Wiskott–Aldrich syndrome protein (WASP) and N-WASP are critical for T cell development

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Although T cell dysfunction and lymphopenia are key features of immunodeficient patients with the Wiskott–Aldrich syndrome and Wiskott–Aldrich syndrome protein (WASP)-deficient mice, T cell development appears relatively normal. We hypothesized that N-WASP, a ubiquitously expressed homologue of WASP, may serve a redundant function with WASP. To examine the unique and redundant activities of WASP and N-WASP, we generated ES cells devoid of WASP and N-WASP [double knockout (DKO)] and used the RAG-2-deficient blastocyst complementation system to generate DKO lymphocytes. Moreover, we mated WASP KO mice with mice containing a conditionally targeted N-WASP allele and used the Cre-loxP system to generate mice lacking WASP and N-WASP in T cells [conditional DKO (cDKO)]. In both systems, N-WASP-deficient cells were indistinguishable from WT cells. In contrast, T cell development in DKO and cDKO mice was markedly altered, as shown by thymic hypocellularity and reduced numbers of peripheral T cells. We found that the combined activity of WASP and N-WASP was important for $CD4^-CD8^-$ double-negative (DN)-to-$CD4^+CD8^+$ double-positive (DP) cell transition, and this may be partly explained by reduced cycling DN3 cells. In addition, decreased migratory responses of $CD4^+CD8^-$ and $CD4^-CD8^+$ single-positive (SP) cells and increased percentage of $CD69^{low}CD24^{low}$ and $CD62L^{low}$ SP cells in cDKO cells imply retention of SP cells in the thymus. In summary, this study suggests that, although WASP serves a unique role for peripheral T cell function, T cell development depends on the combined activity of WASP and N-WASP.

cytoskeleton | thymus | migration | colitis | knockout mice

Lymphoid progenitors enter the thymus to initiate a complex differentiation process resulting in maturation of T cells (1, 2). The well characterized maturation steps of $\alpha\beta$ T cells in the thymus are also governed by trafficking events and positional cues driven by guidance molecules, including chemokines, adhesion molecules, and extracellular matrix components (3–6). Rearrangement of the actin cytoskeleton is regulated during both T cell receptor signaling and T cell migration (7–9), but much less is known about its requirement during T cell development. The small Rho-family GTPases and their guanine nucleotide exchange factors (GEFs), proteins known to regulate the actin cytoskeleton, are clearly required during thymocyte development (10–12). Although these molecules are also known to regulate cell adhesion and migration, their involvement in thymocyte trafficking has not been thoroughly assessed. The small GTPase effector molecule Wiskott–Aldrich syndrome protein (WASP) has also been demonstrated to be a critical regulator of antigen receptor signaling, actin cytoskeletal rearrangements, and lymphocyte migration (13–22). Although WASP deficiency has been correlated with lower numbers of naïve T cells (23), WASP does not seem to play a critical role in T cell development. Moreover, despite a large body of evidence showing a role for WASP in T cell signaling, its role in thymocyte migration has not been studied.

WASP belongs to the WASP family of proteins, including WASP, N-WASP, and WAVE/SCAR molecules 1–3 (24). In particular, N-WASP, which shares 50% homology with WASP, may serve specific and redundant function with WASP in hematopoietic cells. In fact, we have shown that expression of N-WASP in WASP-deficient T cells partly restores CD3-mediated proliferation, implying that WASP and N-WASP might share functions in T cells (25).

Here we sought to explore the importance of cytoskeletal regulation for thymocyte development by examining the unique and redundant roles of WASP and N-WASP. Using two complementary approaches, we analyzed T cells devoid of WASP and N-WASP and demonstrated that thymopoiesis cannot proceed in the absence of WASP and N-WASP.

Results

Deletion of WASP and N-WASP in Lymphocytes Using the RAG-2-Deficient Complementation System Leads to a Block in T Cell Development. We have demonstrated that lymphoid development is normal in WASP knockout (WKO) mice (20). We hypothesized that N-WASP might have some overlapping functions with WASP during lymphopoiesis and sought to investigate the unique and redundant activities for WASP and N-WASP in T cell development and function. To circumvent the embryonic lethality of N-WASP germ-line inactivation in mice (26), we used the RAG2-deficient blastocyst complementation system (27). Blastocysts from RAG-2-deficient mice implanted into foster mothers generate animals that fail to rearrange antigen receptor genes and consequently lack mature B and T cells. Injection of gene-targeted ES cells into RAG-2-deficient blastocysts leads to the generation of somatic chimeras in which all mature B and T cells derive from the injected ES cells. N-WASP-KO (NWKO) and WASP/N-WASP double-KO (DKO) ES cells were isolated from blastocysts resulting from heterozygous matings ($WASP^{+/-}$; $N\text{-}WASP^{neo/+}$) [supporting information (SI) Fig. 6] and injected into RAG2-deficient blastocysts. Lymphoid organs were analyzed from chimeric mice and compared with control WKO and WT mice.

