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IL-18 Acts in Synergy with IL-7 To Promote Ex Vivo Expansion of T Lymphoid Progenitor Cells

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Although IL-18 has not previously been shown to promote T lymphopoiesis, results obtained via a novel data mining algorithm (global microarray meta-analysis) led us to explore a predicted role for this cytokine in T cell development. IL-18 is a member of the IL-1 cytokine family that has been extensively characterized as a mediator of inflammatory immune responses. To assess a potential role for IL-18 in T cell development, we sort-purified mouse bone marrow–derived common lymphoid progenitor cells, early thymic progenitors (ETPs), and double-negative 2 thymocytes and cultured these populations on OP9-Delta-like 4 stromal layers in the presence or absence of IL-18 and/or IL-7. After 1 wk of culture, IL-18 promoted proliferation and accelerated differentiation of ETPs to the double-negative 3 stage, similar in efficiency to IL-7. IL-18 showed synergy with IL-7 and enhanced proliferation of both the thymus-derived progenitor cells and the bone marrow–derived common lymphoid progenitor cells. The synergistic effect on the ETP population was further characterized and found to correlate with increased surface expression of c-Kit and IL-7 receptors on the IL-18–treated cells. In summary, we successfully validated the global microarray meta-analysis prediction that IL-18 affects T lymphopoiesis and demonstrated that IL-18 can positively impact bone marrow lymphopoiesis and T cell development, presumably via interaction with the c-Kit and IL-7 signaling axis. The Journal of Immunology, 2015, 194: 000–000.

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; CLP, common lymphoid progenitor cell; DL, Delta-like; DN, double-negative; DP, double-positive; ETP, early thymic progenitor; GAMMA, global microarray meta-analysis; HSC, hematopoietic stem cell; IRIDESCENT, implicit relationship identification by in silico construction of an entity-based network from text; ISP, immature single-positive; SP, single-positive.

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responses (19). Transgenic overexpression of IL-18 had dramatic effects on the immune system; however, these studies did not focus on the effects on early thymocytes, perhaps due to the important role for this cytokine in Th1 and Th2 differentiation that has kept the spotlight on peripheral immune cell mechanisms (20, 21). Although the immunomodulatory functions of IL-18 are relatively well defined, its potential role in T cell development, as predicted by GAMMA, is not known. Previous studies have demonstrated thymic expression of IL-18, and this cytokine has been shown to promote the differentiation of fetal double-negative (DN) thymocytes to thymic-derived dendritic cells (22, 23). Furthermore, thymocyte stimulation with IL-18 can elicit production of Th1 and Th2 cytokines in the presence of IL-12 and IL-2, respectively (24). These studies demonstrated the potential of IL-18 to signal within the thymic microenvironment and indicated that IL-18 may indeed be a factor capable of influencing early T cell development.

In an attempt to assess the potential role of IL-18 in T cell development, we cocultured mouse bone marrow hematopoietic stem cells (HSCs), common lymphoid progenitor cells (CLPs), thymus early thymic progenitors (ETPs), and DN2 on OP9–Delta-like (DL4) stromal cells, either with IL-18 alone or in conjunction with IL-7, which is traditionally used to promote proliferation and survival of thymocytes in this system (25). We found that IL-18 synergized with IL-7 in promoting strong proliferation of CLP, ETP, and DN2 populations. The effect on the ETP cells was further characterized and found to correlate with increased surface expression of CD127 and CD117 on these cells. Surprisingly, we found that IL-18 alone was capable of promoting ETP proliferation to a magnitude similar to that observed for IL-7. These findings demonstrate a novel role for IL-18 in promoting in vitro T cell development from immature precursors and further validate GAMMA as a method to predict putative phenotype and function for genes.

Materials and Methods

Mice

C57BL/6 mice were bred and housed at the University of Oklahoma–Tulsa Comparative Medicine satellite facility under the oversight of the University of Oklahoma Health Science Center Comparative Medicine Facility (Oklahoma City, OK), an Association for Accreditation and Accreditation of Laboratory Animal Care–approved animal facility. Animal husbandry and all experiments were performed in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council). Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Science Center. Mice used in this study were females ranging from 6 to 12 wk of age. IL-18R1–deficient mice (strain B6.129P2-Hlrbtm1Jj/J) on a C57BL/6 background (26) were purchased from the The Jackson Laboratory (Bar Harbor, ME).

