The Journal of Immunology

Conserved IL-2Rγc Signaling Mediates Lymphopoiesis in Zebrafish

Robert Sertori, Clifford Liongue, Faiza Basheer, Kanako L. Lewis, Parisa Rasighaemi, Dennis de Coninck, David Traver and Alister C. Ward

J Immunol 2016; 196:135-143; Prepublished online 20 November 2015;
doi: 10.4049/jimmunol.1403060
http://www.jimmunol.org/content/196/1/135

Supplementary Material http://www.jimmunol.org/content/suppl/2015/11/19/jimmunol.1403060.DCSupplemental

References This article **cites 96 articles**, 30 of which you can access for free at:
http://www.jimmunol.org/content/196/1/135.full#ref-list-1

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Conserved IL-2Rγc Signaling Mediates Lymphopoiesis in Zebrafish

Robert Sertori,[*,†] Clifford Liongue,[*,†] Faiza Basheer,[*,†] Kanako L. Lewis,[‡] Parisa Rasighaemi,[*,†] Dennis de Coninck,[*,§] David Traver,[‡] and Alister C. Ward[*,†]

The IL-2 receptor γ common (IL-2Rγc) chain is the shared subunit of the receptors for the IL-2 family of cytokines, which mediate signaling through JAK3 and various downstream pathways to regulate lymphopoiesis. Inactivating mutations in human IL-2Rγc result in SCID, a primary immunodeficiency characterized by greatly reduced numbers of lymphocytes. This study used bioinformatics, expression analysis, gene ablation, and specific pharmacologic inhibitors to investigate the function of two putative zebrafish *IL-2Rγc* paralogs, *il-2rγc.a* and *il-2rγc.b*, and downstream signaling components during early lymphopoiesis. Expression of *il-2rγc.a* commenced at 16 h post fertilization (hpf) and rose steadily from 4–6 d postfertilization (dpf) in the developing thymus, with *il-2rγc.a* expression also confirmed in adult T and B lymphocytes. Transcripts of *il-2rγc.b* were first observed from 8 hpf, but waned from 16 hpf before reaching maximal expression at 6 dpf, but this was not evident in the thymus. Knockdown of *il-2rγc.a*, but not *il-2rγc.b*, substantially reduced embryonic lymphopoiesis without affecting other aspects of hematopoiesis. Specific targeting of zebrafish Jak3 exerted a similar effect on lymphopoiesis, whereas ablation of zebrafish Stat5.1 and pharmacologic inhibition of PI3K and MEK also produced significant but smaller effects. Ablation of *il-2rγc.a* was further demonstrated to lead to an absence of mature T cells, but not B cells in juvenile fish. These results indicate that conserved IL-2Rγc signaling via JAK3 plays a key role during early zebrafish lymphopoiesis, which can be potentially targeted to generate a zebrafish model of human SCID. ***The Journal of Immunology*****, 2016, 196: 135–143.**

Interleukin-2 receptor γ common (IL-2Rγc) chain represents the shared component of the receptors for the IL-2 family of cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, each of which regulates different aspects of immune development and function (1–4). Specifically, IL-2 stimulates the growth, differentiation, and activation of various T and NK cell populations (5); IL-4 is involved in B cell proliferation, Ig class switching, and Th2 cell development (6); and IL-7 contributes to the development, survival, and homeostatic proliferation of T cells, especially memory T cells (7). IL-9 exerts a wider range of effects, such as mediating the growth and functional activation of T cells and mast cells, and supporting the differentiation of hematopoietic stem cells (8), whereas IL-15 regulates the proliferation, differentiation, and survival of many cell types, including B cells, NK cells, mast cells, neutrophils, eosinophils, monocytes/macrophages, and dendritic cells (9, 10). Finally, IL-21 stimulates lymphoid cell proliferation and the differentiation of B cells to plasma cells, and it regulates apoptosis in B and NK cells (4).

The IL-2Rγc chain acts as the major signal transduction component of the IL-2 receptor family. Ligand binding stimulates the activation of the tyrosine kinase JAK3 associated with the intracellular region of IL-2Rγc, which activates a number of downstream intracellular pathways, including those involving STAT5, PI3K, and ERK, the latter lying downstream of MEK (11, 12). Inactivating mutations in human IL-2Rγc cause T^-B^+ SCID, characterized by decreased numbers of T cells and a diminished immune response (13). Mice lacking IL-2rγc display a T^-B^- form of SCID (14), with reduced B and T cells (15), and have proved to be an invaluable model for a range of studies, especially relating to immunity and cancer (16–21).

The zebrafish is now established as an important alternate model for the study of vertebrate development and disease, with particular relevance to hematopoiesis and immunity (22, 23). Like mammals, zebrafish undergo distinct phases of hematopoiesis, with lymphoid progenitors seeding the thymus early in development following the establishment of definitive hematopoiesis (24). Zebrafish also shows broad conservation of cytokine receptors and downstream signaling pathway components (25, 26), particularly those known to facilitate the development of blood and immune cells (27–31).

We have previously identified two putative paralogs of the *IL-2Rγc* gene in zebrafish, *il-2rγc.a* and *il-2rγc.b* (25), but their roles have not been elucidated. In this study, we show that *il-2rγc.a*, but not *il-2rγc.b*, is involved in early zebrafish lym-

*School of Medicine, Deakin University, Geelong, Victoria 3216, Australia; [†]Centre for Molecular and Medical Research, Deakin University, Geelong, Victoria 3216, Australia; [‡]Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093; and [§]GROW School for Oncology and Developmental Biology, Maastricht University, Maastricht 6200, the Netherlands

ORCIDs: 0000-0002-1646-4905 (C.L.); 0000-0002-0575-1554 (D.d.C.).

Received for publication December 11, 2014. Accepted for publication October 20, 2015.

This work was supported by an Australian Postgraduate Award (to R.S.), an International Research Scholarship (to F.B.), an Alfred Deakin Postdoctoral Research Fellowship (to C.L.) from Deakin University, and by the resources of the Australian Research Council Linkage Infrastructure Equipment Fund initiative Fish Works: Collaborative Infrastructure *for* Zebrafish Research (to A.C.W.).

R.S., F.B., P.R., K.L.L., and D.d.C. performed experiments; R.S., K.L.L., and F.B. analyzed results and made figures; A.C.W., D.T., and C.L. designed the research and analyzed the results; and R.S., A.C.W., and C.L. wrote the paper, which was approved by all authors.

Address correspondence and reprint requests to Prof. Alister C. Ward, School of Medicine, Deakin University, Pigdons Road, Geelong, VIC 3216, Australia. E-mail address: award@deakin.edu.au

The online version of this article contains supplemental material.

Abbreviations used in this article: CHD, cytokine homology domain; dpf, day postfertilization; hpf, hour postfertilization; IL-2Rγc, IL-2 receptor γ common; QRT-PCR, quantitative RT-PCR; TALEN, transcription activator-like effector nuclease; TSLPR, thymic stromal lymphopoietin receptor; WISH, whole-mount in situ hybridization.

phopoiesis. The *il-2rγc.a* gene is expressed in the developing thymus and adult lymphocytes, with ablation of *il-2rγc.a* leading to a significant and specific reduction in T lymphopoiesis. This phenotype could be mimicked by targeting zebrafish Jak3 by either morpholino-mediated knockdown or pharmacological inhibition, indicating a conserved role for IL-2Rγc/JAK3 signaling across vertebrate lymphopoiesis, with contributions from the STAT5, PI3K, and MEK/ERK pathways also being identified.

Materials and Methods

Nomenclature conventions

Nomenclature rules for zebrafish, fugu, chicken, mouse, and human genes and proteins differ. Gene and protein names are presented according to the respective nomenclature conventions (zebrafish and fugu: *il-2rγc.a*, Il-2rγc.a; human and chicken: *IL-2Rγc*, IL-2Rγc; and mouse: *Il-2rγc*, IL-2Rγc).

Analysis of il-2rγc paralogs from zebrafish

Expressed sequence tags corresponding to the two putative *Danio rerio* (zebrafish) *IL-2Rγc* paralogs, *il-2rγc.a* and *il-2rγc.b* (25), were identified using BLASTX (32), with additional sequences obtained from RT-PCR and 5′RACE products. These sequences were assembled using Sequencher (Gene Codes Corporation) and corresponded to full-length reference sequences deposited at GenBank (http://www.ncbi.nlm.nih.gov/genbank/; accession number NP_001121743.1 *il-2rγc.a*, NP_001116522.1 *il-2rγc.b*). Multiple-sequence alignment of the encoded protein sequences was performed using the CLUSTALX program (33), along with zebrafish thymic stromal lymphopoietin receptor (Tslpr) (CAM88660.1), Il-13rα1 (CAI94933.1) and Il-13rα2 (NP_001107203.1), *Homo sapiens* (human) IL-2Rγc (AAA59145.1), thymic stromal lymphopoietin receptor (TSLPR) (NP_071431.2), IL-13Rα1 (EAW89893.1) and IL-13Rα2 (AAH20739.1), *Mus musculus* (mouse) IL-2Rγc (AAH14720.1), TSLPR (NP_001158207.1), IL-13Rα1 (AAH59939.1) and IL-13Rα2 (EDL14709.1), *Gallus gallus* (chicken) IL-2Rγc (NP_989858.1), TSLPR (XP_416864.3), IL-13Rα1 (XP_420218.3) and IL-13Rα2 (NP_001041543.1), and *Takifugu rubripes* (fugu) Il-2rγc (NP_001129354.1) and Il-13rα2 (XP_003971306.1). A phylogenic tree was derived from this alignment using the Neighbor-Joining algorithm and visualized with NJplot (34). Genomic sequences corresponding to the zebrafish *il-2rγc.a* and *il-2rγc.b* genes were identified using BLASTN, and the positions of intron/exon boundaries were determined by alignment with the corresponding zebrafish *il-2rγc* cDNA sequence, applying the 'GT-AG' rule (35).