T cell development in WKO and NWKO chimeric mice was

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The authors declare no conflict of interest.

Abbreviations: WASP, Wiskott–Aldrich syndrome protein; KO, knockout; WKO, WASP KO; NWKO, N-WASP-KO; DKO, WASP/N-WASP double KO; cDKO, WASP/N-WASP conditional DKO; GEF, guanine nucleotide exchange factor; DN, $CD4^-CD8^-$ double-negative; DP, $CD4^+CD8^+$ double-positive; SP, $CD4^+CD8^-$ and $CD4^-CD8^+$ single-positive.

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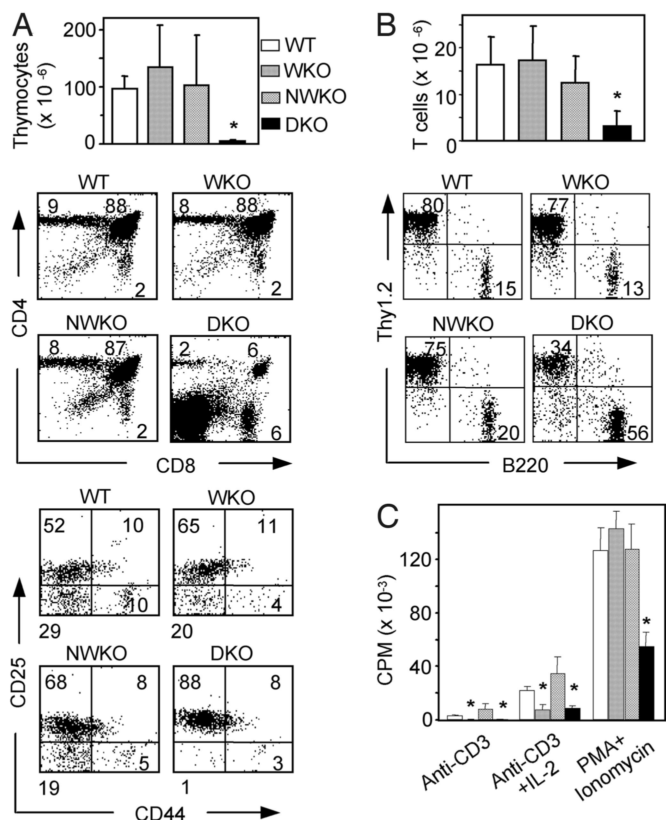

Fig. 1. Deletion of WASP and N-WASP in lymphocytes leads to a block in T cell development. (*A*) Thymocyte numbers are reduced in DKO mice; numbers represent total thymocytes ± SD (n = 4–8 mice per group). Flow cytometric analysis reveals a marked increase in the percentage of total DN thymocytes and of DN3 subset in DKO mice. (*B*) T lymphocyte numbers are reduced in DKO lymph nodes. Absolute numbers represent total Thy1.2$^+$ staining T cells ± SD (n = 4–8 mice per group). *, $P < 0.01$. (*C*) Reduction in proliferative responses of WKO and DKO T cells. Values are representative of four independent experiments. cpm indicates the cpm ([^{3}H]thymidine) ± SD of triplicate wells. *, $P < 0.01$.

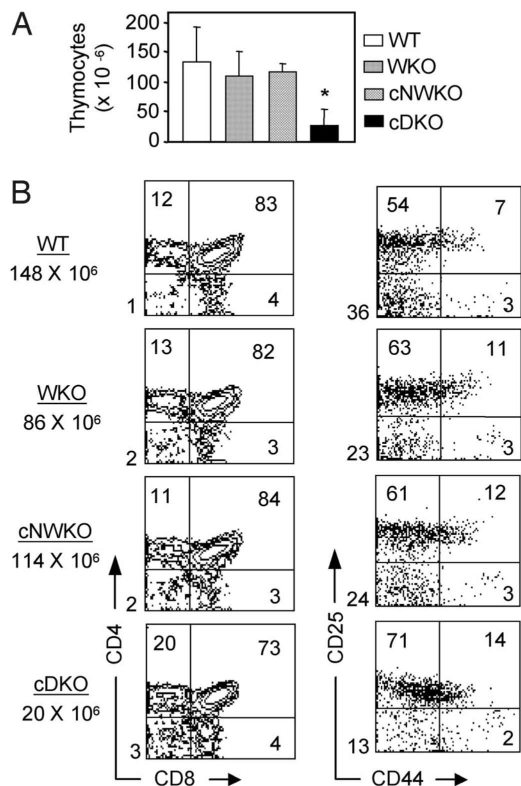

Fig. 2. T cell development is impaired in cDKO T lymphocytes. (*A*) Thymocyte numbers are markedly reduced in cDKO mice. Numbers represent total thymocytes ± SD (n = 4–8 mice per group). *, $P < 0.01$. (*B*) Representative example of flow cytometric analyses demonstrating a modest decrease in the percentage of DP thymocytes and increase in the percentage of DN3 subset in cDKO mice. Overall thymocyte numbers associated with each histogram are noted under each genotype.

