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Global transcriptional analysis delineates the differential inflammatory response interleukin-15 elicits from cultured human T Cells

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Abstract

Objective—Interleukin 15 (IL-15) controls proliferation and survival of T cells, but its effects and the underlying cellular regulation are not well understood. Previous studies have focused on its effects on short-term T-cell cultures. In view of the potential problems associated with using IL-2 alone in adoptive immunotherapy protocols, we investigated the impact of IL-15 on T-cell cultures and the global transcriptional effects it elicits in such cultures.

Methods—DNA microarrays and flow cytometry were used to examine the differential effect of 20 ng/mL IL-15 on primary serum-free T-cell cultures activated and cultured in the presence of IL-2. Quantitative RT-PCR confirmed select microarray data.

Results and Conclusions—IL-15 significantly increased the ex vivo expansion of primary human T cells over the entire 11-day expansions without affecting viability. 1133 genes were consistently differentially expressed among 3 donor samples. Ontological analysis demonstrated that IL-15 increases expression of genes involved in inflammatory response (e.g., TNF-alpha, Oncostatin M, CD40L and CD33) and apoptosis (e.g., TRAIL). IL-15 also induced expression of 4 ‘suppressors of cytokine signaling’ (SOCS) family genes (SOCS1-3, CISH), which are classical negative regulators of cytokine signaling. IL-15 strongly suppressed the expression of inhibitory NK-cell receptor genes (iNKR), including three C-type lectins (KLRB1, KLRC1 and KLRD1) as well as IL-7Ra and Granzyme H. Finally, IL-15 induced differential expression of TNFR superfamily members (CD27 and CD30). These findings suggest that exogenous IL-15 may have a potential role in adoptive immunotherapy by both enhancing proliferation and modulating functionality during ex vivo T-cell expansion.

Introduction

Interleukin-15 (IL-15) is a pro-inflammatory cytokine expressed by activated monocytes and dendritic cells, but not by T cells [1,2]. In vivo, IL-15 is an important cytokine in T-cell survival and homeostasis [3]. It regulates T-lymphocyte proliferation [4] and functions during all 4 phases of the T-cell immune response: initiation, expansion, clonal deletion and memory generation [3]. Upon T-cell activation, IL-15 drives T-cell proliferation, induces effector T

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lymphocytes and stimulates development of memory T lymphocytes [5–7]. IL-15 inhibits activation-induced-cell-death (AICD) using IL-2R γ -chain signaling via Bcl-2 [8]. Because IL-15 stimulates chronic slow cycling of CD8+ memory cells in vivo [9], it may also provide important long-term survival signals in cultures of primary T cells for immunotherapy applications, especially under serum-free conditions [10].

Adoptive immunotherapy uses ex vivo expanded autologous T cells, sometimes in conjunction with stem cell transplantation, to improve clinical outcomes after high-dose chemotherapy [11,12]. Use of either antigen-specific or polyclonally-expanded T cells can be an effective treatment in patients with advanced myeloma and some metastatic cancers [13,14]. Ex vivo generation of large numbers of biologically active T cells through multiple rounds of mitogenic activation remains a significant technological hurdle [15,16]. Use of IL-2 during such expansions is standard [17], but it has also been implicated in activating and expanding T_{reg} cells as well as inducing AICD, both of which negatively affect T-cell function upon reinfusion. It has been hypothesized that other γ -chain cytokines (IL-7, IL-15 and IL-21) could complement or replace IL-2 in adoptive immunotherapy protocols [18,19]. One current problem is that ex vivo expanded T cells can become anergic and unable to initiate an inflammatory immune response upon reinfusion, which is a necessary precursor for an effective adaptive immune response [14]. The main tool for improving functionality of ex vivo expanded T cells is to provide additional ligands during culture to cytokine and co-stimulatory receptors, including both the immunoglobulin (Ig) superfamily and tumor necrosis factor receptor (TNFR) superfamily [20,21]. IL-15 plays a significant role in the host inflammatory response, and induces expression of chemokines and their receptors in resting T cells after short-term exposure [22]. The extent of IL-15's effect on the inflammatory-response gene expression in longer cultures is unknown. Exogenous IL-15-mediated homeostasis during ex vivo T-cell expansions may help correct the decreased functionality observed during T-cell expansions with multiple rounds of activation [23]. We hypothesized that a global transcriptional study of T-cell cultures may also identify expression patterns of other cytokine receptors and co-stimulatory receptors, which may provide additional targets for improving culture conditions.

