilden, Germany). RNA yield and purity were measured by use of the NanoDrop ND-1000 spectrophotometer and the Agilent 2100 bioanalyzer.

Microarray analysis

Microarray experiments were conducted on baseline 6 iDC- and 6 mDC- and 3 dex-iDC- and 6 tol-DC-treated samples by use of Affymetrix Human Genome U133 Plus 2.0 arrays, containing 54,675 probes for 47,000 transcripts (Affymetrix, Maumee, OH, USA). Raw data were normalized by use of the robust multiarray algorithm [20]. Thereupon, we select 31,436 probes after a filtering step, excluding probes not reaching an average log2 signal intensity of 5. For the detection of differentially expressed genes, a linear model was fitted to the data, and empirical Bayes-moderated statistics were calculated with use of the limma package from Bioconductor (Seattle, USA). Adjustment of *P* values was done by the determination of false discovery rates by use of the Benjami-Hochberg procedure [21]. Microarray raw data (.cel files) and processed data have been deposited in the Gene Expression Omnibus of the National Center for Biotechnology Information and are accessible through GEO Series accession number GSE56017.

Real-time qPCR

Microarray expression of selected DC genes was confirmed in aliquots of the same RNA samples by use of qPCR. RNA was reverse transcribed to cDNA by use of the High-Capacity cDNA RT Kit (Applied Biosystems, Carlsbad, CA, USA). Reverse transcription was carried out in a 96-well thermocycler (Veriti 96W, Applied biosystems) in the following conditions: 25°C, 10 min; 37°C, 120 min. TaqMan real-time PCR was used to detect transcripts of *MERTK* and *IL-2*. Primers and probes for each sequence were obtained as inventoried TaqMan gene-expression assays (Applied Biosystems). B-ACTIN was used as a reference gene.

Purified RNA from a naïve $CD4^+$ T cell was reverse transcribed to cDNA by use of the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). *MERTK* mRNA expression was evaluated by qPCR by use of the KAPA SYBR Fast qPCR kit (KapaBiosystems, Wilmington, MA, USA), and reactions were performed on a Stratagene Mx3000 system. Eukaryotic translation elongation factor 1 α1 was used as a housekeeping gene. Amplified products were checked by dissociation curves.

Flow cytometry

MERTK expression, by flow cytometry, was performed with the use of purified or allophycocyanin directly conjugated α-MERTK mAb (2 μg/ml; R&D Systems, Minneapolis, MN, USA) and the appropriate isotype control (Santa Cruz Biotechnology, Heidelberg, Germany; and R&D Systems). Primary antibodies were followed by staining with PE-labeled goat anti-mouse (BD Biosciences, Franklin Lakes, NJ, USA). For intracellular detection of MERTK, cells were fixed with 2% paraformaldehyde, permeabilized with saponin-based permeabilization buffer, and stained with allophycocyanin, directly conjugated α-MERTK mAb (2 μg/ml; R&D Systems).

Activation of $CD4^+$ T cells was analyzed by use of CD4, CD69, CD25, and CD44 antibodies (BD Biosciences). Viability of cells was checked by use of LIVE/DEAD kit (Life Technologies, Carlsbad, CA, USA).

Flow cytometry was performed with the use of BD FACSCanto II and LSR-II (BD Biosciences) or Stratedigm S1000EX (Stratedigm, San Jose, CA, USA) and analyzed with BD FACSDiva 6.1 or FlowJo 7.6.1.

T cell cultures

MLR. Naïve $CD4^+$ T cells were isolated from human PBLs by use of naïve $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec), following the manufacturer's instructions. Allogeneic, naïve $CD4^+$ T cells were cocultured with DCs differently generated in a 96-well microplate at a 20:1 ratio. Purified α-MERTK mAb (R&D Systems) and the appropriate isotype control were added to the culture at 5 μg/ml. In some experiments, polymyxin B (Sigma-Aldrich) was added to the culture at 10 μg/ml.

Naïve $CD4^+$ T cell cultures. Naïve $CD4^+$ T cells were stimulated with α-CD3 antibody (BD Biosciences) and human Mer-Fc (R&D Systems; both prebound to the microplate for 1 h at 37°C, 1 μg/ml). Human rCD36-Fc was used as a negative control (1 μg/ml; R&D Systems). In some experiments, other stimuli were added to the culture, such as α-CD28 antibody (1 μg/ml; BD

Biosciences), human rIL-2 (50 IU/ml; eBioscience, San Diego, CA, USA), α-CD3/CD28 beads at a 1:1 ratio (Life Technologies), or human PROS1 (50 nM; Haematologic Technologies, Essex Junction, VT, USA). For blocking experiments, purified α-MERTK mAb (R&D Systems) or purified α-PROS1 (PS7) mAb (Santa Cruz Biotechnology) was used. For rechallenge experiments, naïve $CD4^+$ T cells were stimulated for 7 days with α-CD3 antibody (1 μg /ml), harvested and washed with PBS, and rechallenged with α-CD3 antibody (0.5 μg/ml) and Mer-Fc (1 μg/ml). T cell activation was analyzed by flow cytometry after 4 days stimulation with α-CD3 antibody and Mer-Fc (1 μg/ml; see Flow cytometry above).

Antigen-specific T cell cultures. For antigen-specific T cell responses, 2 μg/ml bacterial antigen (Fla2; kindly provided by Prometheus Laboratories) was added to PBMC of a CFSE (Invitrogen, Carlsbad, CA, USA)-labeled Crohn's disease patient, cultured, and expanded for 2 weeks in the presence of IL-2 (20 UI/ml). $CFSE^-$ $CD4^+$T cells were sorted by use of BD FACSAria II and restimulated in the presence of Fla2 antigen and autologous, irradiated PBMCs for antigen-specific expansion. After 12 days, Fla2-specific T cells were harvested and cultured with α-CD3 antibody (0.5 μg/ml) and Mer-Fc (1 μg/ml) for 3 days.

T cells were cultured in X-VIVO 15 medium (BioWhittaker), supplemented with 2% AB human serum (Sigma-Aldrich) unless specified otherwise.

Proliferation assay

For all of the experiments, proliferation assay was performed by use of tritiated thymidine (1 μCi/well; Amersham, Cambridge, United Kingdom), and supernatants were obtained and frozen properly. The $[^3H]$thymidine incorporation took place during the last 16 h of culture. Proliferation was also tested by intracellular Ki-67 staining (BD Biosciences) by use of commercial permeabilization and fixation buffers (Invitrogen) and quantified by flow cytometry.

Cytokines

Culture supernatants were collected and frozen at −20°C. IFN-γ and IL-2 were analyzed by ELISA, according to the manufacturer's guidelines.

Western blotting

Cell lysates and Western blot studies were performed by use of standard procedures. Polyvinylidene difluoride membranes were incubated with α-MERTK polyclonal antibody (R&D Systems) and reprobed with actin (Sigma-Aldrich). After washes, membranes were incubated with HRP-conjugated secondary antibody. Proteins were detected by ECL (ImageQuant LAS 4000; GE Healthcare Life Sciences, Barcelona, Spain) by use of ECL Western blotting detection reagent (GE Healthcare Life Sciences).

Statistical analysis

Data are plotted as mean ± SEM. Statistical analysis was performed by use of 2-tailed Student's *t*-test: $*P < 0.05$; $**P < 0.001$; and $***P < 0.0001$.

