Wiskott-Aldrich Syndrome Protein Is Required for Homeostasis and Function of Invariant NKT Cells

Alexander Astrakhan, Hans D. Ochs and David J. Rawlings

*J Immunol* 2009; 182:7370-7380; doi: 10.4049/jimmunol.0804256

http://www.jimmunol.org/content/182/12/7370

References

This article cites 50 articles, 33 of which you can access for free at:
http://www.jimmunol.org/content/182/12/7370.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Wiskott-Aldrich Syndrome Protein Is Required for Homeostasis and Function of Invariant NKT Cells

Alexander Astrakhan,* Hans D. Ochs, † and David J. Rawlings2*†

NKT cells comprise a separate T lineage expressing semi-invariant T cell receptors. Canonical invariant NKT (iNKT) cells specifically recognize lipid Ags presented by CD1d, a MHC class I-like molecule. iNKT cells function, in part, as initial responders to bacterial infection and play a role in immune surveillance and tumor rejection. The Wiskott-Aldrich Syndrome protein (WASP) serves as a crucial link between cellular stimuli and cytoskeletal rearrangements. Although we and others have identified a key role for WASp in homeostasis of T-regulatory and marginal zone B cells, little data exist regarding the role for WASp within the iNKT lineage. Analysis of WASp-expressing cell populations in heterozygous female WASp mice revealed a substantial selective advantage for WASp+/− vs WASp−/− iNKT cells. Although adult WASp-deficient (WASp−/−) mice had normal thymic and bone marrow iNKT numbers, we observed 2- to 3-fold reduction in the numbers of iNKT cells in the spleen and liver. This peripheral iNKT deficit is manifested, in part, due to defective iNKT homeostasis. WASp−/− iNKT cells exhibited reduced levels of integrin surface expression and decreased homing and/or retention within peripheral tissues in a competitive repopulation model. In addition, analysis of young mice showed that WASp is important for both maturation and egress of thymic iNKT cells. WASp−/− iNKT cells also exhibited a marked reduction in Ag-induced proliferation and cytokine production. Our findings highlight the crucial role for WASp in iNKT development, homeostasis, and activation, and identify iNKT dysfunction as an additional factor likely to contribute to the clinical features observed in WAS patients. The Journal of Immunology, 2009, 182: 7370–7380.

Cross-talk between the adaptive and the innate immune systems is critical for mounting an effective immune response to most infectious challenges. Within the adaptive system, B and T lymphocytes uniquely express a rearranged Ag receptor required for generation of long-term immune memory. Distinct subsets of both B and T cells, however, also exhibit characteristics typically associated with innate cells. Within the B cell lineage, marginal zone (MZ)3 and B-1 B cells recognize Ag characteristics typically associated with innate cells. Biological editors, Starkey, and Dragging. 2010. Published by Elsevier B.V.

1 Department of Immunology and 2 Department of Pediatrics, University of Washington School of Medicine and Seattle Children’s Research Institute, Seattle, WA 98101

Received for publication December 18, 2008. Accepted for publication April 7, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a grant from the National Institutes of Health (AI071163; DJR). A.A. is supported by the Cell and Molecular Biology Training Grant (T32GM007270) and the Molecular Medicine fellowship.

2 Address correspondence and reprint requests to Dr. David J. Rawlings, Seattle Children’s Research Institute, 1900 Ninth Avenue, Seattle, WA 98101. E-mail address: drawling@u.washington.edu

3 Abbreviations used in this paper: MZ, marginal zone; WASp, Wiskott-Aldrich Syndrome protein; iNKT, invariant NKT; αGalCer, α-galactosylceramide; Treg, regulatory T cell; WT, wild type; HSC, hematopoietic stem cell; S1P, sphingosine-1-phosphate; LN, lymph node; DC, dendritic cell; MFI, median fluorescence intensity.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0804256
we show that WASp−/− NKT cells fail to compete effectively with WASp+/− NKT cells in vivo. Further, WASp−/− mice exhibit defective development and egress of thymic NKT cells and express lower levels of CD1d1 integrin. Combined, these defects result in significantly reduced numbers of NKT cells within the spleen and the liver of adult mice. WASp+/− NKT cells also display a marked deficit in Ag-induced proliferation and cytokine secretion. Finally, using adoptive transfer studies we show that WASp−/− NKT cells exhibit normal homeostatic proliferation, yet exhibit defects in homing and/or retention within effector tissues. Taken together, these data demonstrate that WASp plays a key role in both NKT homeostasis and function and suggest that impaired NKT function may contribute to the global immune dysregulation observed in WAS patients.

Materials and Methods

**Mice and tissues**

Breeding colonies for wild-type (WT) (C57BL/6) and C57BL/6-WASp−/− (18), and Rag2−/− mice were maintained within the SPF animal facility of Seattle Children’s Research Institute as previously described (4). Animals were analyzed at various ages as noted in results and all studies were conducted according to the guidelines of Seattle Children’s Institutional Animal Care and Use Committee.