indistinguishable from WT mice (Fig. 1*A*), as were the T cell numbers in the periphery (Fig. 1*B*). In contrast, analyses of DKO chimeric mice revealed a profound defect in T cell development with a marked reduction in thymic cellularity (Fig. 1*A*). Flow cytometric analyses showed an increased percentage of CD4$^-$CD8$^-$ double-negative (DN) cells with an increase in DN3 (CD44$^-$CD25$^+$) cells and a decrease in DN4 (CD44$^-$CD25$^-$) cells (Fig. 1*A*). The decrease in DN4 thymocytes was associated with a corresponding decrease in the percentages of CD4$^+$CD8$^+$ double-positive (DP) and CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ single-positive (SP) thymocytes (Fig. 1*A*). A relative increase in CD8 SP cells was observed. These are not immature SP, because they express high levels of TCR (data not shown). This altered thymocyte development was associated with a decreased number of mature T cells in the periphery of DKO mice (Fig. 1*B*). Normal contribution to the B-cell compartment (Fig. 1*B*) argues against chimerism artifacts being responsible for the T cell developmental abnormalities, because the B cell compartment is generally more difficult to populate using this approach (28).

We next investigated TCR-mediated proliferative responses of NWKO and DKO peripheral T lymphocytes. Proliferation of NWKO peripheral T cells was similar to WT cells (Fig. 1*C*). As previously shown, WKO T cells showed a markedly reduced proliferative response upon TCR activation that could be partially rescued by addition of exogenous IL-2 (20, 22). Similar to WKO T cells, DKO T cells exhibited very low proliferative responses (Fig. 1*C*). Notably, DKO cells also had reduced responses to receptor-independent mitogenic stimuli (i.e., phorbol 12-myristate 13-acetate and ionomycin; Fig. 1*C*). In conclusion, deletion of WASP and N-WASP leads to thymic hypocellularity with a profound block at the DN stage, associated with reduced number of T lymphocytes in the periphery.

Conditional Deletion of WASP and N-WASP in Early Thymocytes Results in Aberrant Thymic Development. To gain further insight into the role of WASP and N-WASP in intrathymic T cell differentiation, we specifically inactivated the N-WASP gene in thymocytes of WKO mice using the Cre-*lox*P system (29). Using standard homologous recombination approaches, exon 2 of N-WASP was flanked by *lox*P sites (SI Fig. 7). We determined this exon was essential for N-WASP function (26). We bred N-WASP conditionally targeted WKO mice with transgenic mice expressing the Cre recombinase under the T lymphocyte-specific lck promoter (lck-Cre) to generate WASP/N-WASP conditional DKO mice (termed cDKO hereafter). The use of the lck-Cre transgenic mouse results in homogeneous deletion of *lox*P flanked genes at the DN3 stage (30). Expression of Cre recombinase in cDKO thymocytes was confirmed by intracellular staining for Cre protein (SI Fig. 8*C*). PCR and Western blotting analyses determined nearly complete excision of N-WASP exon 2 and loss of N-WASP protein expression, respectively, in thymocytes expressing Cre (SI Fig. 8 *A*, *B*, and *D*).

Analogous to studies on chimeric mice described above, DN, DP, and SP thymocyte lineages were present in WT, WKO,

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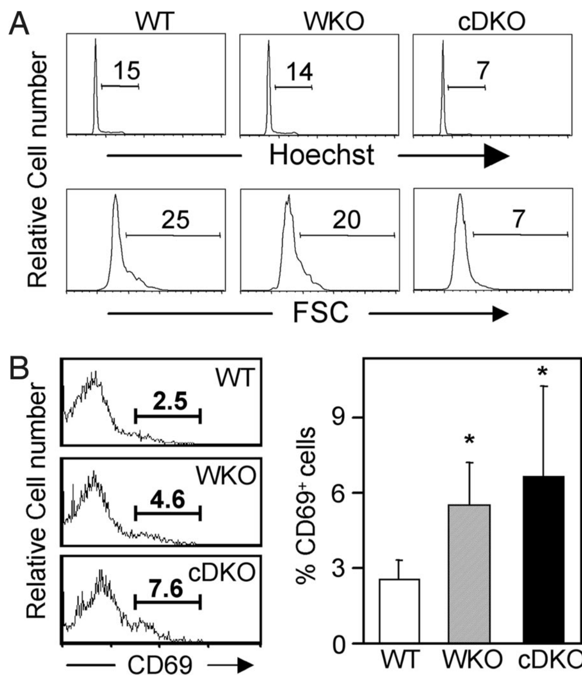