IL-15 studies with cultured T cells have been limited to studies on homeostatic proliferation of murine memory T-cells [9,24,25] and short-term proliferation studies [26,27], all in the presence of serum. One study has used small-scale DNA-microarrays (4604 genes) to investigate the transcriptional profile of the effect of IL-15 in the absence of TCR stimulation in a 48-hour study [28]. Given that serum contains many unknown compounds which could act synergistically or antagonistically with IL-15 and the known pleiotropic effects of serum in cultures of T cells [10], we examined the impact of IL-15 on serum-free T-cell cultures. Following titration and phenomenological studies, we used 18,500-gene microarrays combined with ontological analysis to investigate the transcriptional effects of IL-15 on cultured human T cells. We combined this information with flow-cytometry-based protein-level assays to provide additional insights and relate the data to the observed or previously established phenomenology. Data from a subset of differentially expressed genes were validated using quantitative (Q)-RT-PCR.

Materials and Methods

Cells and culture system

Peripheral blood mononuclear cells (PBMCs) were isolated from 8 donors (L1-L8) as described [29]. CD3+ cells were isolated using the Pan T Cell Isolation Kit II, human (Miltenyi Biotech, Auburn, CA). Immediately following isolation (Day 0), CD3+ T cells were seeded at 10^6 cells/mL in T-flasks using AIM V (Invitrogen, Gaithersburg, MD) serum-free medium supplemented with 100 U/mL IL-2 (Chiron, Emeryville, CA). T cells were activated with anti-CD3/anti-CD28 Mab (BD Biosciences, San Jose, CA) (1:1) coated magnetic beads (500 fmol/

bead) (Dynabeads M-450 Epoxy, Dynal Biotech, Lake Success, NY). The ratio of beads to cells was 3:1. On Day 3, the activation beads were removed using a magnet particle concentrator (MPC-1, Dynal) and cells were split into two groups: 'IL-2 only serum free' ('IL-2') and 'IL-2 + IL-15 serum free' ('IL-2 + IL-15'). The split cultures were reseeded at 3.0×10^5 – 4.0×10^5 into T-flasks using AIM V with both groups receiving 100 IU/mL IL-2. IL-15 was added to the 'IL-15 + IL-2' cultures after Day 3. Cells were allowed to expand to $1.5 - 2 \times 10^6$ cells/mL and then reseeded at $3 - 4 \times 10^5$. Doses of IL-15 and IL-2 were added every second day. Incubator conditions, cell counts, and cell sampling for microarray analysis were previously described [29]. Specific proliferation rates (μ) of cells were calculated as described [29].

Flow cytometry

The following monoclonal antibodies (Mabs) for flow cytometry were purchased from BD Biosciences (San Jose, CA): CD3 (FITC+PE), CD4 PE, CD8 FITC, CD25 (FITC+PE), CD30 PE, CD33 PE, CD69 PE, CD127 PE and CD154 PE. A flow cytometry-based Annexin V assay (Clontech, Palo Alto, CA) was used to assess the fraction of apoptotic cells. Flow cytometry was carried out as described [29]. Briefly, all samples were gated on forward scatter and on propidium iodide negative (PI-) to eliminate debris and dead cells. All subset analyses (e.g., CD40L) were gated on either the CD4+ or CD8+ populations. 10,000 gated events from each tube were acquired using a FACscan (BD Biosciences) and analyzed using CellQuest (BD Biosciences). Quantibrite PE Beads (BD Biosciences) were used, following manufacturer's instructions, to estimate the surface density of the following receptors: CD25, CD30, CD33, CD127 and CD154.

RNA extraction and quality control

Total RNA was extracted from frozen cells using the NucleoSpin RNA II kit (Clontech). RNA samples were resuspended in RNase-free water and stored at -80°C . RNA yield and purity were assessed by UV spectrophotometric measurements at 260 and 280 nm (Biomate 3, Thermo Spectronic, Marietta, OH). RNA integrity was also evaluated using the Bioanalyzer 2100 (Agilent, Palo Alto, CA).

