MerTK is required for apoptotic cell–induced T cell tolerance

Mark A. Wallet,[1] Pradip Sen,[1] Rafael R. Flores,[1] Yaming Wang,[1] Zuoan Yi,[1] Yingsu Huang,[1] Clayton E. Mathews,[5] H. Shelton Earp,[2,3] Glenn Matsushima,[1,2,4] Bo Wang,[1] and Roland Tisch[1,2]

[1]Department of Microbiology and Immunology, [2]UNC Lineberger Comprehensive Cancer Center, [3]Department of Medicine and Pharmacology, [4]UNC School of Medicine Neuroscience Center, University of North Carolina, Chapel Hill, NC 27599
[5]Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA 15213

Self-antigens expressed by apoptotic cells (ACs) may become targets for autoimmunity. Tolerance to these antigens is partly established by an ill-defined capacity of ACs to inhibit antigen-presenting cells such as dendritic cells (DCs). We present evidence that the receptor tyrosine kinase Mer (MerTK) has a key role in mediating AC-induced inhibition of DC activation/maturation. Pretreatment of DCs prepared from nonobese diabetic (NOD) mice with AC blocked secretion of proinflammatory cytokines, up-regulation of costimulatory molecule expression, and T cell activation. The effect of ACs on DCs was dependent on Gas6, which is a MerTK ligand. NOD DCs lacking MerTK expression (NOD.MerTK$^{KD/KD}$) were resistant to AC-induced inhibition. Notably, autoimmune diabetes was exacerbated in NOD.MerTK$^{KD/KD}$ versus NOD mice expressing the transgenic BDC T cell receptor. In addition, β cell–specific CD4$^+$ T cells adoptively transferred into NOD.MerTK$^{KD/KD}$ mice in which β cell apoptosis was induced with streptozotocin exhibited increased expansion and differentiation into type 1 T cell effectors. In both models, the lack of MerTK expression was associated with an increased frequency of activated pancreatic CD11c$^+$CD8α$^+$ DCs, which exhibited an enhanced T cell stimulatory capacity. These findings demonstrate that MerTK plays a critical role in regulating self-tolerance mediated between ACs, DCs, and T cells.

CORRESPONDENCE
Roland Tisch:
rmtisch@med.unc.edu

Abbreviations used: AC, apoptotic cell; BMDC, BM-derived DC; DT, diphtheria toxin; DTR, DT receptor; Gas6, growth arrest–specific protein-6; MerTK, Mer tyrosine kinase; NOD, nonobese diabetic; PLN, pancreatic lymph node; PS, phosphatidylserine; RTK, receptor tyrosine kinase; RPE, retinal pigment epithelial cell; sDC, splenic DC; STZ, streptozotocin.

The processes of tissue remodeling, wound healing, and removal of damaged cells rely on cell death by apoptosis. Apoptosis entails a highly ordered process characterized by cell shrinkage, retention of organelles, nuclear chromatin condensation, and fragmentation, as well as redistribution of phosphatidylserine (PS) on the plasma membrane (1, 2). Under homeostatic conditions, APCs such as macrophages and DCs bind and ingest apoptotic cells (ACs) using a variety of receptors, including Mer tyrosine kinase (MerTK), the PS receptor, scavenger receptor-A (SR-A), CD36, and $\alpha_v\beta_5$ integrin (3–5). In this way, the breakdown and release of potentially immunogenic self-antigens by ACs is avoided (6). Indeed, inefficient clearance of ACs has been linked to autoimmunity (7).

The binding and ingestion of ACs also results in immunoregulation of APCs to further promote self-tolerance. For instance, activation of macrophages is blocked and TGF-β secretion is induced by ACs (8, 9). Similarly, immature DCs become refractory to subsequent stimulation upon encounter with ACs, and maintain a "tolerogenic" phenotype characterized by low levels of MHC, CD40, CD80, and CD86 expression and the lack of proinflammatory cytokine secretion (10–12). Consequently, these DCs are unable to effectively stimulate naive T cells or promote type 1 CD4$^+$ T cell differentiation. In the nonobese diabetic (NOD) mouse model of autoimmune diabetes for example, induction of limited apoptosis of pancreatic β cells prevents progression of the diabetogenic response. Lack of diabetes correlates with the establishment of tolerogenic DCs, and subsequent induction of immunoregulatory T cells (13).

The Tyro3, Axl, and MerTK (TAM) family of receptor tyrosine kinases (RTKs) has been reported to regulate homeostatic activation of macrophages and DCs. Mice lacking all three RTKs exhibit systemic autoimmunity marked by highly activated APCs in vivo (14). MerTK, which

P. Sen's present address is Division of Cell Biology and Immunology, Institute of Microbial Technology, Sector-39A, Chandigarh, 160036, India.

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also is expressed by NKT cells, certain epithelial cell types, and reproductive tissues, is required for efficient phagocytosis of ACs by peritoneal macrophages (5, 15). Lupus-like symptoms detected in aging C57BL/6 mice in which MerTK expression is blocked because of insertion of a neomycin cassette in the kinase domain of the *Mertk* gene ($MerTK^{KD}$), are believed to be, in part, the result of inefficient clearance of ACs by macrophages (5, 16). Investigation of retinal pigment epithelial cells (RPEs) has also shown that engulfment of apoptotic outer segments shed from the photoreceptor is mediated by MerTK via recognition of growth arrest–specific protein-6 (Gas6) and protein S (5, 17–20). Gas6 is ubiquitously expressed and, in addition to binding MerTK, it also serves as a PS opsonin, mediating phagocytosis of ACs (21).

Recently, we demonstrated that AC-induced immunoregulation of DCs is mediated by selective inhibition of the NF-κB pathway, which regulates expression of several genes involved in DC activation and maturation (22). Importantly, upon binding of ACs, MerTK was shown to transduce signals necessary for inhibition of NF-κB activation in BM-derived DCs (BMDCs) and splenic DCs (sDCs) (22). With this in mind, the current study was performed to determine how MerTK regulates DC effector function in response to ACs in vitro and in vivo, and in turn the effect this has on T cell self-tolerance.

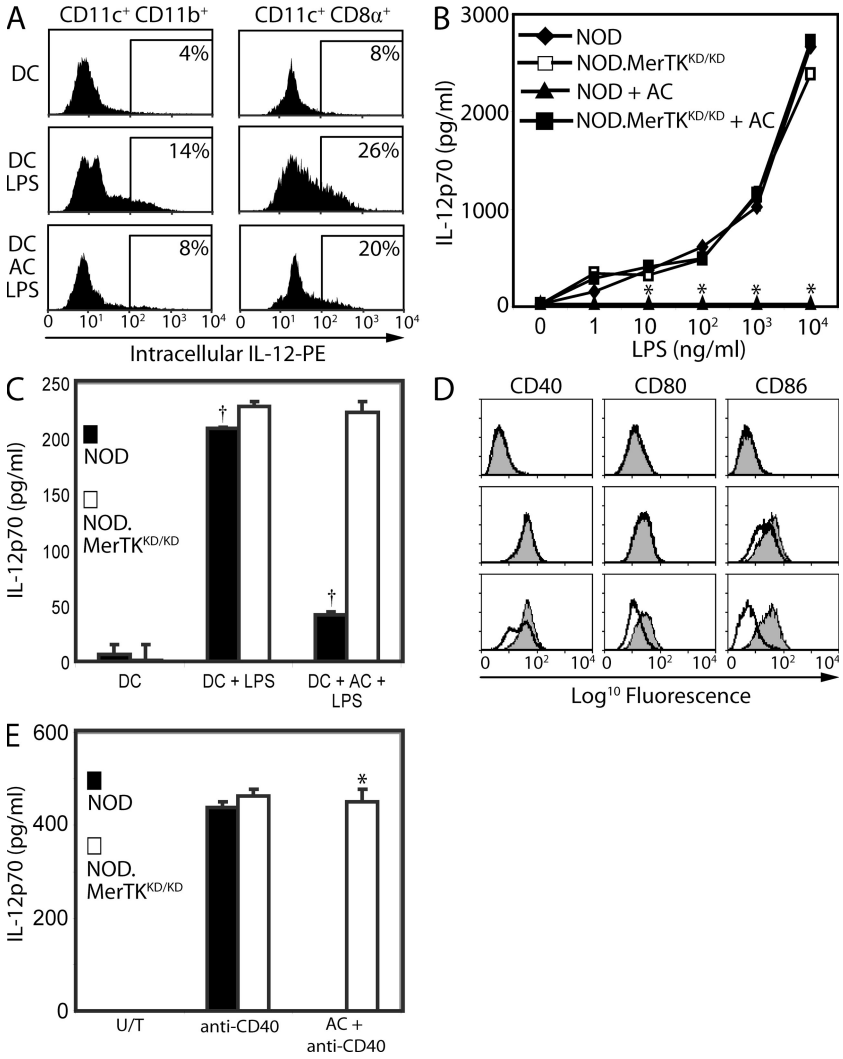

Figure 1. Activation and IL-12 secretion by MerTK-deficient DCs are unaffected by AC pretreatment. (A) Ex vivo NOD sDCs were co-cultured with ACs or left untreated, stimulated for 6 h with IFNγ and 1μg/ml LPS for 24 h, and stained for DC subset surface markers and intracellular IL-12. (B) BMDCs from NOD or $NOD.MerTK^{KD/KD}$ mice were untreated or co-cultured with ACs, stimulated with varying concentrations of LPS for 72 h, and IL-12p70 secretion was measured by ELISA. (C) Ex vivo–derived sDCs from NOD and $NOD.MerTK^{KD/KD}$ mice were untreated or co-cultured with ACs, and then treated with 1 μg/ml LPS for 72 h and IL-12p70 measured. (D) NOD (open histograms) and $NOD.MerTK^{KD/KD}$ (filled histograms) BMDCs were untreated or co-cultured with ACs, stimulated with 100 ng/ml LPS for 24 h, and assessed for costimulatory molecule expression on $CD11c^+$ gated cells. (E) NOD and $NOD.MerTK^{KD/KD}$ BMDCs were untreated or co-cultured with ACs, stimulated by cross-linking CD40 for 72 h, and IL-12p70 secretion measured. All DCs were collected from 10–12-wk-old female mice (4–8 mice per group). Data are representative of two independent experiments. *, $P < 0.001$; †, $P = 0.011$ (Student's *t* test). Error bars represent the mean ± the SEM.

Our data demonstrate that ACs no longer prevent proinflammatory cytokine secretion, costimulatory molecule expression, and T cell stimulation by MerTK-deficient DCs. Additionally, the number and activation status of pancreatic $CD11c^+CD8\alpha^+$ DCs are increased, and T cell–mediated β cell autoimmunity is exacerbated in NOD mice lacking MerTK expression in both TCR-transgenic and T cell adoptive transfer models.