Fig. 3. cDKO DN3 thymocytes fail to progress through the cell cycle. (*A*) cDKO DN3 subset shows decreased frequency of cycling cells (S + G2/M), as determined by DNA content analysis from Hoechst fluorescence, and also lower relative number of large cells, as determined by forward scatter analysis. (*B*) WKO and cDKO DP subset shows increased percentage of $CD69^+$ cells. Displayed are representative FACS histograms (*Left*) and the summary graph (*Right*; average ± SD from 11 to 14 mice per group from six experiments). *, $P < 0.01$ compared with WT.

NWKO, and cDKO mice, whereas overall thymocyte numbers in cDKO mice were markedly reduced (Fig. 2 *A* and *B*). The decreased cellularity cannot be explained by an increase in cell death, because apoptotic cell numbers in cDKO thymus were indistinguishable from WKO and WT thymus (SI Fig. 9*B*). Nonetheless, rapid clearance of apoptotic cells cannot be ruled out. Decreased thymic cellularity in cDKO mice may be related to impaired cell cycle progression in early developing T cells. In this regard, we found a lower number of large and cycling cells in cDKO DN3 (Fig. 3*A* and SI Fig. 9*A*) and, to a lesser degree, DN4 cells. Furthermore, cDKO DN cells showed consistent increased skewing of the DN3-to-DN4 ratio (WT = 0.74 ± 0.45; WKO = 1.49 ± 0.90; cDKO = 3.18 ± 1.89; n = 9–11 mice per group; $P < 0.05$ comparing cDKO with WKO and WT groups; Fig. 2*B*), indicating a more significant deficiency in the developmental progression of DN3 cells. Despite this cycling deficiency, we found that the expression of intracellular TCR β chain in DN3 cells from WT, WKO, and cDKO mice was not significantly different (data not shown).

Thus, impairment in cell cycle progression at DN-to-DP transition with marked reduction in overall thymocyte numbers indicates a specific role for WASP and N-WASP in early thymocyte development.

Accumulation of Mature SP Cells in cDKO Thymus. Because TCR engagement at the DP stage is critical for thymocyte selection, we sought to determine whether TCR activation events were intact at this stage. Signaling at this stage is associated with increased surface expression of the TCR, expression of CD69, and release of calcium from intracellular stores (31). Calcium fluxes after TCR cross-linking occurred with the same kinetics and amplitude in WT, WKO, and cDKO thymocytes (SI Fig. 9*C*). There was also appropriate up-regulation of TCRβ and CD69 (Fig. 3*B* and data not shown); in fact, there were greater percentages of $CD69^{hi}$-expressing DP cells in cDKO and WKO mice when compared with WT mice. Together, these results indicate that cDKO thymocytes can receive activation signals through the TCR at the DP stage. In contrast to higher numbers of $CD69^{hi}$ DP cells in cDKO mice, the number of $CD69^{hi}$-expressing $CD4^+$ and $CD8^+$ SP thymocytes was markedly reduced when compared with both WKO and WT SP cells (Fig. 4*A* and data not shown). Further analysis of the maturation status within cDKO SP thymocytes demonstrated an increased percentage of $CD69^{low}CD24^{low}$ cells (Fig. 4*A*), a phenotype compatible with fully mature thymocytes. However, the majority of SP cells were $CD62L^{low}$ (Fig. 4*A*), a phenotype defining less-mature thymocytes.

Defective Spreading and Migration in cDKO SP Thymocytes. To investigate whether the combined deletion of WASP and N-WASP perturbs thymocyte activation at postselective stages of T cell development, we determined the ability of SP thymocytes to proliferate in response to antigen receptor-induced signaling. As described for total WKO thymocytes (22), WKO and cDKO SP cells failed to proliferate upon CD3 stimulation (Fig. 4*B*). Because WKO and cDKO SP cells were similarly defective, other WASP/N-WASP-dependent signaling events must be responsible for the marked defect in thymocyte development seen only in cDKO mice. Because WASP family members play a critical role for cytoskeletal reorganization and correct trafficking of immune cells, including T cells (19), we hypothesized that the developmental defects in cDKO mice correlated with more profound defects in regulation of the actin cytoskeleton. To model the contact area between a T cell and an antigen-presenting cell, TCR^{high} CD4 or TCR^{high} CD8 SP cells were incubated on surfaces coated with antibodies to CD3 and CD28. The TCR-induced and actin-dependent spreading response of both WKO and cDKO TCR^{high} CD4 or TCR^{high} CD8 SP thymocytes was significantly reduced as compared with WT cells (Fig. 4*C*). This defect was not related to differences in TCR or CD28 expression, because WT, WKO, and cDKO SP thymocytes show similar surface levels of these molecules (data not shown).