DNA-microarray experiments and data analysis

Three experiments (L2, L3, L5) were chosen for microarray transcriptional analysis based on the following criteria: representative expansion characteristics and sufficient extracted RNA of good quality at days 3, 5, 7, 9 and 11. In order to enable comparisons among multiple experiments and multiple timepoints as well as follow the individual culture expression kinetics, a 'reference' design was used [10,30]. Briefly, for each timepoint the RNA sample was Cy3 labeled and a reference RNA was Cy5 labeled. Human Thymus RNA (Cat #7964 Lot 101P0101A, Ambion, Austin, TX) was chosen as global reference [10,30]. Experimental sample and reference RNA were amplified using the 'Low Input Fluorescent Amplification Kit' (Agilent). We used Agilent's Human 1A Oligo Microarrays (V2), which contain approximately 18,500 genes. Hybridizations were carried out at 60°C for 17 hours. After washing in 6X SSC/0.1X SSC, 0.005% Triton X-102, the microarrays were blown dry with nitrogen and scanned using the Agilent Microarray Scanner G2565BA. Agilent's Feature Extraction software (G2567AA, version 7.2) was used to identify spot and feature outliers. Spot quality was assessed and if not discarded, multiple spots of the same gene were averaged (geometric mean). The SNNLERM-algorithm [31] was used to calculate normalized signal intensity ratios and confidence levels. Based on previous experience with transcriptional analysis of primary cell cultures from multiple donors [10,30], in order to minimize patient-specific effects and select genes most consistently differentially expressed between the 'IL-2' and 'IL-15 + IL-2' cultures, we used the following gene-selection criterion. A gene had to have

at least a 1.5-fold difference in at least one timepoint in each experiment in order to be considered for transcriptional analysis. Lowering the fold-difference requirement and increasing the required number of differentially expressed data points yields more robust results by preventing genes exhibiting small fold differences sustained over multiple days and donors from being eliminated. Differentially expressed genes were analyzed using the hierarchical clustering and ontological analysis tools [29] of 'MultiExperiment Viewer (MeV)' from The Institute for Genomic Research [32] (http://www.tigr.org/software/tm4/menu/TM4_Biotechniques_2003.pdf). Ontological analysis is based on the EASE program (version 1.21) (<http://david.niaid.nih.gov/david/ease.htm>) applied to identify over-represented gene groups within a set of genes, using the 'EASE score'[29]. For individual genes, statistical differences between 'IL-2' and 'IL-15 + IL-2' groups were calculated using a two-tailed paired t-test.

Quantitative RT-PCR (Q-RT-PCR)

RNA isolation and reverse transcription was carried out as described [29,31] using the NucleoSpin RNA II kit (Clontech) and TaqMan Reverse Transcription Reagents (Applied Biosystems (ABI), Foster City, CA). Primers (ABI) for the following functionally diverse set of genes were used: Oncostatin M (OSM), Tumor necrosis factor (ligand) superfamily, member 5 (CD40L), Cytokine inducible SH2-containing protein (CISH), Cyclin E2 (CCNE2), Interleukin 7 receptor (IL7R), Granzyme H (GZMH) and Killer cell lectin-like receptor subfamily C, member 1 (KLRC1). Genes were chosen to reflect differentially expressed genes between 'IL-15 + IL-2' and 'IL-2' cultures of a wide range of microarray signal intensities. PCR reactions were carried [29] using the Taqman Universal PCR Master Mix (ABI) in 96-well plates on a Bio-Rad iCycler (Bio-Rad, Hercules, CA). Q-RT-PCR assays for the 7 selected genes were carried out for 32 timepoints. For 5 of the timepoints, no microarray analysis was done.

