WIP is critical for T cell responsiveness to IL-2

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The Wiskott-Aldrich syndrome (WAS) interacting protein (WIP) stabilizes the WAS protein (WASP), the product of the gene mutated in WAS. WIP-deficient T cells have low WASP levels, limiting the usefulness of WIP KO mice in defining the role of WIP in T cell function. To define this role, we compared WIP/WASP double KO (DKO) mice to WASP KO mice on DO11.10 background. T cell development was normal in both strains, but peripheral T cell numbers were significantly decreased in DKO mice. WASP KO T cells proliferated and secreted IL-2 normally in response to OVA peptide (OVAp). In contrast, T cells from DKO mice proliferated poorly in response to OVAp in vitro, and cutaneous hapten hypersensitivity was deficient in these mice. DKO T cells up-regulated CD25 expression and secreted normal amounts of IL-2 after antigen stimulation, but had defective response to IL-2, evidenced by failure to further up-regulate CD25 expression, phosphorylate STAT5, and induce expression of STAT5-dependent genes. DKO, but not WASP KO, T cells had a disrupted subcortical actin cytoskeleton and impaired actin polymerization after T cell antigen receptor (TCR) ligation. These results indicate that WIP is essential for IL-2 signaling and responsiveness in T cells, possibly because of its critical role in TCR-triggered actin cytoskeletal reorganization.

Wiskott-Aldrich syndrome (WAS), an X-linked immunodeficiency caused by mutations in the WAS protein (WASP) gene, is characterized by recurrent infections, eczema, and thrombocytopenia. WASP is the first identified member of a family of proteins involved in signaling and cytoskeletal organization that includes N-WASP and Scar/WASP-family verprolin homologous (WAVE) proteins (1). WASP is expressed only in hematopoietic cells and plays a critical role in cellular function by linking surface receptor signaling to actin reorganization (2).

T cell dysfunction is an important component of WAS and is involved in the susceptibility of WAS patients to recurrent infections, autoimmunity, and development of B cell malignancies. T cells from WAS patients and WASP KO mice fail to spread, cap their T cell antigen receptor (TCR), proliferate, and secrete IL-2 in response to TCR triggering by immobilized anti-CD3 (3, 4). Although WASP localizes with F-actin at the immune synapse (IS) (5), recent data suggest that IS formation may not be impaired in WASP KO T cells, but that WASP is essential for the stability of the IS during its reformation in naïve T cells (6). In addition to its role in T cell activation, WASP plays a role in T cell chemotaxis. T cells from WAS patients have defective chemotaxis in response to stromal cell derived factor-1α (7) and have defective transcellular diapedesis (8). WASP KO T cells have a defect in their ability to home in vivo to spleen and lymph nodes (9).

In T cells, most of WASP is associated with the WASP-interacting protein (WIP) (10). WIP is ubiquitously expressed, but its expression is higher in lymphoid tissues. WIP, like WASP, plays an important role in T cell activation. Our previous work has shown that WIP KO T cells fail to proliferate, secrete IL-2, or increase their F-actin content after TCR ligation with immobilized anti-CD3 and have a homing defect in vivo (9, 11). WASP protein levels, but not mRNA levels, are severely diminished (down to 10% of normal) in T cells from WIP KO mice and WAS patients with mutations that disrupt WIP binding (8). Furthermore, WASP levels can be restored to normal by expressing WIP in WIP KO T cells, indicating that WIP stabilizes WASP in T cells. Similar observations were made in WIP knockdown experiments (12) and WIP-deficient dendritic cells (DCs) (13). WIP also stabilizes actin filaments (14). WIP KO, but not WASP KO, T cells, have defective F-actin increase after TCR ligation, disrupted actin cytoskeleton, and deficient IS formation (6, 8, 11), suggesting a role for WIP in actin-dependent T cell functions.

A better understanding of the individual roles of WIP and WASP in T cell activation is crucial for elucidating the molecular pathology of WAS. WASP-deficient patients and mice have normal levels of WIP and have been instrumental in defining the individual role of WASP in T cell function. The severely-decreased level of WASP in WIP KO mice has limited their usefulness in defining the individual role of WIP in T cell function. To define this role, we generated WIP/WASP double KO (DKO) mice and compared their T cells with those of WASP KO mice. We reasoned that differences between WIP/WASP DKO T cells and WASP KO T cells would reflect WIP functions that are independent of WASP. We demonstrate that WIP plays, independently of WASP, a critical role in T cell activation by antigen, IL-2 signaling and responsiveness, and cytoskeletal rearrangement.