Tissue harvest and cell staining

Thymuses were harvested and placed into complete tumor media as previously described (27). Thymuses were crushed through 70-μm nylon cell strainers to produce single thymocyte suspensions. Cells were treated with RBC lysis buffer (Sigma-Aldrich, St. Louis, MO) and washed into complete tumor media prior to counting. Thymocytes at a concentration of 1 × 10^8 cells/ml were incubated with mAb against mouse CD16/CD32 (Fc Block; BD Biosciences, San Jose, CA) to block potential Fc-mediated Ab binding and then stained at a density of 1 × 10^5 cells/ml with primary mAbs for DN3b, and DN4a sorts, including CD4-bio, CD8-bio, TCR–bio, CD44–allophycocyanin-Cy7, and CD28–FITC, to c-Kit–FITC. To assess the proliferation kinetics, ETPs were labeled with CFSE (Life Technologies, Carlsbad, CA) and then sorted into single-positive (SP) and double-negative (DN)-sorted populations using a BD LSRII four-laser flow cytometer and FACS Diva software (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

OP9-DL4 cocultures

The OP9-DL4 cell line was provided by Juan Carlos Zúñiga-Pflücker and maintained according to the protocols from his laboratory (30). For each experiment a fresh vial was thawed and grown to 60–80% confluence on treated plates; cells were then split and grown again to 60–80% confluence before the final plating on experimental 24-well treated plates. Sorted bone marrow and thymocyte subsets were cocultured in plates with the OP9-DL4 stromal cells in αMEM (Invitrogen, Grand Island, NY) supplemented with 16.5% FBS (Sigma-Aldrich) and penicillin-streptomycin (Sigma-Aldrich) (culture media) and the cytokines indicated in the figures. Note that no FBS was added to any of the cultures. After 7 d, the cells were harvested from the wells, counted, and stained for flow cytometry. Viable cell counts were obtained using 0.4% trypan blue (Lanza, Allendale, NY) staining or annexin V and 7-aminoactinomycin D (7-AAD) staining technique.

Quantitative real-time RT-PCR

Total RNA from sort-purified thymocyte subsets (2 × 10^6 cells) and splenic NK cells (1 × 10^5) were isolated using MinElute columns from Qiagen (Germantown, MD). Total RNA was reverse transcribed to cDNA using a Qiagen Sensiscript reverse transcriptase kit. IL-18R transcript abundance was measured by amplifying cDNA using IL-18R β (Sigma-Aldrich) and penicillin-streptomycin (Sigma-Aldrich) (culture media) and the cytokines indicated in the figures. Note that no FBS was added to any of the cultures. After 7 d, the cells were harvested from the wells, counted, and stained for flow cytometry. Viable cell counts were obtained using 0.4% trypan blue (Lanza, Allendale, NY) staining or annexin V and 7-aminoactinomycin D (7-AAD) staining technique.
neous conditions, it used large-scale literature mining to identify what these coexpressed genes have in common. These commonalities become the inferred functions, roles, and phenotypes for IL-18. Known functions serve as positive controls and, for IL-18, many of its major known roles were correctly predicted on the basis of its coexpressed genes, such as its proinflammatory role in the immune response and its ability to influence cytokine production, as well as genetic associations with other cytokines such as IL-12, IL-10, and IL-1.

Data analysis
Flow cytometry data were analyzed using FACSDiva (BD Biosciences) and FlowJo software (Tree Star). Statistical analysis was performed using Graphpad Prism 6 software, and statistical significance between variables was estimated by performing one-way ANOVA and a Fischer test for multiple comparisons.