Single cell thymus and spleen suspensions were obtained by dissociating tissues with frosted glass slides. For bone marrow (BM) isolation, femur and tibia from a single leg were flushed out using a 25-gauge needle and broken up into a single cell suspension with a 22-gauge needle. Fetal thymic lobes were isolated and digested in 0.1% collagenase Type IV (Worthington), 100 U/ml heparin, and 100 U/ml DNase I (Invitrogen) for 40–60 min at 37°C, filtered through a 40 μm cell strainer, and washed by centrifugation.

**Flow cytometry**

Intracellular staining for WASp was performed as previously described (4) using a peptide-purified rabbit anti-WASP polyclonal Ab (A6053) (25). For surface staining, single cell suspensions were incubated with fluorescently labeled Abs for 15 min at 4°C in staining buffer (PBS w/0.5% BSA). Data were collected on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar). The following Abs were used for staining: CD45.1-PE/Cy7 (clone A20), CD45.2-allophycocyanin (104), CD3-PeCy7 (145-2C11), cd1d-tetramer ( miracles, monocytes, and DCs derived from either the BM or spleen (data not shown), indicating that WASp+ myeloid cells lack a selective advantage despite the documented role for WASp in myeloid migration (14).

We used NK.1.1 and CD3 to analyze selective advantage within the NK and the NKT subsets (Fig. 1A). We observed a selective advantage for WASp−/− NK cells compared with the HSC compartment (Fig. 1, A and B), while NK.1.1+ CD3− NKT cells exhibited an even greater selective advantage, especially within the spleen and liver (Fig. 1B). Because NK.1.1 is expressed by both classical and nonclassical NKT cells, we next used CD1d tetramers loaded with αGalCer to specifically identify invariant NKT cells (11). Within the thymus, tetramer+ NKT cells progress from an immature CD24− to a mature CD24+CD44+ stage of development (27). Similar to HSCs, ~50–55% of thymic CD24+ NKT cells were WASp−, suggesting an initial role for WASp during maturation of thymic NKT cells (Fig. 1C, left). Compared with the thymus, we observed an even more significant selection of CD24+ WASp− NKT cells within the spleen and liver (with an average of 83% WASp− cells in spleen, and 86% in liver). In contrast, we did not observe a difference in selection between the thymus and the BM (Fig. 1C, right). These findings suggested that WASp might be involved in late thymic development as well as access and/or retention of NKT cells in peripheral tissues.

Although female heterozygous carriers represent a useful model to study selection of WASp−/− cells, random X-inactivation might be skewed by lineage extrinsic abnormalities and/or other developmental defects. Therefore, we also used a mixed BM chimera model to evaluate the selection for WASp−/− cells. We used a relatively low WT:WASp−/− cell ratio (1:9) to permit detection of any selective advantage. BM from WT and WASp−/− was transplanted into lethally irradiated WASp−/− animals and recipient mice were analyzed at 5 mo posttransplant. Consistent with the analysis of heterozygous females, we observed no selective advantage for WASp expressing HSC, myeloid or DCs. WASp expression was maintained at the input level of ~5–10% in each of these populations (Fig. 1D and data not shown). In contrast, analysis of NK subsets revealed a progressive increase in the

**CFSE proliferation assay**

Splenocytes were resuspended at 4 × 10^6 cells/ml in 200 μl of complete RPMI 1640 medium and stimulated with αGalCer or PMA/ionomycin. Brefeldin A (final: 1 μg/ml) was added 45 min after start of incubation. After 5–6 h, cells were spun down, stained with CD1d tetramer, fixed, and permeabilized using cytotox/cytperm (BD Biosciences) and stained with Abs against CD4, IL-4, and IFN-γ.

**Results**

WASP−/− NKT cells exhibit a selective advantage in vivo

To assess the role for WASp in NKT development and homeostasis, we initially evaluated selection in WASp+/− heterozygote females using a flow cytometry based assay (4). This approach, based upon assessment of inactivation of the X-chromosome that contains the WAS gene, permits identification of developmental stage(s) where WASp+ cells manifest a selective advantage. As shown in Fig. 1A, hematopoietic stem cells (HSC; Lin−cKit+ ScI−) exhibit a random X-inactivation pattern with ~50% of cells expressing WASp+. This pattern of WASp expression remained unaltered in neutrophils, monocytes, and DCs derived from either the BM or spleen (data not shown), indicating that WASp+ myeloid cells lack a selective advantage despite the documented role for WASp in myeloid migration (14).