RESULTS

MERTK up-regulation in human DCs is controlled by dex

We analyzed microarray gene expression data on in vitro dex-induced human tol-DCs [8] and identified differentially expressed genes in tol-DCs compared with control DCs that could potentially be involved in tolerance induction. Based on the heat map included in **Fig. 1A**, we identified *MERTK*, a member of the tyrosine kinase family known as TAM, as one of the most 50 up-regulated genes expressed in monocyte-derived tol-DCs. In vitro dex treatment increased *MERTK* mRNA expression in iDCs and mDCs by 5.1- and 20.2-fold, respectively, validating the microarray data by qPCR (Fig. 1B). mRNA results were confirmed at the protein level, and MERTK was found to be expressed in in vitro-generated DCs (iDCs, 17.1 ± 3.3%; mDCs, 15.4 ± 3.8%), and the addition of dex resulted in its significant up-regulation (dex-iDCs, 74.4 ± 5.2%; tol-DCs, 59.6 ± 6.9%), as detected by flow cytometry and Western blot (**Fig. 2A** and **B**). Expression kinetics showed >50% of $MERTK^+$ DCs at day 3 upon dex treatment (Supplemental Fig. 1A). It is important to highlight that the majority of MERTK protein was intracellularly detected in the absence of dex (Supplemental Fig. 1B). Moreover, dex-induced MERTK up-regulation was dose

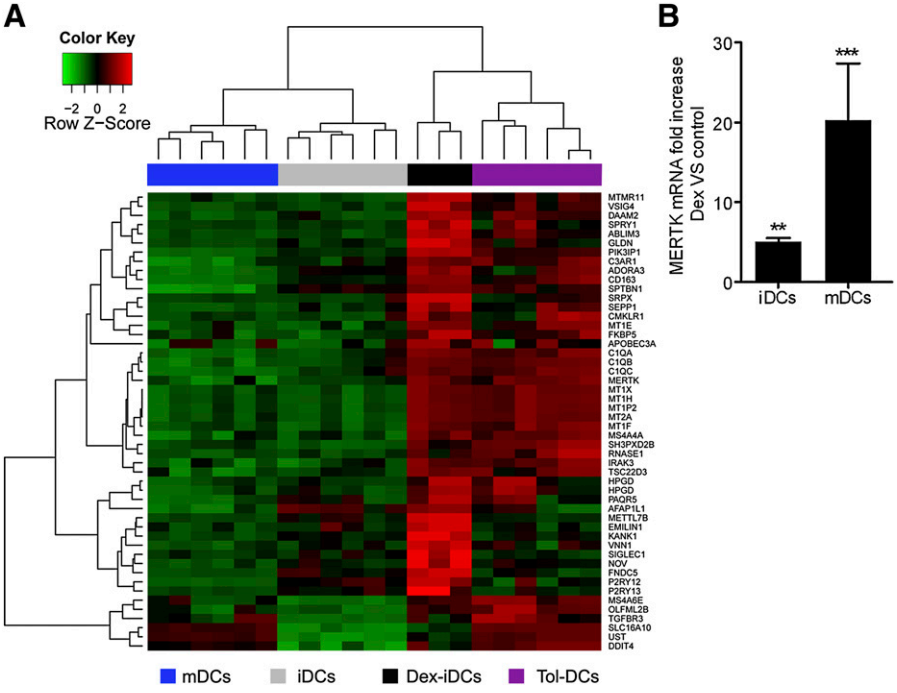

Figure 1. *MERTK* is expressed in human DCs and up-regulated upon dex treatment. (A) Heat map showing clustering (by use of correlation distance and complete method) of the most significant genes among comparisons between untreated human DCs (iDCs), MC-treated DCs (mDCs), dex-treated DCs (dex-iDCs), and dex plus MC-treated DCs (tol-DCs). Results are expressed as a matrix view of gene expression data (heat map), where rows represent genes, and columns represent hybridized samples. The intensity of each color denotes the standardized ratio between each value and the average expression of each gene across all samples. Red pixels correspond to an increased abundance of mRNA in the indicated blood sample, whereas green pixels indicate decreased mRNA levels. (B) Transcripts levels of *MERTK* were determined by real-time PCR by use of *β-ACTIN* as the endogenous reference gene. Data represent dex-treated fold-change induction means ± SEM relative to control DCs [iDCs vs. (VS) dex-iDCs, $n = 3$; mDCs vs. tol-DCs, $n = 8$]. Statistical analysis of *MERTK* expression data of iDCs versus dex-iDCs and mDCs versus tol-DCs was performed with 2-tailed Student's *t*-test: $**P < 0.001$; $***P < 0.0001$.

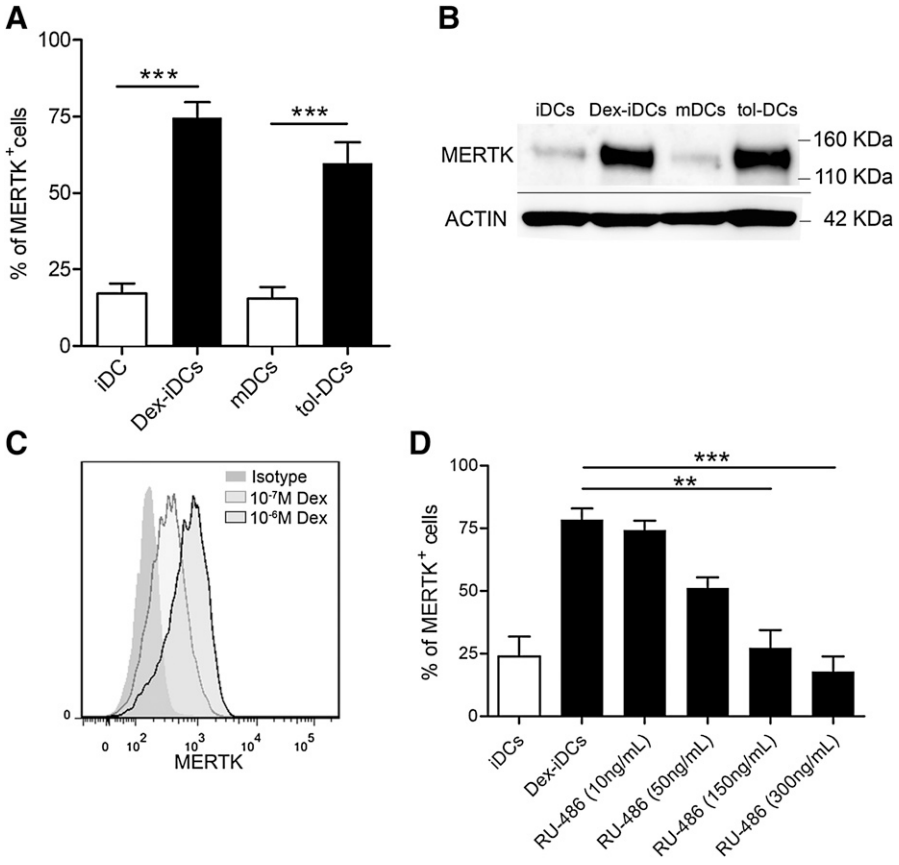

Figure 2. MERTK expression in human DCs is controlled by dex. (A) Flow cytometric analysis of surface MERTK expression on human monocyte-derived DCs ($n \geq 8$). (B) Representative Western blot of MERTK expression in human DCs ($n = 3$). (C) Representative flow cytometry histogram of dex dose-dependent up-regulation of MERTK on DCs: 10^{-7} M (gray line; 56%), 10^{-6} M (black line; 85%), and isotype control (gray, filled histogram). (D) Inhibition of MERTK up-regulation in dex-treated DCs by use of different doses of RU-486 (GR inhibitor). Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $^{**}P < 0.001$; $^{***}P < 0.0001$.

dependent (Fig. 2C), and it was inhibited by RU-486, a specific GR inhibitor (Fig. 2D). We confirmed the involvement of GR in MERTK regulation by use of other glucocorticoids (Supplemental Fig. 1C). When other immunosuppressive agents were tested (vitamin D3, IL-10, and retinoic acid), none of them induced up-regulation of MERTK expression in DC (data not shown).