Results

IL-18 acts in synergy with IL-7 to induce expansion of ETPs on OP9-DL4 stromal cells
An IL-18 dose response was performed to determine the effect of IL-18 on immature thymocytes in culture. Sort-purified ETP cells were cultured for 1 wk on OP9-DL4 stroma with IL-7 added at a concentration of 5 ng/ml in conjunction with IL-18 at concentrations ranging from 0.1 to 100 ng/ml. Supplementing cocultures with IL-18 significantly enhanced the expansion of ETP cells, as determined by total cell yields on day 7, compared with control treatments (Fig. 1B). We observed that the magnitude of ETP expansion in the presence of IL-18 alone was comparable to the ETP expansion observed in the presence of IL-7 alone. Adding IL-7 and IL-18 together to the cocultures greatly increased the cell yields when compared with cultures containing either IL-7 or IL-18 alone. The synergistic effects of IL-18 and IL-7 were evident only at higher doses of IL-18 (>10 ng/ml). Requirement of a higher IL-18 dose for cell response is not unusual, as relatively higher concentrations of IL-18 are known to be required to activate cells in vitro (31). We next tested whether IL-18 influenced expansion of other immature thymocyte subsets, including DN1d/e, DN2, and DN3 populations. We found that neither IL-7 nor IL-18 alone had an apparent effect on the expansion of these thymocytes, although cocultures supplemented with both IL-7 and IL-18 showed a modest effect in promoting expansion of the DN2 population (Fig 1C). These results demonstrate that IL-18 can promote expansion of ETPs and can synergize with IL-7 in a dose-dependent manner.

IL-7 and IL-18 stimulation enhances survival and proliferation of ETPs in OP9-DL4 cocultures
To assess whether increased cell yields in IL-7– and IL-18–stimulated ETP cocultures were due to enhanced survival or increased proliferation, we measured cell viability in cocultures by annexin V/7-AAD staining and monitored cell divisions using a CFSE dilution assay. We observed that the percentage of live cells in ETP cocultures stimulated with cytokines was significantly higher than that observed in unstimulated cocultures (Fig. 2A). IL-7 and IL-18 were equally potent in increasing live cell percentages in 7-d cocultures. There was also no apparent synergistic effect in the IL-7 plus IL-18 condition on cell survival, presumably because there was minimal cell death observed in these cocultures stimulated with IL-7 or IL-18 alone. Because the differences in cell survival among the treatments are small, it is unlikely that enhanced ETP expansion in stimulated cultures was entirely due to enhanced survival. Hence, we compared the proliferation kinetics of unstimulated and stimulated ETP cocultures. CFSE profiles demonstrated that the ETPs had undergone more cell divisions than CFSE staining can reliably detect by day 6, irrespective of the culture conditions (Fig. 2B). However, CFSE profiles on day 4 clearly showed that ETPs stimulated with either IL-7 or IL-18 alone or in
combination experienced more divisions compared with unstimulated ETP cultures. Importantly, the synergistic action of IL-7/IL-18 coadministration was observed on day 4. Taken together, these results support the contention that IL-18 promotes expansion of ETPs by enhancing both survival and proliferation of ETPs.

**IL-18 accelerates the differentiation of immature thymocytes**

To determine whether IL-18 could influence the differentiation of immature thymocytes, sort-purified ETPs and DN2s were cocultured with OP9-DL4 stromal cells in the presence of IL-7 or IL-18 alone or in combination. After 7 d, differentiation of thymocytes in the cocultures was analyzed by discriminating thymocyte populations using surface markers. Both ETP and DN2 cocultures supplemented with IL-7 showed an increase in total cell number after 7 d without any notable changes in the percentages of different thymocyte populations in comparison with untreated cocultures (Fig. 3A, 3B). Similarly, ETP and DN2 cocultures treated with either IL-18 alone or in combination with IL-7 showed an increase in total cell number without any notable changes in the percentages of different thymocyte populations in comparison with untreated cocultures (Fig. 3A, 3B).
without skewing the percentages of any particular thymocyte population as compared with IL-7 alone or untreated cultures. However, when we evaluated differentiation of the ETPs from the CFSE dilution assays at earlier time points (Fig. 3C), we saw that the differentiation of ETPs into DN2 and DN3 subsets occurred at a faster rate in IL-7– and IL-18–stimulated cultures compared with unstimulated controls. The CFSE profiles also showed no preferential expansion or differentiation of a particular thymocyte population in cocultures stimulated in the presence of IL-18 (data not shown).