RESULTS

MerTK is necessary for AC-induced inhibition of DC activation, maturation, and effector function in vitro

Studies have demonstrated that ACs inhibit activation and maturation of DCs. Indeed, AC pretreatment for 3 h blocked LPS-stimulated intracellular IL-12p70 expression in $CD11c^+$ $CD11b^+$ and $CD11c^+CD8\alpha^+$ sDCs prepared from NOD mice (Fig. 1 A). MerTK expression is readily detected on $CD11c^+$ $CD11b^+$ and $CD11c^+CD8\alpha^+$ sDCs, but is reduced on plasmacytoid DCs (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20062293/DC1). To investigate the role of MerTK in AC-induced inhibition of DC effector function, NOD.$MerTK^{KD/KD}$ mice were used as a source of DCs lacking MerTK expression. A 17-cM segment of 129/Ola Chromosome 2 harboring the $MerTK^{KD}$ mutation was introgressed onto the NOD genotype for 11 backcross generations (22). Pretreatment of NOD BMDCs with ACs for 3 h completely inhibited LPS-stimulated IL-12p70 secretion that otherwise was detected in cultures of NOD BMDCs treated with LPS-alone (Fig. 1 B). In contrast, NOD.$MerTK^{KD/KD}$ BMDCs secreted similar levels of IL-12p70 in response to LPS, regardless of AC pretreatment (Fig. 1 B). ACs also blocked LPS-stimulated IL-12p70 secretion by sDCs prepared from NOD but not NOD.$MerTK^{KD/KD}$ mice (Fig. 1 C). Similarly, AC pretreatment inhibited secretion of other proinflammatory cytokines such as TNFα (22) and IL-1α/β (unpublished data) in NOD and not $MerTK^{KD/KD}$ DCs. LPS-stimulated up-regulation of CD40, CD80, and CD86 expression was also inhibited by AC pretreatment of NOD BMDCs (Fig. 1 D, open histograms). In contrast, AC pretreatment had no significant effect on the induction of costimulatory molecule expression by LPS-stimulated NOD.$MerTK^{KD/KD}$ BMDCs (Fig. 1 D, filled histograms).

To rule out the possibility that AC-induced inhibition was specific for LPS stimulation, NOD versus NOD.$MerTK^{KD/KD}$ BMDCs treated with anti-CD40 mAb were compared. Cross-linking with anti-CD40 mAb induced IL-12p70 secretion by NOD BMDCs that was significantly reduced by AC pretreatment ($P < 10^{-3}$; Fig. 1 E).Conversely, anti-CD40 mAb-stimulated IL-12p70 secretion by NOD.$MerTK^{KD/KD}$ BMDCs was not affected by AC pretreatment (Fig. 1 E). These results indicate that in the absence of MerTK expression, DCs are resistant to the inhibitory effect of ACs on secretion of proinflammatory cytokines and up-regulation of costimulatory molecule expression.

Next, the effect of AC pretreatment on the capacity of NOD and NOD.$MerTK^{KD/KD}$ BMDCs to stimulate $CD4^+$ and $CD8^+$ T cells in vitro was investigated. For this purpose, $CD4^+$ and $CD8^+$ T cells were isolated from the spleens of NOD.BDC (23, 24) and NOD.CL4 (25) TCR transgenic mice, respectively. In the absence of ACs, both peptide-pulsed NOD and NOD.$MerTK^{KD/KD}$ BMDCs stimulated high IL-2 secretion by BDC $CD4^+$ (Fig. 2 A) and CL4 $CD8^+$ (Fig. 2 B) T cells. AC pretreatment, however, significantly inhibited the

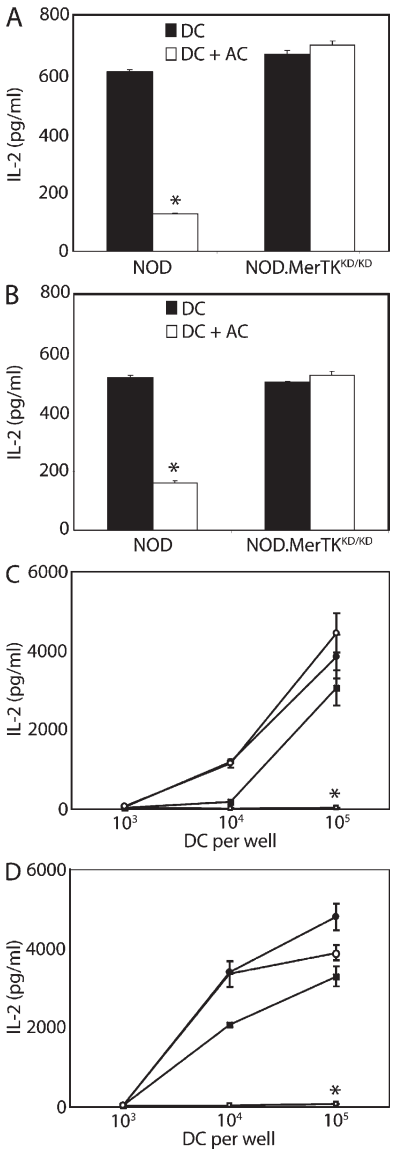

Figure 2. T cell stimulation by MerTK-deficient DCs is not inhibited by ACs. NOD and NOD.$MerTK^{KD/KD}$ BMDCs were co-cultured or not with ACs, plus 0.1 µg/ml BDC peptide and BDC $CD4^+$ T cells (A and C) or 0.1 µg/ml HA peptide and CL4 $CD8^+$ T cells (B and D) for 72 h, and IL-2 secretion was measured by ELISA. Varying numbers of BMDCs were cultured with 10^6 BDC $CD4^+$ (C) or CL4 $CD8^+$ (D) T cells. (C and D) Filled and open squares are NOD BMDCs and NOD BMDCs plus ACs, respectively; filled and open circles are NOD.$MerTK^{KD/KD}$ BMDCs and NOD.$MerTK^{KD/KD}$ BMDCs plus ACs, respectively. BMDCs were isolated from 10–12–wk-old female mice (4–8 mice per group). Data are representative of two independent experiments. *, $P < 0.001$ (Student's *t* test). Error bars represent the mean ± the SEM.

capacity of peptide-pulsed NOD BMDCs to stimulate BDC CD4$^+$ ($P < 10^{-3}$) and CL4 CD8$^+$ ($P < 10^{-3}$) T cells (Fig. 2, A and B). In contrast, AC pretreatment of peptide-pulsed NOD.MerTK$^{KD/KD}$ BMDCs had no effect on IL-2 secretion by either BDC CD4$^+$ or CL4 CD8$^+$ T cells compared with peptide-pulsed only cultures (Fig. 2, A and B). Similar results were obtained when the number of NOD and NOD.MerTK$^{KD/KD}$ BMDCs were titrated (Fig. 2, C and D).

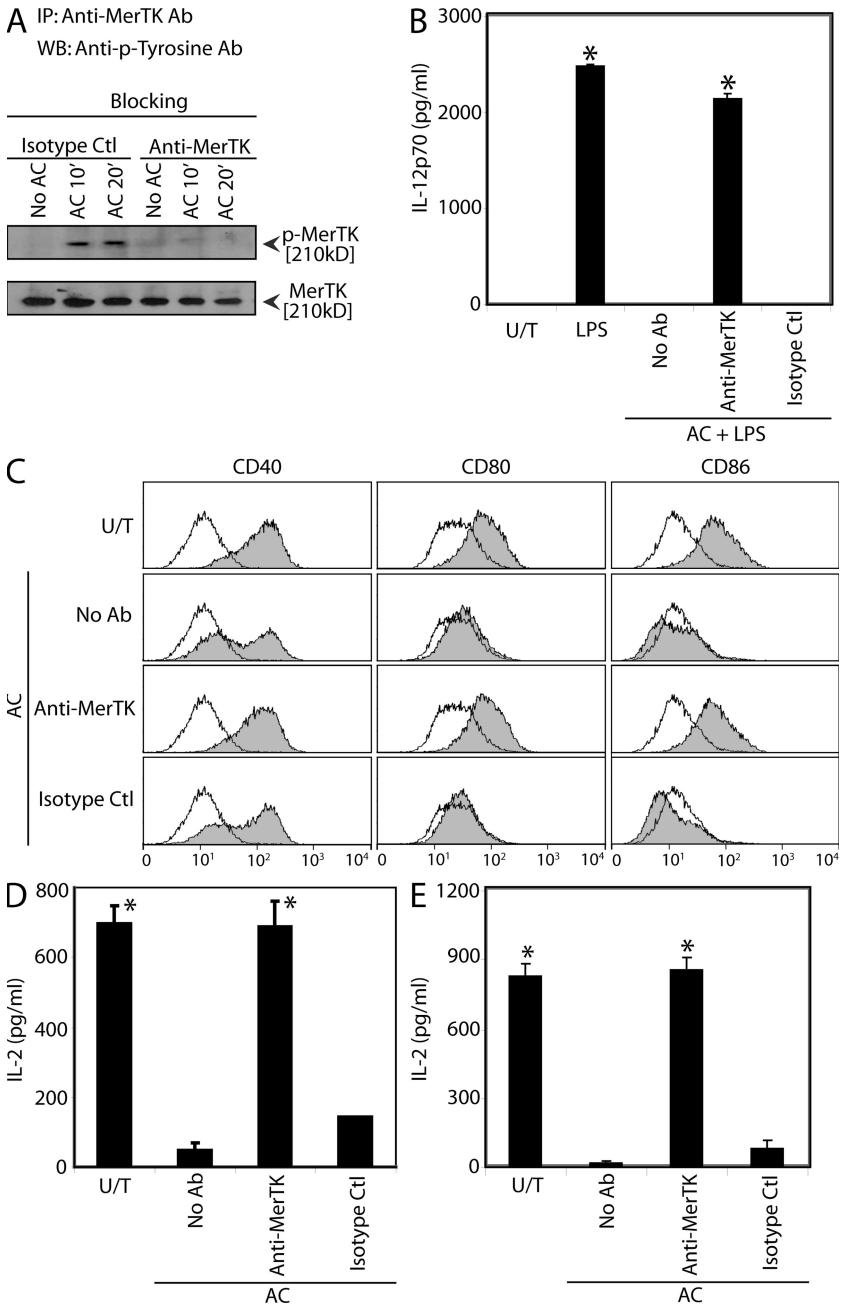

Figure 3. Antibody blockade of MerTK prevents AC-induced inhibition of DCs. (A) NOD BMDCs were pretreated with isotype control or anti-MerTK antibody for 30 min. NOD BMDCs were then treated with ACs for the specified times and MerTK immunoprecipitated from a whole cell lysate with anti-MerTK antibody. Phosphorylation of MerTK (top) was determined by Western blot using an anti–phospho-tyrosine antibody, and the same blot re-probed to detect MerTK protein (bottom). (B–E) NODBMDCs were incubated with either isotype control or anti-MerTK antibody before co-culture with ACs. BMDCs were stimulated with 100 ng/ml LPS and IL-12p70 secretion measured by ELISA 72 h later (B) or costimulatory molecule surface expression measured via FACS 24 h later (C). Open histograms represent untreated DCs and filled histograms represent the indicated treatment group plus LPS. BMDCs were cultured with (D) BDC peptide and BDC CD4$^+$ T cells or (E) HA peptide and CL4 CD8$^+$ T cells for 72 h, and IL-2 secretion measured by ELISA. BMDCs were isolated from 10–12 wk-old female mice (4–8 per group). Data are representative of two independent experiments *, $P < 0.001$ (Student's *t* test). Error bars represent the mean ± the SEM.