Results

IL-15 increases T cell proliferation

In previous cultures of human or murine T lymphocytes, 10 to 100 ng/mL IL-15 was added to serum-containing media [28,33]. From a titration study, we found that 20 ng/mL IL-15 provided the largest expansion in 11-day serum-free cultures. We then completed eight 11-day cultures comparing 'IL-2' cultures to cultures supplemented with 20 ng/mL IL-15 (IL-2 vs. IL-2+IL-15). All eight of these experiments showed a significant positive effect of IL-15 on T-cell expansion (Fig. S1A, Table S1)(Median: 2.2 fold difference, Range: 1.2–38 fold difference, 2-tailed paired t-test: $p = 8.3 \times 10^{-5}$). 20 ng/mL IL-15 affected neither the culture viability (Fig. S1B) nor the fraction of apoptotic T-lymphocytes as measured by the Annexin V assay (data not shown). Although the eight donor samples represented a variety of starting CD4⁺/CD8⁺ ratios, IL-15 did not affect this ratio (data not shown). CD3⁺ purity was greater than 95% throughout all cultures. 10–100 ng/mL IL-15 could not support T-cell expansion in the absence of 100 IU/mL IL-2, and thus 100 IU/mL IL-2 was included in each culture.

Expression profiling of differential IL-15 effects combined with ontological analysis identifies biologically-significant clusters of differentially expressed genes

Expression profiles of 18,500 genes over the 11-day cultures were used to identify genes affected by the addition of 20 ng/mL IL-15 (differential effect of IL-15 over that of IL-2). This global transcriptional analysis allowed us to identify mechanisms behind previously observed phenotypic effects of IL-15 (e.g., inflammation response, survival signaling), but also novel IL-15-regulated genes and processes. Transcriptional analysis was carried on a set of 1133 differentially expressed genes (Table S2, http://www.papoutsakisresearch.northwestern.edu/suppl_mat.htm) that passed the gene-

selection criteria. Seven distinct clusters (A–G) were identified by hierarchical clustering (Fig. 1). The “green” clusters (A, B and E) contain genes that were expressed lower in the ‘IL-15 + IL-2’ cultures. The “red” clusters (F, and to a lesser extent cluster C) contain genes that are expressed higher in IL-15 cultures. Clusters D and G contain genes whose expression alternates between higher expression in the ‘IL-2’ and ‘IL-15 + IL-2’ cultures. Ontological analysis of genes in clusters E and F provided strong evidence that IL-15 induces a unique expression pattern of inflammatory response genes (cluster F), while suppressing genes involved in humoral immune response (cluster E).

IL-15 induces a unique expression pattern of genes involved in T-cell inflammatory response

Cluster F contains 18 genes with significantly higher expression in the ‘IL-15 + IL-2’ cultures and ontologically classified as ‘Immune Response’ (EASE score: 4.27×10^{-3}) (Fig. 2A). These 18 genes fall into three functional groups: genes that play a role in priming T cells to respond to inflammatory cytokines, genes encoding inflammatory cytokines produced by T cells as well as genes involved in T-cell effector function.

Seven of these genes (IL6ST, NFIL3, IL1RN, IL1RAP1, IL1RAP2, CEBPB, and GILT) play a role in priming T-lymphocytes for an inflammatory response. IL-1 and IL-6 are secreted by macrophages. IL-1 increases the access of effector cells to infection sites, while IL-6 induces lymphocyte activation [34]. Cluster F contains 3 receptor signaling elements to IL-6 and IL-1 (‘IL-1 receptor accessory protein’ (IL1RAP), ‘IL-1 receptor agonist’ (IL1RN) and ‘IL-6 signal transducer’ (IL6ST)). CCAAT/enhancer protein- β (CEBPB) is an important regulator of inflammatory response and is known to bind both the IL-1 and IL-6 response elements [35]. ‘Interferon- γ inducible protein 30’ (GILT) is induced by interferon- γ in APCs [36] and T-cells [37].

Five of the ‘Immune Response’ genes in cluster F (OSM, IL8, TNF, IL22 and CSF2)(Fig. 2A) are inflammatory cytokines secreted by T cells. Ontological analysis showed that ‘Cytokine Activity’ genes were overrepresented in cluster F (EASE score 2.93×10^{-4}). Oncostatin M (OSM), an important member of the T-lymphocyte inflammatory response with suggested roles in cell survival, differentiation, proliferation and gene activation [38] was expressed much higher in the ‘IL-15+IL-2’ cultures (Fig. 3A). In the ‘IL-15 + IL-2’ cultures, OSM expression reached a maximum on Day 5 followed by a decrease until Day 7 or 9 and then increased again until the end of the experiment (data not shown). Q-RT-PCR data confirmed the OSM microarray data (Fig. S4G).