The blockage of MERTK in DC–T cell interaction increases naïve CD4$^+$ T cell response

To evaluate the function of MERTK, purified naïve CD4$^+$ T cells were cocultured with DCs in the presence of blocking α-MERTK mAb. Interestingly, T cell proliferation was enhanced significantly in the presence of α-MERTK mAb compared with the isotype control (**Fig. 3A**), revealing a role of MERTK in controlling the immune response. Moreover, when blocking MERTK, IFN-γ production was increased significantly, in concordance with proliferation data (Fig. 3B). Similar results were obtained when whole PBLs were used (Supplemental Fig. 2A and B). The maturation status of DCs did not modify the results observed. When mDCs were incubated with naïve CD4$^+$ T cells in the presence of blocking α-MERTK mAb, proliferation and cytokine secretion were also increased (data not shown). Nonspecific T cell activation, as a result of endotoxin contamination of MERTK antibody, was ruled out by adding polymyxin B to the culture (Supplemental Fig. 2C). To confirm that blocking antibodies were not interfering with T cells, DCs were preincubated with α-MERTK, and unbound antibodies were washed out before culturing MLR experiments. Preincubation of DCs with MERTK-blocking antibodies, followed by wash, also induced increased T cell proliferation and cytokine secretion (data not shown). Furthermore, MERTK not only regulated the magnitude of the alloresponse but also controlled autologous immune response. When *Escherichia coli*-activated DCs were incubated with autologous naïve CD4$^+$ T cells in the presence of α-MERTK mAb, T cell proliferation was also increased (Supplemental Fig. 2D), suggesting that MERTK expression in human DCs plays an important role in regulating naïve T cell activation.

MERTK inhibits naïve CD4$^+$ T cell activation

To investigate the direct function of MERTK in T cell activation, we used a Mer-Fc to mimic the effect of MERTK expressed on human DCs. Stimulated, naïve CD4$^+$ T cells with α-CD3 mAb were cultured in the presence of Mer-Fc for 7 days. Surprisingly, Mer-Fc significantly suppressed naïve CD4$^+$ T cell proliferation (67%) and IFN-γ production (from 963 ± 363 to 204 ± 116 pg/ml; **Fig. 4A** and **B**). An irrelevant rFc protein (CD36-Fc) did not alter T cell proliferation or cytokine production (Supplemental Fig. 3A). When Mer-Fc was blocked by use of α-MERTK mAb, the proliferative response to α-CD3 was restored (Supplemental Fig. 3B), confirming the direct suppressive effect of MERTK on T cell activation. To explore the mechanism by which Mer-Fc inhibited T cell activation, we analyzed IL-2 secretion in response to α-CD3. Consistent with our previous data, IL-2 production was reduced significantly when T cells were incubated with Mer-Fc

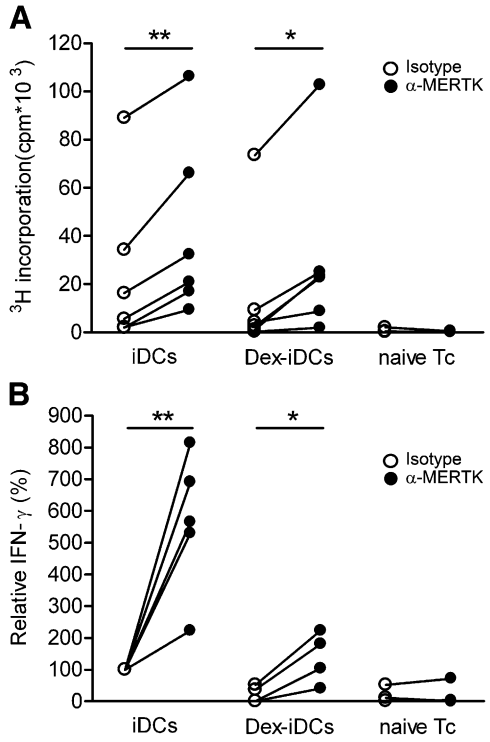

Figure 3. The blockage of MERTK increases allogenic T cell response. DCs were cocultured with naïve CD4$^+$ T cells (ratio 1:20), and T cell activation was measured after 7 days. (A) Proliferation of T cells cultured with iDCs, dex-iDCs, or alone (naïve Tc) in the presence of α-MERTK mAb (black-filled dots) or with an isotype control (empty dots); $n \geq 5$. (B) Relative IFN-γ production in MLR supernatant in the presence of α-MERTK mAb (black-filled dots) compared with isotype control (empty dots); $n \geq 4$. For normalization, T cell IFN-γ production, induced by iDCs in the presence of isotype control antibody of each experiment, was set at 100, and relative IFN-γ production in the presence of α-MERTK mAb was calculated. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's t-test: $^*P < 0.05$; $^{**}P < 0.001$.

(from 266 ± 65 to 100 ± 30 pg/ml; Fig. 4C). *IL-2* mRNA was down-regulated rapidly after overnight stimulation with α-CD3 and Mer-Fc (Supplemental Fig. 3C), which correlated with the low levels of IL-2 cytokine production in the supernatants. To investigate further the mechanism of MERTK-mediated suppression, the expression of early TCR activation markers (CD69, CD44, and CD25) was measured after T cell activation. In the presence of Mer-Fc, percentage of positive cells and MFI of all activation markers were diminished (Fig. 4D) without compromising cell viability (Supplemental Fig. 3D). To test further the inhibitory potential of this receptor, we added soluble α-CD28 mAb or rIL-2 to naïve CD4$^+$ T cell culture, described previously in Fig. 4A. Remarkably, in the presence of stronger costimulatory signals, such as α-CD3α-CD28 stimulation or exogenous IL-2, T cell inhibition by Mer-Fc was still significant (Fig. 4E and Supplemental Fig. 3E and F).

MERTK suppresses antigen-specific memory T cell activation

To evaluate the ability of MERTK to suppress previously activated CD4$^+$ T cells, we stimulated naïve CD4$^+$ T cells with α-CD3 mAb in vitro for 1 wk, washed cells, and rechallenged with α-CD3 mAb plus rMer-Fc. In line with our previous results, Mer-Fc significantly suppressed 77% of T cell proliferation (**Fig. 5A**) and reduced IFN-γ (86%) and IL-2 (83%) production (Fig. 5B and C) of activated T cells. To test the immunosuppressive capacity of Mer-Fc in memory T cells, sorted flagellin-specific CD4$^+$ T cells from Crohn's disease patients (unpublished results) were expanded and rechallenged with α-CD3 mAb plus Mer-Fc. Interestingly, a proliferation assay revealed again a potent suppressive effect of MERTK (38%) in this model of memory T cell response, shown in Fig. 5D. Not only proliferation but also their ability to produce IFN-γ (58%) was strongly impaired by Mer-Fc (Fig. 5E). The same results were observed with tetanus toxoid-specific T cells isolated from healthy donors (data not shown).