To determine the capacity for further development along the T cell lineage, we evaluated DN3a thymocyte subsets generated in vitro from ETP/OP9-DL4 cocultures and fresh ex vivo DN3a thymocytes sort-purified from the thymus for their potential to develop into DP cells. We found that all in vitro–generated DN3a subsets, irrespective of the source and treatment conditions, differentiated into DP cells when cocultured on OP9-DL4 stromal cells for 8 d (Fig. 3D) in the absence of IL-7. However, in the presence of IL-7 there were significantly fewer DP thymocytes, as evidenced by the percentage of cells within the DP quadrants, from DN3a populations generated from ETP cocultures as well as from DN3a populations from thymus. This effect is not surprising, as IL-7 has been shown to inhibit the transition of DN2/DN3 thymocytes to the DP stage (32). These results collectively suggest that IL-18 increased the expansion of immature thymocytes without interfering with their differentiation into more mature thymocytes.

**ETP and DN2 subsets express IL-18 receptor transcript but not discernible levels of IL-18 receptor protein as assessed by flow cytometry**

To further characterize the mechanisms by which IL-18 exerts its effect on developing T cells, surface expression of the IL-18R1 (CD218a) was assessed on the four main thymocyte populations, DN, DP, SP4, and SP8 (Fig. 4A). Only a small percentage of the DN population and the SP4 population stained positively for the IL-18R1. Subdivision of the DN compartment revealed that only the DN1 population contained IL-18R1–expressing cells. Further parsing of the DN1 compartment by CD24 and c-Kit staining showed IL-18R1 surface expression to be restricted to the DN1e population. This is somewhat surprising given that the DN1a/b population (ETPs) expanded in the presence of IL-18, as did both ETPs and DN2 cells with the combination of both IL-7 and IL-18 as shown in Fig. 1C.

Because surface expression of the IL-18R1 was not detectable in the IL-18–responsive ETP and DN2 populations using flow cytometry, we assessed IL-18 receptor expression on ETP, DN1e/d, DN2, and DN3 thymocytes using real-time RT-PCR. Both ETPs and DN2 expressed low levels of IL-18R1 and IL-18R accessory protein mRNA compared with DN1e/d populations and positive control splenic NK cells (Fig. 4B). The OP9-DL4 stromal cells did not express IL-18 receptor either at transcript level or at surface expression (data not shown).

**The IL-18 effect on thymocyte expansion is absent in cells from IL-18R1–deficient mice**

Although we did not detect surface expression of IL-18 receptors on ETPs or DN2 thymocytes, the presence of IL-18 receptor transcripts in these cells suggest the possibility for very low levels of receptor expression. To determine whether the IL-18 effects in the cultures were the direct result of IL-18 receptor engagement, we repeated the OP9-DL4 cocultures described above comparing thymocytes from IL-18R1–null mice to wild-type thymocytes. ETPs from either C57BL/6 mice or IL-18R1–null mice (Fig. 5) were plated with no cytokine, IL-7, IL-18, or a combination of IL-7 and IL-18. As expected, the IL-18R1–null mice did not show expansion in response to IL-18. The synergy between IL-18 and IL-7 was also absent in the null mice. This indicated that the IL-18 effects were mediated through the IL-18 receptor even though we were unable to detect IL-18R1 on the surface of the ETPs via flow cytometry.