Results and Discussion

Thymic Development Is Normal, but the Number of Peripheral T Cells Is Reduced in DKO Mice. We analyzed T cell development in DKO, WASP KO, and WT mice bred on the BALB/c DO11.10 background. Thymus cellularity was comparable in all 3 strains of mice (Fig. 1*A*). FACS analysis of thymocytes showed that the percentages of thymic cell populations that included $CD4^-CD8^-$ double negative (DN) cells, $CD4^+CD8^+$ double positive (DP) cells, $CD4^+$ single positive (SP) cells, and $CD8^+$ SP cells were similar between WT, WASP KO, and DKO mice (Fig. 1*B* and Table S1). T cell development was similarly unaffected in DKO and WASP KO mice on nontransgenic background. These results indicate that WIP is not essential for T cell development.

Analysis of spleen and peripheral lymph nodes (PLN) in DKO, WASP KO, and WT mice revealed a comparable number of cells in spleens of all 3 strains, but a significantly decreased number of cells in PLNs and number of lymphocytes in peripheral blood of DKO mice (Fig. 1*A* and Fig. S1). As previously shown (3), the percentages of $CD3^+$, $CD4^+$, and $B220^+$ cells in the spleens and PLNs of WASP KO mice were comparable with those in WT controls (Fig. 1 *C* and *D* and (Table S1). In contrast, the percentage of $CD3^+$ and $CD4^+$ T cells in spleens, PLN, and peripheral blood (Fig. S1) was markedly decreased in DKO mice. DKO mice had an increased percentage of B cells in PLN, but

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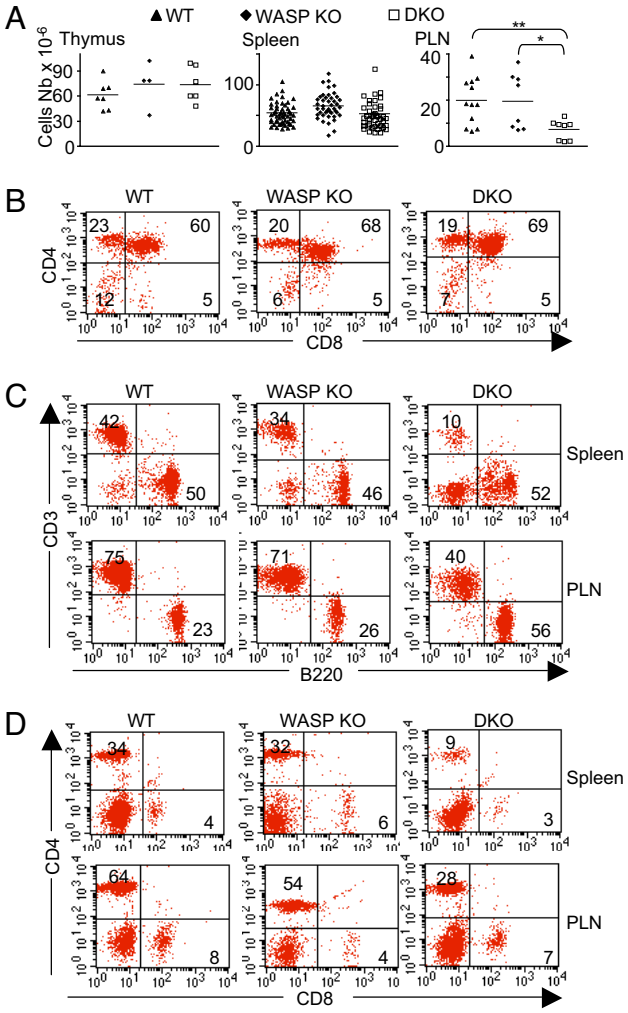

Fig. 1. Normal thymic development but diminished number of peripheral T cells in DKO mice. (*A*) Cellularity of thymus, spleen and PLNs. *, $P < 0.1$; **, $P < 0.01$. (*B*) FACS analysis of CD4 and CD8 expression by thymocytes. These are representative profiles of 4–7 mice analyzed per group. (*C* and *D*) FACS analysis of CD3 and B220 expression (*C*) and CD4 and CD8 expression (*D*) by lymphocytes from spleen and PLN. Representative profiles of 6 mice analyzed per group. The numbers in the quadrants of *B–D* represent percentages.

not in spleen. These results indicate that WIP is important for maintaining normal numbers of peripheral $CD4^+$ T cells. The previously demonstrated defect in the homing of DKO T cells to spleen and LN, which is more severe than that of WASP KO T cells (9), may have contributed to the T cell lymphopenia of DKO mice.

DKO T Cells Proliferate Poorly in Response to Antigen in Vitro and Have Impaired T Cell Function in Vivo. We examined the ability of T cells to proliferate in response to antigen stimulation in vitro. Purified splenic T cells from WT, WASP KO, and DKO mice were stimulated with $OVA_{323–339}$ peptide (OVAp; 0.5 μg/mL) in the presence of WT irradiated T cell-depleted splenocytes as antigen-presenting cells (APCs). There was no significant difference in proliferation to OVAp between WASP KO and WT T cells. In contrast, DKO T cells exhibited severely impaired proliferation to OVAp over a peptide concentration range from 0.1 to 5 μg/mL (Fig. 2 *A* and *B*). T cells from DO11.10 WIP KO mice also proliferated poorly to OVAp (Fig. S2). These data indicate that WIP, but not WASP, is critical for the proliferation of T cells to antigen.