Zebrafish husbandry and manipulation

Wild-type as well as Tg(*lck*:*lck*-EGFP)^{cz1} (Lck:eGFP) (36) and Tg(Cau.*ighv*-*ighm*:EGFP)^{sd19} (IgM1:eGFP) (37) transgenic zebrafish were maintained using standard husbandry practices (38). Wild-type embryos at the 1–8-cell stage were injected with anti-sense morpholinos diluted in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $CaCl_2$, 5.0 mM HEPES; pH 9). Morpholinos used were: il-2rγc.a^{SS1} (5′-CTTGTTTCATTAATACATACCCGCC, 3 mM), il-2rγc.a^{SS2} (5′-CTTCACTGTTTAACATCACAGTAAC, 7 mM), il-2rγc.b^{SS} (5′- GCAGCACTAAACAAAATATGATGCA, 5 mM), jak3^{SS} (5′-TTAAATGTGTTAGTGTCTCACCACT, 2 mM), stat5.1^{SS} (5′-GTGAACTTGTGACTTACCAGAGTTG, 1 mM), stat5.2^{SS} (5′ GTTGTCATCTGGTGCTCATACCTTC, 1 mM) and standard control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA, 1-7 mM as appropriate). In some experiments, embryos were coinjected with 100 pg/nl mRNA encoding eGFP or constitutively active version of zebrafish Jak3 A572V (this study) or Stat5.1 (H298R/N714F) (39) and standard control and il-2rγc.a^{SS1} morpholinos. Alternatively, 1-cell embryos were injected with 100 pg/nl mRNA encoding transcription activator-like effector nucleases (TALENs) (40) targeting exon 3 of *il-2rγc.a*, raised to adulthood intercrossed and progeny screened by RFLP with *NdeI* to identify mutants. Embryos were also treated with the JAK3 inhibitor tofacitinib (41) at 30 and 60 μM, MEK inhibitor PD98059 (42, 43) at 25 μM, and PI3K inhibitor LY294002 (44) at 15 μM from 56 h postfertilization (hpf) and then fixed at the appropriate time points. Single-cell suspensions were prepared from the thymus of Lck:eGFP and kidney of IgM1:eGFP transgenic zebrafish, as described (45). Sytox Red Dead Cell Stain was used for live cell discrimination (Molecular Probes), and cell sorting was performed with a FACSAria II (BD Biosciences). National and institutional guidelines for the care and use of laboratory animals were followed in all studies.

RT-PCR, quantitative RT-PCR, and 5′RACE

Total RNA was extracted from 30 zebrafish embryos using Trizol reagent (Life Technologies) following the manufacturer's recommendations and then resuspended in nuclease-free water. This RNA was subjected to semiquantitative RT-PCR with the following primers: *il-2rγc.a* 5′-CAGGCGTCAGGACCACATACAG and 5′- CTCTCACTATCACTGCTGGACTGG (time course/sequencing), 5′-AGAAGTGCGTTATGTGACCCTG and 5′- TCTGGTCAGTCCTGTAACGAAC (SS1 morpholino titration), 5′- CGAAGACTGTCCTGAATATGAGAC and 5′- AGACTCACTCCACTCGCTCCAG (SS2 morpholino titration and TALEN sequencing), 5′- AGAAGTGCGTTATGTGACCCTG and 5′- TCTGGTCAGTCCTGTAACGAAC (sequencing), 5′- TATGCTGAAAGAATATGTGAAG and 5′-AGACTCACTCCACTCGCTCCAG (sequencing), 5′- CGTCACTGGTCTTGTATGCTG and 5′- GTCGTTTTTCCTCATCAATCTGC (sequencing), *il-2rγc.b* 5′-TGGAACGAGCACAGCGACAC and 5′- GAAGAACCGCAGGAATCAGC (time course/sequencing), 5′- CAGTCATTTGTCACTCAGACGCTC and 5′- GATGCAGGTTTTACGGAGAGGT (morpholino titration), 5′- CAGTCATTTGTCACTCAGACGCTC and 5′- GAAGAACCGCAGGAATCAGC (sequencing), 5′- CGTCATACAGTGTGTCTCCAGTCTC and 5′- ACAGTATGGATGAGATGAGGATGG (sequencing), *jak3* 5′- AACTCAGAGACCACCTTCAGCA and 5′- GTGTGACCACCCTTCCTTCC (morpholino titration), *stat*5.1 5′- CAGGGAGATGCTCTACACCAG and 5′- CTCCGACTTGATGCTCTGC (morpholino titration), *stat*5.2 5′- CAGCACTTCCCCATTGAGG and 5′- CTCGTGTCAGCCAGGTCTC (morpholino titration), and *β-actin* 5′-TGGCATCACACCTTCTAC and 5′-AGACCATCACCAGAGTCC. RNA was also subjected to 5′RACE to generate cDNA using a 5′ RACE kit (Life Technologies), according to the manufacturer's protocol. Gene-specific primers were *il-2rγc.a* 5′-AGGCTTTTTCAGTTCC (gsp1), 5′- AGACTCACTCCACTCGCTCCAG (gsp2), *il-2rγc.a* 5′- AGACTCACTCCACTCGCTCCAG (gsp3), 5′GTTGTCGTTCTTCGTAACATTC (gsp4), 5′- AACCTTTCGCTGTGG (new gsp1), *il-2rγc.b* 5′- AAAGACTGGCTTGGGT (gsp1), *il-2rγc.b* 5′- GATGCAGGTTTTACGGAGAGGT (gsp2) ,and *il-2rγc.b* 5′-GAAAGTGTGTCGCTGTGCTC (gsp3).

Total RNA was also extracted from zebrafish embryos, larvae, and pooled adult zebrafish tissues with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol for animal tissues. This was subjected to semiquantitative RT-PCR with previously published primers for TCR-β chains (Vb1.5/17.5, Vb12) and Ig heavy chains (igVH1, igVH4) (46, 47). Total RNA was also subjected to quantitative RT-PCR (QRT-PCR) with the following primers: *il-2rγc.a* 5′-GTCACTGGTCTTGTATGCTGT and 5′-GCTCTCACTATCACTGCTGG, *il-2rγc.b* 5′-AGAAAGACCCAAGCCAGT and 5′-ATCTTTTTCCTCTCACAGTACC, *jak3* 5′-AACAGAGCGAGCAGCAGAGAG and 5′-GTGTGACCACCCTTCCTTCC, *pik3cγ* 5′-AGGGGCACTTGTGATTGAG and 5′-CTTCACTATTTCAATCATTCCAA, *β-actin* 5′-TGGCATCACACCTTCTAC and 5′-AGACCATCACCAGAGTCC and *map2k1* and *map2k2* (48). Data were normalized to *β-actin*, and fold change was calculated using the ΔΔCt method.

Total RNA was extracted from sorted cells using Trizol reagent and DirectZol RNA MiniPrep spin columns (ZymoResearch). cDNA was prepared using QuantiTect Reverse Transcription Kit (Qiagen) and subjected to QRT-PCR with *il-2rγc.a*, *il-2rγc.b*, *lck*, *ighm*, and *ef1a* primers (37). Each primer set flanked splice sites such that amplification of contaminating genomic DNA would produce considerably larger fragments in each case. Data were normalized to *ef1a*, and fold change was calculated using the ΔΔCt method. Each primer set flanked splice sites such that amplification of contaminating genomic DNA would produce considerably larger fragments in each case.

Genomic DNA analysis

Genomic DNA was obtained from pooled F1 embryos with QuickExtract following the manufacturer's instructions. This DNA was subjected to PCR with specific primers for *il-2rγc.a* and analyzed by RFLP with *NdeI* and by Sanger sequencing at the Australian Genome Research Facility.

Whole-mount in situ hybridization (WISH) and histochemistry

Embryos were dechorionated and fixed for 1-2 d in 4% (w/v) paraformaldehyde at 4°C prior to whole-mount in situ hybridization (WISH) with DIG-labeled anti-sense probes, as described (49, 50). Freshly anesthetized embryos were subjected to *O*-dianisidine staining of hemoglobin, as described (51). Quantitation was achieved by measuring the area of staining relative to eye diameter as determined using CellSens Dimension 1.6 software (Olympus) or counting of individual cells on ∼30 embryos. Data were analyzed for significance with a Student *t* test, with Welch's correction used where necessary to account for variation between the data groups.