We used NK.1.1 and CD3 to analyze selective advantage within the NK and the NKT subsets (Fig. 1A). We observed a selective advantage for WASp−/− NK cells compared with the HSC compartment (Fig. 1, A and B), while NK.1.1+ CD3− NKT cells exhibited an even greater selective advantage, especially within the spleen and liver (Fig. 1B). Because NK.1.1 is expressed by both classical and nonclassical NKT cells, we next used CD1d tetramers loaded with αGalCer to specifically identify invariant NKT cells (11). Within the thymus, tetramer+ NKT cells progress from an immature CD24− to a mature CD24+CD44+ stage of development (27). Similar to HSCs, ~50–55% of thymic CD24+ NKT cells (~70%) were WASp−, suggesting an initial role for WASp during maturation of thymic NKT cells (Fig. 1C, left). Compared with the thymus, we observed an even more significant selection of CD24+ WASp− NKT cells within the spleen and liver (with an average of 83% WASp− cells in spleen, and 86% in liver). In contrast, we did not observe a difference in selection between the thymus and the BM (Fig. 1C, right). These findings suggested that WASp might be involved in late thymic development as well as access and/or retention of NKT cells in peripheral tissues.

Although female heterozygous carriers represent a useful model to study selection of WASp−/− cells, random X-inactivation might be skewed by lineage extrinsic abnormalities and/or other developmental defects. Therefore, we also used a mixed BM chimera model to evaluate the selection for WASp−/− cells. We used a relatively low WT:WASp−/− cell ratio (1:9) to permit detection of any selective advantage. BM from WT and WASp−/− was transplanted into lethally irradiated WASp−/− animals and recipient mice were analyzed at 5 mo posttransplant. Consistent with the analysis of heterozygous females, we observed no selective advantage for WASp expressing HSC, myeloid or DCs. WASp expression was maintained at the input level of ~5–10% in each of these populations (Fig. 1D and data not shown). In contrast, analysis of NK subsets revealed a progressive increase in the

**CFSE proliferation assay**

Splenocytes were washed 2× in PBS and resuspended at 5 × 10^6 cells/ml with 250 nM CFSE (Invitrogen). Cells were incubated for 8–9 min at 37°C, shaking periodically, and washed with complete RPMI 1640 medium. Cells were washed 3× with complete medium and incubated at 1 × 10^6 cells/ml in complete medium supplemented with αGalCer (1–100 ng/ml), IL-15 (100 ng/ml), or PMA (10 ng/ml)/ionomycin (500 ng/ml). Cells were stained and analyzed at 72 or 96 h poststimulation.

**Cell transfer experiments**

Total BM suspensions from WT (CD45.2+) and WASp−/− (CD45.1+) mice were mixed at a ratio of 1:9 and transferred into irradiated (1050 rad) WASp−/− recipients at a dose of 5 × 10^6 cells/recipient. For iNKT
percentage of WASp<sup>+</sup> cells in the spleen and liver compared with the BM. Even stronger skewing was again observed within the NKT lineage with an increased proportion of WASp<sup>+</sup> CD3<sup>+</sup> NK1.1<sup>+</sup> NKT cells in all tissues (Fig. 1, D and E). Nearly identical data were obtained using congenically marked BM (comprised of a 1:9 mixture of CD45.1<sup>+</sup> WT and CD45.2<sup>+</sup> WASp<sup>+</sup> cells) transplanted into WASp<sup>−/−</sup> recipients (data not shown). Combined, these observations further supported the interpretation that WASp-dependent function(s) impact both late thymic iNKT cell development and peripheral iNKT cells homeostasis.

Reduced peripheral iNKT numbers in WASp<sup>−/−</sup> mice

We next analyzed WASp<sup>−/−</sup> mice to directly assess the consequences of WASp deficiency on iNKT development and function.

We analyzed tissues from 8 to 14 wk old WT and WASp<sup>−/−</sup> mice using costaining with CD1d tetramers, CD3, CD24, NK1.1 and CD44 (Fig. 2A). The absolute number of immature CD24<sup>+</sup> iNKT cells within the thymus did not differ significantly between WT and WASp<sup>−/−</sup> mice (Fig. 2B). Although there was a trend for an increase in CD24<sup>+</sup> iNKT cell numbers in WASp<sup>−/−</sup> mice, this difference did not reach statistical significance in adult animals. In addition, we observed normal numbers of mature CD24<sup>+</sup> CD44<sup>+</sup> iNKT cells within both the thymus and BM, indicating that early iNKT development in adult animals was not significantly perturbed by WASp-deficiency in the absence of competing WT cells (Fig. 2, A and C). Consistent with these findings, we also observed similar levels of CD1d expression on DP thymocytes from WT and WASp<sup>−/−</sup> mice (data not shown).
Despite normal numbers of thymic iNKT cells in adult WASp−/− animals, analysis of peripheral tissues revealed a significant decrease in total iNKT cells in the spleen and liver (Fig. 2, A and C). iNKT cells were reduced by at least 2-fold in the spleen and >3-fold in the liver of WASp−/− animals. The mature iNKT compartment can be further divided into CD4+ and CD4− subsets, which diverge at an early developmental stage and exhibit unique functional activities (28). Both CD4+ and CD4− iNKT subsets were decreased in the spleen and liver of WASp−/− animals, with CD4+ iNKT cells showing the greatest numerical decline (>3-fold reduction relative to WT mice, Fig. 2C; bottom panels). The observed reduction in peripheral iNKT numbers did not appear to reflect a maturation defect, as expression of developmental markers including both NK1.1 and CD44 were not altered in WASp−/− iNKT cells (Fig. 2D).