**IL-18–stimulated ETPs significantly upregulate c-Kit and IL-7Rα receptor expression**

c-Kit signaling has been shown to play an important role in promoting proliferation and differentiation of DN1 and DN2 thymocyte populations (33). Additionally, Zhou et al. (34) showed that rIL-18 can positively regulate the expression of c-Kit in human melanocytes. To evaluate a potential mechanistic concurrence
in our model, we tested the effects of IL-18 on regulation of c-Kit and IL-7Rα expression on immature thymocytes differentiating in the OP9-DL4 cocultures. ETPs cultured in the presence of IL-7 or IL-18 alone for 7 d demonstrated significantly upregulated c-Kit expression on the resulting DN2 and DN3 cells, although the effect was modest in comparison with the effect of adding both cytokines simultaneously (Fig. 6). ETPs exposed to IL-7 plus IL-18 showed robust increases in surface c-Kit expression (∼13.5-fold over control), which was significantly greater than what was observed in ETP cultures exposed to IL-7 or IL-18 alone (Fig. 6A). Because IL-18 had a synergistic effect with IL-7, we also assessed the IL-7 receptor expression on these cells when treated with IL-18. As expected, stimulating ETPs with IL-18 caused a significant upregulation of surface IL-7Rα in differentiating thymocytes after 7 d of coculture (Fig. 6B). ETP cultures exposed to IL-7 with or without IL-18 showed minimal IL-7Rα expression on the differentiating thymocytes, presumably owing to activation-induced receptor internalization.

**IL-18 promotes the expansion of immature progenitor cells in OP9-DL4 cocultures**

Lack of IL-18–mediated proliferative effects in DN1e/d despite IL-18 receptor expression by these cells suggested that IL-18 receptor expression alone is not sufficient for the proliferative effects. Alternatively, we found that cells responding to IL-18 both express c-Kit and IL-7R, as well as upregulate these receptors after stimulation with IL-18. Hence, we hypothesized that the IL-18 proliferative effects are not restricted to immature thymocytes and may be present in other progenitor cells expressing c-Kit and IL-7 receptors. To test this hypothesis we further investigated the proliferative effects of IL-18 on HSCs and CLPs isolated from mouse bone marrow, which also express the c-Kit receptor. We observed that supplementing cultures with IL-7 and IL-18 significantly enhanced the proliferation of both HSCs and CLPs cocultured on the OP9-DL4 stromal cells (Fig. 7), although the proliferative effects of IL-7 and IL-18 combination are modest in cocultures started with HSCs compared with CLPs.

**Discussion**

Humans have ∼25,000 genes and, although their positions have been known since the completion of the draft genome in 2000, about a third of them still have yet to be characterized. Algorithmic approaches to predicting function on the basis of transcriptional network analysis, such as the GAMMA approach used in the present study, enable function and phenotype to be predicted for genes (Fig. 8). In the case of IL-18, much was already known; however, what we have shown in this study is that there can still be unknown/unexplored functions for known genes that...
can be inferred on the basis of their close neighbors in the transcriptional network.

As detailed in Results, IL-18 dramatically influenced T cell development in our in vitro model. IL-18 augmented thymocyte proliferation and accelerated differentiation capacity. Interestingly, the proliferative effects of IL-18 are not restricted to T cell progenitors in the thymus but also are seen in more immature progenitor cells such as HSCs and in CLPs in bone marrow that give rise to all lymphoid-derived cells, suggesting the possibility that IL-18 can influence lymphopoiesis more broadly. Although IL-18 has well-established roles in a number of other processes, its effects in augmenting thymocyte expansion are mediated through positive regulation of a c-Kit and IL-7 signaling axis. IL-18 effects in augmenting thymocyte proliferation are mediated by positively regulating a c-Kit and IL-7 signaling axis.

Consistent with these studies, we observed higher levels of both IL-7R and c-Kit play important roles in promoting the proliferation and survival of immature thymocytes (33, 43). There is ample evidence suggesting that physiological stressors such as infection and inflammation can cause thymic atrophy due to apoptosis of DN and DP subsets (39, 40), the key players in restoring thymic homeostasis postinfection or inflammation remain poorly defined. Because IL-18 showed effects similar to IL-7 in promoting proliferation and survival of ETPs, one could predict that IL-18 can act as a compensatory mechanism to boost thymic progenitors during an infection or inflammation and to restore thymic homeostasis. However intriguing, the complexities associated with exploring this hypothesis are beyond the scope of the present study. Previous studies have demonstrated dendritic cell potential of DN1d and DN1e subsets in vivo (41) and the ability of IL-18 to drive differentiation of fetal DN1 cells to dendritic cells (22). However, in our OP9-DL4 cocultures, IL-18–induced proliferative effects appeared restricted to DN1a/b (ETP) and DN2 cells that progressed toward DN3, as we observed no expansion of the DN1d/e cells in this system (Fig. 4). In any case, the experiments presented in the present study clearly demonstrate that under controlled conditions, IL-18 can potentiate ETP proliferation and differentiation toward the T lineage.