The lack of sensitivity of NOD.MerTK$^{KD/KD}$ DCs to AC pretreatment could be caused by an artifact of DCs developing in the absence of MerTK expression, and/or the genetic background of the mice. To rule out these possibilities, the effect of a blocking anti-MerTK polyclonal antibody on NOD and BALB/c BMDCs was assessed. The inhibitory activity of the polyclonal antibody was verified by demonstrating that phosphorylation of MerTK induced by ACs was inhibited by treating NOD DCs with anti-MerTK, but not an isotype control antibody (Fig. 3 A). Despite AC pretreatment, LPS-stimulated IL-12p70 secretion (Fig. 3 B) and up-regulation of costimulatory molecule expression (Fig. 3 C) were readily detected in NOD BMDCs incubated with anti-MerTK antibody. Furthermore, ACs failed to inhibit stimulation of BDC CD4$^+$ (Fig. 3 D) and CL4 CD8$^+$ (Fig. 3 E) T cells by NOD BMDCs incubated with the anti-MerTK, but not the isotype control antibody.

Next, BALB/c DCs were treated with blocking anti-MerTK or isotype control antibody before AC treatment. Similar to NOD DCs, ACs prevented LPS-stimulated up-regulation of costimulatory molecule expression (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20062293/DC1) and IL-12p70 secretion (Fig. S2 B), and inhibited T cell stimulation (Fig. S2 C) by BALB/c BMDCs. Anti-MerTK, but not isotype control antibody, however, blocked AC-induced inhibition of IL-12p70 and T cell stimulation by BALB/c DCs (Fig. S2, A–C). Together, these findings demonstrate that AC-induced inhibition of activation, maturation, and effector function of BMDCs and sDCs in vitro is dependent on MerTK.

AC-induced inhibition of DCs is Gas6 dependent

Gas6 binds MerTK, and some evidence indicates that protein S is also a MerTK ligand (26–29). Other yet to be identified ligands have also been proposed. Initially, protein S, which is found in serum, was investigated. Protein S mRNA expression by AC was not detected via RT-PCR (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20062293/DC1), and LPS-stimulated IL-12p70 secretion by NOD BMDCs was inhibited by ACs despite serum-free culture conditions (Fig. S3 B). These results indicate that protein S is not necessary for MerTK binding of ACs, and they are consistent with recent observations by our group that Tyro3, and not MerTK, binds protein S (30). Whether Gas6 functioned as a MerTK ligand for ACs was then tested. Gas6 has been reported to bind PS (31), which would be exposed on the inverted plasma membrane of ACs. Accordingly, Gas6 surface expression on apoptotic thymocytes induced in vitro via γ irradiation or prepared directly from the thymus was measured by FACS. Apoptotic thymocytes were identified by costaining with a VAD-FMK-FITC conjugate. Gas6 was detected on the surface of apoptotic thymocytes regardless of whether apoptosis was induced in vitro or in vivo (Fig. 4 A). Furthermore, Gas6 protein expression as determined by Western blot was induced in a temporal manner in irradiated thymocytes cultured under serum-free conditions (Fig. 4 B). Because protein S may bind to thymocytes in vivo and therefore be present on ACs even under serum-free culture conditions, a direct role for Gas6 in AC-mediated inhibition of DCs was established by using a blocking anti-Gas6 polyclonal antibody. Pretreatment of ACs with anti-Gas6, but not an isotype control antibody, blocked the capacity of ACs to inhibit LPS-stimulated IL-12p70 secretion by NOD BMDCs (Fig. 4 C).

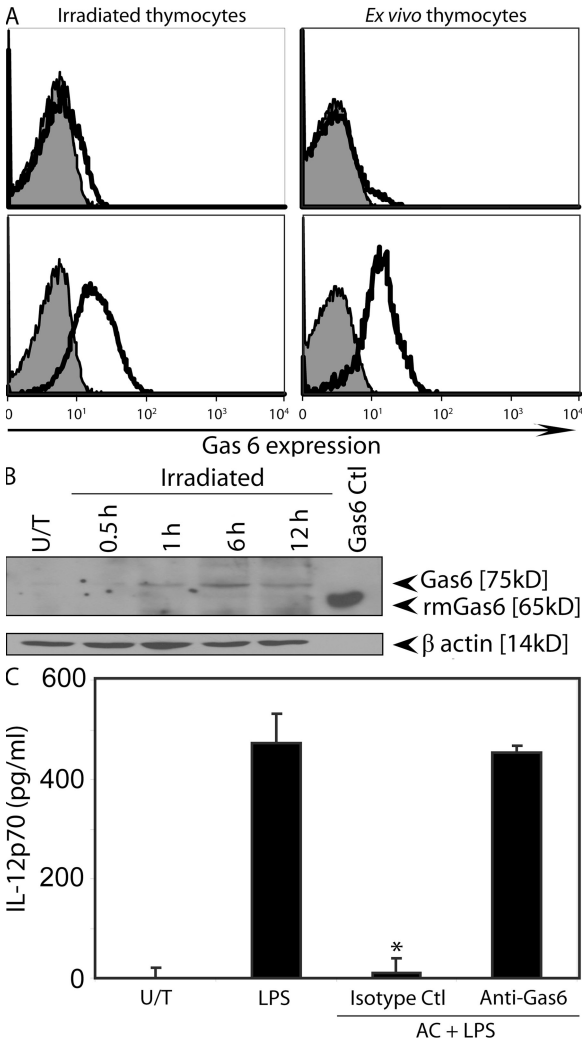

Figure 4. AC-expressed Gas6 is necessary for DCs inhibition. (A) FITC-VAD-FMK–negative (nonapoptotic) thymocytes (filled histograms) and FITC-VAD-FMK–positive (apoptotic) thymocytes (open histograms) were surface-stained with isotype control (top) or anti-Gas6 antibody (bottom). (B) Gas6 (top) and β actin (bottom) protein was detected via Western blot in whole-cell lysates prepared from untreated (U/T) thymocytes or apoptotic thymocytes at various times after γ irradiation. 10 ng of recombinant murine Gas6 protein (rmGas6) was loaded as a positive control (note that a truncation causes rmGas6 to have a reduced molecular weight compared with endogenous Gas6; see Materials and methods). (C) NOD BMDCs were pretreated with ACs incubated with anti-Gas6 or isotype control antibody, and LPS-induced (100 ng/ml) IL-12p70 secretion was measured via ELISA 72 h later. Thymocytes were isolated from 3–4 wk-old female NOD mice. BMDCs were isolated from 10–12-wk-old female NOD mice (4 per group). Data are representative of two independent experiments. *, P = 0.005 (Student's *t* test). Error bars represent the mean ± the SEM.

These results demonstrate that Gas6 expression is induced on the surface of ACs, and that it is required for MerTK-mediated inhibition of DCs by ACs.

MerTK is required to maintain T cell tolerance to apoptotic β cells

The lack of sensitivity of MerTK$^{KD/KD}$ DCs to AC-induced inhibition in vitro would be expected to promote autoimmunity in vivo. To test this hypothesis, TCR transgenic NOD.BDC mice were bred with NOD.MerTK$^{KD/KD}$ animals, and the development of diabetes was compared in female mice lacking MerTK expression (NOD.MerTK^{KD}.BDC) or expressing wild-type levels of MerTK (NOD.BDC). Although only 10% (1/10) of NOD.BDC female mice developed diabetes over 52 wk (Fig. 5 A), which is consistent with the diabetes incidence reported for other NOD.BDC colonies, diabetes onset was accelerated, and the incidence of diabetes significantly increased (7/11; P = 0.038, χ^2) in NOD.MerTK^{KD}.BDC female mice (Fig. 5 A). Furthermore, ELISPOT demonstrated a significantly (P = 0.002, Student's *t* test) increased frequency of IFNγ-secreting CD4$^+$ T cells in the pancreatic lymph nodes (PLNs) of 4 wk-old NOD.MerTK^{KD}.BDC versus NOD.BDC female mice (Fig. 5 B).

One possible explanation for exacerbated β cell autoimmunity in NOD.MerTK^{KD}.BDC mice is that apoptotic β cells failed to inhibit DC activation and/or maturation, leading to enhanced activation of BDC CD4$^+$ T cells. Analysis of DCs infiltrating the pancreas of 4-wk-old mice demonstrated that the number of CD11c$^+$CD8α$^+$ DCs was increased in NOD.MerTK^{KD}.BDC mice (Fig. 5 C). Furthermore, an increased frequency of CD86^{HI} and CD80^{HI} CD8α$^+$ DCs was found among pancreatic CD11c$^+$CD8α$^+$ DCs in NOD.MerTK^{KD}.BDC when compared with NOD.BDC female mice (Fig. 5 C). No difference, however, was detected in the activation status of pancreatic CD11c$^+$CD11b$^+$ DCs (Fig. 5 C) in the two groups of mice. These findings demonstrate that in the absence of MerTK expression, enhanced β cell autoimmunity correlated with increased activation and numbers of pancreatic CD11c$^+$CD8α$^+$ DCs.