PROS1 regulates T cell proliferation through MERTK

To investigate whether the expression of MERTK is regulated during T cell stimulation, naïve CD4$^+$ human T cells were activated by use of α-CD3/CD28 beads, and MERTK expression was evaluated. As shown in **Fig. 6**, *MERTK* mRNA levels were increased after 72 h of stimulation (Fig. 6A). We confirmed the expression of MERTK at protein level by flow cytometry (Fig. 6B).

Based on the fact that MERTK and PROS1 are expressed upon T cell activation, we hypothesized that PROS1 could favor T cell proliferation. To assess whether PROS1 was involved in CD4$^+$ T cell suppression mediated by MERTK on DCs, we neutralized soluble PROS1 from the culture media by use of mAb. Interestingly, our results revealed that the blockage of PROS1 abrogated T cell proliferation (82%) and IFN-γ production (from 375 ± 67 to 99 ± 58 pg/ml) induced by α-CD3 stimulation (**Fig. 7A** and **B**). As PROS1 is present in human serum, we performed experiments in serum-free media to confirm the proproliferative effect of PROS1 on T cells. In these settings, the addition of human PROS1 to activated naive CD4$^+$ T cells for 5 days showed a significant increase of proliferation by use of [^{3}H]thymidine incorporation and Ki-67 intracellular staining (Fig. 7C and D). We did not observe any changes in T cell proliferation when PROS1 was added to unstimulated T cells (data not shown). Moreover, PROS1 was able to rescue the proliferation of activated T cells in the presence of Mer-Fc (Fig. 7E). To understand better the mechanism by which PROS1 was regulating T cell proliferation, we neutralized MERTK on activated T cells and added PROS1 to the culture. As shown in Fig. 7F, we remarkably found that the blocking of MERTK on T cells significantly reduced the ability of PROS1 to induce proliferation of activated T cells.

DISCUSSION

Arising from the importance of tol-DCs being currently used in clinical trials to treat human immune-based diseases [22], it would be of great interest to define better the molecules and mechanisms that mediate their tolerogenic function. In taking advantage of in vitro generation of dex-induced tol-DCs [8], we identified MERTK as a highly up-regulated receptor expressed in

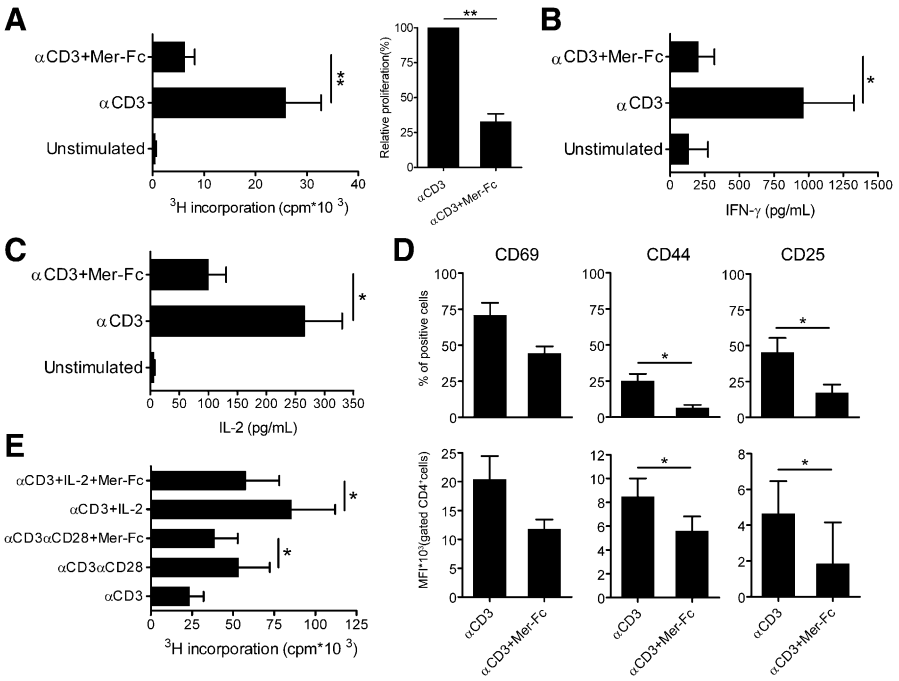

Figure 4. Mer-Fc inhibits naïve CD4$^+$ T cell activation and proliferation. (A, left) Proliferation of naïve CD4$^+$ T cell measured by [^{3}H]thymidine incorporation ($n = 11$). (Right) Relative proliferation; maximum proliferation (T cell proliferation induced by α-CD3 activation) was set at 100, and relative proliferation (T cell proliferation induced by α-CD3 plus Mer-Fc) was calculated per experiment. (B) IFN-γ and (C) IL-2 production in culture supernatants ($n \geq 9$). (D) Expression of T cell activation markers (CD69, CD44, and CD25) analyzed by flow cytometry after stimulation of naïve CD4$^+$ T cells with α-CD3 or α-CD3 plus Mer-Fc for 4 days ($n = 4$). (Upper) Percentage of positive cells compared with isotype control. (Lower) MFI of 4 independent experiments. (E) Proliferation of naïve CD4$^+$ T cell upon α-CD3α-CD28 stimulation or exogenously added rIL-2 plus Mer-Fc; $n \geq 4$. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $^*P < 0.05$; $^{**}P < 0.001$.

monocyte-derived tol-DCs. Our results show that MERTK is expressed in human DCs, and it is up-regulated specifically at mRNA and protein level by dex, therefore, establishing MERTK as a characteristic feature to identify tol-DCs generated with glucocorticoids, even though its expression is not restricted to tol-DCs, as revealed with the MERTK presence in iDC and mDCs. These results are in agreement with the presence of GR binding sites in the *MERTK* gene locus [23]. Interestingly, its expression was induced by a broad variety of corticosteroids but not by other immunosuppressive agents, such as IL-10, vitamin D3, or retinoic acid. The fact that other immunosuppressant drugs did not induce MERTK up-regulation revealed a specific expression pattern of molecules, according to each tolerogenic agent used in the generation of tol-DCs. Besides, it also explains the diversity of functional consequences that may lead to tolerance induction [24].