It has been reported that the proliferation of T cells from AND TCR transgenic WASP KO to moth cytochrome C peptide is partially reduced (15). The normal proliferation of WASP KO DO11.10 T cells to OVAp may reflect differences in the affinity of the transgenic TCRs for their cognate MHC class II peptides and/or differences in genetic background and is consistent with normal proliferation of peripheral blood mononuclear cells from WAS patients to alloantigens (16) and in some cases to antigens (Fig. S3*B*) and normal T cell dependent antibody responses in WASP KO mice (3). Moreover, we found that splenocytes from 2,4,6,-trinitrophenyl–keyhole limpet hemocyanin (KLH)-immunized non-TCR transgenic WASP KO mice proliferate vigorously in response to KLH (Fig. S3*A*).

It has been well documented that T cells from WAS patients and WASP KO mice proliferate poorly to immobilized anti-CD3 (3, 4, 16). WASP KO DO11.10 T cells did not proliferate to anti-CD3 immobilized on plastic plates (Fig. 2*C*). However, they proliferated normally to soluble anti-CD3 presented by APCs. In contrast, DKO T cells failed to proliferate to both immobilized and soluble anti-CD3. These results suggest that there is a fundamental difference in the response of WASP KO T cells to TCR triggering by anti-CD3 on an immobilized surface versus TCR triggering by antigen or soluble anti-CD3 presented by APCs. This discrepancy could be caused by defective spreading of WASP KO T cells over anti-CD3 coated surfaces (17), which may be required for effective TCR signaling. The requirement for T cell spreading may be bypassed when APCs present antigen or soluble anti-CD3 to the T cells.

To define the basis of the defective proliferation of DKO T cells to OVAp, we analyzed cell cycle progression in T cells after antigen stimulation. Fig. 2*D* shows that ≈30% of WT T cells and WASP KO T cells progressed through the S phase after antigen stimulation. In contrast, DKO T cells were blocked in the G_0/G_1 phase and did not progress through the S phase. DKO T cells did not show a substantial increase of cell death (represented by the fraction of cells in sub-G_1) when compared with WT and WASP KO T cells. Staining of OVAp-stimulated T cell with annexin V and propidium iodide (PI) revealed little increase in apoptosis or cell death in DKO T cells (Fig. S4), indicating that the impaired proliferation of DKO T cells is caused by a defect in cell cycle progression rather than an increase in the rate of cell death.

We investigated T cell function in vivo by examining contact hypersensitivity (CHS) to hapten, a classic example of an in vivo response that involves T cell immunity. Oxazolone- or dinitrofluorobenzene (DNFB)-sensitized WT mice challenged by painting the hapten on ear skin exhibited ear swelling that peaked 24 h after hapten challenge and subsided 96 h later (Fig. 2*E*). Ear swelling in response to hapten challenge was comparable in WT and WASP KO mice, but was severely impaired in DKO mice. These results suggest that T cell function in vivo is impaired in DKO mice. The normal CHS in WASP KO mice is consistent with normal proliferation of their T cells to antigen and with the normal function of their APCs (ref. 18 and Fig. S4*A*). Moreover, non-TCR transgenic WASP KO mice also exhibited a normal CHS to DNFB (Fig. S3*C*) and normal migration of skin DCs to draining LN (DLN) (Fig. S5*B*). Previous work examining WASP KO mice on 129Sv and C57BL/6 background reported a partial defect in CHS in response to DNFB at 24 h, but not at 48 h after challenge (19) and in the migration of skin DCs (20) and s.c.-injected in vitro-labeled DCs (21, 22). The differences observed between our study and those studies may be explained by differences in backgrounds and experimental techniques. The defective CHS in DKO mice is unlikely to be caused by defective APC function, because DCs purified from the spleens of DKO mice were comparable to WT DCs in their ability to support proliferation and IL-2 secretion by DO11.10 WT T cells in response to OVAp (Fig. S5*A*). Furthermore, migration of skin DCs to DLN was intact in DKO mice (Fig. S5*B*). In view of these