Defective generation of thymic iNKT cells in young WASp−/− mice

The initial burst of iNKT expansion occurs at ~2–3 wk post birth, starting in the thymus and culminating in the periphery (29). Immature CD24+ iNKT cells develop into mature CD24− CD44+ cells that subsequently progress from an NK1.1− to an NK1.1+ state.

**FIGURE 2.** WASp−/− mice generate normal thymic iNKT cell numbers but exhibit a deficit in peripheral iNKT cells. A, Representative FACS plot showing the gating strategy used to identify iNKT cell subsets in various tissues in WT vs WASp−/− mice. B, WASp−/− mice exhibit normal numbers of immature thymic iNKT cells. C, Reduced mature iNKT numbers in WASp−/− spleen and the liver. Error bars represent data from three independent experiments using six, 8- to 12-wk-old animals/experiment. D, Representative FACS data showing normal expression of CD44 and NK1.1 on splenic and thymic iNKT cells. At least ten animals were examined per genotype. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
developmental stage within both the thymus and periphery (29). Interestingly, analysis of 15–16 day old mice using Abs directed against CD44, CD3, CD24, CD4, and NK1.1 revealed significantly reduced numbers of mature CD24− iNKT cells within the thymus of young WASp−/− animals (Fig. 3A). This change impacted all subsequent stages of mature iNKT development as identified by CD44/NK1.1/CD4 staining (data not shown). In contrast, thymic CD24+ immature iNKT cell numbers were significantly increased in WASp−/− mice suggesting that WASp activity was important for the proliferative expansion of iNKT cells at the CD24+ to CD24− transition (Fig. 3A, left). Similar to 6–12 wk old animals, iNKT cell numbers were also reduced within the spleen of young animals (Fig. 3A). Although total iNKT numbers in the liver were also reduced, these data did not reach statistical significance likely reflecting the much smaller number of iNKT cells present in liver at this age and limited numbers of animals analyzed. Similar to older WASp−/− animals, CD4+ iNKT cell numbers were significantly reduced within the liver (data not shown). These findings, in association with our observations in heterozygous female carriers, suggest a role for WASp in the proliferative expansion of immature thymic iNKT cells.

Partial correction of peripheral iNKT composition in aged WASp−/− mice

We next analyzed tissues from older animals to determine whether peripheral iNKT numbers in WASp−/− mice might normalize over time. Six-month-old mice had equivalent numbers of iNKT cells within the spleen and the BM, yet exhibited reduced iNKT numbers within the thymus (Fig. 3B). The reduction in the thymic iNKT compartment is consistent with an overall reduction in thymic cellularity observed in aged WASp−/− animals (data not shown). Similar to 8- to 12-wk-old mice, we observed reduced numbers of iNKT cells within the liver; however, this difference did not reach statistical significance perhaps due to small sample size. Similar results were also obtained in 1-year-old mice (data not shown). Combined with findings from 2-wk-old mice, our observations suggest that a reduced rate of iNKT production in WASp−/− mice results in lower numbers of splenic iNKT cells that are partially rescued over time.

Additionally, we determined whether WT vs WASp−/− mice exhibited any differences in cell surface phenotype within the mature iNKT cell compartment. Mature iNKT cells express a variety of memory-associated cell surface markers, including CD69 and CD122 (IL-2Rα) (30). Although WT and WASp−/− splenic and liver iNKT cells expressed equivalent levels of CD122 (data not shown), we observed a significant up-regulation of CD69 expression on WASp−/− iNKT cells derived from BM, spleen, and liver of 6-mo-old mice (Fig. 3C). Higher levels of CD69 expression were also observed in liver iNKT cells from 15-day-old mice (data not shown). This increase in CD69 expression was cell intrinsic, as it was also observed in WASp−/− iNKT cells in heterozygous WASp carriers (data not shown). Because WASp-deficient iNKT cells expressed WT-like levels of NK1.1, CD44, and CD122, the overall iNKT maturation status appeared to be relatively unperturbed in WASp−/− mice. Rather, we anticipate that increased CD69 expression in WASp−/− cells reflect the outcome of reduced signaling through the sphingosine-1-phosphate (S1P) receptor. Binding of S1P to S1P-receptors regulates iNKT thymic egress.
and CD69 serves as a negative regulator of S1P-mediated signaling (32). Higher CD69 expression levels may therefore correlate with reduced S1P responsiveness and inefficient thymic egress of WASp−/−/H11002/NKT cells, as previously shown for WASp-deficient marginal zone B cells (4, 5).