Two of the ‘Immune Response’ genes in cluster F (CD40L and TRAIL) are involved in inflammation-related effector functions. ‘TNF-related apoptosis inducing ligand’ TRAIL is expressed by CTLs and preferentially induces apoptosis [39]. Microarray analysis showed that IL-15 also increased expression of CD40L (CD154) (Fig. S2B). This result was confirmed with Q-RT-PCR (Figs. S4B). The change in CD40L expression was almost entirely in the CD4 + cells (Fig. S2A). At the protein level, ‘IL-15 + IL-2’ cultures consistently showed ca. 50% higher CD40L surface density in CD4+ T cells (data not shown). IL-15 also increased IL-2R α (CD25) expression at both the transcriptional and protein levels (Figs. S2C,D).

IL-15 induces expression of suppressors of cytokine signaling (SOCSs)

Cluster F also contains an overrepresentation of ‘regulation of cell growth’ genes (EASE score: 8.39×10^{-3}). Included in this group are CISH, SOCS1 and SOCS2, which were all expressed significantly higher in the ‘IL-15 + IL-2’ cultures (Fig. 3B,C,D). SOCS3, not contained in Cluster F, also exhibited higher expression in ‘IL-15 + IL-2’ cultures between Days 5–7 (Fig. 3E), although the magnitude of differential expression was generally smaller than in CISH, SOCS1 and SOCS2. CISH Q-RT-PCR data match the microarray data (Fig. S4C). IL-15 had

the most immediate effect on SOCS1 and SOCS2 expression with both genes being upregulated between Day 3 and Day 5 while both genes were downregulated in the 'IL-2' cultures during the same period (data not shown). CISH was sharply downregulated in all three 'IL-2' cultures between Day 3 and Day 7 while expression remained constant or increased in 'IL-15 + IL-2' cultures. The upregulation of SOCS genes was sustained over the entire 11-day expansions.

IL-15 suppresses transcription of IL-7R α (CD127), inhibitory NK-cell receptors (iNKRs), Granzyme H and genes involved in humoral immune response

The most differentially expressed gene in cluster A (Fig. 1) is IL-7R α (CD127). IL-15 suppresses expression of IL-7R α at both the transcriptional and protein level (Fig. 4A,B). In addition to decreasing the fraction of T cells expressing IL-7R α (Fig. 4A), IL-15 also decreased IL-7R α + receptor density (data not shown). Cluster E also contains 32 genes that are ontologically classified as 'Immune Response' genes (EASE score 3.18×10^{-4}) (Fig. 2B) with higher expression in 'IL-2' cultures. Of these 32 genes, six are inhibitory NK-cell receptors (iNKR), including all three iNKR families (LIRs, KIRs and C-type lectins). It contains 1 KIR (KIR3DL2), 1 LIR (LILRB1) and 4 C-type lectins (CD160, KLRD1, KLRC1, KLRB1). The higher mRNA expression of KLRC1 was confirmed by Q-RT-PCR (Fig. S4F). Furthermore, IL-15 strongly suppressed Granzyme H expression as measured using both microarrays and Q-RT-PCR (Fig. S3A,S4D). The 'Immune Response' genes of cluster E also contains genes involved in humoral immune response such as IL-4 [40], IL-26 [41] and CD1D [42]. Cluster E also contains two genes involved in complement system: complement component 8, β -polypeptide (C8B) and H factor (complement)-like 1 (HFL1).

IL-15 induces CD33 expression

Cluster F also contained an overrepresentation of 'Cell Proliferation' genes (EASE score: 4.27×10^{-3}). The highest differentially expressed gene in this group was CD33, a cell adhesion molecule of the immunoglobulin superfamily. IL-15 increased CD33 expression at both the transcriptional and protein levels (Fig. 4C,D). A greater fraction of CD4+ cells expressed CD33 than CD8+ cells under both 'IL-2' and 'IL-15 + IL-2' conditions with IL-15 increasing the fraction of CD4+ T lymphocytes expressing CD33 (Figs. 7C). IL-15 consistently increased the CD33 receptor density (data not shown).