Transient expression and analysis in human 293T cells

Human 293T cells were grown to 50–80% confluency before transfection with pBKCMV or pBKCMV expressing zebrafish Jak3 and/or Stat5.1 using lipofectamine reagent (Life Technologies). After incubation at 37°C in 10% (v/v) CO_2 for 2 d, a total cell lysate was prepared and subjected to Western blot analysis with anti-phospho Stat5.1 (Millipore 05-495) and anti-GAPDH (Millipore CB1001).

Results

Two IL-2Rγc paralogs in zebrafish

We have previously identified two putative zebrafish paralogs of the mammalian *IL-2Rγc* gene, called *il-2rγc.a* and *il-2rγc.b* (25). The full-length sequences of these genes were assembled from expressed sequence tags and predicted mRNA sequences present in gene databases, supplemented by sequencing of RT-PCR and 5′RACE products, and the encoded zebrafish protein sequences deduced. A phylogenetic tree was generated using the human, mouse, chicken, fugu, and zebrafish IL-2Rγc sequences along with those of the closely related thymic stromal lymphopoietin receptor (TSLPR), with the more divergent IL-13Rα sequences used as an out-group (Fig. 1A). This analysis grouped the zebrafish Il-2rγc.a and Il-2rγc.b sequences in a clade with the other IL-2Rγc chains supported by strong bootstrapping values, which was distinct from the related TSLPR clade. Of the two zebrafish sequences, Il-2rγc.b was the more divergent.

Alignment of the human, mouse, chicken, fugu, and zebrafish IL-2Rγc chains confirmed the conservation of key domains in both zebrafish sequences including an extracellular cytokine homology domain, a transmembrane domain and an intracellular domain (data not shown), including a Box 1 motif (Fig. 1B). Importantly, Il-2rγc.a showed conservation of three of the four intracellular tyrosines present in mammalian counterparts, with one additional nonconserved tyrosine, whereas Il-2rγc.b possessed only one conserved tyrosine and showed considerable divergence in its extended cytoplasmic region.

Expression of zebrafish IL-2Rγc paralogs

RT-PCR and QRT-PCR were used to characterize the expression of *il-2rγc.a* and *il-2rγc.b* during zebrafish embryogenesis. Expression of *il-2rγc.a* was first apparent at 16 hpf and then increased particularly from 4 days postfertilization (dpf) during the establishment of lymphopoiesis (Fig. 1C) (24). Conversely, *il-2rγc.b* showed biphasic expression with strong expression at 8–24 hpf, which waned before increasing from 5 dpf to maximal expression at 6 dpf (Fig. 1D). WISH was performed using probes for *il-2rγc.a* and *il-2rγc.b*. Distinct expression within the thymus was only evident for *il-2rγc.a* (Fig. 1F), which was not observed for *il-2rγc.b* (Fig. 1H) or sense controls (Fig. 1E, 1G). In adult zebrafish, *il-2rγc.a* was broadly expressed, but with highest expression in the thymus, kidney, and spleen (Fig. 1I), which represent key lymphoid organs (45, 52), whereas *il-2rγc.b* expression was highest in the spleen (Fig. 1J). Further analysis in transgenic zebrafish lines revealed that expression of both *il-2rγc.a* and *il-2rγc.b* was higher in the lck^+ T cell population within the thymus (Fig. 1K) and the igm^+ B cell population within the kidney (Fig. 1L).

Knockdown of il-2rγc.a but not il-2rγc.b affects lymphopoiesis

To investigate the functions of zebrafish *il-2rγc.a* and *il-2rγc.b*, a morpholino-mediated knockdown strategy was used (53), with morpholinos designed to interfere with splicing of the premRNA in the region encoding the cytokine homology domain (Supplemental Fig. 1A–C). The level of gene knockdown was determined by RT-PCR, which confirmed robust knockdown in embryos injected with 3 mM il-2rγc.a^{SS1}, 7 mM il-2rγc.a^{SS2}, and 5 mM il-2rγc.b^{SS} morpholino, compared with those injected with standard control morpholino at the equivalent concentration (Supplemental Fig. 1D, 1E). Sequence analysis of alternate transcripts seen with il-2rγc.a^{SS1} and il-2rγc.a^{SS2} confirmed that these would encode severely truncated proteins in each case (Supplemental Fig. 1F).

Morpholino-injected embryos showed no overt phenotypes; however, analysis by WISH with an early lymphocytic marker, *ikaros* (54), revealed significantly decreased expression from 3 dpf in the thymus of embryos injected with il-2rγc.a^{SS1} (Fig. 2B, 2E) and il-2rγc.a^{SS2} (Fig. 2C, 2F) compared with those injected with the standard control morpholino (Fig. 2A), which continued at 5 dpf (Fig. 2I, 2K, and data not shown). In contrast, *ikaros* expression in the thymus was not significantly altered in il-2rγc.b^{SS}-injected embryos at any time point tested (Fig. 2D, 2G, 2J, 2L).

To confirm these effects, embryos were analyzed with a range of other lymphocyte markers. Significantly decreased expression of *rag1*, a marker of more mature lymphoid cells (55), was also observed 4–6 dpf in embryos injected with il-2rγc.a^{SS1} (Fig. 2N, 2Q, 2U, 2W, and data not shown) and il-2rγc.a^{SS2} (Fig. 2O, 2R, and data not shown), but not those injected with il-2rγc.b^{SS} (Fig. 2P, 2S, 2V, 2X). This effect on lymphopoiesis was confirmed with the decreased expression of other lymphocyte-specific markers, *tcrα* and *lck* (36, 56), at 5 dpf in embryos injected with il-2rγc.a^{SS1} (Fig. 2Z, 2D') or il-2rγc.a^{SS2} (Fig. 2A', 2E') relative to controls (Fig. 2Y, 2C'), which was not observed with il-2rγc.b^{SS} (Fig. 2B', 2F').

To analyze the effects of *il-2rγc.a* ablation on mature T and B cells, TALEN-mediated genome targeting was used because the effects of morpholinos are only transient (57, 58). Specific TALENs directed to exon 3 (Supplemental Fig. 2A) were injected into one-cell embryos, which were raised to adulthood and intercrossed. One pair produced ~25% progeny that recapitulated the loss of *rag1* at 5 dpf (Supplemental Fig. 2E–H compared with Supplemental Fig. 2B–D), which were shown to have both *il-2rγc.a* alleles mutated by RFLP (Supplemental Fig. 2I) and sequence analysis (Supplemental Fig. 2J). Siblings from the cross were raised to 28 dpf and analyzed for the expression of rearranged TCR-β and Ig genes, as markers of mature T and B cells, respectively (46) (Fig. 2G'), and also genotyped (Fig. 2H'). This demonstrated the presence of mature T and B cells in wild-type larvae, as expected. In contrast, larvae carrying two *il-2rγc.a* mutant alleles lacked mature T cells, although mature B cells were present, albeit less consistently than in wild-type larvae.

It was important to determine whether the role of il-2rγc.a was confined to lymphopoiesis or whether it also exerted an effect on hematopoiesis more generally. Therefore, embryos were stained with *O*-dianisidine to visualize hemoglobin as a measure of erythrocytes (59) and subjected to WISH with *lysozyme* (*lyz*) to mark leukocytes (60). No significant differences in either the extent of *O*-dianisidine staining (Supplemental Fig. 3A, 3B and data not shown) or the number of lyz^+ cells (Supplemental Fig. 3C, 3D) were observed in embryos injected with either il-2rγc.a^{SS1} or il-2rγc.a^{SS2} compared with standard control morpholino. Similarly, expression of *c-myb*, a marker of hematopoietic stem–progenitor cells (61), at 3 dpf, was equivalent between il-2rγc.a morphants and controls (Supplemental Fig. 3E, 3F, and data not shown). Collectively, this result indicates a lymphocyte-specific function for Il-2rγc.a.