We also considered the possibility that the reduced number of iNKT cells in spleen and peripheral tissues might be due to sequestration of iNKT cells within the lymph nodes (LN). As previously reported (14), WASp-deficiency is associated with moderate lymphadenopathy, and this correlated with a modest increase in the number of iNKT cells within WASp−/−/H11002/LN. However, LN resident iNKT cells (∼5–10 × 10⁵) represent only a minor fraction of total iNKT cells within the liver and spleen compartments (2 × 10⁶ and 5 × 10⁵, respectively; Fig. 3B) in WASp−/− mice, suggesting that LN sequestration is unlikely to be a major factor in the observed alterations in iNKT cell numbers and distribution.

Reduced Ag-induced proliferation in WASp−/− iNKT cells

Mature iNKT cells proliferate and secrete cytokines following Ag receptor engagement. We used the model galactolipid, αGalCer, to determine the role of WASp in the functional responses of iNKT cells. Total splenocytes derived from either WT or WASp-deficient mice were labeled with CFSE, stimulated with αGalCer, and analyzed for both relative percentage of tetramer+ cells and CFSE dilution to assess iNKT cell proliferation. We observed a clear expansion of tetramer+ cells in αGalCer-treated cultures containing WT iNKT cells. This population increased 6-fold compared with untreated controls with the vast majority of cells undergoing one or more cell divisions (Fig. 4A). In contrast, there was no increase in the proportion of tetramer+ cells in αGalCer-treated cultures containing WASp-deficient tetramer+ cells. Furthermore, only 50% of WASp-deficient tetramer+ cells exhibited CFSE dilution compared with 80% in WT controls (Fig. 4A). These differences in proliferation were evident at 72 h poststimulation and especially pronounced at 96 h poststimulation. The proliferative defect in WASp-deficient tetramer+ cells could not be rescued even with high doses of αGalCer (up to 100 ng/ml, data not shown).

To eliminate the possibility that the reduced proliferative response of WASp-deficient iNKT cells might be secondary to inefficient Ag processing via WASp−/− DCs, we also conducted...
studies using mixtures containing an equal number of CFSE-labeled CD45.1^+ WT and CD45.2^+ WASp^-/- splenocytes. Cells were CFSE-labeled and stimulated with αGalCer as above. Cells were stained with anti-CD45.1 and anti-CD45.2 and proliferation of tetramer^+ cells was analyzed by flow cytometry (Fig. 4B). As above, αGalCer treatment resulted in 4- to 7-fold expansion of WT cells, but failed to significantly expand WASp^-/- tetramer^+ cells (Fig. 4B, right panels). Notably, under these culture conditions a larger percentage of WASp^-/- tetramer^+ cells exhibited a diluted CFSE signal (~50–70% compared with ~50%; compare Fig. 4A to 4B, right). However, this cycling population still exhibited substantially higher CFSE levels indicating that they sustained fewer cell divisions compared with WT cells (data not shown).

Impaired IL-15 receptor signaling results in significant reduction in thymic and peripheral NKT numbers (33). Therefore, we also analyzed the relative response of WASp-deficient NKT cells to IL-15. Total splenocytes from WT or WASp^-/- mice were labeled with CFSE and stimulated with IL-15. Under these conditions, the relative proportion of tetramer^+ NKT cells remained essentially identical in WT and WASp^-/- cultures (Fig. 4C). Both populations also exhibited similar levels of IL-15 induced CFSE dilution (Fig. 4C, right). Of note, the IL-15 response was much greater in tetramer^+ cells and this response was also indistinguishable in cultures containing WT vs WASp^-/- cells. The more robust response of tetramer^+ cells is consistent with previous data regarding the IL-15 response of WT primary T and NK cells (34). Notably, both the WT and WASp^-/- NKT cells also exhibited an equivalent proliferative response to PMA/ionomycin stimulation (data not shown), consistent with the interpretation that WASp deficiency primarily impacts a proximal signal(s) initiated following Ag-receptor engagement. Together, these data support the conclusion that WASp^-/- NKT cells exhibit a cell-intrinsic TCR signaling deficit.

**WASp^-/- NKT cells display defective cytokine secretion**

The invariant NKT subset is characterized by its ability to rapidly produce large amounts of cytokines upon Ag receptor engagement (2). Previous studies have shown that WASp-deficient T and NK cells exhibit marked deficits in receptor-triggered cytokine production (14). Therefore, we also sought to determine whether cytokine production by WASp^-/- NKT cells was similarly impaired. Splenocytes from WT or WASp^-/- mice were stimulated with either 10 or 100 ng/ml αGalCer for 5–6 h and cytokine production was analyzed using intracellular flow cytometry. As anticipated, a substantial proportion of both CD4^+ and CD4^- NKT cells produced similar levels of both cytokines in response to low level Ag (10 ng/ml) stimulation; and this deficit was only partially rescued using this approach (data not shown). Together, these data demonstrate that WASp plays a critical role in promoting Ag-induced, effector cytokine production by NKT cells.
Wasp promotes iNKT CD11a expression and tissue localization