We also examined the migratory response of SP thymocytes to CCL19, a chemokine identified to guide thymocytes into the medulla and out of the thymus (32, 33). Using an *in vitro* Transwell chemotaxis chamber assay, WKO SP thymocytes showed reduced migratory response as compared with WT SP cells (Fig. 4*D*). Importantly, this defect was even more pronounced in cDKO SP cells. Because migratory abnormalities may also be associated with the developmental defects notable at the DN-to-DP transition, we evaluated the ability of cDKO DN thymocytes to migrate in response to CXCL12, which is chemotactic for immature thymocytes. Similar to SP cells, DN cDKO were more defective in migration than DN WKO or DN WT thymocytes (Fig. 4*E*). Together, these results suggest that the thymocyte developmental defects associated with N-WASP and WASP double deficiency occur as a result of the coupling of TCR-induced proliferative defects, aberrant cytoskeletal reorganization, and impaired migration.

Peripheral T Cells of cDKO Mice Are Reduced in Number and Associated with the Early Onset of Colitis. We expected that the decreased number of thymocytes in cDKO mice would be associated with decreased numbers of peripheral T cells. Indeed, similar to our data on chimeric mice (Fig. 1*B*), the numbers of $CD4^+$ and $CD8^+$ T lymphocytes were significantly reduced in cDKO mice when compared with both WKO and WT mice (Fig. 5*A*). As noted for cDKO SP thymocytes, peripheral T cells from cDKO mice proliferated poorly to antigen receptor stimulation (Fig. 5*B*), spread at much lower rates (Fig. 5*C*), and had lower migratory responses as compared with WT T cells (Fig. 5*D*). Furthermore,