IL-2Rγc signaling components are conserved

Having identified a conserved functional role for the Il-2rγc.a paralog in zebrafish lymphopoiesis, it was of interest to determine

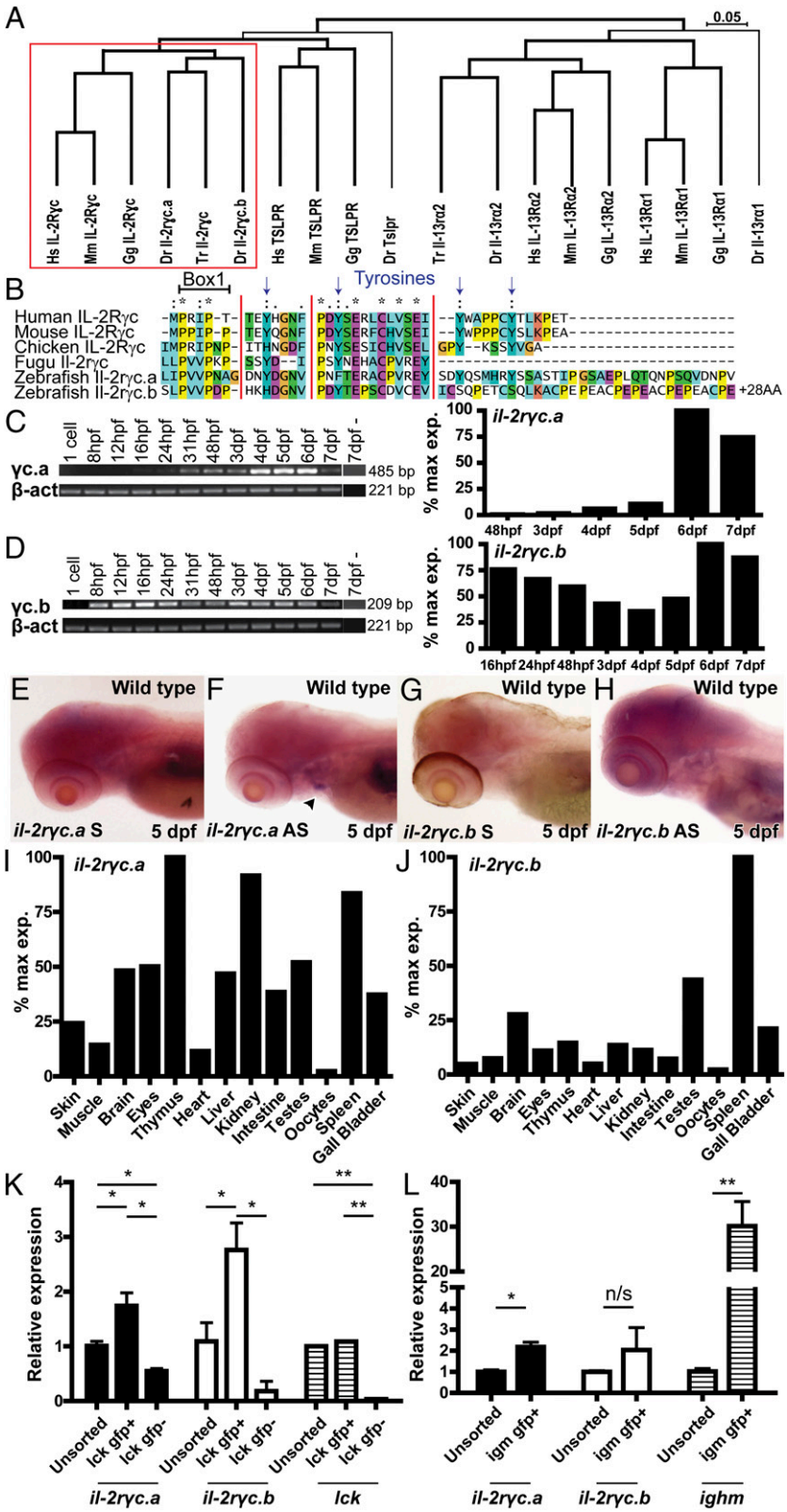

FIGURE 1. Characterization of zebrafish *il-2rγc* genes and their targeting with morpholinos. (**A**) Phylogenetic analysis of IL-2Rγc proteins. A phylogenetic tree was constructed from a ClustalX multiple alignment of zebrafish (Dr) Il-2rγc.a, Il-2rγc.b, Il-13rα1, Il-13rα2, and Tslpr along with the human (Hs), mouse (Mm), chicken (Gg), and fugu (Tr) IL-2Rγc, IL-13Rα1, IL-13Rα2, and TSLPR using the Neighbor-Joining algorithm with 1000 replicates. The IL-2Rγc clade is highlighted with a red box and bootstrap values >90% are bolded. (**B**) Conserved elements within IL-2Rγc intracellular domains. The intracellular regions of human, mouse, chicken, fugu, and zebrafish IL-2Rγc sequences were aligned using ClustalX. The sequences around specific motifs are shown with similar residues colored the same, and conservation across the six sequences indicated (identical *, highly similar:, similar .). (**C** and **D**) Expression of *il-2rγc* genes during zebrafish embryogenesis. Embryos were harvested during embryogenesis at the times indicated and analyzed by RT-PCR (*left*) and QRT-PCR (*right*) with primers specific for *il-2rγc.a* (C) or *il-2rγc.b* (D) along with *β-actin* controls with the sizes of amplified products indicated. Representative RT-negative controls are shown (7 dpf-) to confirm the absence of genomic contamination in RT-PCR, with QRT-PCR displayed as a percentage of maximal expression. (**E**–**H**) WISH expression of *il-2rγc.a* and *il-2rγc.b*. Embryos at 5 dpf were subjected to WISH with *il-2rγc.a* and *il-2rγc.b* sense (S) and antisense (AS) RNA probes. Staining in the thymus with *il-2rγc.a* is indicated with an arrow. (**I** and **J**) Adult expression of *il-2rγc.a* and *il-2rγc.b*. Adult zebrafish tissues were analyzed with QRT-PCR with primers specific for *il2rγc.a* (I) or *il2rγc.b* (J), with levels displayed as a percentage of maximal expression. (**K** and **L**) Expression of *il-2rγc.a* and *il-2rγc.b* in adult lymphoid cells. GFP^+ lymphocytes were sorted from the thymus of Lck:eGFP (K) and kidney of IgM1:eGFP (L) transgenic fish. Shown are the normalized relative transcript levels of *il-2rγc.a* (black bars), *il-2rγc.b* (white bars), and *lck* or *ighm* (checked bars), relative to unsorted cells, as determined with QRT-PCR (mean ± SEM of three technical replicates). $*p < 0.05$, $**p < 0.001$.

whether the downstream signaling pathways were also conserved. IL-2Rγc signals via JAK3, with zebrafish previously shown to possess a single, highly conserved *jak3* gene (26). This signaling component, which was also strongly expressed in the developing thymus (Supplemental Fig. 4), was targeted by two approaches. In the first approach, embryos were treated with the JAK3-specific inhibitor tofacitinib (41) compared with a DMSO control from 56 hpf, prior to the onset of lymphopoiesis (62) (Fig. 3A–3D). Alternatively, embryos were injected with a morpholino targeting a splice site within *jak3* (Fig. 3E–3H). Both approaches substantially reduced expression of *rag1* (Fig. 3A–3G), along with other lymphocyte markers (data not shown).

IL-2Rγc/JAK3 stimulates various downstream pathways, including those involving STAT5, PI3K, and ERK (12, 63, 64).