As shown above, Wasp deficiency exerts the greatest peripheral impact on liver resident iNKT cells. Notably, signaling through the CD11a integrin is required for retention of iNKT cells within the liver (35). We therefore assessed CD11a expression in iNKT cells derived from WT vs Wasp<sup>+/−</sup> animals to determine whether the reduction in tissue resident cells might correlate with altered CD11a expression. Consistent with this idea, we observed a marked reduction in CD11a expression in Wasp-deficient iNKT cells (Fig. 6A). This decrease was present in all mature iNKT cells including cells derived from the thymus, spleen, BM, and liver (data not shown). It was also present in both CD4<sup>+</sup> and CD4<sup>−</sup> mature iNKT cells. Furthermore, decreased CD11a expression was also readily apparent in CD24<sup>+</sup> immature thymic iNKT cells (data not shown), suggesting that the absence of Wasp results in reduced CD11a expression at multiple stages of iNKT development.

We also evaluated heterozygote Wasp carriers to determine whether altered CD11a expression levels were likely to represent a cell-intrinsic defect. Analysis using CD11a, iNKT markers and Wasp expression again revealed a specific reduction in relative CD11a expression in Wasp-deficient iNKT cells within the thymus, BM, spleen, and liver (Fig. 6B and data not shown). This change was specific to iNKT cells, as similar levels of CD11a were present on conventional, splenic CD4<sup>+</sup> T cells derived from WT and Wasp-deficient animals (Fig. 6A, right panel).

Both Wasp<sup>−/−</sup> Tregs and Wasp<sup>−/−</sup> mature B cells exhibit impaired homeostasis and decreased fitness in vivo (4, 5, 17–19). To determine whether Wasp<sup>−/−</sup> iNKT cells might also exhibit altered fitness in vivo we conducted a competitive analysis of WT vs Wasp<sup>−/−</sup> iNKT cells in lymphopenic mice. This approach allowed us to assess both relative fitness and tissue distribution in the absence of any developmental inputs. We enriched for thymic iNKT cells by magnetically depleting CD8<sup>+</sup> and CD24<sup>+</sup> cells, resulting in a 100-fold enrichment of the iNKT population (with iNKT comprising at least 15% of input cells, Fig. 6C, top panel).

These enriched cell populations were labeled with CFSE, mixed at 50:50 ratio of WT and Wasp<sup>−/−</sup> cells, and transferred into unmanipulated Rag2<sup>−/−</sup> mice. Recipient animals were evaluated at 7 days posttransplant for the relative proportion of engrafted cells in various lymphoid and tissue compartments. Equivalent numbers of WT and Wasp<sup>−/−</sup> iNKT cells were present within the BM. In contrast, the relative proportion of WT cells increased substantially within the spleen and, particularly, within the liver (Fig. 6C). This skewing did not appear to be due to decreased proliferative capacity,
as both WT and WASp-deficient iNKT cells exhibited similar levels of CFSE dilution (Fig. 6C, histogram). Thus, WASp-deficiency results in impaired iNKT homeostasis by modulating iNKT migration and/or retention within secondary effector tissues.

**Discussion**

As part of this work and previous studies, we have completed extensive analyses of the relative selective advantage for WASp+ cells in distinct myeloid and lymphoid cell subsets. Our current and previous data (4, 19) fail to demonstrate any selective advantage WT vs WASp− multipotent HSC) in mice. In addition, we have observed no significant differences among various myeloid-derived subsets (data not shown). In contrast, there is a pronounced enrichment for WASp+ cells within lymphoid populations, including various T and B cell subsets, as well as in the NK lineage. Our findings are consistent with a recently published report that used a similar methodology to examine multiple hematopoietic cell populations (5). The authors of that study also failed to identify any selective advantage for WT HSC or myeloid cells but demonstrated a pronounced selective advantage of WASp+ cells within lymphoid subsets, including peripheral T cell populations and MZ B cells. Surprisingly, while these authors were the first to report a selective advantage for thymic and splenic iNKT cells, they did not detect a significant advantage for WASp+ splenic NK cells. This latter difference may reflect the smaller number of animals examined in the previous study Consistent with our findings, we and others have previously observed selective outgrowth of WASp+ Tregs and NK cells in patients with revertant mutations within the WAS gene (19, 36, 37). Thus, our current findings and other previously published data (5, 38–40) clearly demonstrate the significant selective advantage for WASp+ cells within nearly all mature lymphoid subsets in human WAS patients and murine models of WAS.

In the current work, we focused on the marked selective advantage observed for mature WASp+ iNKT cells and also conducted studies to assess the functional impact of WASp deficiency within this subset. Using heterozygous carrier female mice, mixed BM chimera studies, and adoptive cell transfer experiments we demonstrate that WASp+ iNKT cells exhibit a strong selective advantage in comparison with WASp− iNKT cells in the thymus, spleen, and liver. Our combined data also indicate that WASp is partially required for late-stage thymic iNKT development and egress; is essential for Ag-dependent iNKT function; and is important for peripheral iNKT homeostasis.