To better define events going on between apoptotic β cells, DCs, and CD4$^+$ T cells, a model system was established in which naive BDC CD4$^+$ T cells were transferred into either 4 wk-old NOD or NOD.MerTK$^{KD/KD}$ female recipients and β cell apoptosis synchronized by treatment with a single dose of streptozotocin (STZ). This approach was necessary because analysis of NOD.BDC and NOD.MerTK^{KD}.BDC mice is limited by heavy islet infiltration at an early age (i.e., by 3 wk of age), and the observation that β cell apoptosis is not synchronized. At 4 wk of age, both NOD and NOD.MerTK$^{KD/KD}$ mice lack islet infiltration. NOD and NOD.MerTK$^{KD/KD}$ mice received a single i.p. injection of 120 mg/kg of STZ or citrate saline as a vehicle control. STZ at 120 mg/kg induces only moderate β cell apoptosis, and the treated NOD mice remain diabetes free (13). Equivalent levels of Gas6 were detected on the surface of the few apoptotic β cells detected in untreated mice, and on apoptotic β cells in the STZ-treated

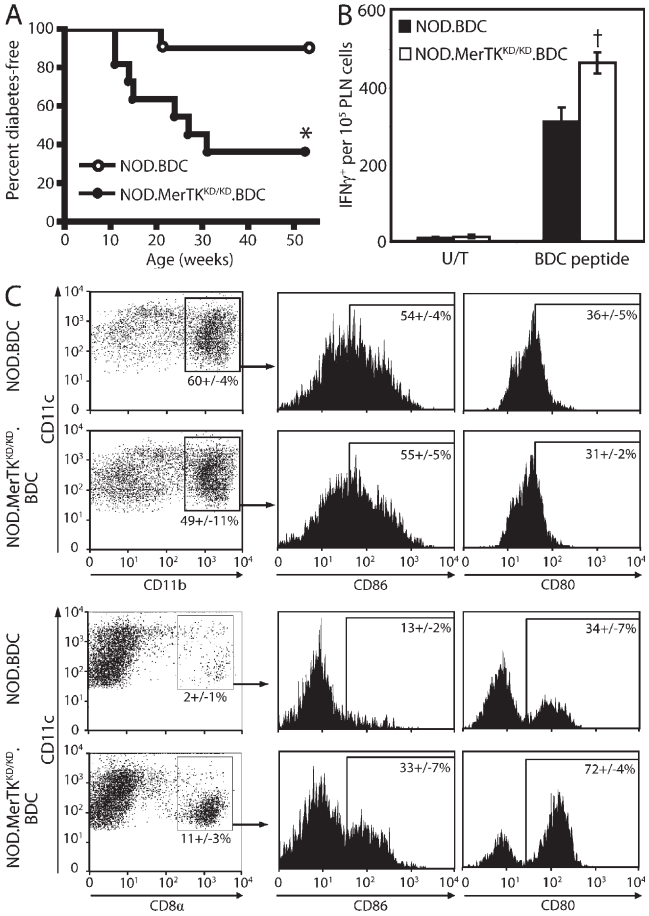

Figure 5. MerTK-deficient mice expressing a diabetogenic TCR transgene exhibit aggressive insulitis and exacerbated T1D. (A) NOD.BDC (*n* = 10) and NOD.MerTK^{KD}.BDC (*n* = 11) female mice were monitored for diabetes. *, P = 0.013 (Kaplan-Meir log rank). (B) The frequency of IFNγ-producing cells within the PLN of NOD.BDC and NOD.MerTK^{KD}.BDC mice was determined by ELISPOT. All mice were 4–5-wk-old (5 mice per group). Data are representative of two independent experiments. †, P = 0.002 (Student's *t* test). Error bars represent the mean ± the SEM. (C) Pancreatic DCs prepared from 4-wk-old female NOD.BDC and NOD.MerTK^{KD}.BDC mice were analyzed by FACS. Percentages for the gated cell populations are the average of three independent experiments.

animals (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20062293/DC1). Furthermore, a kinetic analysis of apoptotic β cell clearance/persistence showed no difference between STZ-treated NOD and NOD.MerTK$^{KD/KD}$ mice (Fig. S5). CFSE-labeled BDC CD4$^+$ T cells were adoptively transferred, and 72 h later, proliferation was measured in the draining PLNs. Only limited proliferation of BDC CD4$^+$ T cells was detected in NOD recipients treated with either STZ or citrate saline (Fig. 6 A). Similarly, the majority CFSE$^+$ BDC CD4$^+$ T cells (71%) had not proliferated in citrate saline–treated NOD.MerTK$^{KD/KD}$ recipients (Fig. 6 A). In contrast, BDC CD4$^+$ T cells exhibited significant proliferation in NOD.MerTK$^{KD/KD}$ mice treated with STZ (Fig. 6 A). Additionally, the frequency of CFSE$^+$ IFNγ-producing cells was

markedly increased in STZ-treated NOD.MerTK$^{KD/KD}$ recipients (36%) compared with STZ-treated NOD or citrate saline–treated NOD and NOD.MerTK$^{KD/KD}$ recipients (~2 to 3%; Fig. 6 B). Consistent with these latter findings, the total number of CFSE$^+$IFNγ^+ BDC CD4$^+$ T cells in the PLN of STZ-treated NOD.MerTK$^{KD/KD}$ recipients increased with time relative to the other groups. At 72 h after transfer, no significant difference in the number of CFSE$^+$ BDC CD4$^+$ T cells among the four treatment groups was detected (Fig. 6 C), although the number of CFSE$^+$IFNγ^+ T cells was increased in STZ-treated NOD.MerTK$^{KD/KD}$ recipients (Fig. 6 D). At 96 h after transfer, the number of CFSE$^+$ BDC CD4$^+$ T cells significantly declined among STZ- and citrate saline–treated NOD mice, and citrate saline–treated NOD.MerTK$^{KD/KD}$ mice. In contrast, both the number of CFSE$^+$ and CFSE$^+$IFNγ^+ BDC CD4$^+$ T cells continued to increase in STZ-treated NOD.MerTK$^{KD/KD}$ recipients (Fig. 6, C and D). Notably, the increased T cell reactivity of transferred CFSE$^+$ BDC CD4$^+$ T cells in STZ-treated NOD.MerTK$^{KD/KD}$ recipients was DC-dependent, based on experiments using NOD.MerTK$^{KD/KD}$ mice expressing the diphtheria toxin (DT) receptor (DTR) transgene driven by the CD11c promoter as recipients. The CD11c-DTR transgenic model has been used to transiently deplete DCs in vivo upon DT injection (32). NOD.MerTK.DTR mice were treated with DT or left untreated, and 24 h later, mice received STZ and CFSE$^+$ BDC CD4$^+$ T cells. As demonstrated in Fig. 6 E, DT treatment, which induces a >10-fold reduction of PLN DCs, significantly reduced proliferation and the frequency of CFSE$^+$IFNγ^+ BDC CD4$^+$ T cells compared with the non-DT treated group of mice.

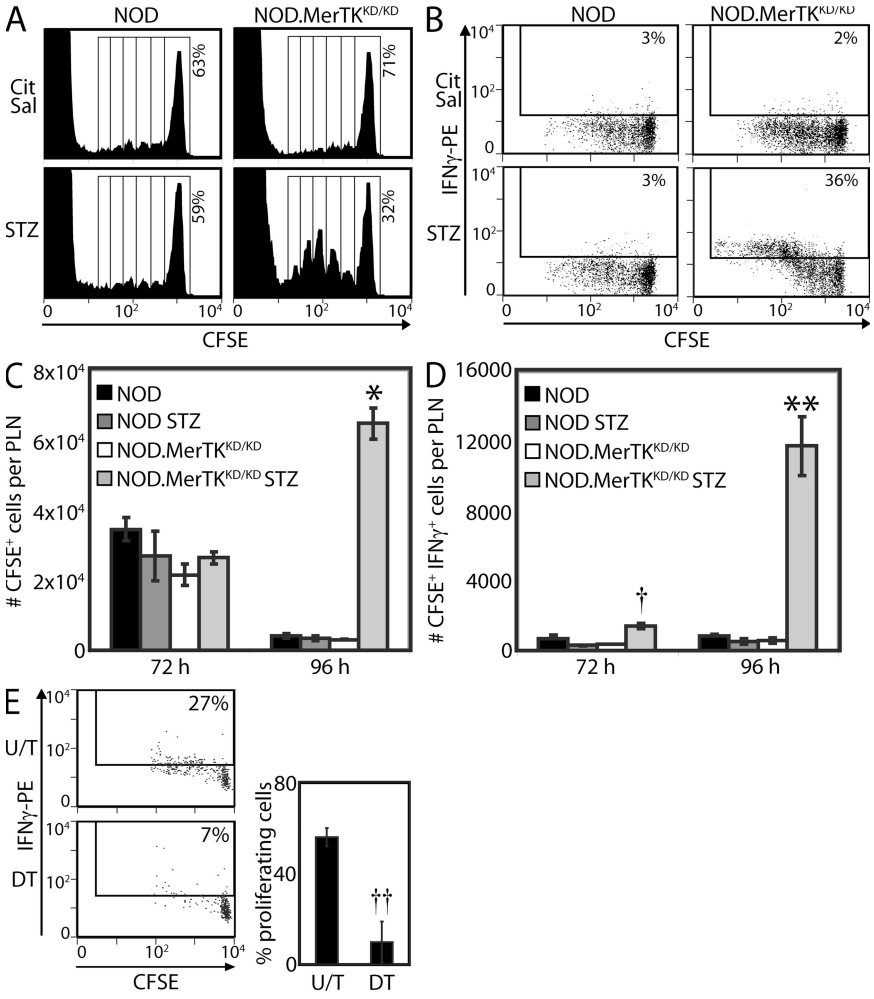

Figure 6. β Cell apoptosis in MerTK-deficient mice induces CD4$^+$ T cell proliferation, and type 1 differentiation that is DC dependent. NOD or NOD.MerTK$^{KD/KD}$ 4–6 wk-old female mice were treated with citrate saline control (Cit Sal) or 120 mg/kg STZ. 2.5 × 10^6 CFSE-labeled BDC CD4$^+$ T cells were transferred into each mouse, and 72 h later, PLNs were harvested and cell division (A and B) and intracellular IFNγ expression (B) were determined via FACS. The percentage of CFSE$^+$CD4$^+$ T cells is provided for each column in A. The total number of CD4$^+$ (C) CFSE$^+$ and (D) CFSE$^+$ IFNγ$^+$ T cells was determined at 72 and 96 h after transfer. (E) NOD.MerTK.DTR mice were treated with DT (4 ng/g body weight) or left untreated, and 24 h later they received 120 mg/kg STZ and 10^6 CFSE$^+$ BDC CD4$^+$ T cells. The percentage of IFNγ$^+$ (left) and frequency of proliferating CFSE$^+$ T cells in the PLN were assessed 72 h later. Results are the mean of two experiments with groups of four mice each. *, $P < 0.001$; **, $P = 0.007$; †, $P = 0.005$; ††, $P = 0.002$ (Student's *t* test). Error bars represent the mean ± the SEM.