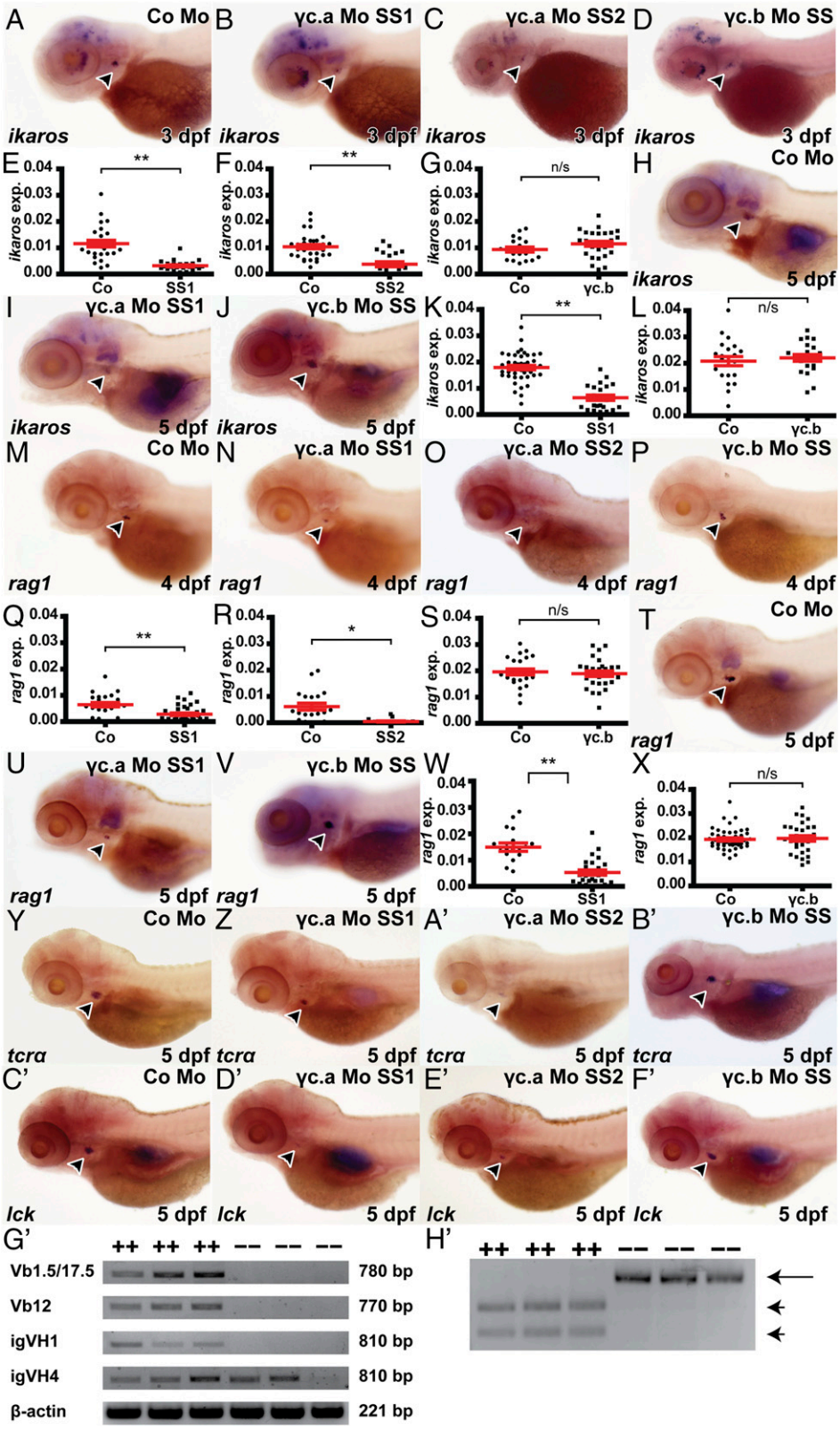

FIGURE 2. Effect of il-2rγc ablation on lymphoid cells. (**A–F'**) WISH analysis of morphants embryos. Embryos injected with standard control (Co Mo), il-2rγc.a^{SS1} (γc.a Mo SS1), il-2rγc.a^{SS2} (γc.a Mo SS2), or il-2rγc.b^{SS} (γc.b Mo SS) morpholinos were subjected to WISH with *ikaros* (A–L), *rag1* (M–X), *tcrα* (Y–B'), and *lck* (C'–F') at the times indicated. The extent of *ikaros* and *rag1* staining in the thymi (shown with the arrows) was determined as a ratio to eye size, averaged for individual embryos, with mean and SEM shown in red for il-2rγc.a^{SS1} (E, K, Q, and W), il-2rγc.a^{SS2} (F and R), and il-2rγc.b^{SS} (G, L, S, and X). (**G'** and **H'**) RT-PCR analysis of mutant larvae. Expression of TCR β-chain rearrangements V(D)J-C$_\beta$ Vb1.5 and Vb12, Ig H chain gene rearrangements VH1 and VH4 and *β-actin* as a control were examined in wild-type (++) and *il-2rγc.a* mutant (−−) zebrafish by RT-PCR for expression of TCR and Ig (G'). RT-negative controls yielded no products (data not shown). Genomic DNA was extracted from these larvae and was subjected to PCR with primers surrounding the mutation site and digested with *NdeI* for genotyping (H'). The arrowheads indicate the cleaved products produced from wild-type alleles, and the arrow shows uncleaved products in embryos carrying mutant alleles. Statistically significant differences are indicated. *$p < 0.05$, ** $p < 0.001$. n/s, not significant.

Zebrafish possess two *STAT5* paralogs, *stat5.1* and *stat5.2* (65), which were ablated separately with morpholinos (Fig. 3I–3L). Only knockdown of Stat5.1 resulted in a significant, albeit small, decrease in *rag1* expression compared with the controls. Finally, zebrafish also possess both PI3K and ERK (66, 67), which were investigated with LY294002, a PI3K inhibitor (68), and PD98059, an inhibitor of MEK, which lies upstream of ERK (69). The extent of *rag1* expression was significantly decreased for both inhibitors compared with a DMSO control (Fig. 3M–3P). Notably the JAK3 inhibitor reduced the expression of *jak3* (1.5 ± 0.1-fold; $p = 0.029$), as would be expected because this is a marker of developing lymphocytes (Supplemental Fig. 4). The PI3K inhibitor also reduced expression of *pi3kcg* (3.0 ± 0.3-fold; $p < 0.0001$), which is

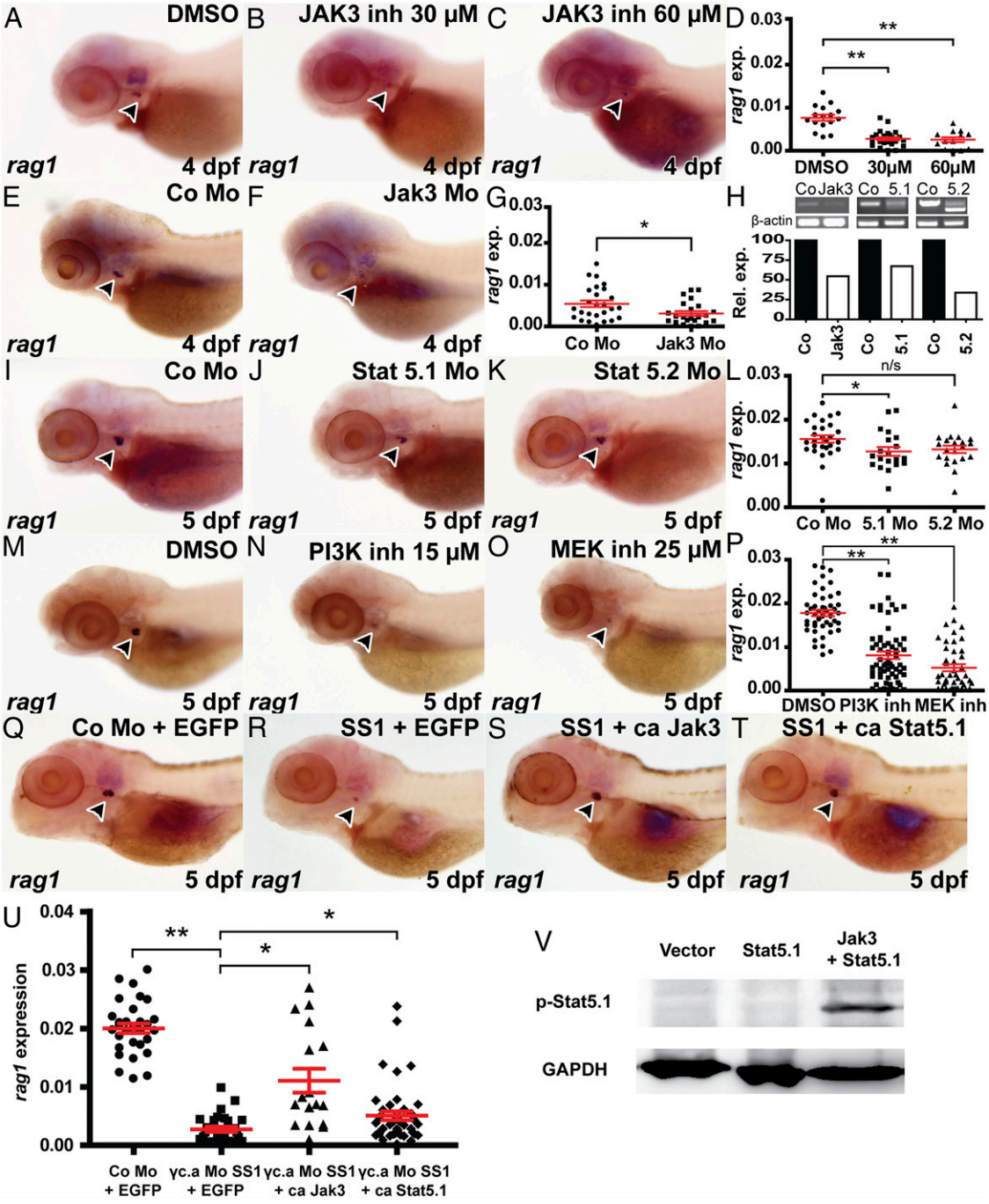

FIGURE 3. Effect of disruption of downstream pathways on lymphoid cells. (**A**–**G**, **I**–**U**) WISH analysis of manipulated embryos. Embryos at 56 hpf were bathed in DMSO vehicle control (A and M) or inhibitors for JAK3 (B and C), PI3K (N), or MEK (O) or injected at the 1–8-cell stage with standard control (E and I), *jak3* (F), *stat5.1* (J), or *stat5.2* (K) morpholinos or standard control morpholino plus EGFP mRNA (Q) or il-2rγc.a^{SS1} morpholino plus EGFP mRNA (R) or mRNA encoding constitutively active Jak3 (S) or Stat5.1 (T) and subjected to WISH with *rag1* at the times indicated. The *rag1* expression in the thymi is indicated with arrows and quantified relative to eye size for embryos in which Jak3 (D and G), Stat5.1 and Stat5.2 (L), PI3K and MEK (P), or Il-2rγc.a (U) were targeted. Each symbol represents the average ratio for individual embryos, with mean and SEM shown in red. (**H**) Analysis of *jak3*, *stat5.1*, and *stat5.2* morpholino targeting. Total RNA was prepared from embryos injected with standard control (Co) or *jak3* (Jak3) morpholinos at 4 dpf, or *stat5.1* (5.1) or *stat5.2* (5.2) morpholinos at 5 dpf, and subjected to RT-PCR with gene specific primers (*upper panels*) and *β-actin* (*middle panels*), with the levels of residual wild-type products indicated for each morphant (*lower panel*). (**V**) Phosphorylation of zebrafish Stat5.1 by Jak3. Total cell lysates prepared from HEK293T cells transfected with empty vector or vector expressing Stat5.1 or Jak3 plus Stat5.1 and subjected to Western blot analysis with α-pStat5 (*upper panel*) or α-GAPDH (*lower panel*). Statistically significant differences are indicated. *$p < 0.05$, **$p < 0.001$. n/s, not significant.

known to be highly expressed in lymphocytes (70). In contrast, the MEK inhibitor had no effect on expression of *map2k1* (1.00 ± 0.04-fold; $p = 0.7662$) and increased the expression of *map2k2* (1.2-fold ± 0.02; $p = 0.002$).