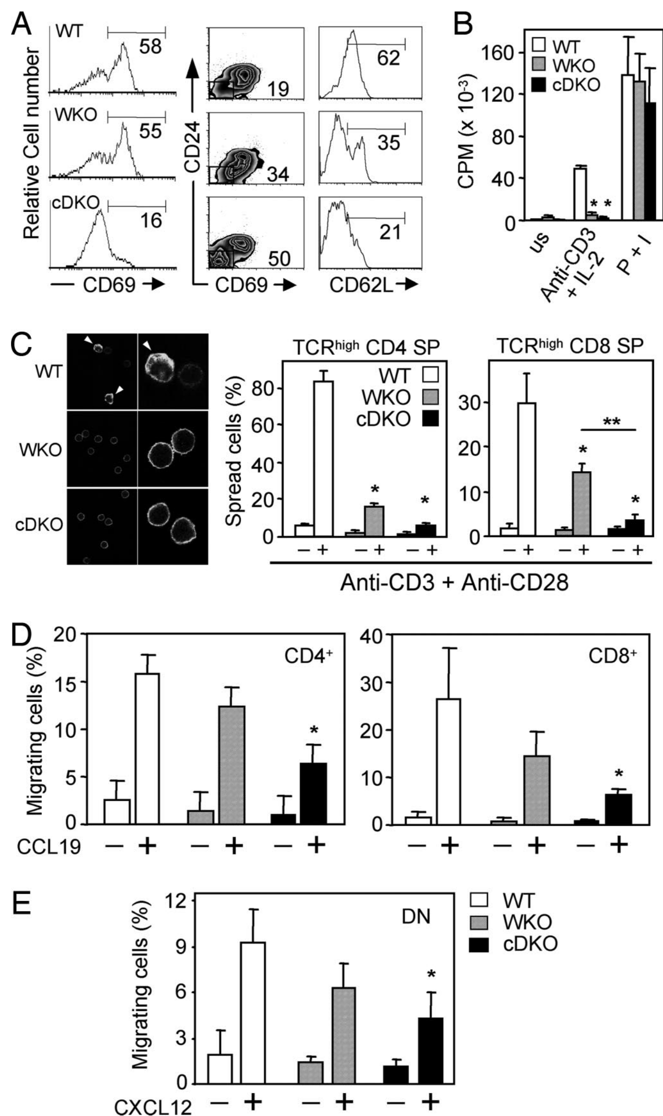

Fig. 4. Reduced CD69 up-regulation, proliferation, spreading, and migration in cDKO SP thymocytes. (*A*) Representative FACS profiles show decreased CD69 and CD62L expression and increased percentage of $CD69^{low}CD24^{low}$ subset within cDKO $CD4^+$ SP thymocytes. (*B*) Proliferative response to anti-CD3 plus IL-2 stimulation is similarly reduced in WKO and cDKO SP thymocytes. Bars are representative mean values of cpm ([^{3}H]thymidine) ± SD of triplicate wells from one of three independent experiments. *, $P < 0.01$ compared with WT. (*C*) Antigen-receptor-induced spreading is markedly decreased in both WKO and cDKO SP thymocytes. (*Left*) Phalloidin staining [×40 (*Top*) and ×100 (*Bottom*)] depicts the formation of large sheet-like lamellipodia of spread WT SP thymocytes (white arrowheads), which are absent in the representative fields from WKO and cDKO cells. (*Right*) Graphs show the average of relative numbers of spread TCR^{high} CD4 and TCR^{high} CD8 SP thymocytes (±SD) representative of two experiments. *, $P < 0.01$ compared with WT. (*D*) CCL19-driven migratory activity of cDKO SP thymocytes is reduced. The percentage of migrating cells is shown as mean values (±SD) from at least two experiments (n = 3–4 mice per group). *, $P < 0.05$ when compared with WT. (*E*) CXCL12-driven transmigration of cDKO DN cells is reduced. The percentage of migrating cells is shown as mean values (±SD) from two experiments (n = 3–4 mice per group). *, $P < 0.05$ compared with WT.

$CD4^+$ T cells from cDKO mice were predominantly $CD44^+CD62L^-$, a phenotype of effector/memory cells (SI Fig. 10*A*). Recently, we identified a reduction in the number and function of naturally occurring regulatory T cells ($CD4^+CD25^+$ $FoxP3^+$) in WKO mice (34). In cDKO mice, we observed a similar defect when compared with WT mice [percent $CD25^+$ cells in $CD4^+$ SP thymocytes: cDKO, 2.25 ± 1.21 (n = 6); WKO, 2.59 ± 0.38 (n = 4); WT, 5.81 ± 0.53 (n = 4)]. Because the total numbers of $CD4^+$ SP thymocytes are markedly reduced in cDKO, the absolute numbers of nTregs in cDKO mice were dramatically reduced in comparison with WT and WKO (data not shown). Interestingly, these aberrant numbers of effector and regulatory T cells were associated with more severe earlier onset of colitis in cDKO mice when compared with WKO mice, with the majority of animals developing clinical (SI Fig. 10*B*) and histopathologic (Fig. 5*E*) signs of colitis by 2 months of age.