MERTK belongs to the tyrosine kinase family known as TAM (including 3 members: TYRO-3, AXL, and MERTK). It has been shown previously that TAM receptors are notably expressed by monocytes and their derivatives, emphasizing the involvement of MERTK on the clearance of apoptotic bodies [13, 14]. TAM receptors have also been described as pleiotropic-negative regulators of TLRs and cytokine receptor signaling in murine DCs [11]. It has been described how steroids regulate expression of MERTK and PROS1 and enhance their activity in AC clearance in human macrophages [25], as well as in DCs [26]. Although the importance of MERTK in the engulfment and efficient clearance of AC in humans has been investigated, little is known about the immunomodulatory role of MERTK in humans, as it may differ from animal models. In this report, we reveal an unknown function of MERTK in regulating T cell response. Interestingly, by adding α-MERTK blocking mAb to MLR or an autologous response, CD4$^+$ T cell proliferation and IFN-γ production were enhanced significantly, revealing a role of MERTK in controlling a naïve T cell response. Although MERTK is highly expressed in dex-iDCs, we observed a more pronounced effect of blocking the

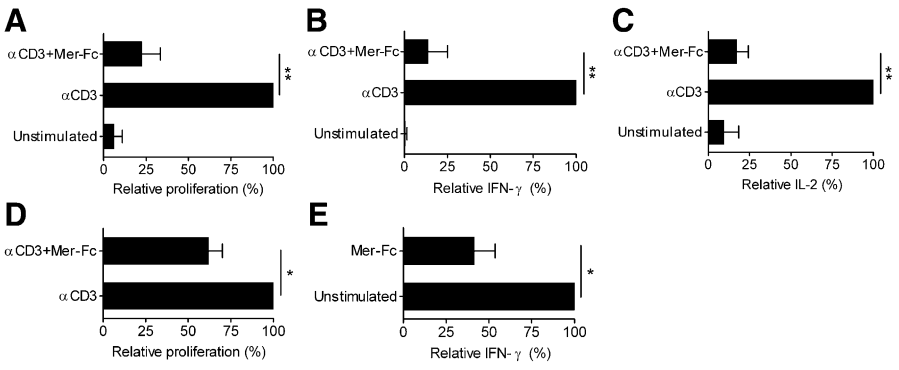

Figure 5. Mer-Fc inhibits antigen-specific memory T cell response. (A) Stimulated CD4$^+$ T cells were harvested, washed, and rechallenged further with α-CD3 and Mer-Fc for 72 h; $n = 4$. Relative proliferation was measured by [^{3}H]thymidine incorporation. (B) Relative IFN-γ and (C) relative IL-2 production in culture supernatants ($n \geq 3$). (D) Sorted Fla2-specific CD4$^+$ T cells were stimulated with α-CD3 and Mer-Fc for 72 h; $n = 3$. Relative proliferation was measured. (E) Relative IFN-γ production in culture supernatants; $n = 3$. All graphs show relative values normalized as described previously in Fig. 4. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $^*P < 0.05$; $^{**}P < 0.001$.

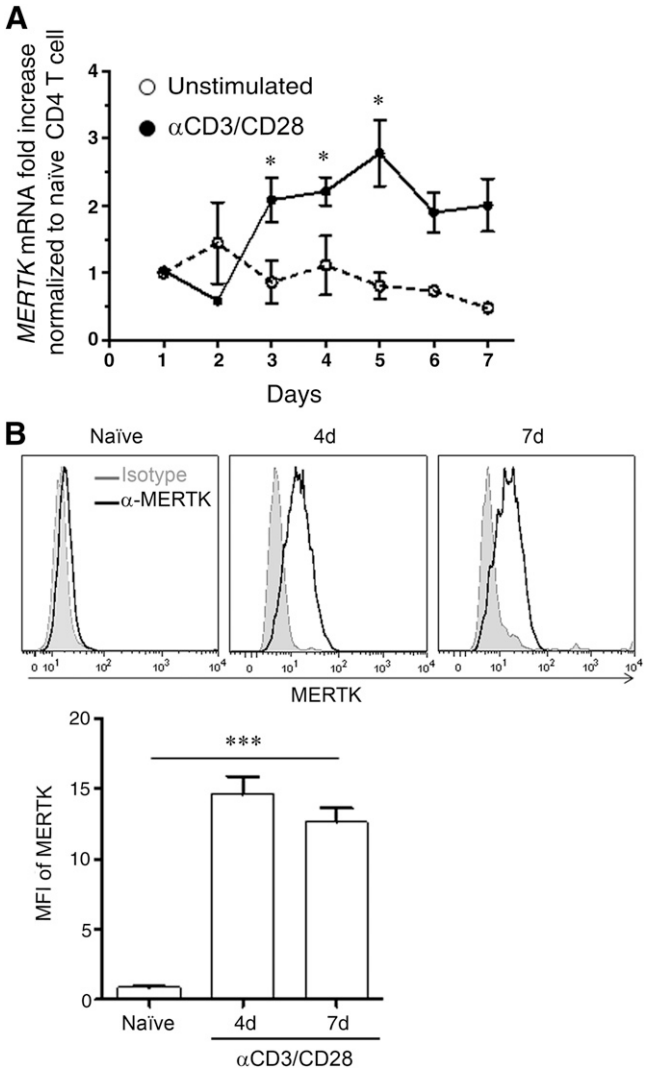

Figure 6. MERTK is expressed in activated human T cells. (A) Naïve $CD4^+$ T cells were isolated and in vitro stimulated with α-CD3/CD28 beads in a 1:1 ratio. Cells were harvested at different time-points, and *MERTK* mRNA was evaluated by qPCR. Fold induction was normalized to acutely isolated, naïve $CD4^+$ T cells ($n = 5$). (B) Surface MERTK expression on acutely isolated, 4 days (4d) and 7 days (7d) postactivated, naïve $CD4^+$ T cells was evaluated by flow cytometry. Representative histogram and MFI analysis of 4–6 independent samples are shown. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $*P < 0.05$; $***P < 0.0001$.

receptor in iDCs. This could be explained by the lower immunogenic capacity of dex-iDCs as a result of the glucocorticoid-induction of other inhibitory mechanisms, such as IL-10 [8]. Although high MERTK expression is observed in dex-iDCs, the tolerogenic properties of these cells may impede the evaluation of the inhibitory effect of MERTK itself. Nevertheless, the fact that the blocking of MERTK in dex-iDCs alloresponse results in an enhanced proliferation suggests that this receptor contributes to their tolerogenic properties.

A pronounced suppressive effect of GAS6 [27] and PROS1 [19] (natural ligands) on DCs via MERTK has already been shown. However, the role of MERTK in regulating T cell activation has not yet been explored. Our results demonstrate the importance of MERTK in controlling T cell proliferation and cytokine production; henceforth, we wondered whether MERTK is involved in T cell-priming regulation.

MERTK induction on DCs constitutes a self-regulatory mechanism to restrain an ongoing immune response [11]. Notwithstanding, a neutralizing effect of MERTK on T cell proliferation would highlight the importance of this receptor in regulating the adaptive immune response. To test the direct function of MERTK in T cell activation, we used Mer-Fc to mimic the effect of MERTK expressed on human DCs. Remarkably, we demonstrate that MERTK suppresses memory and naïve $CD4^+$ T cell activation, proliferation, IFN-γ, and IL-2 secretion by use of human rMer-Fc. Naïve T cell IL-2 production is essential for $CD4^+$ and $CD8^+$ T cell growth, proliferation, and differentiation. Indeed, one of the most rapid consequences of T cell activation is the de novo synthesis of IL-2; followed by expression of a high-affinity IL-2R, it permits the expansion of effector T cell populations activated by antigen [28]. We hypothesize that MERTK on DCs might be inhibiting T cell expansion, impairing IL-2 production to negatively regulate T cell proliferation, thus leading to an intrinsic negative feedback to down-regulate CD25 (α subunit of IL-2R) and IFN-γ production on T cells. This may contribute to maintain the physiologic balance of immune activation against pathogens yet evading exacerbated inflammation. It is important to highlight that MERTK capacity to inhibit $CD4^+$ T cells (in a cell-nonautonomous manner) has not been described previously. Wallet et al. [29] investigated the effect of NOD mice DCs lacking MERTK in mediating AC-induced inhibition of DC activation and therefore, T cell proliferation. However, these authors did not observe a direct effect of MERTK in T cell activation, independently of AC engulfment by DCs, revealing significant differences of MERTK function between human and mice [29].