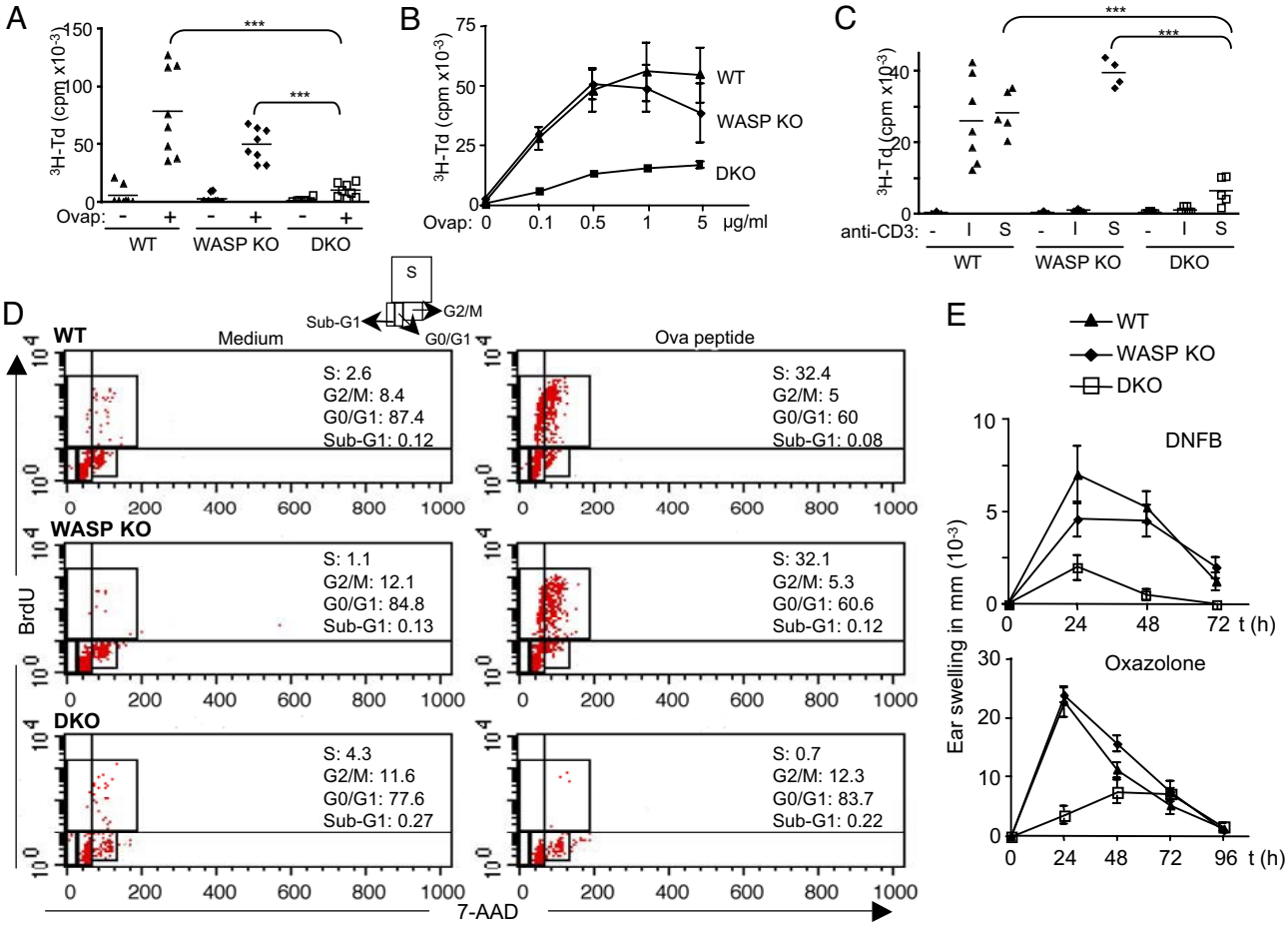

Fig. 2. Impaired antigen-specific T cell function in DKO mice in vitro and in vivo. (*A*) T cell proliferation to 0.5 μg/mL OVAp. ***, $P < 0.001$. (*B*) OVAp dose–response of T cell proliferation. (*C*) T cell proliferation to immobilized (I) and soluble (S) anti-CD3 antibody. ***, $P < 0.001$. (*D*) FACS analysis of cell cycle in T cells stimulated with 0.5 μg/mL OVAp using BrdU/7-AAD staining. Numbers indicate percentages of cells in each stage of the cycle. (*E*) Hapten-induced contact hypersensitivity. The results represent the difference in thickness between hapten- and vehicle-challenged ears. Significant differences were determined by 2-way ANOVA (DNFB: F = 3.69, Dfn = 6, Dfd = 35, $P = 0.006$; Oxazolone: F = 10.81, Dfn = 12, Dfd = 90, $P < 0.0001$).

findings, our results suggest that impaired CHS in DKO mice is likely caused by defective T cell function (9). We cannot rule out a possible defect in NK cell function, because NK cells may contribute to CHS (23).