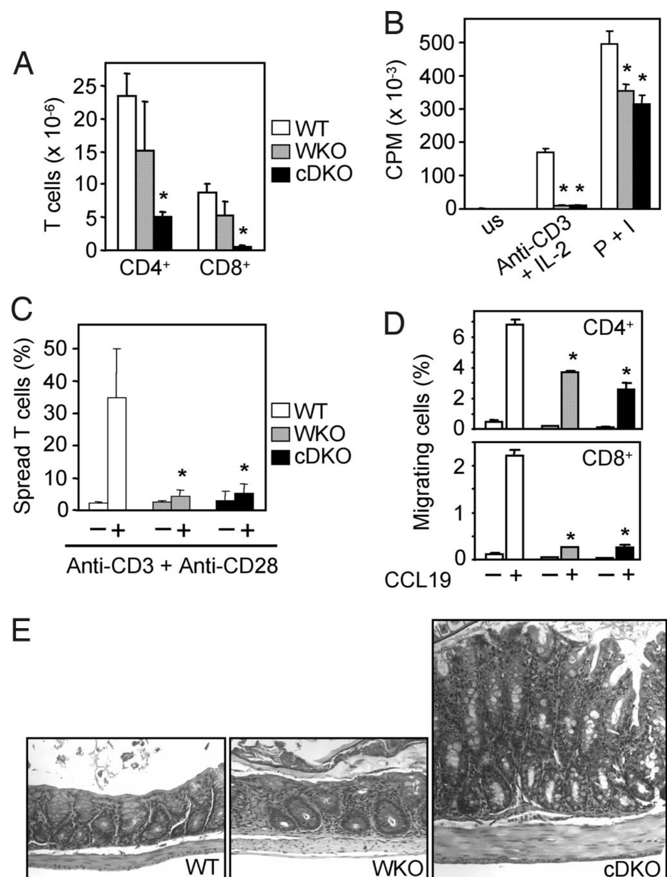

Fig. 5. Reduced number and activation of T cells in the periphery and early onset of colitis in cDKO mice. (*A*) $CD4^+$ and $CD8^+$ T cell numbers in spleens of cDKO mice are significantly reduced. Bars represent average of absolute lymphocyte numbers (±SD) (n = 4–6 mice per group). *, $P < 0.01$ compared with WT and WKO. (*B*) T cells purified from lymph nodes of WKO and cDKO mice show marked decrease in proliferative response to anti-CD3 plus IL-2 stimulation. Bars indicate mean values of cpm ([^{3}H]thymidine) ± SD of triplicate wells from one of four experiments. *, $P < 0.01$ compared with WT. (*C*) Antigen-receptor-induced spreading is markedly decreased in WKO and cDKO peripheral T cells. Bar graphs show the average of relative numbers of spread T cells from triplicate samples (±SD) representative of three experiments. *, $P < 0.01$ compared with WT. (*D*) CCL19-induced chemotaxis of peripheral T cells is significantly diminished in both WKO and cDKO mice. Data are shown as average ± SD of triplicates from one of two experiments. (*E*) Histological analyses of distal colons show signs of severe inflammation with crypt elongation and mucosal thickening in samples from cDKO mice. Age-matched (2 months) WT and WKO mice show no sign of colitis in this representative experiment.

Discussion

In this study, we sought to determine the unique and redundant functions of WASP and N-WASP in T cell development. Because WASP plays a critical role in peripheral T cell activation and cytoskeletal regulation, it seemed somewhat surprising to us that

T cell development is relatively normal in WAS patients and in WASP-deficient mice. We hypothesized that N-WASP might play redundant roles with WASP in some of the signaling events required for T cell development.

We have deleted WASP and N-WASP in T cells by using two different gene-targeting approaches, RAG-2-deficient blastocyst complementation assay and conditional deletion by Cre recombinase. Deletion of WASP and N-WASP severely compromised T cell development with reduced numbers of thymocytes and peripheral T cells. In contrast, T cell numbers were similar to normal in WKO and NWKO mice, suggesting that WASP and N-WASP have overlapping functions during T cell development. We found slight differences in results using the two approaches for deletion. The RAG-2 chimeric animals showed a block in progression from the DN3-to-DN4 stage. Our data with the conditional deletion approach also showed a relative block in the DN3-to-DN4 transition, but the defect was less pronounced. Although we failed to detect N-WASP by Western blotting of total thymocytes in cDKO mice, some residual N-WASP expression might be present at the DN3 and DN4 stage, because the lck promoter seems to drive homogeneous and higher expression of Cre recombinase at the DN3 stage (30). Thus, whereas deletion of N-WASP occurs within the thymus in cDKO mice, WASP and N-WASP are deleted in lymphoid progenitors before their arrival in the thymus in the RAG-2 chimeric mice. Notably, WASP-deficient bone marrow-derived hematopoietic stem cells were shown to have defective chemotaxis and homing properties (35).

Our study suggests a complex regulation of T cell maturation by WASP and N-WASP. Despite marked hypocellularity and a partial block in progression at the DN stage in cDKO mice, the existing DP cells responded to TCR activation, as determined by expression of CD69, TCRβ, and calcium fluxes. In marked contrast, cDKO SP cells exhibited pronounced deficiency in TCR-triggered proliferation, spreading, and chemokine-driven migration. These results support a fundamental role for WASP and N-WASP for correct overall T cell development, whereas their activity may be overlapping with other proteins for some of the receptor-mediated signaling events at different stages during development. In this context, many functional activities are quite distinct during the stages of DP and SP cells. For example, PKC-θ is crucial for mature T cell activation but dispensable for TCR-dependent thymocyte development (36). Moreover, DP thymocytes seem to form an immunological synapse pattern distinct from that of mature T cells (37). Single-positive cells respond to TCR triggering by spreading, but freshly isolated DP cells fail to polarize actin and spread (38). Likewise, the migratory properties of cortical thymocytes change dramatically upon TCR engagement, changing from random and slow migration patterns to rapid and inwardly directed to the medulla (39).

Although WKO thymocytes have defective TCR-induced proliferative response and spreading, thymocytes undergo fairly normal development and maturation (20, 22). Thymocytes from cDKO mice also have abolished proliferation and spreading, but in addition have more pronounced defects in migratory responses and development. Our data are consistent with recent data demonstrating that the thymocyte expression of a verprolin homology, cofilin homology, and acidic (VCA) domain-deleted WASP resulted in a severe block in T cell development with defective DN3 progression and impaired positive selection (40). However, in addition to reduced cell cycle progression at DN-to-DP transition in cDKO thymus, we also observed an accumulation of CD69^{low}CD24^{low} and CD62L^{low} SP cells that might indicate retention of mature cells with reduced migratory function (41). One potential unifying hypothesis is that deletion of both WASP and N-WASP results in defective intrathymic migration, preventing cells from reaching specific locations to receive appropriate stimulating signals and to egress from the thymus (42).

