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Ontogeny and localization of the cells produce IL-2 in healthy animals

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ABSTRACT

IL-2 is a growth factor for activated T cells and is required for maintenance of naturally arising regulatory T cells (nTregs). Mice defective in IL-2/IL-2 receptor signaling pathways have impaired nTregs and suffer from lymphoproliferative disorders, suggesting that IL-2 is present and functional in healthy animals. However, the cellular source of IL-2 is currently unknown. To determine which cells produce IL-2 in healthy animals, we established mice carrying *cre* gene knock in at the *il-2* locus (termed IL-2^{cre}). When IL-2^{cre} mice were crossed with EGFP reporter mice, EGFP was exclusively expressed by a fraction of CD4 T cells present in both lymphoid and non-lymphoid tissues. Live imaging of IL-2^{cre} mice that carry the luciferase reporter showed concentrated localization of luciferase^{+} cells in Peyer's patches. These cells were not observed in new born mice but appeared within 3 days after birth. Reduction of antigen receptor repertoire by transgene expression reduced their number, indicating that recognition of environmental antigens is necessary for generation of these IL-2 producers in healthy animals. A substantial fraction of EGFP^{+} cells also produce IL-10 and IFN-γ, a characteristic profile of type 1 regulatory T cells (Tr1). The data suggest that a group of Tr1 cells have addition roles in immune homeostasis by producing IL-2 along with other cytokines and help maintaining Tregs.

1. Introduction

IL-2 is a major growth factor for activated T cells and it is required for differentiation of activated naïve CD4 T cells into effector and memory CD4 T cells [1,2]. Naïve CD4 T cells become activated after antigen recognition and begin to produce IL-2. Expression of IL-2 is tightly regulated in naïve CD4 T cells and requires an extensive period of antigen stimulation [3]. Most activated CD4 T cells die after 5–7 days of stimulation by activation induced cell death (AICD), and IL-2 also plays a critical role in AICD to induce cell death [4]. Moreover, IL-2 is essential for functional naturally arising regulatory T cells (nTregs) [5,6]. *il-2* Knock-out mice as well as *il-2rα* (known as CD25), *il-2rβ* (known as CD122), and *stat5* (a signal transducer through IL-2-receptor) knock-out mice have profound defects in nTreg populations and undergo lymphoproliferation followed by lethal autoimmunity (reviewed by Refs. [5,7]). Since nTregs do not secrete IL-2, they depend on IL-2 from paracrine sources but the identity of cells that produce IL-2 in healthy animals and maintain functional nTregs is unknown.

Production of IL-2 *in vivo* has been studied using several different systems. One approach was the use of *egfp* knock-in at the *il-2* locus [8]. In this system, the *egfp* gene was inserted in exon 1 of the *il-2* gene. T cells from these mice expressed *egfp* in a manner indistinguishable from that of endogenous *il-2* gene expression. Yet, these studies did not detect IL-2 expression when mice were not immunized with antigens, likely due to the lack of sensitivity of the system. Expression of IL-2 was also studied using transgenic mice that express EGFP under the control of the IL-2 promoter [9,10]. These mice expressed IL-2 in response to TCR stimulation but the expression of *egfp* was somewhat different from the expression of the endogenous *il-2* gene and again, these studies did not determine the presence of cells that express IL-2 *in vivo* under un-immunized conditions. Moreover, fate or localization of cells that produced IL-2 is currently unknown.

Abbreviations: EGFP, enhanced green fluorescent protein; nTregs, naturally arising regulatory T cells; SPF, specific pathogen free.

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To identify which cells produce IL-2 in healthy animals, we established IL-2^{cre} mice in which the *cre* gene was inserted into the *il-2* locus. In IL-2^{cre} mice, *ex vivo cre* gene expression was limited to activated T cells and the pattern of Cre expression mirrored endogenous *il-2* gene expression. When IL-2^{cre} mice were crossed with EGFP reporter mice, we found that EGFP is exclusively expressed in CD4 T cells *in vivo*. These EGFP^{+} CD4 cells, when restimulated *ex vivo*, produced IL-2 along with IFN-γ and IL-10, but not IL-4, resembling the characteristics of IL-10-producing type 1 regulatory T cells (Tr1).

2. Materials and methods

2.1. Generation of IL-2^{cre} mice

IL-2^{cre} Knock-in mice were generated by inserting *cre* cDNA [11] into the *il-2* gene just upstream of the first codon using genomic DNA fragment isolated from C57.BL/6 genomic DNA (liver) library. We placed an internal ribosomal entry sequence (IRES) into the 3′ of the *cre* gene to allow for the co-translation of the *il-2* mRNA along with the *cre* mRNA. Genomic DNA extracted from tail was screened for genotyping by TaKaRa Ex Taq PCR system (TaKaRa, Otsu, Japan). Wild type *il-2* allele was amplified with primers IL-2-5′: 5′-TGCCACACAGGTAGACTCTT-3′and IL-2^{cre} -3′: 5′-GCTGTAGAGCTTGAAGTGGA-3′, in the following heating cycle: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; for 35 cycles. *il-2^{cre}* allele was amplified with primers IL-2-5′ and IL-2-3′: 5′-ACGTTCTC CTTGCGGATGCG-3′, in the following heating cycle: 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min; for 35 cycles. All primers used in this study were synthesized by Integrated DNA Technology (Coralville, IA). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. IL-2^{cre}-PGK-neo^{r} mice were crossed with FLPeR mice on C57BL/6 background [purchased from Jackson Laboratory (Bar Harbor, ME)] to create the IL-2^{cre} mice removed PGK-neo^{r} gene. IL-2^{cre} mice were crossed with EGFP reporter Z/EG mice on C57BL/6 background (purchased from Jackson Laboratory to create the IL-2^{cre}: Z/EG mice. For *in vivo* live imaging of T cells, IL-2^{cre} mice were bred to luciferase reporter mice [FVB.129S6(B6)*Gt(ROSA)26Sor$^{tm1(Luc)Kael}$*/J, termed R26^{luc} herein] from Jackson laboratory.