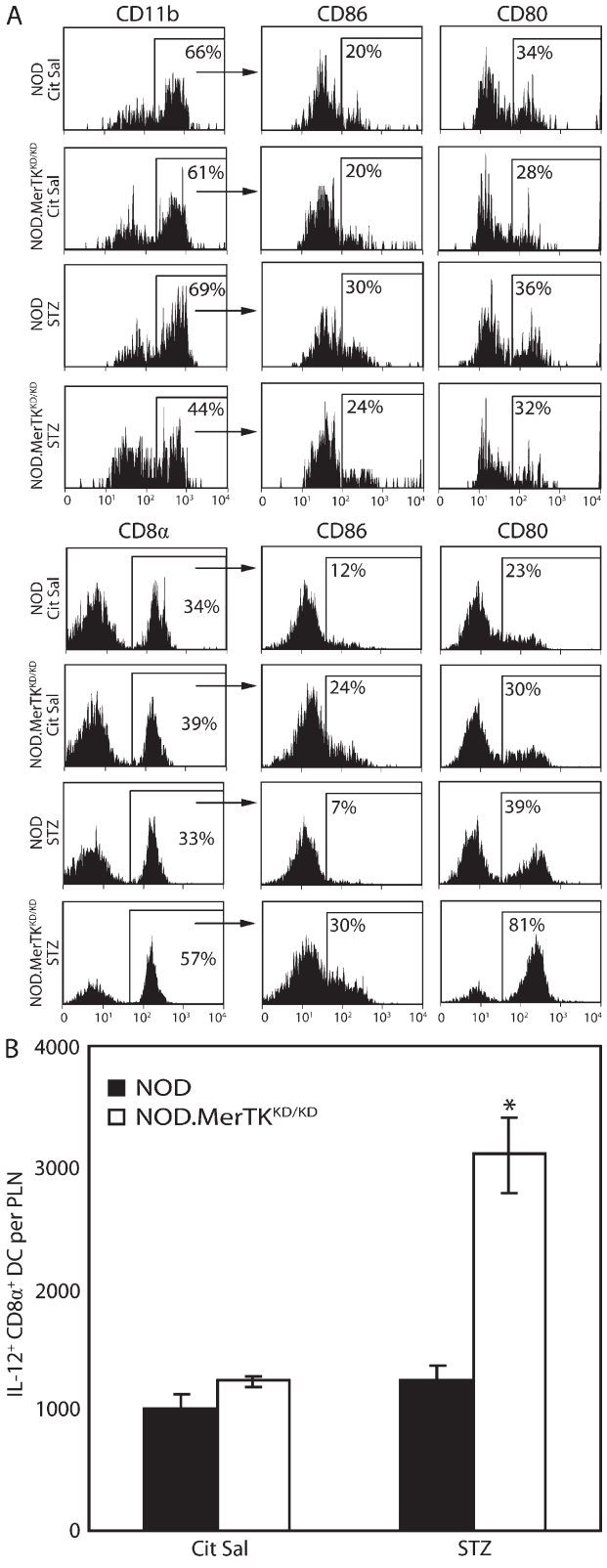

Figure 7. An increased frequency of activated CD11c$^+$CD8α$^+$ DCs is detected in STZ-treated MerTK-deficient recipient mice. (A and B) 5 × 10^6 BDC CD4$^+$ T cells were transferred, and after 72 h, CD11c$^+$CD11b$^+$ and CD11c$^+$CD8α$^+$ DCs were isolated from the pancreas and stained for CD11b, CD8α, CD80 and CD86 (A), or PLN CD11c$^+$CD8α$^+$ DCs stained for intracellular IL-12 p40/p70, and the total number of IL-12 expressing CD11c$^+$CD8α$^+$ DCs determined per PLN (B). *, P = 0.026 (Student's *t* test). Error bars represent the mean ± the SEM.

CD11c$^+$CD8α$^+$ DCs from STZ-treated MerTK-deficient NOD mice have an increased capacity to stimulate T cells

Analysis of pancreatic DCs showed no significant difference in the frequency of CD11c$^+$CD11b$^+$ and CD11c$^+$CD8α$^+$ DCs in citrate saline–treated NOD and NOD.MerTK$^{KD/KD}$ recipients, or STZ-treated NOD recipients (Fig. 7 A). In contrast, an increase in the frequency of pancreatic CD11c$^+$CD8α$^+$ DCs was detected in STZ-treated NOD.MerTK$^{KD/KD}$ recipients (Fig. 7 A). The frequency of CD11c$^+$CD8α$^+$ DCs expressing elevated surface CD80 and CD86 in the pancreas (Fig. 7 A), and intracellular IL-12 in the PLN (Fig. 7 B) was also increased in STZ-treated NOD.MerTK$^{KD/KD}$ recipients relative to the other three groups. These findings indicate that enhanced proliferation and IFNγ expression by CFSE$^+$ BDC CD4$^+$ T cells transferred into STZ-treated NOD.MerTK$^{KD/KD}$ mice is DC dependent and correlates with an increased frequency of activated CD11c$^+$CD8α$^+$ DCs.

To determine whether acquisition of β cell antigens in vivo by NOD.MerTK$^{KD/KD}$ DCs promoted β cell–specific T cell expansion, mice were treated with STZ as above, DCs were sorted via FACS from the pancreas, and the sorted DCs were cultured with CFSE$^+$ BDC CD4$^+$ T cells (without exogenous peptide). As demonstrated in Fig. 8 A, a significant increase in proliferation was detected in cultures prepared with DCs isolated from STZ-treated NOD.MerTK$^{KD/KD}$ as compared with NOD mice (P = 0.025, Student's *t* test). Next, pancreatic CD11c$^+$CD11b$^+$ and CD11c$^+$CD8α$^+$ DCs were sorted, and the capacity to stimulate BDC CD4$^+$ T cells in vitro was tested, again, in the absence of exogenous antigen. Similar to results obtained with a pool of sorted pancreatic DCs (Fig. 8 A), no increase in CFSE$^+$ BDC CD4$^+$ T cell proliferation was seen for either CD11c$^+$CD11b$^+$ or CD11c$^+$CD8α$^+$ DCs prepared from STZ- versus citrate saline–treated NOD mice (Fig. 8 B). Strikingly, a 9.7-fold increase in BDC CD4$^+$ T cell proliferation was observed for CD11c$^+$CD8α$^+$ DCs sorted from STZ-treated compared with citrate saline–treated NOD.MerTK$^{KD/KD}$ mice (Fig. 8 B). On the other hand, only a marginal increase (1.8-fold) in CD4$^+$ T cell proliferation was detected for CD11c$^+$CD11b$^+$DCs sorted from the pancreas of NOD.MerTK$^{KD/KD}$ mice treated with STZ versus citrate saline (Fig. 8 B). These data demonstrate that DCs lacking MerTK expression, upon encounter with apoptotic β cells, drive expansion of β cell–specific CD4$^+$ T cells. Furthermore, β cell–specific CD4$^+$ T cell stimulation is predominately mediated by CD11c$^+$CD8α$^+$ DCs in NOD.MerTK$^{KD/KD}$ mice.

DISCUSSION

ACs provide a continuous source of self-antigen, which can be ingested, processed, and presented by DCs (4). Autoimmune reactivity to these self-antigens, however, is blocked by

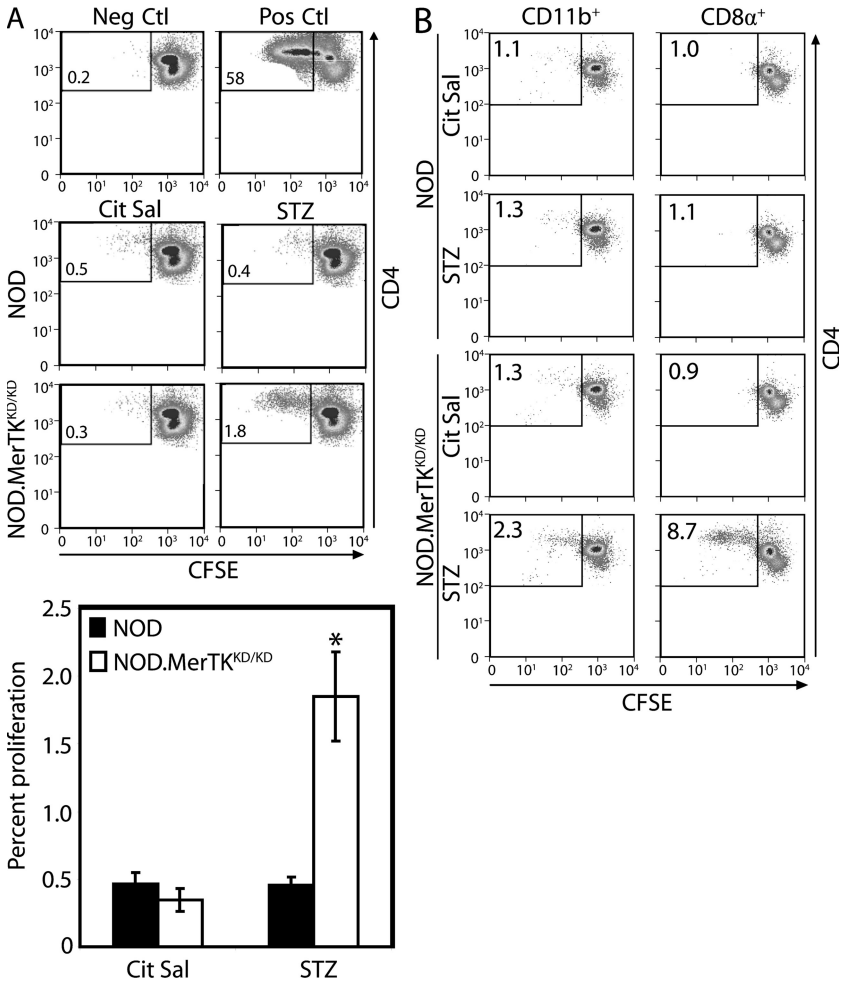

Figure 8. CD11c⁺CD8α⁺ DCs from STZ-treated NOD.MerTK$^{KD/KD}$ mice exhibit an increased T cell stimulatory capacity. NOD or NOD.MerTK$^{KD/KD}$ mice were treated with citrate saline or 120 mg/kg STZ, and 18 h after treatment, pancreatic DCs (A) or CD11c⁺CD11b⁺ or CD11c⁺CD8α⁺ DC subsets (B) were purified and co-cultured with CFSE⁺ BDC CD4⁺ T cells (without exogenously added peptide) for 72 h. Negative control (BDC CD4⁺ T cells only) and positive control (BDC CD4⁺ T cells + BDC peptide-pulsed DCs) were included. Data represent 3 replicate experiments, with 5 4–5-wk-old female mice per treatment group in each experiment. *, P = 0.025 (Student's *t* test). Error bars represent the mean ± the SEM.

the capacity of ACs to inhibit subsequent DC activation and maturation (10–12, 30, 33). MerTK is involved in phagocytosis of ACs by certain subsets of macrophages (5, 15, 34) and RPEs (35). In addition, MerTK and TAM family members Axl and Tyro3 have been shown to regulate homeostatic activation of innate effector cells (14, 16). More recently, we have demonstrated that ACs immunoregulate DCs in part by preventing activation of the NF-κB pathway via MerTK-mediated signaling events (22). Accordingly, the role for MerTK in AC-induced immunoregulation of DCs and self-tolerance was investigated.

In vitro analyses of BMDCs and ex vivo sDCs demonstrate an essential role for MerTK in AC-induced inhibition of DC activation, maturation, and effector function. AC pretreatment of NOD, but not NOD.MerTK$^{KD/KD}$ DCs or DCs incubated with anti-MerTK antibody inhibited proinflammatory cytokine secretion, up-regulation of costimulatory molecule expression, and T cell stimulation (Figs. 1–3 and S2). Furthermore, Gas6 expression was detected on the surface of apoptotic thymocytes and β cells (Figs. 4 A and S4), and a direct role for this PS opsonin was shown by blocking the inhibitory effect of ACs via anti-Gas6 antibody (Fig. 4 C). Importantly, in the absence of AC pretreatment, NOD and NOD.MerTK$^{KD/KD}$ DC activation and APC function did not significantly differ (Figs. 1 and 2). Based on these data, we conclude that MerTK primarily serves to bind ACs and transduce signals that block subsequent activation and maturation in DCs. In contrast to RPE and certain subsets of macrophages, however, DCs do not require MerTK for efficient phagocytosis of ACs (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20062293/DC1) (16, 30).