Interestingly, we provide further evidence that MERTK is able to regulate negatively the immune response, even though T cells had been activated previously by α-CD3 or in antigen-specific memory T cells from Crohn's disease patients recently characterized by our group (unpublished data). The fact that MERTK suppresses the proliferation of these specific populations contributes to speculate in novel therapies to treat autoimmune or immune-based diseases considering TAM receptors, in particular, MERTK, as potential candidates. Indeed, a recent publication showed the therapeutic efficacy of TAM tyrosine kinase agonists in collagen-induced arthritis by the administration of plasmids coding for GAS6 and PROS1 [30].

MERTK overexpression has been reported in a variety of human cancers, including B- and T-acute lymphocytic leukemia, indicating the involvement of MERTK in intrinsic cell proliferation [31]. Surprisingly, in contrast to what has been reported in mice, the expression of MERTK by human T cells may represent an autonomous mechanism of regulation that remains to be understood.

Although 2 main ligands have been described for MERTK—GAS6 and PROS1 [32]—the ubiquitous location of these pleiotropic proteins in vivo makes it difficult to study their role in TAM receptors-mediated-immune regulation, particularly

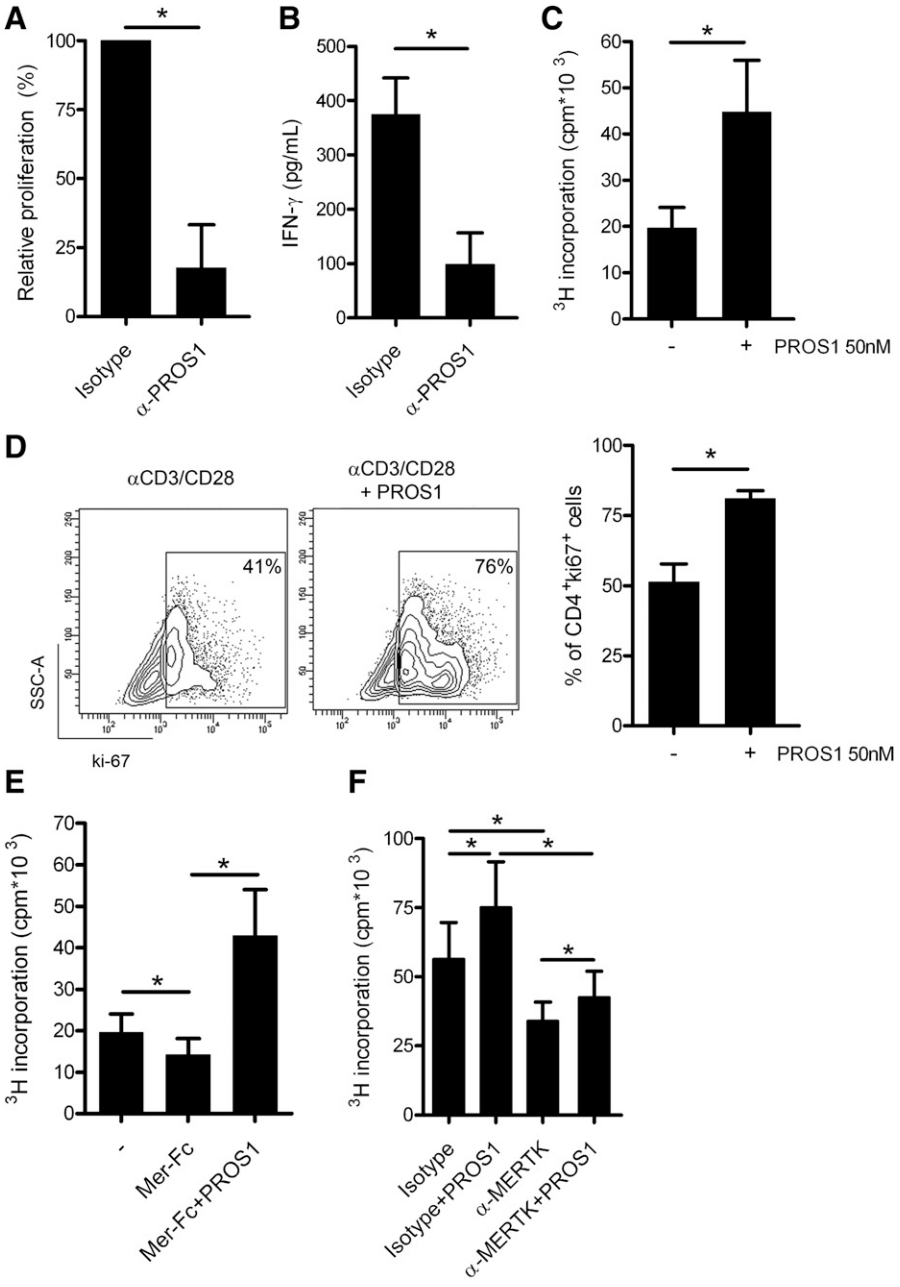

Figure 7. PROS1 regulates T cell proliferation through MERTKR. (A) Relative proliferation of α-CD3-stimulated, naïve $CD4^+$ T cells treated with isotype control or α-PROS1 mAb. Maximum proliferation (T cell proliferation induced by α-CD3 activation, treated with isotype control) was set at 100, and relative proliferation (T cell proliferation induced by α-CD3 plus α-PROS1) was calculated per experiment; $n = 4$. (B) IFN-γ production in culture supernatants ($n = 5$). (C) Naïve $CD4^+$ T cells were cultured in serum-free media and activated with α-CD3/CD28 beads. Human PROS1 (50 nM) was added to the culture for 5 days, and T cell proliferation was measured by [^{3}H]thymidine incorporation; $n = 5$. (D) Naïve $CD4^+$ T cells were cultured in serum-free media and activated with α-CD3/CD28 beads. Human PROS1 (50 nM) was added to the culture for 5 days, and T cell proliferation was evaluated by Ki-67 intracellular staining. Flow cytometry plots show representative Ki-67 staining, gated on live $CD4^+$ cells. (Right) Percentage of $CD4^+Ki\text{-}67^+$ cells; $n = 3$. SSC-A, Side-scatter-area. (E) Naïve $CD4^+$ T cells were cultured in serum-free media and activated with α-CD3/CD28 beads. Human PROS1 (50 nM) and Mer-Fc were added to the culture for 5 days, and T cell proliferation was measured by [^{3}H]thymidine incorporation; $n = 5$. (F) Naïve $CD4^+$ T cells were cultured in serum-free media and activated with α-CD3/CD28 beads. α-MERTK mAb or isotype control was added to the culture for 30 min before addition of human PROS1 (50 nM). After 5 days, T cell proliferation was measured by [^{3}H]thymidine incorporation; $n = 6$. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's t-test: $^*P < 0.05$.

in humans. Recently, Carrera Silva et al. [19] reported that activated human $CD4^+$ T cells produce PROS1 that acts locally at the DC–T cell interface limiting DC activation. It has been shown that overexpression of GAS6 and PROS1 is correlated with poor prognosis in a variety of cancers [33, 34]. We wondered whether PROS1 was involved in $CD4^+$ T cell suppression mediated by MERTK on DCs to asses that we neutralized soluble PROS1 from the culture media by use of mAb. Proliferation was abrogated by α-PROS1 mAb, suggesting that PROS1 has a proproliferative effect in activated T cells. It is tempting to speculate that MERTK expressed by DCs might be neutralizing PROS1 and therefore, avoiding the autocrine effect on T cells mediated by MERTK. PROS1, produced by hepatocytes and endothelial cells, is found in large amounts in the blood [35]. We demonstrate further that the addition of human PROS1 to serum-free media-cultured T cells enhanced T cell proliferation significantly through MERTK expressed on T cells. These findings confirmed the direct proproliferative effect of PROS1 on T cells.