DKO T Cells Express IL-2 Receptor (IL-2R) and Secrete IL-2 Normally After Antigen Stimulation, but Fail to Respond to IL-2. Antigen stimulation of T cells up-regulates CD25 (IL-2Rα chain) expression and results in IL-2 synthesis and secretion, which drives T cell proliferation. Given the defective proliferation of DKO T cells to antigen, we examined whether they are impaired in their ability to express CD25 and/or secrete IL-2. Fig. 3*A Left* and *B* shows that after stimulation for 48 h with OVAp presented by WT APCs DKO T cells up-regulated CD25 expression and secreted IL-2 to an extent comparable to that of WT and WASP KO T cells. IL-2 secretion by DKO T cells was normal over a range of OVAp concentrations from 0.1 to 5 μg/mL (Fig. 3*C*). Normal IL-2R expression and IL-2 production in response to antigen stimulation strongly suggests that TCR signaling pathways are intact in DKO T cells. This hypothesis is supported by the observation that phosphorylation of Erk and up-regulation of CD69 surface expression in response to TCR/CD3 ligation were normal in DKO T cells. Nuclear factor of activated T cells (NFAT) activation has been reported to be partially decreased in anti-CD3-stimulated Jurkat cells in which WIP has been knocked down (12), suggesting that WIP is important for IL-2 production. Although we have not examined NFAT activation in DKO T cells, our finding of normal IL-2 production suggests that any potential decrease in NFAT activation does not affect IL-2 secretion by these cells.

Interaction of IL-2 with its high-affinity receptor induces further up-regulation of CD25 and delivers critical proliferation signals (24). WT and WASP KO T cells that had been first activated with OVAp then washed and stimulated with exogenous IL-2 further up-regulated CD25 expression (Fig. 3 *A Right* and *D*). In contrast, OVAp-activated DKO T cells were not able to further up-regulate CD25 expression in response to IL-2. Unresponsiveness of DKO T cells to IL-2 was supported by the observation that addition of exogenous IL-2 failed to correct their defective proliferation to OVAp (Fig. 3*E*). These results indicate that WIP is essential for T cells to respond to IL-2.

IL-2R Signaling Is Defective in DKO T Cells. IL-2 binding to its receptor activates a variety of pathways that include the activation of JAK tyrosine kinases and subsequent phosphorylation of the transcription factor STAT5. IL-2 stimulation induced STAT5 phosphorylation in WT and to a slightly lesser extent in WASP KO T cells (Fig. 4*A*). In contrast, IL-2 caused no detectable STAT5 phosphorylation in DKO T cells. STAT5 level relative to actin was comparable in WT, WASP KO, and DKO T cells (Fig. 4*A*). After its phosphorylation in response to IL-2 stimulation, p-STAT5 dimerizes and translocates to the nucleus (25, 26). We

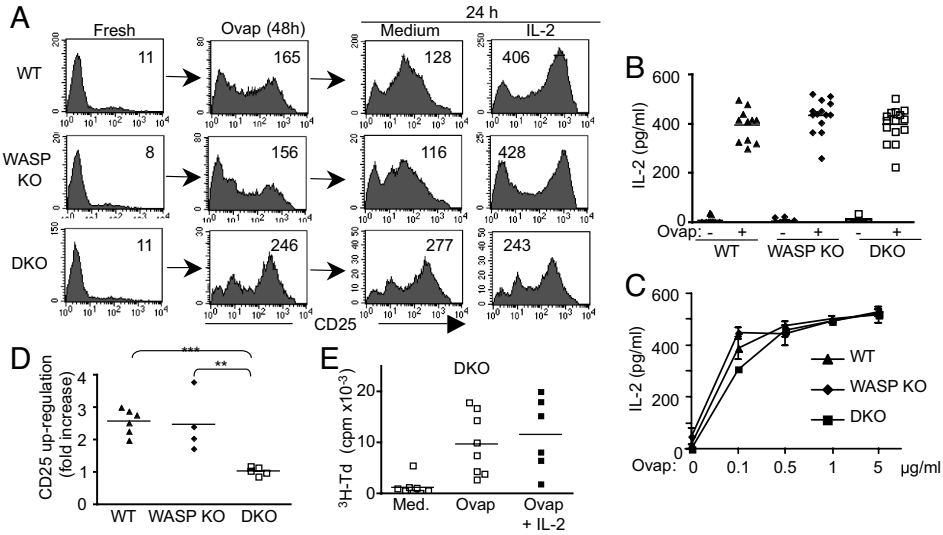

Fig. 3. DKO T cells express CD25 and secrete IL-2 normally in response to antigen stimulation, but fail to respond to IL-2. (*A*) Representative ($n = 3$) FACS analysis of CD25 expression by T cells after OVAp stimulation for 48 h (*Left*) followed by IL-2 stimulation for another 24 h (*Right*). The numbers shown indicate mean fluorescence intensities (MFIs). (*B*) IL-2 secretion by T cells in response to 0.5 μg/mL OVAp. (*C*) OVAp dose–response of IL-2 secretion. (*D*) Fold increase of CD25 up-regulation induced by IL-2 (40 ng/mL) in OVAp-activated T cells. Fold increase was calculated by dividing the MFIs of CD25 staining in OVAp-activated T cells stimulated by IL-2 by the MFI of CD25 in Ovap-activated T cells. **, $P < 0.01$; ***, $P < 0.001$. (*E*) Effect of exogenous IL-2 on the proliferation of DKO T cells to OVAp.