The roles of and mechanisms by which TAM RTKs regulate innate cell activation and/or function are complex, and clearly cell-type dependent. Recent work by Ivashkiv et al. demonstrated that IFNα-mediated inhibition of TNFα secretion in macrophages is caused by Axl binding of Gas6 or

ACs, even though MerTK and Tyro3 are expressed and bind these ligands (36). Here, IFNα was found to up-regulate Axl, which in turn differentially induced the transcriptional suppressor Twist (36). Interestingly, Axl-induced Twist blocked TNFα expression by preventing NF-κB binding to the TNFα gene promoter. As noted above, MerTK binding of ACs in DCs also regulates NF-κB–dependent transcription, but in this case via inhibition of upstream IκB kinase activation in a phosphatidylinositol 3-kinase–dependent manner (22). These findings suggest that NF-κB is a common target of the TAM family in macrophages and DCs and that the mechanism by which NF-κB activity is regulated is dependent on the particular RTK, the context of the signaling events, and cell type.

The inability of ACs to block activation and maturation of DCs lacking MerTK expression would be expected to break T-cell tolerance in vivo. In fact, this was observed in the two in vivo model systems studied. In NOD.MerTK^{KD}.BDC mice, overt diabetes developed at an enhanced frequency and accelerated onset, which corresponded with an increased frequency of IFNγ-secreting CD4$^+$ T cells in the PLN compared with NOD.BDC mice (Fig. 5, A and B). Furthermore, BDC CD4$^+$ T cells transferred into STZ-treated NOD.MerTK$^{KD/KD}$ exhibited enhanced expansion and IFNγ expression, resulting in a significantly increased number of type 1 effectors at 96 h after transfer compared with the other groups of recipients (Fig. 6). Importantly, this effect was dependent on the induction of β cell apoptosis because expansion and IFNγ expression by BDC CD4$^+$ T cells in citrate saline–treated NOD.MerTK$^{KD/KD}$ recipients was limited, and mimicked that of STZ- or citrate saline–treated NOD recipient mice (Fig. 6). Surprisingly, the total number of CFSE$^+$ BDC CD4$^+$ T cells in the PLN of STZ-treated NOD.MerTK$^{KD/KD}$ recipients at 72 h after transfer was similar to the other 3 groups (Fig. 6 C), despite increased proliferation (Fig. 6 A). This discrepancy was caused by enhanced trafficking of BDC CD4$^+$ T cells from the PLN into the islets of the STZ-treated NOD.MerTK$^{KD/KD}$ recipients (unpublished data). The marked decrease in the number of BDC CD4$^+$ T cells observed between 72 and 96 h after transfer for STZ- or citrate saline–treated NOD recipients (Fig. 6, C and D) is typical of antigen-induced tolerance, in which T cells undergo expansion and then contraction. This result is consistent with a report by the Glaichenhaus group demonstrating that STZ-induced β cell apoptosis in young NOD mice induces tolerogenic DCs that prevent the expansion and development of pathogenic T effector cells (13). Indeed, pancreatic DCs from STZ-treated NOD.MerTK$^{KD/KD}$ recipients exhibited a typical "proinflammatory" phenotype based on increased expression of costimulatory molecules and IL-12 relative to STZ-treated NOD recipients (Figs. 7 and 8). Furthermore, real-time PCR analysis showed that TNFα and IL-1β mRNA expression was increased 9.5- and 9.9-fold, respectively, in DCs sorted from the pancreas of STZ-treated NOD.MerTK$^{KD/KD}$ versus NOD recipients (unpublished data). The latter is noteworthy in view of the cytotoxic effect TNFα and IL-1β have on β cells (37, 38). Importantly, the frequency of apoptotic β cells was similar in STZ-treated NOD and NOD.MerTK$^{KD/KD}$ mice (Fig. S5) ruling out the possibility that "antigen-load" influenced BDC CD4$^+$ T cell expansion and IFNγ expression in a disparate manner among the respective groups. Therefore, the lack of MerTK expression results in the development of pathogenic β cell–specific type 1 T effector cells once β cells are undergoing apoptosis.

Several observations indicate that MerTK-deficient CD11c$^+$ CD8α$^+$ DCs contribute to β cell autoimmunity in the two model systems studied. For instance, in both NOD.MerTK^{KD}.BDC mice and STZ-treated NOD.MerTK$^{KD/KD}$ mice, the frequency and activation status of CD11c$^+$CD8α$^+$ DCs was increased relative to CD11c$^+$CD11b$^+$ DCs (Fig. 5 C and Fig. 7 A). Furthermore, pancreatic CD11c$^+$CD8α$^+$ DCs from STZ-treated NOD.MerTK$^{KD/KD}$ mice exhibited an increased T cell stimulatory capacity in vitro compared with CD11c$^+$ CD11b$^+$ DCs from the same mice, or CD11c$^+$CD8α$^+$ and CD11c$^+$CD11b$^+$ DCs from the other experimental groups (Fig. 8 B). Importantly, T cell stimulation was directly dependent on the capacity of the respective DCs to endocytose, process, and present antigen derived from STZ-induced apoptotic β cells in vivo. These results are similar to reports indicating that CD11c$^+$CD8α$^+$ DCs are highly efficient at presenting antigens derived from ACs (39, 40), and are potent producers of IL-12p70 (41, 42). Furthermore, CD11c$^+$CD8α$^+$ DCs have been implicated in driving β cell autoimmunity (43).

Although MerTK-expressing macrophages and NKT cells were not directly examined, it is unlikely that these innate effectors play a significant role in promoting β cell autoimmunity in the two model systems. For instance, no difference in the number and activation status of CD11b$^+$ F4/80$^+$ macrophages was detected in the PLN or pancreas of NOD.BDC versus NOD.MerTK^{KD}.BDC mice or NOD versus NOD.MerTK$^{KD/KD}$ recipients (unpublished data). The observation that clearance of apoptotic β cells over a 24-h period was similar in STZ-treated NOD or NOD.MerTK$^{KD/KD}$ recipients (Fig. S5) further suggests no significant defect in MerTK-deficient macrophages; macrophages are generally considered to be the most efficient cell type for removal of ACs (6, 44, 45). NKT cells have been shown to have an immunoregulatory role in T1D, and reduced numbers of NKT cells in NOD mice has been linked to diabetes progression (43). Using CD1d tetramers complexed with an α-galactosylceramide peptide, no difference in the number of NKT cells was detected under the various conditions tested, regardless of MerTK expression (unpublished data). Although work by Behrens et al. (46) suggests that MerTK is required for activation of NKT cells in vitro, the lack of MerTK expression appears to have no significant effect on an already diminished pool of NKT cells in NOD mice. Finally, results obtained with NOD.MerTK.DTR mice in which proliferation and IFNγ expression of adoptively transferred BDC CD4$^+$ T cells in the PLNs were significantly reduced after DT injection and in vivo DC depletion (Fig. 6 E), further demonstrate a primary role for DCs in mediating enhanced β cell–specific T cell reactivity in MerTK-deficient NOD mice. Importantly, the frequency of

$CD11b^+F4/80^+$ macrophages and CD1d tetramer-staining NKT cells in the PLNs of recipient mice was not affected by DT treatment (Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20062293/DC1).

Our results suggest that pancreatic $CD11c^+CD8\alpha^+$ DCs lacking MerTK expression are no longer sensitive to the inhibitory effects of ACs. These DCs, upon encountering apoptotic β cells, process and present antigens, but maintain the capacity to be activated and in turn promote β cell–specific T cell reactivity. A reduced frequency of $CD11c^+CD11b^+$ DCs, which may have a protective role in NOD mice under certain conditions, may also indirectly promote β cell autoimmunity in the respective models. Interestingly, specific polymorphisms within the *MERTK* gene have been identified in patients with systemic lupus erythematosus (47). Furthermore, *MERTK* and *Mertk* are located within the T1D-susceptibility intervals *IDDM12/13* and *Idd13* both in human populations and mice, respectively (48–51). Sequence analysis of cDNAs encoding MerTK from a variety of mouse strains have identified at least three allelic variants (unpublished data). Therefore, it is possible that allelic variants of *MERTK* and *Mertk* encode proteins that alter the response of DCs or other innate effectors (i.e., macrophages and NKT cells) to ACs, leading to the promotion or prevention of autoimmunity.

Noteworthy is that, despite a periphery that is "proinflammatory" (Figs. 6–8), NOD.MerTK$^{KD/KD}$ female (and male) mice remain diabetes-free. This is caused by increased efficiency of thymic negative selection in NOD.MerTK$^{KD/KD}$ mice and effective purging of the β cell–specific T cell repertoire (unpublished data). Consequently, insulitis is significantly reduced, and NOD.MerTK$^{KD/KD}$ mice fail to develop diabetes. In marked contrast, BDC clonotypic thymocytes in NOD.MerTK$^{KD/KD}$.BDC mice are not deleted, and the high frequency of mature T cells that enter the periphery readily differentiate into pathogenic effectors and drive β cell autoimmunity (Fig. 5 A). Similarly, transferred BDC $CD4^+$ T cells proliferate and differentiate into type 1 effectors in STZ-treated NOD.MerTK$^{KD/KD}$, but not NOD recipients (Fig. 6). Together, these findings suggest that MerTK influences the "normal" progression of β cell autoimmunity in at least two mutually nonexclusive ways: (a) in the thymus, by regulating the efficiency of negative selection by thymic DCs, and (b) in the periphery, by effecting DC responses to ACs and in turn influencing the general proinflammatory milieu. Efforts are ongoing to further define the roles of MerTK in T1D. In summary, our findings demonstrate that MerTK is necessary for establishing T cell tolerance to AC-expressed proteins, and that altered function of this RTK may, in addition to T1D, contribute to other T cell–mediated autoimmune diseases.

MATERIALS AND METHODS

Mice. NOD/LtJ (NOD), NOD.Cg-Tg(TcraBDC2.5)1Doi Tg(TcrbBDC2.5)2Doi (NOD.BDC), NOD-Tg(TcraCl4, TcrbCl4; NOD.CL4), and BALB/c mice were maintained and bred under specific-pathogen free conditions. Establishment of NOD.MerTK$^{KD/KD}$ mice has been described (22). The CD11c-DTR transgene (32) was backcrossed 13 generations onto the NOD genetic background; NOD.DTR mice develop diabetes at a similar time of onset and frequency as wild-type NOD mice. Use of mice was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Preparation of BMDCs and sDCs. BMDCs and sDCs were prepared from male or female mice between 8–12 wk of age as described (52). In brief, BM cells were flushed from tibias and femurs, and RBCs were lysed. BM cells were cultured for 8 d in RPMI 1640 complete media (10% FBS, penicillin/streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids, and 2-mercaptoethanol) with 10 ng/ml GM-CSF (PeproTech) and 10 ng/ml IL-4 (PeproTech). On day 8, BMDCs were washed and cultured in RPMI complete media (without cytokines) for experiments.