WASp+ iNKT cells exhibit a modest selective deficit during the final stage of mature thymic iNKT (CD24hi to CD24−CD44dim) development. This deficit is only apparent, however, in two week old animals or within the context of competing WASp+ iNKT cells. In contrast, adult WASp−/− animals generate normal numbers of mature thymic iNKT cells. Our findings suggest that WASp−/− iNKT cells transition normally through positive selection, but exhibit a reduced proliferative burst during the transition from the CD24hi to CD24−CD44dim developmental stage. This decreased maturation rate is readily detected in very young animals and in heterozygous WASp carriers where WASp+/− iNKT cells are partially out-competed in the presence of WASp+ mature thymic iNKT cells. Transition into the mature (CD24−) iNKT stage is mediated via TCR and SLAM-family receptor signaling (41–43) and WASp has previously been shown to impact both of these signaling cascades in primary T cells (7, 44). In addition, our data clearly show that WASp deficiency impacts TCR signaling in mature iNKT cells. Based on our data, we anticipate that the more limited impact of WASp-deficiency on thymic iNKT numbers in adult WASp−/− mice reflects inefficient egress and resultant accumulation of thymic iNKT cells. Emigration of thymic iNKT cells is regulated by SIP signaling (31). CD69 acts as a negative regulator of these events; and S1P-receptor engagement also promotes down-regulation of CD69 surface expression (32). We and others have shown that WASp mediates S1P signaling in marginal zone B cells (4, 5), suggesting that increased CD69 expression on WASp−/− peripheral iNKT may represent inefficient S1P signaling in WASp−/− iNKT cells. Together, these findings suggest that WASp plays a role in both the initial development of CD24− iNKT cells and in the egress of mature iNKT cells from the thymus to the periphery.

The most significant changes observed in our study were the alterations in peripheral iNKT compartment in WASp−/− mice. WASp deficiency specifically impacted cells within the liver and spleen but not the BM. Interestingly, the numbers of splenic, but not liver, iNKT cells reached WT levels in older animals. This tissue and age-specific phenotype suggests several roles for WASp in peripheral iNKT homeostasis. First, reduced egress of thymic iNKT cells likely leads to a diminished pool of peripheral iNKT cells and slower seeding of peripheral tissues. Second, WASp−/− iNKT exhibit a peripheral homeostatic defect most likely due to altered tissue-specific migration and/or retention. We observed a marked selective advantage for WASp+ iNKT cells within the spleen and the liver (compared with the BM) in both WASp heterozygous carriers and in Rag2-KO mice transplanted with iNKT-enriched thymocytes. The migration and retention of iNKT cells within peripheral tissues is mediated by a complex interplay between integrins (CD11a; Ref. 35), chemokines (CXCL16; Ref. 45) and costimulatory factors (ICOS; Ref. 46). We observed significantly reduced levels of CD11a integrin expression on WASp-deficient iNKT cells derived from all tissues; and this defect was cell-intrinsic, as analysis of WASp heterozygous mice also revealed reduced CD11a levels specifically in WASp−/− iNKT cells. Signaling through CD11a is crucial for iNKT migration to the liver (35) and we have recently demonstrated that WASp-deficient marginal zone B cells are impaired in their ability to aggregate CD11a and form a functional immune synapse (4). Notably, we did not observe lower CD11a levels on WASp−/− MZ B cells (4) or naive CD4+ T cells, suggesting differential regulation of CD11a expression in iNKT vs conventional T cells or MZ B cells. Our combined observations suggest that WASp-mediated signals might be required for sustained CD11a expression in vivo and that altered CD11a signals are likely to impact iNKT homeostasis in the liver. Altered signaling through the chemokine receptor, CXCR6, may also impact this process since CXCR6 signaling, via interaction with CXCL16, is essential for iNKT migration to the liver (45). Additional experiments will be required to determine whether WASp-deficient iNKT cells exhibit altered CXCR6 expression and/or reduced migration to CXCL16.

Notably, previous studies have shown that CD11a-deficient or CXCR6-deficient mice do not exhibit deficits in splenic iNKT numbers (35, 45). Thus, while we observed reduced CD11a on iNKT cells derived from all the tissues, this defect cannot explain the altered cell numbers within the spleen in WASp−/− animals. In addition to chemokines and integrins, peripheral iNKT homeostasis is dependent on a steady provision of costimulatory signals. Although thymic iNKT development depends on CD28-B7 interactions (47), peripheral survival relies on costimulatory signals delivered through ICOS/ICOS-L interaction (46). Although WASp is involved in CD28-mediated signaling (48), its role in ICOS signaling has not yet been established.

Notably, we observed a marked selective advantage for WASp+ iNKT cells within the spleen and the liver, we observed similar levels of CFSE dilution in WASp−/− vs WT iNKT cells in
transplanted Rag2-/- mice. This observation strongly suggests that, unlike the positioning signals described above, the events mediating iNKT homeostatic proliferation are intact in the absence of WASp. Consistent with this view, we also observed no difference in the proliferative response of WT vs WASp-/- iNKT cells to IL-15, the primary mediator of iNKT homeostatic proliferation in vivo (33).