Notch signaling plays a crucial role in directing T cell development by tightly regulating various developmental steps, including commitment, selection, proliferation, and survival of differentiating thymocytes (42). For instance, Notch can promote the proliferation and survival of DN populations by positively regulating the growth-promoting signal pathways mediated by IL-7R and c-Kit (33, 43). There is ample evidence suggesting that both IL-7R and c-Kit play important roles in promoting the proliferation and survival of immature thymocytes (33, 44, 45). Consistent with these studies, we observed higher levels of both IL-7R and c-Kit on the surface of thymocytes expanded in the presence of IL-18 and IL-7 (Fig. 6). These two receptor signaling pathways reportedly directly interact with each other (46) and positively regulate STAT5 signaling. Hence, it is plausible that the IL-18 effects in augmenting thymocyte expansion are mediated through positive regulation of a c-Kit and IL-7 signaling axis. However, further studies are essential to confirm this contention and to explore whether IL-18 modulates c-Kit and IL-7R expression directly using transcriptional or posttranscriptional mechanisms or indirectly by modulating notch signaling, which is known to modulate the expression of these receptors on the surface.

Perhaps most importantly, we have demonstrated that IL-18 can substitute for IL-7 in early thymic development processes and can
synergize with IL-7 in promoting proliferation. This finding warrants further investigation to determine whether coadministration of IL-18 and IL-7 will be valuable for therapeutic purposes. Administration of rIL-7 has shown promise in clinical trials due to its ability to promote lymphopoiesis under lymphopenic conditions (47, 48). However, use of IL-7 has limitations such as its bias toward homeostatic expansion of peripheral lymphocytes and its marginal effects on enhancing progenitor cell populations that give rise to lymphocyte diversity (49, 50). Furthermore, the duration and dose of IL-7 necessary for effect pose a risk of graft-versus-host disease due to its ability to enhance T cell functions (48, 51). IL-18 has also been put into the spotlight for its potential role in cancer treatment and is showing promising results in preclinical and clinical studies (52–54). Based on the robust synergistic effects observed in our assays and the recently reported effects of IL-18 in driving homeostatic expansion of naive CD8 T cells in lymphopenic mice (37), it is possible that combining IL-18 and IL-7 as a combination therapy in humans could overcome the limitations of IL-7 by enhancing the IL-7 effects on bone marrow and thymus progenitor cells and decreasing the dose and duration of IL-7 needed for lymphocyte reconstitution in clinical scenarios of lymphopenia. Alternatively, this synergy could be exploited to expand progenitor cells ex vivo for reconstitution into lymphopenic hosts. As IL-7 has been characterized to be absolutely required for “normal” T cell development, it will be interesting to further characterize and compare the differences in phenotypically identical cells that have been raised in IL-7 versus IL-8 environments. The value of these studies lies in the ability to potentially co-opt these cells that have been raised in IL-7 versus IL-18 environments. The characterization and comparison of differences in phenotypically identical genitor cells and decreasing the dose and duration of IL-7 needed for driving homeostatic expansion of naive CD8 T cells in lymphopenic mice (37), it is possible that combining IL-18 and IL-7 as a combination therapy in humans could overcome the limitations of IL-7 by enhancing the IL-7 effects on bone marrow and thymus progenitor cells and decreasing the dose and duration of IL-7 needed for lymphocyte reconstitution in clinical scenarios of lymphopenia. Alternatively, this synergy could be exploited to expand progenitor cells ex vivo for reconstitution into lymphopenic hosts.

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Disclosures

A provisional patent disclosure has been filed for use of IL-8 and IL-7 for treatment of lymphopenia. The following authors are listed on the provisional patent: T.K.T., S.K.G., J.H.M., J.D.W., C.J.V.D.W., C.T., and A.A.T. The remaining authors have no financial conflicts of interest.

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