analyzed the localization of pSTAT5 in the cytoplasm versus nucleus before and after IL-2 stimulation of T cells (Fig. 4*B*). A small and comparable amount of STAT5, but not pSTAT5, was detectable in the nucleus of unstimulated WT and WASP KO T cells, consistent with the previously demonstrated shuttling of STAT5 in a cytokine-independent manner (27). After IL-2 stimulation, all p-STAT5 translocated to the nucleus in WT and WASP KO T cells (Fig. 4*B Left*), with none being detected in the cytoplasm (Fig. 4*B Right*). Nuclear extracts from unstimulated DKO T cells contained no detectable STAT5. Consistent with their failure to phosphorylate STAT5, IL-2-stimulated DKO T cells had no detectable p-STAT5 in either nuclear or cytoplasmic

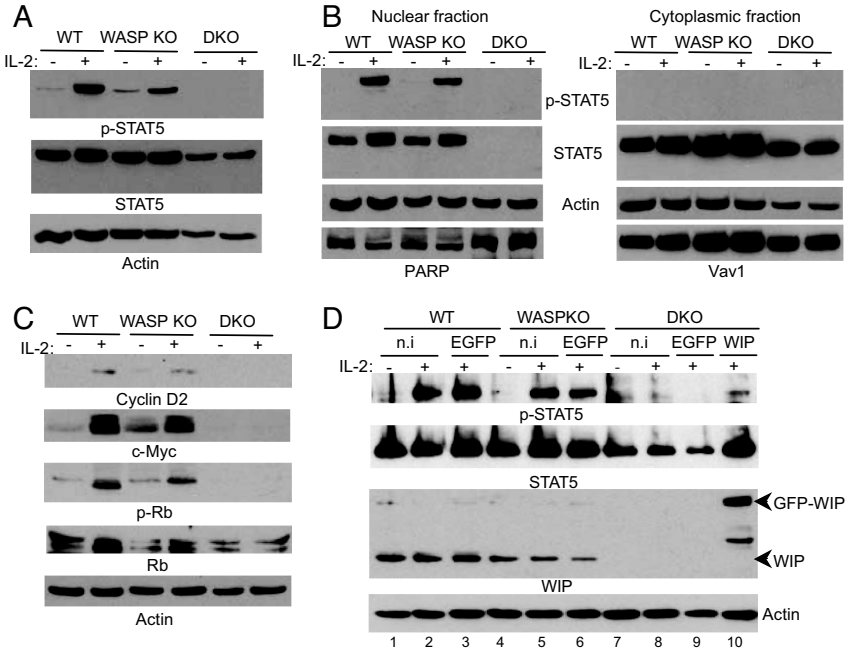

Fig. 4. IL-2R signaling is defective in DKO T cells. (*A*) Western blot analysis of STAT5 phosphorylation in T cells before and 20 min after stimulation with IL-2 (50 ng/mL). STAT5 and actin were used as loading controls. (*B*) Western blot analysis of localization of STAT5 and pSTAT5 in T cells before and after stimulation for 20 min with IL-2. PARP and Vav1 were used as controls for the cytoplasmic and nuclear fractions, respectively. Actin was used as loading control. (*C*) Western blot analysis of induction of Cyclin D2 and c-Myc expression and phosphorylation of Rb after IL-2 stimulation of OVAp-activated T cells. (*D*) WIP overexpression partially restores STAT5 phosphorylation. T cells were either noninfected (n.i) (lanes 1, 2, 4, 5, 7, and 8) or infected with control Lenti-EGFP (lanes 3, 6, and 9) or Lenti-EGFP-WIP (lane 10). After 48 h, STAT5 phosphorylation induced by IL-2 stimulation was analyzed by Western blot. Results shown are representative of 3 experiments.