Taken together, our findings suggest a dual role for WASp in peripheral iNKT homeostasis: WASp is important for iNKT migration to the peripheral tissues and is involved in promoting cellular retention within the tissues, in part by regulating integrin surface expression. Although WASp is required for the homeostasis of both iNKT and Tregs, the mechanism(s) involved appear to be distinct for each subset. Unlike iNKT cells, WASp-/- mice generate normal numbers of splenic Tregs (18, 19). However, in contrast to WASp-/- iNKT cells, WASp-/- Tregs exhibit a markedly reduced rate of homeostatic proliferation and this correlates with progressive loss of this population when placed in direct competition with WT Tregs (19). These contrasting phenotypes suggest separate and nonoverlapping functions for WASp in promoting homeostasis of specific T cell subsets: WASp is involved in both Ag-driven homeostatic proliferation and in chemokine and integrin-mediated tissue migration and retention. Maintenance of Tregs and other Ag-dependent T cell lineages requires WASp activity for persistent signaling through the Ag receptor. In noncanonical subsets such as iNKT cells, homeostatic proliferation is driven by cytokines rather than Ag, and our data suggest that this subset requires WASp for entry and retention in secondary lymphoid tissues and, possibly, also for key costimulatory signals.

Finally, our data demonstrate, not surprisingly, that WASp is essential for Ag-induced proliferation and cytokine production in iNKT cells. As previously reported for mutant CD4 T cells (14–16), our findings imply that WASp-deficient iNKT cells manifest a proximal, cell-intrinsic deficit in Ag-receptor signaling. Consistent with this view, WT and WASp-/- iNKT cells expand equivalently in response to signals (including PMA/ionomycin stimulation) that bypass these proximal events. Notably, the proliferation defect in WASp-deficient CD4+ T cells correlates with markedly reduced IL-2 production; and can be rescued by provision of exogenous IL-2 (15, 16). In contrast, provision of WT iNKT in trans failed to rescue the sustained cycling defect in WASp-/- iNKT cells. This observation suggests that, unlike CD4 T cells, Ag-driven iNKT activation is less dependent upon autocrine signals. The cytokine production defect observed in Ag-stimulated WASp-deficient iNKT cells is also consistent with previous work in both T and NK cells (15, 49). As with proliferation, the defect in cytokine production appears to reflect inefficient proximal signaling, as PMA/ionomycin stimulation resulted in equivalent cytokine production by WT and WASp-deficient cells. In peripheral tissues, iNKT cells serve as immune sentinels, proliferating and producing large amounts of cytokines following triggering of the Ag receptor (2). WASP-deficient iNKT cells are unable to properly perform both of these crucial tasks. These findings imply that WASp-/- animals should manifest compromised iNKT response in vivo and be at increased risk for specific viral and intracellular infections. Further work using lineage specific targeting will be required to directly test this idea.

Of note, a report published during the review of this manuscript also highlights the crucial role for WASp in Ag-mediated proliferation and cytokine production of iNKT cells (50). Consistent with our in vitro data, in vivo Ag-challenged WASp-/- iNKT cells exhibit defective cytokine production and proliferation. In contrast to our data, however, the authors imply a crucial role for WASp in the transition from the NK1.1- to NK1.1+ mature iNKT cell stage. These authors reported a near complete absence of CD44+ NK1.1+ cells in both the thymus and periphery of WASp-/- animals (50). In contrast, as described herein, while we observed decreased peripheral iNKT numbers, these cells exhibited WT levels of CD44, NK1.1, and all other maturation markers (with the exception of CD69). Notably, the WASp-/- mice used in our study were generated independently (15) and have been backcrossed onto the B6 background for >10 generations. Because NK1.1 is expressed on the B6 but not the 129 background (51), is seems most likely that background strain differences are responsible for the aberrant NK1.1 expression observed by Loci and colleagues (50). The iNKT maturation defect implicated by these authors might be clarified by analysis based upon additional maturation markers.

Combined, our findings suggest a model in which WASp is important for both homeostasis and function of iNKT cells. Our data would predict that WAS patients would initially lag behind age-matched normal counterparts in percentage of peripheral iNKT cells. However, this initial defect is likely to be rescued over time. Combined with the overall immunodeficiency and immune dysfunction observed in WAS patients, reduced iNKT functionality may contribute to both the elevated infection rate and increased autoimmune incidence observed in this complex disorder.

Acknowledgments
We thank Mitchell Kronenberg and the National Institutes of Health tetramer facility for providing the CD1d tetramers and Jit Khim for help with mouse work.

Disclosures
The authors have no financial conflict of interest.

References
WASP REGulates NKT HOMeostasis AND FUNCTION

23. Cava, A. L., L. V. Kaer, and Fu-Dong-Shi. 2006. CD4
27. Benlagha, K., D. G. Wei, J. Veiga, L. Teyton, and A. Bendelac. 2005. Charac-