IL-15 affects the expression pattern of co-stimulatory molecules

There are two major classes of co-stimulatory molecules: the immunoglobulin superfamily (CD28 and 'inducible T-lymphocyte co-stimulator' (ICOS)) and the tumor-necrosis factor receptor (TNFR) superfamily (CD27, CD30, OX40, 4-1BB, HVEM) [21]. Among these, IL-15 induced expression of ICOS ($p=.022$) while suppressed CD28 ($p=.038$) and CD27 ($p=.006$) expression (data not shown), although the fold differences were small. We also found that IL-15 enhanced CD30 ($p=.0009$) expression at the protein and to a lesser extent at the transcriptional level (Fig. S3B,C,D). CD30 was not expressed on unactivated T cells, but its expression exceeded 80% on Day 5 and then decreased through Day 11 (Fig. S3A). IL-15 slowed the downregulation of CD30 between Days 5 and 11. IL-15 also increased the fraction of T lymphocytes expressing CD30 and CD30 surface density (Fig. S3C).

Q-RT-PCR versus DNA-microarray data

Q-RT-PCR experiments were carried out for 7 target genes on 13 timepoints from the 3 experiments used for microarray analysis. Figure S4 shows a direct comparison between 'IL-15 + IL-2' and 'IL-2' cultures. Q-RT-PCR data were normalized and are presented using two different housekeeping genes: 18S rRNA (18S) and β -glucuronidase 2 (GUSB). There is excellent directional agreement between the Q-RT-PCR data and the microarray data for all seven target genes. Disagreement between the two methods only occurred when the measured

microarray fold differences were small. We conclude that the Q-RT-PCR data confirm the microarray data, with the microarray data giving a more conservative estimate of gene expression ratios.

Discussion

From previous studies, strong evidence exists that IL-15 controls T-cell viability and proliferation *in vivo* (see [3,6,43] for reviews). In this study, we found that 20 ng/mL IL-15 increased T-cell expansion by a median of 2.2-fold in 11-day cultures. Previous reports on the effect of IL-15 on ex vivo T-cell expansion have been mixed. Lubong et al. [44] reported no expansion advantage when 1 ng/mL IL-15 was added to anti-CD3 activated HIV+ CTLs in RPMI + 10% FCS. In contrast, increased proliferation of ConA-activated T lymphocytes in the presence of 20 ng/mL IL-15 in RPMI with 10% FCS has also been reported [27]. Both of these studies were done in the presence of serum, which introduces other cytokines and growth factors in the cultures, thus complicating data interpretation.

Few studies have used microarray analysis as an integral part of developing protocols for adoptive immunotherapy. In our global transcriptional analysis, the data revealed that in addition to increasing T-cell expansion, IL-15 induced a distinct expression pattern of inflammation, cytokine receptor and co-stimulatory receptor genes. IL-15 has long been associated with inflammatory response and is currently a drug target for rheumatoid arthritis and inflammatory bowel disease [45]. We presented a distinct IL-15 induced expression pattern of inflammation genes (Fig. 2A), including the five 'Immune Response' genes in cluster F (OSM, IL8, TNF, IL22 and CSF2). OSM (Fig. 3A) belongs to the IL-6 cytokine family and is produced by activated T lymphocytes as part of an inflammatory response [46,47]. OSM secretion is involved in T-lymphocyte inflammatory response, which it regulates by activating both the Janus Kinase-Signal Transducers and Activators of Transcription (JAK/STAT) and the Mitogen-Activated Protein Kinase (MAPK) signaling pathway. CD40 ligand (CD40L, CD154) (Fig. S2A,B) is a member of the TNF superfamily and can stimulate production of cytokines like IL-12 by APCs [48]. IL-8 is an inflammatory cytokine secreted by T-lymphocytes as a chemoattractant to induce transendothelial migration of leukocytes [36], while TNF α is a powerful cytokine secreted by T cells undergoing an inflammation response [37]. IL-22 is a class2 alpha-helical cytokine with known function in modulating inflammatory response while 'Colony stimulating factor 2' (GM-CSF) is a proinflammatory cytokine secreted by T-lymphocytes to attract monocytes and granulocytes *in vivo* [38,39]. 'Triggering receptor expressed on myeloid cells-like 2' (TREM2) has also been reported to be an important mediator of adaptive immune response and is upregulated in response to inflammation [49]. Taken together these results suggest that IL-15 enhances the transcription of proinflammatory cytokines. Increased expression of these inflammation genes as well as the T-cell effector gene TRAIL and T-cell activation markers CD25 suggest that IL-15 improves the robustness of T-cell activation and may improve T-cell functionality upon reinfusion during immunotherapy protocols by priming T cells to respond to inflammation.