Finally, coinjection of mRNA encoding a constitutively active Jak3 and to a lesser extent Stat5.1 rescued *rag1* expression in embryos injected with il-2rγc.a^{SS1} morpholino (Fig. 3Q–3U), and zebrafish Jak3 could phosphorylate Stat5.1 in vitro (Fig. 3V).

Discussion

The IL-2R family has been studied extensively in mammals, where it has an important role in immune cell development (7), with mutations of the shared *IL-2Rγc* signaling chain leading to a T^-B^+ SCID in humans (14) and a T^-B^- SCID in mice (15). A range of bioinformatic approaches confirmed the presence of two *IL-2Rγc* paralogs in zebrafish, *il-2rγc.a*, and *il-2rγc.b*. The Il-2rγc.a protein showed higher sequence conservation, particularly within the intracellular region. Peak expression of *il-2rγc.a* also occurred coincidentally with embryonic lymphopoiesis, when it was evident in the developing thymus, whereas *il-2rγc.b* was not expressed at this site. The *il-2rγc.a* gene was broadly expressed in adult tissue, but showed highest expression in the spleen, thymus, and kidney with its expression demonstrated in the lymphoid cell populations of these thymus and kidney, whereas *il-2rγc.b* showed peak expression in the spleen. Knockdown of Il-2rγc.a—but not Il-2rγc.b—substantially inhibited embryonic T lymphopoiesis, but did not affect other aspects of hematopoiesis. Mature T cells were also absent in *il-2rγc.a* ablated larvae, although mature B cells were present, recapitulating human SCID. This finding suggests that Il-2rγc.a has a conserved role in lymphopoiesis, with the function of Il-2rγc.b currently unknown, consistent with the divergent roles observed for many teleost genes (71–73).

Mammalian IL-2Rγc signals specifically through JAK3 (74–76), which then activates a number of downstream pathways. Zebrafish Il-2rγc.a possessed a conserved JAK3 docking site and was coexpressed with Jak3 in the developing thymus. In addition, ablation of zebrafish Jak3 recapitulated the effects of *il-2rγc.a* knockdown, consistent with JAK3 loss also causing SCID in both humans and mice (77–79), whereas a constitutively active Jak3 could rescue the effects of *il-2rγc.a* knockdown. Lying downstream of IL-2Rγc/JAK3 are a number of signaling molecules, including STAT5, PI3K, and ERK, each of which have been shown to contribute to lymphopoiesis. Thus, *Stat5a*$^-$*/Stab5b*$^-$ mice showed reduced B lymphocytes because of decreased proliferative responses to IL-2 (80–83), *Erk1*$^-$*/Erk2*$^-$ mice exhibited defective thymic T cell maturation (84, 85), whereas PI3K knockout mice displayed a range of immune defects, including impaired proliferation of T cells in p85α or p110δ knockouts and a reduction in thymocyte numbers in p110γ knockouts (86). Ablation or inhibition of these molecules in zebrafish also produced a reduction in lymphocytes suggesting multiple pathways contribute to lymphocyte development. Of the two zebrafish STAT5 proteins, Stat5.1 appeared to play the key role in this regard, whereas a constitutively active Stat5.1 was able to rescue il-2rγc.a morphants partially, with zebrafish Jak3 shown to phosphorylate Stat5.1.

Having determined the importance of Il-2rγc.a/Jak3 signaling in zebrafish lymphopoiesis, it remains of interest to determine which receptor complexes are involved. In zebrafish, ligand-specific receptor subunits have been identified for all IL-2R family components, except IL-2Rα and IL-9Rα (25). Motifs important for downstream pathways are also conserved on several receptor subunits, including the conserved STAT5 docking sites (YXXV/L/M) on Il-2rβ, Il-4rα, and Il-7rα (87–89), and a docking site for SHC, which contributes to activation of the PI3K and ERK pathways, on Il-2rβ (64) (data not shown). Ligands of the IL-2R family identified in zebrafish include Il-4, Il-15, Il-15–like, and Il-21 (29, 90), with potential Il-2 (91–93) and Il-7 ligands (94) characterized in other teleosts, but not Il-9 (95). Therefore, the phenotypes observed following knockdown of *il-2rγc.a* and *jak3* could potentially be due to defective signaling via several cytokines. Zebrafish Il-4 has been shown to stimulate B cell proliferation and Ab production in adult fish (29), but its role in T cell development has not been determined. However, zebrafish carrying an *il-7rα* mutation showed decreased *rag1* expression at 5 dpf (28), indicating that Il-7–mediated signaling represents an important contributor for early T cell lymphopoiesis. This article also showed contributions from both zebrafish Jak1 and Jak3 in mediating these effects, consistent with the data presented in this study.

Finally, disruption of zebrafish Il-2rγc.a signaling resulted in ablation of lymphopoiesis with similar characteristics to human SCID (74, 96). This result provides important proof-of-principle evidence that the generation of a zebrafish SCID model by specific gene targeting is feasible. Although a number of husbandry challenges need to be overcome, such a SCID zebrafish would represent an invaluable new resource to investigate aspects of immunity and provide a platform for transplantation and cancer studies.

Acknowledgments

We thank the Deakin University Animal House staff for superb aquarium management.

Disclosures

The authors have no financial conflicts of interest.

References

1. Alves, N. L., F. A. Arosa, and R. A. W. van Lier. 2007. Common γ chain cytokines: dissidence in the details. *Immunol. Lett.* 108: 113–120.
2. Malek, T. R. 2008. The biology of interleukin-2. *Annu. Rev. Immunol.* 26: 453–479.
3. Meazza, R., B. Azzarone, A. M. Orengo, and S. Ferrini. 2011. Role of common-gamma chain cytokines in NK cell development and function: perspectives for immunotherapy. *J. Biomed. Biotechnol.* 2011: 861920.
4. Spolski, R., and W. J. Leonard. 2008. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu. Rev. Immunol.* 26: 57–79.
5. Liao, W., J. X. Lin, and W. J. Leonard. 2013. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. *Immunity* 38: 13–25.
6. Jiang, H., M. B. Harris, and P. Rothman. 2000. IL-4/IL-13 signaling beyond JAK/STAT. *J. Allergy Clin. Immunol.* 105: 1063–1070.
7. Rochman, Y., R. Spolski, and W. J. Leonard. 2009. New insights into the regulation of T cells by γ(c) family cytokines. *Nat. Rev. Immunol.* 9: 480–490.
8. Goswami, R., and M. H. Kaplan. 2011. A brief history of IL-9. *J. Immunol.* 186: 3283–3288.
9. Budagian, V., E. Bulanova, R. Paus, and S. Bulfone-Paus. 2006. IL-15/IL-15 receptor biology: a guided tour through an expanding universe. *Cytokine Growth Factor Rev.* 17: 259–280.
10. Huntington, N. D., N. Legrand, N. L. Alves, B. Jaron, K. Weijer, A. Plet, E. Corcuff, E. Mortier, Y. Jacques, H. Spits, and J. P. Di Santo. 2009. IL-15 trans-presentation promotes human NK cell development and differentiation *in vivo*. *J. Exp. Med.* 206: 25–34.
11. Malek, T. R., and I. Castro. 2010. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity* 33: 153–165.
12. Lai, Y. G., M. S. Hou, A. Lo, S. T. Huang, Y. W. Huang, H. F. Yang-Yen, and N. S. Liao. 2013. IL-15 modulates the balance between Bcl-2 and Bim via a Jak3/1-PI3K-Akt-ERK pathway to promote CD8αα+ intestinal intraepithelial lymphocyte survival. *Eur. J. Immunol.* 43: 2305–2316.
13. Leonard, W. J. 1994. The defective gene in X-linked severe combined immunodeficiency encodes a shared interleukin receptor subunit: implications for cytokine pleiotropy and redundancy. *Curr. Opin. Immunol.* 6: 631–635.
14. Noguchi, M., H. Yi, H. M. Rosenblatt, A. H. Filipovich, S. Adelstein, W. S. Modi, O. W. McBride, and W. J. Leonard. 1993. Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 73: 147–157.
15. Bosma, G. C., R. P. Custer, and M. J. Bosma. 1983. A severe combined immunodeficiency mutation in the mouse. *Nature* 301: 527–530.
16. Bastide, C., C. Bagnis, P. Mannoni, J. Hassoun, and F. Bladou. 2002. A Nod Scid mouse model to study human prostate cancer. *Prostate Cancer Prostatic Dis.* 5: 311–315.
17. Goya, M., S. Miyamoto, K. Nagai, Y. Ohki, K. Nakamura, K. Shitara, H. Maeda, T. Sangai, K. Kodama, Y. Endoh, et al. 2004. Growth inhibition of human prostate cancer cells in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice by a ligand-specific antibody to human insulin-like growth factors. *Cancer Res.* 64: 6252–6258.
18. Wu, T. T., R. A. Sikes, Q. Cui, G. N. Thalmann, C. Kao, C. F. Murphy, H. Yang, H. E. Zhau, G. Balian, and L. W. K. Chung. 1998. Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. *Int. J. Cancer* 77: 887–894.
19. Chen, X., E. Sievers, Y. Hou, R. Park, M. Tohme, R. Bart, R. Bremner, J. R. Bading, and P. S. Conti. 2005. Integrin α v β 3-targeted imaging of lung cancer. *Neoplasia* 7: 271–279.