We hypothesize that the availability of soluble PROS1 during DC–T cell interactions will define the result of the immune response. Although this concept seems counterintuitive, our results reveal that MERTK function varies depending on the expressing cell type. Thereby, this receptor restrains activation on DCs, and by contrast, it has a proproliferative function on T cells. Carrera-Silva et al. [19] showed that T cell-derived PROS1 functions locally at the DC–T cell interface and engages TAM

signaling within DCs to limit their activation. It is well known that MERTK is up-regulated in human APCs upon tolerogenic treatment, and several studies showed its anti-inflammatory role, especially in AC clearance [36]. On the other hand, MERTK is ectopically expressed or overexpressed in hematologic and epithelial malignant cells acting as a tumor oncogene [37]. Recently, Knubel et al. [38] described that specific MERTK inhibition profoundly limits human glioma growth, and several other studies are considering the inhibition of MERTK as a therapeutic approach to treat cancer. However, the mechanisms by which increased MERTK signaling contributes to tumor malignancy remain unknown. Interestingly, our results reveal a hitherto-unknown dual function for MERTK, depending on whether it is expressed in human DCs or T cells.

Collectively, our study gives novel insights into the molecular basis for regulating T cell activation, considering MERTK as a key player for the suppression of a T cell response. We demonstrate that MERTK expression on tol-DCs contributes to their tolerogenic function by directly regulating the adaptive immune response. MERTK is identified as a novel immune-suppressive receptor, which is expressed in human DCs and up-regulated by glucocorticoids. Therefore, it is conceivable that MERTK on the DC membrane suppresses T cell responses by neutralizing PROS1 produced by human T cells and inhibiting an autocrine and proproliferative effect of PROS1 in MERTK-expressing T cells. Interestingly, experiments by use of Mer-Fc demonstrated that the new inhibitory function of MERTK is not dependent on the activation state of DCs.

Additionally, MERTK expression in cancer cell lines, beside its role as a tumor oncogene [37, 39], could be involved in silencing T cell responses as a mechanism for tumor-immune escape. The proposed immune-evading effect of MERTK in tumors is in agreement with the immunosuppressive effect demonstrated in the current study. We provide evidence that this receptor not only acts regulating DC activation [19], but it also regulates naïve and memory T cell responses. Targeting this molecule may provide an interesting approach to induce or inhibit tolerance effectively for the purpose of immunotherapy.

AUTHORSHIP

R.C. designed and performed research, analyzed and interpreted data, and wrote the manuscript. E.A.C.S. performed research and analyzed and interpreted data. G.F.-G., A.E.E., E.C-G., and C.E. performed research. J.J.L. contributed vital analytical tools. E.R. and J.P. interpreted data. C.R. designed research and interpreted data. D.B.-R. designed research, analyzed and interpreted data, and wrote the manuscript.

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DISCLOSURES

The authors have no conflicting financial interests.

REFERENCES

1. Medzhitov, R. (2007) Recognition of microorganisms and activation of the immune response. *Nature* **449,** 819–826.
2. Figdor, C. G., de Vries, I. J., Lesterhuis, W. J., Melief, C. J. (2004) Dendritic cell immunotherapy: mapping the way. *Nat. Med.* **10,** 475–480.
3. Cabezón, R., Benítez-Ribas, D. (2013) Therapeutic potential of tolerogenic dendritic cells in IBD: from animal models to clinical application. *Clin. Dev. Immunol.* **2013,** 789814.
4. Stoop, J. N., Harry, R. A., von Delwig, A., Isaacs, J. D., Robinson, J. H., Hilkens, C. M. (2010) Therapeutic effect of tolerogenic dendritic cells in established collagen-induced arthritis is associated with a reduction in Th17 responses. *Arthritis Rheum.* **62,** 3656–3665.
5. Steinman, R. M., Hawiger, D., Nussenzweig, M. C. (2003) Tolerogenic dendritic cells. *Annu. Rev. Immunol.* **21,** 685–711.
6. Suciu-Foca Cortesini, N., Piazza, F., Ho, E., Ciubotariu, R., LeMaoult, J., Dalla-Favera, R., Cortesini, R. (2001) Distinct mRNA microarray profiles of tolerogenic dendritic cells. *Hum. Immunol.* **62,** 1065–1072.
7. Vizzardelli, C., Pavelka, N., Luchini, A., Zanoni, I., Bendickson, L., Pelizzola, M., Beretta, O., Foti, M., Granucci, F., Nilsen-Hamilton, M., Ricciardi-Castagnoli, P. (2006) Effects of dexamethazone on LPS-induced activationand migration of mouse dendritic cells revealed by a genome-wide transcriptional analysis. *Eur. J. Immunol.* **36,** 1504–1515.
8. Cabezón, R., Ricart, E., España, C., Panés, J., Benitez-Ribas, D. (2012) Gram-negative enterobacteria induce tolerogenic maturation in dexamethasone conditioned dendritic cells. *PLoS ONE* **7,** e52456.
9. Lai, C., Lemke, G. (1991) An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. *Neuron* **6,** 691–704.
10. Lu, Q., Gore, M., Zhang, Q., Camenisch, T., Boast, S., Casagranda, F., Lai, C., Skinner, M. K., Klein, R., Matsushima, G. K., Earp, H. S., Goff, S. P., Lemke, G. (1999) Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. *Nature* **398,** 723–728.
11. Rothlin, C. V., Ghosh, S., Zuniga, E. I., Oldstone, M. B., Lemke, G. (2007) TAM receptors are pleiotropic inhibitors of the innate immune response. *Cell* **131,** 1124–1136.
12. Sen, P., Wallet, M. A., Yi, Z., Huang, Y., Henderson, M., Mathews, C. E., Earp, H. S., Matsushima, G., Baldwin, Jr., A. S., Tisch, R. M. (2007) Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. *Blood* **109,** 653–660.
13. Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., Matsushima, G. K. (2001) Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature* **411,** 207–211.
14. Zizzo, G., Hilliard, B. A., Monestier, M., Cohen, P. L. (2012) Efficient clearance of early apoptotic cells by human macrophages requires M2c polarization and MerTK induction. *J. Immunol.* **189,** 3508–3520.
15. Gohlke, P. R., Williams, J. C., Vilen, B. J., Dillon, S. R., Tisch, R., Matsushima, G. K. (2009) The receptor tyrosine kinase MerTK regulates dendritic cell production of BAFF. *Autoimmunity* **42,** 183–197.
16. Graham, D. K., Dawson, T. L., Mullaney, D. L., Snodgrass, H. R., Earp, H. S. (1994) Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. *Cell Growth Differ.* **5,** 647–657.
17. Behrens, E. M., Gadue, P., Gong, S. Y., Garrett, S., Stein, P. L., Cohen, P. L. (2003) The mer receptor tyrosine kinase: expression and function suggest a role in innate immunity. *Eur. J. Immunol.* **33,** 2160–2167.
18. Angelillo-Scherrer, A., de Frutos, P., Aparicio, C., Melis, E., Savi, P., Lupu, F., Arnout, J., Dewerchin, M., Hoylaerts, M., Herbert, J., Collen, D., Dahlbäck, B., Carmeliet, P. (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat. Med.* **7,** 215–221.
19. Carrera Silva, E. A., Chan, P. Y., Joannas, L., Errasti, A. E., Gagliani, N., Bosurgi, L., Jabbour, M., Perry, A., Smith-Chakmakova, F., Mucida, D., Cheroutre, H., Burstyn-Cohen, T., Leighton, J. A., Lemke, G., Ghosh, S., Rothlin, C. V. (2013) T Cell-derived protein S engages TAM receptor signaling in dendritic cells to control the magnitude of the immune response. *Immunity* **39,** 160–170.
20. Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., Speed, T. P. (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* **31,** e15.