IL-15 induced expression of SOCS-family proteins (Fig. 3B-E). Expression of CISH and SOCS1-3 are known to be activated by IL-2, IFN γ and anti-CD3 mAb [50,51]. The current model for CISH and SOCS1-3 predicts that upon cytokine ligation, SOCS are upregulated and, by a negative feedback loop, inactivate JAK-STAT signaling [52-54]. This JAK/STAT regulation makes SOCSs a regulator of inflammatory response, since inflammatory cytokines such as TNF α , GM-CSF, IL-1, IL-18, IL-6 and IL-2 transcription are activated by JAK/STAT signaling [55,56]. We were unable to find any reports describing IL-15-induced SOCS expression. It is possible that the observed increase in SOCS expression is the result of increased expression of Oncostatin M (OSM) and its signaling receptor in the presence of IL-15. OSM is known to quickly upregulate CISH, SOCS1 and SOCS3 [57]. Upregulation of

SOCS genes was sustained over the entire 11-day expansions, although it slightly decreased after day 9. This is a significant finding because it demonstrates that IL-15, unlike IL-2 alone, can induce a long-term SOCS-mediated inflammatory response, which may improve the final T-cell functionality.

CD30 (Fig. S3B-D) represents a good target for improving T-cell expansion since CD30 expression peaks when proliferation from the original activation begins to slow down, as shown here and earlier [58]. CD30/CD30L interactions promote T-cell survival and proliferation when taking place after TCR-activation [21]. CD30 ligation during T-cell expansions in the presence of IL-15, after an initial TCR-based activation, represents a possibility to increase overall expansion without resorting to multiple rounds of TCR-based activation.

CD33 expression (Fig. 4C,D) is normally associated with myeloid cells, but lymphoid expression has also been reported on chronically activated human T cells [48] and on CD4+ cells infected with 'human T-cell leukemia virus type-1 (HTLV-1) [49]. CD33 expression by normal T cells has been hypothesized to be part of a general inflammatory response [48]. Although CD33's exact biological function in T cells remains unresolved, our data (Fig. 4C,D) show that IL-15 induces CD33 expression, especially in CD4+ cells after Day 7. CD33 has been reported to be cytokine inducible and expressed on anti-CD3 activated T-lymphocytes [59]. Several studies suggest that CD33 may be a negative regulator of cell proliferation [60, 61]. Limited studies have addressed the effect of CD33 ligation, though it has been linked to the secretion of pro-inflammatory cytokines [62]. Our data support the possibility that CD33 expression in T cells may be part of a larger IL-15 induced inflammatory response.

We found that IL-15 suppressed expression of inhibitory natural killer (NK) cell-associated receptors (iNKR)(Fig. 2B). iNKRs are normally expressed on NK cells, but also negatively regulate T-cell activation and survival [63,64]. When these data are viewed together with the pro-activation genes (e.g., CD25) induced by IL-15 (Fig. S2C,D), it suggests that IL-15 increases T-cell activation by shifting the balance of pro and anti-activation genes. *In vivo*, iNKRs can control the ability of the immune system to eradicate viruses and reject tumors [65]. A previous study has reported that surface expression of KLRD1 and KLRC1 are easily upregulated on T lymphocytes by 20 ng/mL IL-15 in the absence of TCR stimulation [66]. The study was done with 10% Human AB serum, which may explain the differences from what was observed in our study. T cells expressing iNKRs are characterized as having diminished proliferation [67,68]. These findings are consistent with increased proliferation and decreased iNKR expression of the IL-15 cultures observed here. Granzyme H (Fig. S3A), which had a very similar expression profile to the iNKRs, is known to complement granzyme B function in NK cells [69]. Taken together, these data suggest that IL-15 downregulates NK-cell like functions in T cells.

Our data show that IL-15 suppresses IL-7R α expression up to 14 days after activation (Fig. 4A,B). One previous study has reported that 100 ng/mL IL-15 reduces IL-7R α mRNA and surface expression in naïve murine T lymphocytes [70]. Increased IL-7R α has been correlated to decreased T-cell *ex vivo* expansion as well as downregulation of the lymph node homing adhesion molecules CCR7 and CD62L [71].