To fix the specificity of the TCR repertoire in CD4 T cells, IL-2^{cre}: Z/EG mice were crossed with RAG-1 knock-out mice (Jackson Laboratory) and OT-II (Jackson Laboratory) TCR-transgenic (Tg) mice. CD4 T cells from OT-II mice express specific TCRs for OVA peptide 323–339.

All mice were maintained and bred under specific pathogen-free conditions under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Medical College of Georgia and Loyola University Medical Center, Chicago.

2.2. Isolation of lymphoid and myeloid cells

To prepare single cell suspensions from thymus, spleen, mesenteric lymph nodes (mLNs) and Peyer's patches (PPs), the collected organs were incised and treated with 0.5% FCS-RPMI1640 medium containing 2 mg/ml collagenase D (Roche, Indianapolis, IN) and 30 μg/ml DNase I (Roche) for 30 min at 37 °C. The cells were suspended in 5% FCS-RPMI1640 medium containing 5 mM EDTA and were passed through 40 μm cell strainer. Bone marrow cells were gently flushed into a tube using a syringe and a 25-gauge needle with 10 ml of 0.5% FCS-RPMI1640 medium. Red blood cells were removed by treatment with ACK buffer (Invitrogen, Carlsbad, CA).

For isolation of intraepithelial lymphocytes (IELs), small intestines were cut away from PP and the fat, and were opened longitudinally, washed to remove fecal content, and shaken in RPMI1640 medium containing 10% FCS and 1 mM DTT (Fluka, part of Sigma–Aldrich, St. Louis, MO) for 30 min at 37 °C. IEL fractions were passed through a stainless steel tea strainer. For isolation of lamina propria lymphocytes (LPLs), the remaining intestines were cut into small pieces and incubated with serum-free RPMI1640 medium containing 0.5 mg/ml collagenase type 3 (Worthington, Lakewood, NJ) for 20 min at 37 °C. The digested pieces including LPL or IEL fractions were passed through 40 μm cell strainer and resuspended in 6 ml of 40% Percoll (GE Healthcare, Piscataway, NJ) and overlaid on 6 ml 70% Percoll in a 15 ml tube. Percoll gradient separation was performed by centrifugation at 800*g* for 30 min at 25 °C without braking. IEL or LPL were collected at the intermediate layer of the Percoll gradient, and were washed with RPMI1640 medium containing 10% FCS. For isolation of lung lymphocytes, lung lobes were dissected out, incised, and incubated in RPMI1640 medium containing 5% FCS and 5 mM EDTA with gentle shake for 1 h at room temperature, then were treated with collagenase D (2 mg/ml, Roche) and DNaseI (30 μg/ml, Roche) for 1 h at 37 °C. Digested pieces of lungs were passed through 40 μm cell strainer and lymphoid cells were isolated by Percoll gradient performed as described above.

2.3. In vivo live imaging

Luciferase reporter mice (R26^{luc}) with or without IL-2^{cre} knock in allele were first shaved on the ventral side, and then cleaned with 70% ethanol. Mice were injected with 100 μl of 25 mg/ml of luciferin (D-Luciferin Firefly potassium salt, Caliper Life Sciences, Hopkinton, MA USA) intraperitoneally. Mice were anesthetised with 2% isoflurane and placed in the Xenogen IVIS 100 (Caliper LifeSciences, Hopkinton, MA USA) 10 min after injection. Live images were taken at 5 min intervals. To determine the location of sites emitting light, mice were euthanized and the abdominal cavity was exposed.

2.4. Cell sorting and cell culture

Naïve CD4^{+}CD62L^{+} T cells were purified from spleen using a mouse CD4^{+}CD62L^{+} T cell isolation kit II (Miltenyi Biotec, Auburn, CA: purity > 95%). To collect EGFP^{+} CD4 T cells (CD3^{+}CD4^{+}EGFP^{+}), memory CD4 T cells (CD3^{+}CD4^{+}CD62L^{low} EGFP^{-}) and naïve CD4 T cells (CD3^{+}CD4^{+}CD62L^{high} EGFP^{-}), each fraction was collected from spleen using FACS Aria (Becton Dickinson, San Jose, CA: purity > 97%).

The sorted naïve CD4 T cells were stimulated with plate-bound anti-CD3 antibodies (5 μg/ml) and soluble anti-CD28 antibodies (2 μg/ml) for 2 days, and were further cultured without stimulation and with exogenous IL-2 (10 ng/ml). The induced EGFP^{+} CD4 T cells were analyzed by Accuri C6 flow cytometer (Accuri cytometers, Ann Arbor, MI) or FACS Canto (Becton Dickinson). The induced EGFP^{+} and EGFP^{-} CD4 T cells were purified by FACS Aria (Becton Dickinson).

2.5. Cell lysis and Western blotting

The cells were lysed in SDS sample buffer (containing 2%SDS, 5% beta-mercaptoethanol, 5% glycerol, and 62 mM Tris, pH 6.5), boiled, and frozen at −80 °C until analysis. Lysates from an equal number of cells were loaded in each well, followed by electrophoresis and blotting onto PVDF membrane. Membranes were probed with antibodies specific for Cre (Novagen, Madison, WI) and β-actin (Sigma, St. Louis, MO), followed by the appropriate HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) and enhanced chemiluminescence (GE Healthcare).