The importance of cytoskeletal polarization and remodeling for lymphocyte activation has been highlighted (7, 43). Molecules that regulate the actin cytoskeleton upstream of WASP family members have been implicated as critical for thymocyte development. Mice lacking Vav-1, a guanine exchanging factor for Rho GTPases, share many features of WASP/N-WASP double-deleted mice by having thymic hypocellularity associated with a block at the DN3 stage of development (44). Likewise, cells that escape the DN3 block show inefficient selection, resulting in reduced numbers of peripheral T cells. Similar to our findings on cDKO SP cells, T cells from Vav-1-deficient mice have defective receptor-induced actin polymerization (45–47). Interestingly, Vav-1 deficiency can be largely corrected by expression of activated Rac-1 (48), and Rac activation of actin rearrangements is mediated by WAVE proteins, molecules closely related to WASP and N-WASP (49). Together with our results, these studies provide compelling evidence for the importance of regulated actin remodeling for correct thymopoiesis.

Materials and Methods

See also *SI Text*.

Animal Handling. Mice were housed at Children's Hospital and Massachusetts General Hospital under specific pathogen-free conditions. Animal experiments were carried out after approval and in accordance with guidelines from the Subcommittee on Research Animal Care of Massachusetts General Hospital.

Proliferation Analysis. Purified T cells (1×10^5 per well) were added to 96-well tissue culture plates and cultured in RPMI medium 1640, supplemented with 2 mM L-glutamine/1 mM sodium pyruvate/100 units/ml penicillin/streptomycin/50 μM 2-mercapthoethanol/10% FCS. Murine IL-2 (5 ng/ml; BD Biosciences, San Jose, CA) was added to the wells where anti-CD3ε stimulating antibody (10 μg/ml; clone 145-2C11; BD Biosciences) was previously allowed to bind (1 h at 37°C). To assess receptor-independent activation, cells were stimulated with phorbol-myristate-acetate and ionomycin (Sigma–Aldrich, St. Louis, MO) at 10 ng/ml and 0.5 μM, respectively. T cells were cultured for 56–60 h and pulsed with 1 μCi [^{3}H]thymidine (1 Ci = 37 GBq) for an additional 12–16 h, harvested, and scintillation counted.

Migration Assay. The migration of thymocytes to CCL19 and CXCL12 (R&D Systems, Minneapolis, MN) was determined in a Transwell chemotaxis chamber (5 μm; Corning, Lowell, MA). Total thymocytes (5×10^4) were diluted in RPMI medium 1640 supplemented with 0.5% BSA and added to the upper chamber; 25 nM CCL19 or 20 nM CXCL12 was added to the lower chamber. Cells that migrated after 1.5 h at 37°C were collected and counted by flow cytometry with unlabeled caliBRITE beads (BD Biosciences) as reference counting. Cells were labeled with anti-CD4 and -CD8 to determine the percentage of DP and SP cells.

Thymocyte-Spreading Assay. To determine the spreading response of TCR^{high} CD4 and TCR^{high} CD8 SP cells (isolated by FACS) stimulating antibodies to CD3 and CD28 (clones 145-2C11 and 37.51, respectively, both at 10 μg/ml; BD Biosciences) were allowed to bind for 10 min at 37°C onto glass slides (precoated with poly-L-lysine). Cells were fixed with 3.8% paraformaldehyde for 10 min at room temperature (RT) and permeabilized with PBS/0.1% Triton X-100 for 5 min at RT. Actin was stained with AlexaFluor 488-conjugated phalloidin (Molecular Probes/Invitrogen, Carlsbad, CA) for 30 min at RT. Spread cells were defined as having a flattened appearance and formation of

actin-rich broad lamellipodia as compared with nonspread round cells. The percentage of spread cells of the total number of cells was calculated by counting 300–400 cells per condition in triplicate or quadruplicate at ×100 magnification.

Statistical Analyses. Statistical analyses were performed by using raw data or arcsin-transformed percentage data with the GraphPad Prism program (ver. 4.00, GraphPad, San Diego, CA). Comparison among all groups was done by one-way ANOVA or the Kruskal–Wallis test, and differences among group pairs were determined by unpaired Student's *t* test or the Mann–Whitney test. Differences were considered significant when $P < 0.05$.

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