FACS. The following monoclonal antibodies used for FACS were purchased from BD Biosciences: anti–CD40-FITC, –CD86-FITC, –CD80-FITC, –CD11c-PE, –CD11c-APC, and –IFNγ-PE. Polyclonal affinity purified goat anti-MerTK (AF591), anti-Gas6, and normal goat IgG were purchased from R&D Systems. Biotinylated anti–goat IgG was obtained from Vector Laboratories. Anti-CD16/32 for Fc blocking, PerCP-streptavidin and PE-streptavidin were purchased from BD Biosciences. Stained cells were analyzed on a FACScan or FACSCalibur (BD Biosciences) using Summit Software (Dako).

Pretreatment of DCs with ACs. Thymocytes prepared from 4–6-wk-old mice were adhered to plastic for 2 h to remove DCs and macrophages, γ irradiated at 600 R, and cultured in base medium for 12 h. FACS demonstrated >90% apoptotic and <10% necrotic thymocytes based on Annexin V and propidium iodide staining. Apoptosis was confirmed via DNA fragmentation analysis. DCs were co-cultured with ACs at a ratio of 1:10 (DC:AC) for 3 h. In some experiments, DCs were treated with anti-MerTK antibody before AC incubation. In brief, DCs (5×10^6/well) were incubated with anti–mouse FcγIII/II (BD Biosciences) in 6-well ultra-low cluster plates for 0.5 h at 37°C to block Fc receptor binding. DCs were treated for 1 h at 37°C with 20 μg of goat anti-MerTK antibody or goat IgG, which is an isotype control. Alternatively, some AC samples were pretreated with 20 μg polyclonal anti-Gas6 or goat IgG for 30 min before co-culture with DCs. In other experiments, DCs were co-cultured with ACs in serum-free HL1 media (Cambrex) to eliminate serum-derived protein S.

Measurement of IL-12 production from DCs. DCs (9×10^5/well) were pretreated with or without ACs for 3 h, washed, and stimulated with LPS for 72 h. Supernatants were collected and assayed for IL-12p70 in triplicate using an ELISA kit (BD Biosciences) following the manufacturer's instructions. Intracellular IL-12 staining was performed using a Cytofix/Cytoperm cell permeabilization kit (BD Biosciences) and PE-labeled anti–mouse IL-12p40/70. Alternatively, DCs were stimulated by cross-linking CD40 with 10 μg/ml of anti-CD40 IgM (HM40-3; BD Biosciences) and anti-IgM antibody (G188-2; BD Biosciences) for 30 min.

DC:T cell stimulation assay. DCs were pretreated (or not) with ACs for 3 h, washed, and plated in 24-well ultra-low cluster plates at 3×10^4 DCs/well. BDC $CD4^+$ or CL4 $CD8^+$ T cells (3×10^5/well) plus 0.1 μg/ml of the $H2A^{g7}$-restricted BDC mimotope (RTRPLWVRME) or H2-K^d–restricted influenza virus HA peptide (IYSTVASSL) were added, and cultures were incubated for 72 h. Alternatively, varying numbers of DCs were cultured with 10^6 BDC $CD4^+$ or CL4 $CD8^+$ T cells. Supernatants were harvested, and IL-2 concentration was measured in triplicate by a sandwich ELISA using the JES6-1A12 (eBioscience), and biotinylated JES6-EH4 anti–IL-2 Abs, and streptavidin-HRP (BD Biosciences). Concentration of IL-2 was determined using a standard curve. Splenic BDC $CD4^+$ and CL4 $CD8^+$ T cells were prepared by depleting DCs, macrophages, and B cells via magnetic cell sorting using anti-CD11c, anti-CD11b, and anti-CD19 antibody-magnetic microbeads (Miltenyi Biotec), respectively. BDC $CD4^+$ and CL4 $CD8^+$ T cells were then positively selected using anti-CD4 or -CD8 antibody-magnetic

microbeads (Miltenyi Biotec). Purity of BDC $CD4^+$ and CL4 $CD8^+$ T cells was routinely >98%.

STZ treatment and in vivo analysis of adoptively transferred T cells. 4-wk-old NOD or NOD.MerTK^{KD} mice were treated with 120 mg/kg STZ (Sigma-Aldrich) or citrate saline buffer alone. 4 h later, 5×10^6 BDC $CD4^+$ CFSE-labeled T cells were injected i.v. PLNs were harvested, dissociated, and stained for FACS analysis of T cell proliferation and IFNγ production. Alternatively, NOD.MerTK.DTR female mice 4–5 wk of age were treated with DT (4 ng/g body weight; Sigma-Aldrich), and 24 h later they were treated with 120 mg/kg STZ and 5×10^6 CFSE-labeled BDC $CD4^+$ T cells.

ELISPOT. Opaque 96-well MultiScreen plates (Millipore) were coated with purified anti-IFNγ monoclonal antibody (BD Biosciences). Irradiated NOD splenocytes (5×10^5), PLN (5×10^3), and BDC peptides were cultured at 37°C for 72 h. IFNγ spot-forming cells were detected with biotinylated monoclonal anti-IFNγ, streptavidin-HRP (BD Biosciences), and 3-amino-9-ethyl-carbaazol substrate, and quantitated using an Immuspot plate reader and software (Cellular Technology Ltd.).

Isolation of pancreatic DCs. The pancreas was removed from 4-wk-old mice, incubated for 1 h at 37°C in RPMI medium with 10% FCS, 0.5 mg/ml collagenase D (Roche), and 5 μg/ml DNase I (Stratagene), and further disrupted by vigorous pipetting in PBS with 2 mM EDTA. DCs were enriched using anti-CD11c magnetic beads (Miltenyi Biotec). In some experiments, individual pancreatic DCs stained with anti-CD11c-APC were sorted using a MoFlo FACS sorter (Dako Cytomation).

In vitro T cell stimulation. BDC $CD4^+$ T cells (5×10^5 cells) isolated with a $CD4^+$ T cell enrichment kit (BD Biosciences) were CFSE labeled and cultured in 96-well ultra-low cluster plates with FACS-sorted pancreatic DCs (10^4) for 72 h and analyzed via FACS. Alternatively, 10^4 individually FACS-sorted $CD11c^+CD11b^+$ or $CD11c^+CD8\alpha^+$ DCs were cultured with 5×10^5 CFSE-labeled BDC $CD4^+$ T cells.

Detection of Gas6 protein. Fresh thymocytes or γ-irradiated thymocytes were stained with VAD-FMK-FITC (Promega) and polyclonal anti-Gas6 antibody, followed by biotinylated anti-goat IgG and PerCP-streptavidin. Alternatively, mice were treated with 120 mg/kg STZ, and 2 h later pancreatic islets were harvested as previously described (53). Islets were cultured for 12 h in low-glucose DME with 10% FBS, dissociated with enzyme-free dissociation solution (Sigma-Aldrich), and stained for Gas6. In addition, Gas6 was detected by Western blot. In brief, thymocytes were harvested from 4-wk-old NOD mice and either left untreated or irradiated (600R) to induce apoptosis and cultured in serum-free HL1 medium (Cambrex) for up to 12 h. Thymocytes were lysed in RIPA with protease inhibitor cocktail (Roche), and 40 μg of protein from each sample or 10 ng of recombinant murine Gas6 (rmGas6; R&D Systems) was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with polyclonal anti-Gas6 antibody, HRP anti–goat IgG (Santa Cruz Biotechnology), and ECL Plus substrate (GE Healthcare). Note that rmGas6 is truncated by 92 amino acid residues and consequently is ~10 kD smaller in comparison to native Gas6.

Analysis of protein S expression in ACs. Apoptosis was induced in thymocytes by γ irradiation (600R), and RNA was prepared using TRIZOL Reagent (Invitrogen). cDNA was synthesized using oligo-dT primers and SuperScript II reverse transcription (Stratagene). Protein S and β actin transcript were detected by PCR using the following primers: protein S 5′ GAGGCGGTATCTCTGTGCTC 3′ and 5′ GGTGTGGCACTGAAGGAAAT 3′; β actin 5′ AGAGGGAAATCGTGCGTGAC 3′ and 5′ CAATAGTGATGACCTGGCCGT 3′.

Phagocytosis of ACs. Apoptotic thymocytes were stained with 2.5 μM Cell Tracker Green (Invitrogen) and co-cultured with BMDC at a ratio of 5:1 for 6 h at 37 or 4°C. Alternatively, NOD BMDCs were pretreated with 10 μg of anti-MerTK or isotype control antibody before AC pretreatment. Cells were then cultured for 10 min in PBS with 2 mM EDTA and washed vigorously to remove noningested but bound AC, which was confirmed by confocal microscopy. BMDCs were stained with anti–CD11c-APC and analyzed for ingestion of fluorescent ACs by FACS.

In vivo AC clearance assay. Mice received a single i.p. injection of 120 mg/kg STZ in citrate saline. Pancreases were harvested and digested with collagenase, and islets were isolated (53). Purified islets were dissociated in PBS, plus 2 mM EDTA for 20 min at room temperature, followed by vigorous pipetting. Islet cells were fixed in 70% ethanol, permeabilized with 0.1% Triton X-100, and stained with propidium iodide. Cells were analyzed for DNA content by FACS, and subdiploid cells were considered to be apoptotic.

Online supplemental material. Fig. S1 demonstrates surface expression of MerTK on BMDCs and sDCs. Fig. S2 shows that antibody blockade of MerTK on BALB/c BMDCs prevents AC-induced inhibition. Fig. S3 demonstrates that protein S has no role in AC-induced inhibition. Fig. S4 shows Gas6 surface expression on apoptotic β cells. Fig. S5 shows that no significant difference exists in in vivo clearance of apoptotic β cells in NOD and NOD.MerTK$^{KD/KD}$ mice. Fig. S6 demonstrates that MerTK is not required for AC phagocytosis by BMDCs. Fig. S7 shows that DT treatment of NOD.MerTK.DTR mice depletes DCs but not macrophages or NKT cells in the PLN. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20062293/DC1.

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REFERENCES

1. Kerr, J.F., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* 26:239–257.
2. Fadok, V.A., D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, and P.M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148:2207–2216.
3. Wallet, M.A., P. Sen, and R. Tisch. 2005. Immunoregulation of dendritic cells. *Clin. Med. Res.* 3:166–175.
4. Albert, M.L., S.F. Pearce, L.M. Francisco, B. Sauter, P. Roy, R.L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 188:1359–1368.
5. Scott, R.S., E.J. McMahon, S.M. Pop, E.A. Reap, R. Caricchio, P.L. Cohen, H.S. Earp, and G.K. Matsushima. 2001. Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature.* 411:207–211.
6. Savill, J., V. Fadok, P. Henson, and C. Haslett. 1993. Phagocyte recognition of cells undergoing apoptosis. *Immunol. Today.* 14:131–136.
7. Gaipl, U.S., S. Franz, R.E. Voll, A. Sheriff, J.R. Kalden, and M. Herrmann. 2004. Defects in the disposal of dying cells lead to autoimmunity. *Curr. Rheumatol. Rep.* 6:401–407.
8. Reidy, M.F., and J.R. Wright. 2003. Surfactant protein A enhances apoptotic cell uptake and TGF-beta1 release by inflammatory alveolar macrophages. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 285:L854–L861.
9. Huynh, M.L., V.A. Fadok, and P.M. Henson. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J. Clin. Invest.* 109:41–50.