20. Boyle, M. J., M. Connors, M. E. Flanigan, S. P. Geiger, H. Ford, Jr., M. Baseler, J. Adelsberger, R. T. Davey, Jr., and H. C. Lane. 1995. The human HIV/peripheral blood lymphocyte (PBL)-SCID mouse. A modified human PBL-SCID model for the study of HIV pathogenesis and therapy. *J. Immunol.* 154: 6612–6623.
21. Vaughan, A. M., S. H. Kappe, A. Ploss, and S. A. Mikolajczak. 2012. Development of humanized mouse models to study human malaria parasite infection. *Future Microbiol.* 7: 657–665.
22. Traver, D., P. Herbomel, E. E. Patton, R. D. Murphey, J. A. Yoder, G. W. Litman, A. Catic, C. T. Amemiya, L. I. Zon, and N. S. Trede. 2003. The zebrafish as a model organism to study development of the immune system. *Adv. Immunol.* 81: 253–330.
23. Yoder, J. A., M. E. Nielsen, C. T. Amemiya, and G. W. Litman. 2002. Zebrafish as an immunological model system. *Microbes Infect.* 4: 1469–1478.
24. Chen, A. T., and L. I. Zon. 2009. Zebrafish blood stem cells. *J. Cell. Biochem.* 108: 35–42.
25. Liongue, C., and A. C. Ward. 2007. Evolution of Class I cytokine receptors. *BMC Evol. Biol.* 7: 120.
26. Liongue, C., L. A. O'Sullivan, M. C. Trengove, and A. C. Ward. 2012. Evolution of JAK-STAT pathway components: mechanisms and role in immune system development. *PLoS One* 7: e32777.
27. Ito, K., F. Takizawa, Y. Yoshiura, M. Ototake, and T. Nakanishi. 2008. Expression profile of cytokine and transcription factor genes during embryonic development of zebrafish *Danio rerio*. *Fish. Sci.* 74: 391–396.
28. Iwanami, N., F. Mateos, I. Hess, N. Riffel, C. Soza-Ried, M. Schorpp, and T. Boehm. 2011. Genetic evidence for an evolutionarily conserved role of IL-7 signaling in T cell development of zebrafish. *J. Immunol.* 186: 7060–7066.
29. Zhu, L. Y., P. P. Pan, W. Fang, J. Z. Shao, and L. X. Xiang. 2012. Essential role of IL-4 and IL-4Rα interaction in adaptive immunity of zebrafish: insight into the origin of Th2-like regulatory mechanism in ancient vertebrates. *J. Immunol.* 188: 5571–5584.
30. Paffett-Lugassy, N., N. Hsia, P. G. Fraenkel, B. Paw, I. Leshinsky, B. Barut, N. Bahary, J. Caro, R. Handin, and L. I. Zon. 2007. Functional conservation of erythropoietin signaling in zebrafish. *Blood* 110: 2718–2726.
31. Aggad, D., C. Stein, D. Sieger, M. Mazel, P. Boudinot, P. Herbomel, J. P. Levraud, G. Lutfalla, and M. Leptin. 2010. *In vivo* analysis of Ifn-γ1 and Ifn-γ2 signaling in zebrafish. *J. Immunol.* 185: 6774–6782.
32. Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389–3402.
33. Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. J. Gibson. 1998. Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* 23: 403–405.
34. Perrière, G., and M. Gouy. 1996. WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie* 78: 364–369.
35. Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp. 1986. Splicing of messenger RNA precursors. *Annu. Rev. Biochem.* 55: 1119–1150.
36. Langenau, D. M., A. A. Ferrando, D. Traver, J. L. Kutok, J. P. Hezel, J. P. Kanki, L. I. Zon, A. T. Look, and N. S. Trede. 2004. *In vivo* tracking of T cell development, ablation, and engraftment in transgenic zebrafish. *Proc. Natl. Acad. Sci. USA* 101: 7369–7374.
37. Page, D. M., V. Wittamer, J. Y. Bertrand, K. L. Lewis, D. N. Pratt, N. Delgado, S. E. Schale, C. McGue, B. H. Jacobsen, A. Doty, et al. 2013. An evolutionarily conserved program of B-cell development and activation in zebrafish. *Blood* 122: e1–e11.
38. Lawrence, C. 2007. The husbandry of zebrafish (*Danio rerio*): A review. *Aquaculture* 269: 1–20.
39. Lewis, R. S., S. E. Stephenson, and A. C. Ward. 2006. Constitutive activation of zebrafish Stat5 expands hematopoietic cell populations in vivo. *Exp. Hematol.* 34: 179–187.
40. Dahlem, T. J., K. Hoshijima, M. J. Jurynec, D. Gunther, C. G. Starker, A. S. Locke, A. M. Weis, D. F. Voytas, and D. J. Grunwald. 2012. Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. *PLoS Genet.* 8: e1002861.
41. Changelian, P. S., M. E. Flanagan, D. J. Ball, C. R. Kent, K. S. Magnuson, W. H. Martin, B. J. Rizzuti, P. S. Sawyer, B. D. Perry, W. H. Brissette, et al. 2003. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. *Science* 302: 875–878.
42. Dudley, D. T., L. Pang, S. J. Decker, A. J. Bridges, and A. R. Saltiel. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* 92: 7686–7689.
43. Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* 270: 27489–27494.
44. Vlahos, C. J., W. F. Matter, K. Y. Hui, and R. F. Brown. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269: 5241–5248.
45. Wittamer, V., J. Y. Bertrand, P. W. Gutschow, and D. Traver. 2011. Characterization of the mononuclear phagocyte system in zebrafish. *Blood* 117: 7126–7135.
46. Schorpp, M., M. Bialecki, D. Diekhoff, B. Walderich, J. Odenthal, H. M. Maischein, A. G. Zapata, and T. Boehm. 2006. Conserved functions of Ikaros in vertebrate lymphocyte development: genetic evidence for distinct larval and adult phases of T cell development and two lineages of B cells in zebrafish. *J. Immunol.* 177: 2463–2476.
47. Petrie-Hanson, L., C. Hohn, and L. Hanson. 2009. Characterization of rag1 mutant zebrafish leukocytes. *BMC Immunol.* 10: 8.
48. Nguyen, A. T., A. Emelyanov, C. H. Koh, J. M. Spitsbergen, S. H. Lam, S. Mathavan, S. Parinov, and Z. Gong. 2011. A high level of liver-specific expression of oncogenic Kras(V12) drives robust liver tumorigenesis in transgenic zebrafish. *Dis. Model. Mech.* 4: 801–813.
49. Schulte-Merker, S., R. K. Ho, B. G. Herrmann, and C. Nüsslein-Volhard. 1992. The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* 116: 1021–1032.
50. Thisse, C., and B. Thisse. 2008. High-resolution *in situ* hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* 3: 59–69.
51. Lieschke, G. J., A. C. Oates, M. O. Crowhurst, A. C. Ward, and J. E. Layton. 2001. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood* 98: 3087–3096.
52. Willett, C. E., A. Cortes, A. Zuasti, and A. G. Zapata. 1999. Early hematopoiesis and developing lymphoid organs in the zebrafish. *Dev. Dyn.* 214: 323–336.
53. Nasevicius, A., and S. C. Ekker. 2000. Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* 26: 216–220.
54. Willett, C. E., H. Kawasaki, C. T. Amemiya, S. Lin, and L. A. Steiner. 2001. Ikaros expression as a marker for lymphoid progenitors during zebrafish development. *Dev. Dyn.* 222: 694–698.
55. Willett, C. E., J. J. Cherry, and L. A. Steiner. 1997. Characterization and expression of the recombination activating genes (*rag1* and *rag2*) of zebrafish. *Immunogenetics* 45: 394–404.
56. Danilova, N., V. S. Hohman, F. Sacher, T. Ota, C. E. Willett, and L. A. Steiner. 2004. T cells and the thymus in developing zebrafish. *Dev. Comp. Immunol.* 28: 755–767.
57. Ekker, S. C., and J. D. Larson. 2001. Morphant technology in model developmental systems. *Genesis* 30: 89–93.
58. Hogan, B. M., H. Verkade, G. J. Lieschke, and J. K. Heath. 2008. Manipulation of gene expression during zebrafish embryonic development using transient approaches. *Methods Mol. Biol.* 469: 273–300.
59. de Jong, J. L., and L. I. Zon. 2005. Use of the zebrafish system to study primitive and definitive hematopoiesis. *Annu. Rev. Genet.* 39: 481–501.
60. Yang, C. T., C. J. Cambier, J. M. Davis, C. J. Hall, P. S. Crosier, and L. Ramakrishnan. 2012. Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. *Cell Host Microbe* 12: 301–312.
61. Zhang, Y., H. Jin, L. Li, F. X. Qin, and Z. Wen. 2011. cMyb regulates hematopoietic stem/progenitor cell mobilization during zebrafish hematopoiesis. *Blood* 118: 4093–4101.
62. Davidson, A. J., and L. I. Zon. 2004. The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. *Oncogene* 23: 7233–7246.
63. Ghoreschi, K., A. Laurence, and J. J. O'Shea. 2009. Janus kinases in immune cell signaling. *Immunol. Rev.* 228: 273–287.
64. Yu, A., L. Zhu, N. H. Altman, and T. R. Malek. 2009. A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. *Immunity* 30: 204–217.
65. Lewis, R. S., and A. C. Ward. 2004. Conservation, duplication and divergence of the zebrafish stat5 genes. *Gene* 338: 65–74.
66. Snaar-Jagalska, B. E., S. F. Krens, I. Robina, L. X. Wang, and H. P. Spaink. 2003. Specific activation of ERK pathways by chitin oligosaccharides in embryonic zebrafish cell lines. *Glycobiology* 13: 725–732.
67. Pozios, K. C., J. Ding, B. Degger, Z. Upton, and C. Duan. 2001. IGFs stimulate zebrafish cell proliferation by activating MAP kinase and PI3-kinase-signaling pathways. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 280: R1230–R1239.
68. Finkielsztein, A., and G. M. Kelly. 2009. Altering PI3K-Akt signalling in zebrafish embryos affects PTEN phosphorylation and gastrulation. *Biol. Cell* 101: 661–678, 4, 678.
69. Shaul, Y. D., and R. Seger. 2007. The MEK/ERK cascade: from signaling specificity to diverse functions. *Biochim. Biophys. Acta* 1773: 1213–1226.
70. Su, A. I., T. Wiltshire, S. Batalov, H. Lapp, K. A. Ching, D. Block, J. Zhang, R. Soden, M. Hayakawa, G. Kreiman, et al. 2004. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc. Natl. Acad. Sci. USA* 101: 6062–6067.
71. Gorissen, M., N. J. Bernier, S. B. Nabuurs, G. Flik, and M. O. Huising. 2009. Two divergent leptin paralogues in zebrafish (*Danio rerio*) that originate early in teleostean evolution. *J. Endocrinol.* 201: 329–339.
72. Powell, G. T., and G. J. Wright. 2012. Genomic organisation, embryonic expression and biochemical interactions of the zebrafish junctional adhesion molecule family of receptors. *PLoS One* 7: e40810.
73. McClintock, J. M., R. Carlson, D. M. Mann, and V. E. Prince. 2001. Consequences of Hox gene duplication in the vertebrates: an investigation of the zebrafish Hox paralogue group 1 genes. *Development* 128: 2471–2484.
74. Kovanen, P. E., and W. J. Leonard. 2004. Cytokines and immunodeficiency diseases: critical roles of the γ(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways. *Immunol. Rev.* 202: 67–83.
75. O'Sullivan, L. A., C. Liongue, R. S. Lewis, S. E. M. Stephenson, and A. C. Ward. 2007. Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease. *Mol. Immunol.* 44: 2497–2506.
76. Sugamura, K., H. Asao, M. Kondo, N. Tanaka, N. Ishii, K. Ohbo, M. Nakamura, and T. Takeshita. 1996. The interleukin-2 receptor γ chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu. Rev. Immunol.* 14: 179–205.
77. Notarangelo, L. D., P. Mella, A. Jones, G. de Saint Basile, G. Savoldi, T. Cranston, M. Vihinen, and R. F. Schumacher. 2001. Mutations in severe combined immune deficiency (SCID) due to JAK3 deficiency. *Hum. Mutat.* 18: 255–263.