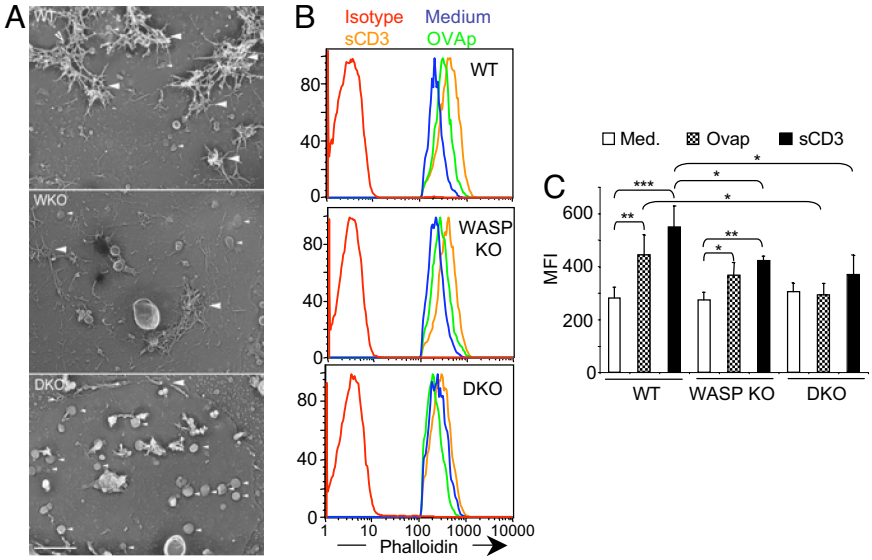

Fig. 5. TCR triggered cytoskeletal rearrangement is defective in DKO T cells. (*A*) Representative structure of the actin-membrane interfaces in T cells stimulated by adherence to anti-CD3-coated coverslips. The larger arrowheads indicate actin filament foci on the cytoplasmic membrane surface; small arrows indicate coated pits. Note the paucity of filament foci in the DKO cells. (Bar: 0.5 μm.) (*B*) F-actin content of gated CD4$^+$ cells T cells stimulated for 48 h with medium, soluble anti-CD3 (sCD3), or OVAp in the presence of APCs. Results shown are representative of 3 experiments. (*C*) MFI values pooled from 3 different experiments. *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

extracts. Failure of IL-2 to activate STAT5 in DKO T cells likely underlies their inability to further up-regulate CD25 and proliferate in response to IL-2, because STAT5 is critical for these events (25, 26).

IL-2 activates STAT5-dependent expression of cyclin D2 and cMyc and causes phosphorylation of the E2F inhibitor retinoblabtoma protein (Rb). To verify that IL-2 signaling via STAT5 is defective in DKO T cells, T cells were preactivated for 2 days with OVAp presented by WT APCs, then washed and incubated with IL-2 for 1 day. Fig. 4*C* shows that IL-2 induced cyclin D2 and c-Myc protein expression and Rb phosphorylation to a comparable degree in WT and WASP KO T cells. In contrast, IL-2 failed to induce these events in DKO T cells. We examined whether reconstitution of DKO T cells with WIP reverses the defect in IL-2 signaling. T cells were infected with lentivirus that encoded EGFP-WIP or EGFP alone as control. Fig. 4*D* shows that introduction of EGFP-WIP (lane 10), but not EGFP (lane 9), partially restored IL-2-induced STAT5 phosphorylation in DKO T cells. Incomplete restoration of STAT5 phosphorylation is likely because only a fraction of the T cells (≈60%) expressed EGFP-WIP. The small numbers of purified T cells that could be obtained from DKO spleens precluded examination of purified EGFP$^+$ cells. These findings indicate that WIP is important for STAT5 phosphorylation and activation after IL-2 stimulation.

Cytoskeletal Rearrangement Is Defective in DKO T Cells. An intact cytoskeleton is important for IL-2R signaling and STAT5 activation by prolactin and epidermal growth factor (28–30). We compared the actin cytoskeleton in T cells from WT, WASP KO, and DKO mice. Fig. 5*A* shows that the cytoplasmic surface of the plasma membrane of freshly-isolated WT T cells, captured by removing the apical cell surface of cells spread on anti-CD3-coated coverslips, was decorated with actin foci from which F-actin radiated. A general background of filaments was also observed. Although this actin organization was somewhat attenuated in WASP KO T cells, membrane F-actin assemblies and F-actin were not found in DKO T cells, as previously observed in WIP KO T cells (11).

We also examined the F-actin content of T cells that have been cultured for 2 days with soluble anti-CD3, OVAp, or medium in the presence of APCs, to mimic the conditions used to demonstrate the defect in IL-2 responsiveness of DKO T cells. Fig. 5 *B* and *C* shows that F-actin content of T cells cultured in medium was comparable in all 3 strains. After stimulation with soluble anti-CD3 or OVAp, F-actin robustly increased in WT T cells and to a slightly lesser extent in WASP KO T cells with a significant difference in cells stimulated with anti-CD3. Compensation by N-WASP may explain the modest impairment of F-actin rise in WASP KO T cells. In contrast, DKO T cells completely failed to increase their F-actin content after stimulation with OVAp and exhibited a minimal increase in F-actin in response to soluble anti-CD3. These results indicate that WIP is important for TCR-mediated actin cytoskeleton reorganization.