10. Steinman, R.M., S. Turley, I. Mellman, and K. Inaba. 2000. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J. Exp. Med.* 191:411–416.
11. Voll, R.E., M. Herrmann, E.A. Roth, C. Stach, J.R. Kalden, and I. Girkontaite. 1997. Immunosuppressive effects of apoptotic cells. *Nature.* 390:350–351.
12. Morelli, A.E., A.T. Larregina, W.J. Shufesky, A.F. Zahorchak, A.J. Logar, G.D. Papworth, Z. Wang, S.C. Watkins, L.D. Falo Jr., and A.W. Thomson. 2003. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood.* 101:611–620.
13. Hugues, S., E. Mougneau, W. Ferlin, D. Jeske, P. Hofman, D. Homann, L. Beaudoin, C. Schrike, M. Von Herrath, A. Lehuen, and N. Glaichenhaus. 2002. Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. *Immunity.* 16:169–181.
14. Lu, Q., and G. Lemke. 2001. Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. *Science.* 293:306–311.
15. Cohen, P.L., R. Caricchio, V. Abraham, T.D. Camenisch, J.C. Jennette, R.A. Roubey, H.S. Earp, G. Matsushima, and E.A. Reap. 2002. Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. *J. Exp. Med.* 196:135–140.
16. Camenisch, T.D., B.H. Koller, H.S. Earp, and G.K. Matsushima. 1999. A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. *J. Immunol.* 162:3498–3503.
17. D'Cruz, P.M., D. Yasumura, J. Weir, M.T. Matthes, H. Abderrahim, M.M. LaVail, and D. Vollrath. 2000. Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat. *Hum. Mol. Genet.* 9:645–651.
18. Gal, A., Y. Li, D.A. Thompson, J. Weir, U. Orth, S.G. Jacobson, E. Apfelstedt-Sylla, and D. Vollrath. 2000. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. *Nat. Genet.* 26:270–271.
19. Duncan, J.L., M.M. LaVail, D. Yasumura, M.T. Matthes, H. Yang, N. Trautmann, A.V. Chappelow, W. Feng, H.S. Earp, G.K. Matsushima, and D. Vollrath. 2003. An RCS-like retinal dystrophy phenotype in mer knockout mice. *Invest. Ophthalmol. Vis. Sci.* 44:826–838.
20. Duncan, J.L., H. Yang, D. Vollrath, D. Yasumura, M.T. Matthes, N. Trautmann, A.V. Chappelow, W. Feng, H.S. Earp, G.K. Matsushima, and M.M. LaVail. 2003. Inherited retinal dystrophy in Mer knockout mice. *Adv. Exp. Med. Biol.* 533:165–172.
21. Ishimoto, Y., K. Ohashi, K. Mizuno, and T. Nakano. 2000. Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. *J. Biochem. (Tokyo).* 127:411–417.
22. Sen, P., M.A. Wallet, Z. Yi, Y. Huang, M. Henderson, C.E. Mathews, H.S. Earp, G. Matsushima, A.S. Baldwin Jr., and R.M. Tisch. 2006. Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-κB activation in dendritic cells. *Blood.* 109:653–660.
23. Katz, J.D., B. Wang, K. Haskins, C. Benoist, and D. Mathis. 1993. Following a diabetogenic T cell from genesis through pathogenesis. *Cell.* 74:1089–1100.
24. Haskins, K., M. Portas, B. Bergman, K. Lafferty, and B. Bradley. 1989. Pancreatic islet-specific T-cell clones from nonobese diabetic mice. *Proc. Natl. Acad. Sci. USA.* 86:8000–8004.
25. Morgan, D.J., R. Liblau, B. Scott, S. Fleck, H.O. McDevitt, N. Sarvetnick, D. Lo, and L.A. Sherman. 1996. CD8+ T cell-mediated spontaneous diabetes in neonatal mice. *J. Immunol.* 157:978–983.
26. Chen, J., K. Carey, and P.J. Godowski. 1997. Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. *Oncogene.* 14:2033–2039.
27. Godowski, P.J., M.R. Mark, J. Chen, M.D. Sadick, H. Raab, and R.G. Hammonds. 1995. Reevaluation of the roles of protein S and Gas6 as ligands for the receptor tyrosine kinase Rse/Tyro 3. *Cell.* 82:355–358.
28. Varnum, B.C., C. Young, G. Elliott, A. Garcia, T.D. Bartley, Y.W. Fridell, R.W. Hunt, G. Trail, C. Clogston, R.J. Toso, et al. 1995. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. *Nature.* 373:623–626.
29. Hall, M.O., M.S. Obin, M.J. Heeb, B.L. Burgess, and T.A. Abrams. 2005. Both protein S and Gas6 stimulate outer segment phagocytosis by cultured rat retinal pigment epithelial cells. *Exp. Eye Res.* 81:581–591.
30. Seitz, H.M., T.D. Camenisch, G. Lemke, H.S. Earp, and G. Matsushima. 2007. Macrophages and dendritic cells use different Axl/Mertk/Tyro3 receptors in clearance of apoptotic cells. *J. Immunol.* 178:5635–5642.
31. Wu, Y., N. Tibrewal, and R.B. Birge. 2006. Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol.* 16:189–197.
32. Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, et al. 2002. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity.* 17:211–220.
33. Verbovetski, I., H. Bychkov, U. Trahtemberg, I. Shapira, M. Hareuveni, O. Ben-Tal, I. Kutikov, O. Gill, and D. Mevorach. 2002. Opsonization of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. *J. Exp. Med.* 196:1553–1561.
34. Wu, Y., S. Singh, M.M. Georgescu, and R.B. Birge. 2005. A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. *J. Cell Sci.* 118:539–553.
35. Hall, M.O., A.L. Prieto, M.S. Obin, T.A. Abrams, B.L. Burgess, M.J. Heeb, and B.J. Agnew. 2001. Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. *Exp. Eye Res.* 73:509–520.
36. Sharif, M.N., D. Sosic, C.V. Rothlin, E. Kelly, G. Lemke, E.N. Olsen, and L.B. Ivashkiv. 2006. Twist mediates suppression of inflammation by type I IFNs and Axl. *J. Exp. Med.* 203:1891–1901.
37. Chong, M.M., H.E. Thomas, and T.W. Kay. 2002. Suppressor of cytokine signaling-1 regulates the sensitivity of pancreatic β cells to tumor necrosis factor. *J. Biol. Chem.* 277:27945–27952.
38. McKenzie, M.D., N.L. Dudek, L. Mariana, M.M. Chong, J.A. Trapani, T.W. Kay, and H.E. Thomas. 2006. Perforin and Fas induced by IFNγ and TNFα mediate β cell death by OT-I CTL. *Int. Immunol.* 18:837–846.
39. Schnorrer, P., G.M. Behrens, N.S. Wilson, J.L. Pooley, C.M. Smith, D. El-Sukkari, G. Davey, F. Kupresanin, M. Li, E. Maraskovsky, et al. 2006. The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc. Natl. Acad. Sci. USA.* 103: 10729–10734.
40. Iyoda, T., S. Shimoyama, K. Liu, Y. Omatsu, Y. Akiyama, Y. Maeda, K. Takahara, R.M. Steinman, and K. Inaba. 2002. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J. Exp. Med.* 195:1289–1302.
41. Ohteki, T., T. Fukao, K. Suzue, C. Maki, M. Ito, M. Nakamura, and S. Koyasu. 1999. Interleukin 12-dependent interferon gamma production by CD8alpha+ lymphoid dendritic cells. *J. Exp. Med.* 189:1981–1986.
42. Hochrein, H., K. Shortman, D. Vremec, B. Scott, P. Hertzog, and M. O'Keeffe. 2001. Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. *J. Immunol.* 166:5448–5455.
43. Naumov, Y.N., K.S. Bahjat, R. Gausling, R. Abraham, M.A. Exley, Y. Koezuka, S.B. Balk, J.L. Strominger, M. Clare-Salzer, and S.B. Wilson. 2001. Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets. *Proc. Natl. Acad. Sci. USA.* 98:13838–13843.
44. Fadok, V.A., D.L. Bratton, A. Konowal, P.W. Freed, J.Y. Westcott, and P.M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* 101:890–898.
45. Wyllie, A.H., J.F. Kerr, and A.R. Currie. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251–306.
46. Behrens, E.M., P. Gadue, S.Y. Gong, S. Garrett, P.L. Stein, and P.L. Cohen. 2003. The mer receptor tyrosine kinase: expression and function suggest a role in innate immunity. *Eur. J. Immunol.* 33: 2160–2167.

47. Cheong, H.S., S.O. Lee, C.B. Choi, Y.K. Sung, H.D. Shin, and S.C. Bae. 2006. MERTK polymorphisms associated with risk of haematological disorders among Korean SLE patients. *Rheumatology (Oxford)*. 46:209–214.
48. Serreze, D.V., M. Bridgett, H.D. Chapman, E. Chen, S.D. Richard, and E.H. Leiter. 1998. Subcongenic analysis of the Idd13 locus in NOD/Lt mice: evidence for several susceptibility genes including a possible diabetogenic role for beta 2-microglobulin. *J. Immunol.* 160:1472–1478.
49. Jerez-Timaure, N.C., E.J. Eisen, and D. Pomp. 2005. Fine mapping of a QTL region with large effects on growth and fatness on mouse chromosome 2. *Physiol. Genomics*. 21:411–422.
50. Graham, D.K., G.W. Bowman, T.L. Dawson, W.L. Stanford, H.S. Earp, and H.R. Snodgrass. 1995. Cloning and developmental expression analysis of the murine c-mer tyrosine kinase. *Oncogene*. 10: 2349–2359.
51. Graham, D.K., T.L. Dawson, D.L. Mullaney, H.R. Snodgrass, and H.S. Earp. 1994. Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. *Cell Growth Differ.* 5:647–657.
52. Bhattacharyya, S., P. Sen, M. Wallet, B. Long, A.S. Baldwin Jr., and R. Tisch. 2004. Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and of IκB kinase activity. *Blood*. 104:1100–1109.
53. Wong, C.P., L. Li, J.A. Frelinger, and R. Tisch. 2006. Early autoimmune destruction of islet grafts is associated with a restricted repertoire of IGRP-specific CD8+ T cells in diabetic nonobese diabetic mice. *J. Immunol.* 176:1637–1644.