78. Russell, S. M., N. Tayebi, H. Nakajima, M. C. Riedy, J. L. Roberts, M. J. Aman, T. S. Migone, M. Noguchi, M. L. Markert, R. H. Buckley, et al. 1995. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science* 270: 797–800.
79. Park, S. Y., K. Saijo, T. Takahashi, M. Osawa, H. Arase, N. Hirayama, K. Miyake, H. Nakauchi, T. Shirasawa, and T. Saito. 1995. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity* 3: 771–782.
80. Yao, Z., Y. Cui, W. T. Watford, J. H. Bream, K. Yamaoka, B. D. Hissong, D. Li, S. K. Durum, Q. Jiang, A. Bhandoola, et al. 2006. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc. Natl. Acad. Sci. USA* 103: 1000–1005.
81. Imada, K., E. T. Bloom, H. Nakajima, J. A. Horvath-Arcidiacono, G. B. Udy, H. W. Davey, and W. J. Leonard. 1998. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. *J. Exp. Med.* 188: 2067–2074.
82. Nakajima, H., X. W. Liu, A. Wynshaw-Boris, L. A. Rosenthal, K. Imada, D. S. Finbloom, L. Hennighausen, and W. J. Leonard. 1997. An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor α chain induction. *Immunity* 7: 691–701.
83. Snow, J. W., N. Abraham, M. C. Ma, B. G. Herndier, A. W. Pastuszak, and M. A. Goldsmith. 2003. Loss of tolerance and autoimmunity affecting multiple organs in STAT5A/5B-deficient mice. *J. Immunol.* 171: 5042–5050.
84. Pagès, G., S. Guérin, D. Grall, F. Bonino, A. Smith, F. Anjuere, P. Auberger, and J. Pouysségur. 1999. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science* 286: 1374–1377.
85. Chan, G., S. Gu, and B. G. Neel. 2013. Erk1 and Erk2 are required for maintenance of hematopoietic stem cells and adult hematopoiesis. *Blood* 121: 3594–3598.
86. Koyasu, S. 2003. The role of PI3K in immune cells. *Nat. Immunol.* 4: 313–319.
87. Imbert, V., R. Rezzonico, P. Reichenbach, and M. Nabholz. 2002. Induction of interleukin-2 receptor alpha (IL-2Ralpha) expression by interleukin-2: important role of the interleukin-2 receptor beta chain region between the two Stat5 docking sites. *Eur. Cytokine Netw.* 13: 331–339.
88. Jiang, Q., W. Q. Li, R. R. Hofmeister, H. A. Young, D. R. Hodge, J. R. Keller, A. R. Khaled, and S. K. Durum. 2004. Distinct regions of the interleukin-7 receptor regulate different Bcl2 family members. *Mol. Cell. Biol.* 24: 6501–6513.
89. Friedrich, K., W. Kammer, I. Erhardt, S. Brändlein, W. Sebald, and R. Moriggl. 1999. Activation of STAT5 by IL-4 relies on Janus kinase function but not on receptor tyrosine phosphorylation, and can contribute to both cell proliferation and gene regulation. *Int. Immunol.* 11: 1283–1294.
90. Gunimaladevi, I., R. Savan, K. Sato, R. Yamaguchi, and M. Sakai. 2007. Characterization of an interleukin-15 like (IL-15L) gene from zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* 22: 351–362.
91. Bird, S., J. Zou, T. Kono, M. Sakai, J. M. Dijkstra, and C. Secombes. 2005. Characterisation and expression analysis of interleukin 2 (IL-2) and IL-21 homologues in the Japanese pufferfish, *Fugu rubripes*, following their discovery by synteny. *Immunogenetics* 56: 909–923.
92. Blohm, U., E. Siegl, and B. Köllner. 2003. Rainbow trout (*Oncorhynchus mykiss*) sIgM- leucocytes secrete an interleukin-2 like growth factor after mitogenic stimulation *in vitro*. *Fish Shellfish Immunol.* 14: 449–465.
93. Díaz-Rosales, P., S. Bird, T. H. Wang, K. Fujiki, W. S. Davidson, J. Zou, and C. J. Secombes. 2009. Rainbow trout interleukin-2: cloning, expression and bioactivity analysis. *Fish Shellfish Immunol.* 27: 414–422.
94. Kono, T., S. Bird, K. Sonoda, R. Savan, C. J. Secombes, and M. Sakai. 2008. Characterization and expression analysis of an interleukin-7 homologue in the Japanese pufferfish, *Takifugu rubripes*. *FEBS J.* 275: 1213–1226.
95. Secombes, C. J., T. Wang, and S. Bird. 2011. The interleukins of fish. *Dev. Comp. Immunol.* 35: 1336–1345.
96. Leonard, W. J. 1996. The molecular basis of X-linked severe combined immunodeficiency: defective cytokine receptor signaling. *Annu. Rev. Med.* 47: 229–239.