Conclusion

This study presents 4 important findings. First, WIP is critical for T cell proliferation to antigen. WIP is not required for transmission of TCR signals that result in up-regulation of CD25 and IL-2 synthesis, but it is essential for activation of STAT5 and IL-2 responsiveness. Second, WIP, but not WASP, is critical for actin cytoskeletal reorganization in T cells in response to TCR ligation. Because the actin cytoskeleton is thought to be important for IL-2R signaling, this may underlie the requirement for WIP in IL-2 responsiveness. Third, there is a fundamental difference in the requirement for WASP between T cell proliferation to immobilized anti-CD3 and T cell proliferation in response to more physiologic stimulation by antigen. WASP is necessary for T cell spreading over anti-CD3-coated surfaces, which may be required to collect sufficient TCR/CD3 signals to exceed the T cell activation threshold. In contrast, WASP plays little role in antigen presentation by APCs, a process that may not require T cell spreading. Fourth, the WASP/WIP complex itself may not be important for the T cell response to antigen, because WASP KO T cells, which lack this complex but have normal levels of WIP, proliferate quite normally in response to antigen. In sum, our results demonstrate that WIP, independently of WASP, is critical for T cell activation.

Materials and Methods

Mice. Generation of WASP KO, WIP KO, and DKO mice has been described (3, 9, 11). All strains were backcrossed at least 8 times with BALB/c DO11.10 mice. WT littermates were used as controls. Mice were housed under pathogen-free conditions and studies were performed in accordance with Children's Hospital policies and procedures.

Flow Cytometry. Cells from thymus, spleen, and PLN were stained and analyzed on a FACS Calibur flow cytometer (Becton Dickinson) using CellQuest software. For direct staining, the following conjugated antibodies were used: anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-CD3 (145–2C11), anti-B220 (RA3–6B2) and anti-CD25 (PC 61) (all from BD Pharmingen). Intracellular F-actin content was measured in permeabilized cells by using phalloidin-TRITC as described (11).

Proliferation, IL-2 Secretion, and Cell Cycle Analysis. Splenic T cells from WT, WASP KO, and DKO mice were purified with a mouse pan T cell isolation kit, and splenocytes were depleted of T cells with CD90 microbeads by using an autoMacs (Miltenyi Biotec). T cells (10^5) were stimulated with immobilized anti-CD3 mAb (145–2C11; eBiosciences; 10 μg/mL in coating solution), 1 μg/mL soluble anti-CD3 mAb or 0.5 μg/ml of OVAp (323–339; Bachem) in the presence of irradiated (3,000 rad) T cell-depleted splenocytes (5×10^5) for 2 days. Proliferation was measured by adding 1 μCi of [^{3}H]thymidine for 16 h. IL-2 levels in supernatants were measured by ELISA. The cell cycle assay was performed by using the FITC-BrdU/7-aminoactinomycin D (7-AAD) flow kit (BD Biosciences).

Hapten-Induced Contact Hypersensitivity. Development of contact hypersensitivity to oxazolone and to DNFB were measured as described (23, 31).

Western Blot Analysis. Cells were pretreated and stimulated as indicated, after which they were washed in ice-cold PBS and harvested in lysis buffer containing vanadate and proteases inhibitors. Nuclear extracts were prepared by using a Nuclear Extract Kit from Active Motif. Proteins were separated by SDS/PAGE, transferred to Immobilon-P membrane (Millipore), and blotted with the specified antibodies as shown in *Results*. Antibody to phospho-STAT5 that recognizes both phosphorylated STAT5a and STAT5b isoforms and antibodies to STAT5 (C17), PARP, phospho-Rb, Rb, and c-Myc antibodies were from Cell Signaling Technology. Actin mAb was from Chemicon International. Cyclin D2 mAb was from Invitrogen. Vav1 mAb was from Abcam, EGFP mAb (clone JL-8) was from Clontech, and WIP (3D10) mAb was as described (32).

Lentiviral Infection. EEGFP and EEGFP-WIP were cloned into pHAGE-fEF1a-IzsGW vector. HIV-based lentivirus vectors were packaged at the Harvard Gene Therapy Initiative. A total of 3×10^6 T cells were cultured overnight in media containing lentivirus at a multiplicity of infection of 2, polybrene (8 μg/mL), and phytohemagglutinin (3 μg/mL). The cells were washed the next day, and the experiment was performed 48 h after infection.

Electron Microscopy. Cells were adhered to anti-CD3-coated coverslips by centrifugation at $280 \times g$ for 5 min at 4 °C, incubated at 37 °C for 30 min, and mechanically opened to reveal the basal cytoplasmic surface by attaching a polylysine-coated coverslip to the apical cell surface and removing it in PHEM buffer as described (11).

Statistical Analysis. Two-tailed Student's *t* test was used to compare the differences between groups (Figs. 1–3 and 5). The development of CHS was analyzed by 2 way ANOVA using GraphPad PRISM software (Fig. 2*E*).

Additional Methods. For more details see *SI Text*.

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