21. Smyth, G. K. (2005) *limma: Linear Models for Microarray Data*. Springer, New York.
22. Harry, R. A., Anderson, A. E., Isaacs, J. D., Hilkens, C. M. (2010) Generation and characterisation of therapeutic tolerogenic dendritic cells for rheumatoid arthritis. *Ann. Rheum. Dis.* **69,** 2042–2050.
23. Paakinaho, V., Kaikkonen, S., Makkonen, H., Benes, V., Palvimo, J. J. (2014) SUMOylation regulates the chromatin occupancy and anti-proliferative gene programs of glucocorticoid receptor. *Nucleic Acids Res.* **42,** 1575–1592.
24. Naranjo-Gómez, M., Raïch-Regué, D., Oñate, C., Grau-López, L., Ramo-Tello, C., Pujol-Borrell, R., Martínez-Cáceres, E., Borràs, F. E. (2011) Comparative study of clinical grade human tolerogenic dendritic cells. *J. Transl. Med.* **9,** 89.
25. McColl, A., Bournazos, S., Franz, S., Perretti, M., Morgan, B. P., Haslett, C., Dransfield, I. (2009) Glucocorticoids induce protein S-dependent phagocytosis of apoptotic neutrophils by human macrophages. *J. Immunol.* **183,** 2167–2175.
26. Hodrea, J., Majai, G., Doró, Z., Zahuczky, G., Pap, A., Rajnavölgyi, É., Fésüs, L. (2012) The glucocorticoid dexamethasone programs human dendritic cells for enhanced phagocytosis of apoptotic neutrophils and inflammatory response. *J. Leukoc. Biol.* **91,** 127–136.
27. Alciato, F., Sainaghi, P. P., Sola, D., Castello, L., Avanzi, G. C. (2010) TNF-alpha, IL-6, and IL-1 expression is inhibited by GAS6 in monocytes/macrophages. *J. Leukoc. Biol.* **87,** 869–875.
28. Lenardo, M., Chan, K. M., Hornung, F., McFarland, H., Siegel, R., Wang, J., Zheng, L. (1999) Mature T lymphocyte apoptosis—immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* **17,** 221–253.
29. Wallet, M. A., Sen, P., Flores, R. R., Wang, Y., Yi, Z., Huang, Y., Mathews, C. E., Earp, H. S., Matsushima, G., Wang, B., Tisch, R. (2008) MerTK is required for apoptotic cell-induced T cell tolerance. *J. Exp. Med.* **205,** 219–232.
30. Van den Brand, B. T., Abdollahi-Roodsaz, S., Vermeij, E. A., Bennink, M. B., Arntz, O. J., Rothlin, C. V., van den Berg, W. B., van de Loo, F. A. (2013) Therapeutic efficacy of Tyro3, Axl, and Mer tyrosine kinase agonists in collagen-induced arthritis. *Arthritis Rheum.* **65,** 671–680.
31. Brandao, L. N., Winges, A., Christoph, S., Sather, S., Migdall-Wilson, J., Schlegel, J., McGranahan, A., Gao, D., Liang, X., Deryckere, D., Graham, D. K. (2013) Inhibition of MerTK increases chemosensitivity and decreases oncogenic potential in T-cell acute lymphoblastic leukemia. *Blood Cancer J.* **3,** e101.
32. Stitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., Radziejewski, C., Mattsson, K., Fisher, J., Gies, D. R., Jones, P. F., Masiakowski, P., Ryan, T. E., Tobkes, N. J., Chen, D. H., DiStefano, P. S., Long, G. L., Basilico, C., Goldfarb, M. P., Lemke, G., Glass, D. J., Yancopoulos, G. D. (1995) The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell* **80,** 661–670.
33. Hutterer, M., Knyazev, P., Abate, A., Reschke, M., Maier, H., Stefanova, N., Knyazeva, T., Barbieri, V., Reindl, M., Muigg, A., Kostron, H., Stockhammer, G., Ullrich, A. (2008) Axl and growth arrest-specific gene 6 are frequently overexpressed in human gliomas and predict poor prognosis in patients with glioblastoma multiforme. *Clin. Cancer Res.* **14,** 130–138.
34. Saraon, P., Musrap, N., Cretu, D., Karagiannis, G. S., Batruch, I., Smith, C., Drabovich, A. P., Trudel, D., van der Kwast, T., Morrissey, C., Jarvi, K. A., Diamandis, E. P. (2012) Proteomic profiling of androgen-independent prostate cancer cell lines reveals a role for protein S during the development of high grade and castration-resistant prostate cancer. *J. Biol. Chem.* **287,** 34019–34031.
35. Burstyn-Cohen, T., Heeb, M. J., Lemke, G. (2009) Lack of protein S in mice causes embryonic lethal coagulopathy and vascular dysgenesis. *J. Clin. Invest.* **119,** 2942–2953.
36. Seitz, H. M., Camenisch, T. D., Lemke, G., Earp, H. S., Matsushima, G. K. (2007) Macrophages and dendritic cells use different Axl/Mertk/Tyro3 receptors in clearance of apoptotic cells. *J. Immunol.* **178,** 5635–5642.
37. Cook, R. S., Jacobsen, K. M., Wofford, A. M., DeRyckere, D., Stanford, J., Prieto, A. L., Redente, E., Sandahl, M., Hunter, D. M., Strunk, K. E., Graham, D. K., Earp III, H. S. (2013) MerTK inhibition in tumor leukocytes decreases tumor growth and metastasis. *J. Clin. Invest.* **123,** 3231–3242.
38. Knubel, K. H., Pernu, B. M., Sufit, A., Nelson, S., Pierce, A. M., Keating, A. K. (2014) MerTK inhibition is a novel therapeutic approach for glioblastoma multiforme. *Oncotarget* **5,** 1338–1351.
39. Schlegel, J., Sambade, M. J., Sather, S., Moschos, S. J., Tan, A. C., Winges, A., DeRyckere, D., Carson, C. C., Trembath, D. G., Tentler, J. J., Eckhardt, S. G., Kuan, P. F., Hamilton, R. L., Duncan, L. M., Miller, C. R., Nikolaishvili-Feinberg, N., Midkiff, B. R., Liu, J., Zhang, W., Yang, C., Wang, X., Frye, S. V., Earp, H. S., Shields, J. M., Graham, D. K. (2013) MERTK receptor tyrosine kinase is a therapeutic target in melanoma. *J. Clin. Invest.* **123,** 2257–2267.

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