This study represents the first exploration of IL-15 effects on the expression of co-stimulatory molecules. Our results suggest that a more comprehensive study to examine how combinations of cytokines and co-stimulatory ligands may improve T-cell adoptive immunotherapy protocols with the eventual goal of eliminating the need for multiple rounds of TCR-based activation. The most recent report on the successful use of genetically engineered T-cells to treat metastatic melanoma [16] points out the need for devising suitable *ex vivo* expansion protocols for culturing T cells which retain their functional properties over a long time period

after transplantation. Thus, understanding how ex vivo culture conditions affect T-cell functionality remains an important issue in the development of robust cellular-immunotherapy protocols. In this context, IL-15 is a promising cytokine for the ex vivo expansion of T cells for adoptive-immunotherapy protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Hierarchical clustering of 1133 differentially expressed genes between 'IL-15 + IL-2' and 'IL-2' cultures. Experiments L2, L3, and L5 were ordered temporally left-to-right for each experiment. A white column separates each experiment. Rows show expression ratios (IL-15 + IL-2/IL-2) for all timepoints and experiments for a particular gene. Genes in red are expressed higher in 'IL-15 + IL-2' cultures, whereas green color indicates genes with higher expression in 'IL-2' cultures. Gray spots indicate missing expression ratios. Gene clusters A–G represent genes with similar expression patterns.

FIGURE 2. Genes were grouped together based on ontological definition and were hierarchically clustered. Experiments L2, L3, and L5 were ordered temporally left-to-right for each experiment. A white column separates each experiment. Rows show expression ratios (IL-15 + IL-2/IL-2) for all timepoints and experiments for a particular gene. Genes in red are expressed higher in 'IL-15 + IL-2' cultures, whereas green color indicates genes with higher expression in 'IL-2' cultures. Gray spots indicate missing expression ratios. P-value represents the results of a two-tailed paired t-test comparing the 'IL-2' and 'IL-15 + IL-2' cultures. **A:** 'Immune Response Genes' from cluster F (Fig. 1). Median Fold Difference (IL-15 + IL-2/IL-2) represents the median expression ratio of the 10 time points (columns) in the study. Maximum Fold Difference (IL-15

+ IL-2/IL-2) represents maximum expression ratio of the 10 time points (columns) in the study.
B: 'Immune Response Genes' from cluster E (Fig. 1). Median Fold Difference (IL-2/IL-15 + IL-2) represents the median expression ratio of the 10 time points (columns) in the study.
Maximum Fold Difference (IL-2/IL-15 + IL-2) represents maximum expression ratio of the 10 time points (columns) in the study.

FIGURE 3.

IL-15 induces expression of Oncostatin M (OSM) and 'suppressors of cytokine signaling' (CISH, SOCS1-3). mRNA ratios $[(IL-15 + IL-2)/(IL-2)]$ for all three blood samples tested (L2 (■) L3 (□) and L5 (▨)) by DNA-microarray analysis. A positive expression ratio indicates higher expression in the 'IL-15 + IL-2' cultures, while a negative expression ratio indicates higher expression in the 'IL-2' cultures. **A:** OSM ($p=.001$). **B:** CISH ($p=.003$). **C:** SOCS1 ($p=.001$). **D:** SOCS2 ($p=.0004$). **E:** SOCS3 ($p=.039$).

FIGURE 4.

IL-15 downregulates CD127 (IL-7R) (p-value = .004) expression and induces expression of CD33 (p-value = .054). A positive expression ratio indicates higher expression in the 'IL-15 + IL-2' cultures, while a negative expression ratio indicates higher expression in the 'IL-2' cultures. **A:** Representative result (L7) from a study of CD127 surface expression in 'IL-2' ($\diamond$) and 'IL-15 + IL-2' ($\blacksquare$) cultures. **B:** CD127 expression ratios from all three experiments tested (L2, L3 and L5) [(IL-15 + IL-2)/(IL-2)] from DNA-microarray analysis. **C:** Representative result (L6) of CD33 surface expression on CD4+ cells. **D:** CD33 expression ratios [(IL-15 + IL-2)/(IL-2)] from DNA-microarray analysis for all three experiments tested (L2, L3 and L5).