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IL-2 Regulates Expression of C-MAF in Human CD4 T Cells

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Blockade of IL-2R with humanized anti-CD25 Abs, such as daclizumab, inhibits Th2 responses in human T cells. Recent murine studies have shown that IL-2 also plays a significant role in regulating Th2 cell differentiation by activated STAT5. To explore the role of activated STAT5 in the Th2 differentiation of primary human T cells, we studied the mechanisms underlying IL-2 regulation of C-MAF expression. Chromatin immunoprecipitation studies revealed that IL-2 induced STAT5 binding to specific sites in the C-MAF promoter. These sites corresponded to regions enriched for markers of chromatin architectural features in both resting CD4 and differentiated Th2 cells. Unlike IL-6, IL-2 induced C-MAF expression in CD4 T cells with or without prior TCR stimulation. TCR-induced C-MAF expression was significantly inhibited by treatment with daclizumab or a JAK3 inhibitor, R333. Furthermore, IL-2 and IL-6 synergistically induced C-MAF expression in TCR-activated T cells, suggesting functional cooperation between these cytokines. Finally, both TCR-induced early IL-4 mRNA expression and IL-4 cytokine expression in differentiated Th2 cells were significantly inhibited by IL-2R blockade. Thus, our findings demonstrate the importance of IL-2 in Th2 differentiation in human T cells and support the notion that IL-2R–directed therapies may have utility in the treatment of allergic disorders. The Journal of Immunology, 2011, 187: 3721–3729.

Signal transducers and activators of transcription proteins are activated by a variety of cytokines, growth factors, and hormones. They comprise an evolutionarily conserved family of seven proteins in the mammalian genome (1). These proteins regulate vital cellular functions such as proliferation, survival, and differentiation. The two STAT5 proteins, STAT5a and STAT5b, are activated by members of the γ chain family of cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), which together regulate lymphoid development, differentiation, and survival of various components of the immune system (2). Studies with knockout and transgenic mice have shown that Stat5a/5b are essential for the development and/or homeostatic maintenance of T cells, including CD8, γδ TCR, CD4+CD25+Foxp3+ regulatory T cells (Tregs), and, most importantly for our studies, the selective differentiation of CD4 Th cells (3–8).

T cell differentiation involves epigenetic changes in lineage-associated genes by covalent modifications of DNA and histones and the histone variant H2A.Z (9). Transcriptionally active regions of chromatin are generally enriched with several modifications of histones such as mono-, di-, and trimethylylation of H3K4 (8, 10, 11) and H2A.Z (12), which differentially demarcate promoter and enhancer regions or regions of nucleosomal instability, respectively. A number of different STAT proteins, including Stat5, interact with transcriptional regulatory regions and are known to regulate T cell differentiation by enhancing or repressing key genes involved in these processes (13). Th2 differentiation in both mouse and human CD4 T cells is critically dependent on IL-2 (14, 15). Consistently, Stat5a knockout mice show defective Th2 responses and decreased IL-4 production, whereas a constitutively active Stat5a mutant can restore IL-4 production in IL-2–deficient CD4 T cells and Th2 differentiation in IL-4Rα–deficient CD4 T cells (6, 16). IL-2–activated Stat5 is necessary for increased transcription and cell surface expression of IL-4R in differentiating Th2 cells (17), as well as for appropriate chromatin remodeling to enhance accessibility of the murine Il4 locus (16, 18). Additionally, genome-wide analysis of Stat5 DNA binding in fully differentiated murine Th2 cells reveals several probable Stat5 binding sites, suggesting that Stat5 can potentially regulate numerous Th2-associated factors (17).

The c-maf proto-oncogene was the first lineage-specific factor identified for Th2 cells and belongs to the AP-1 family of proteins (19). It binds to a Maf recognition element (MARE) site in the Il4 promoter and directly transactivates Il4 gene transcription (19). Overexpression of c-maf in murine Th1 clones induces low levels
of endogenous IL-4 synthesis, whereas transgenic mice over-expressing CD4-specific c-maf preferentially develop a Th2 phenotype and have attenuated production of the Th1 cytokine IFN-γ (20). Recent studies have also shown that c-maf is required for the efficient development of murine T follicular helper and Th17 lineages, as well as for the production of IL-10 by Th17 cells (21–23). Thus, c-maf plays essential roles in the differentiation and function of multiple effector T cell lineages. In murine T cells, c-maf expression is regulated by IL-6–activated Stat3 (24). However, relatively little is known about the regulation of C-MAF transcription during early human T cell activation prior to differentiation.

In this study, to our knowledge we show for the first time that in primary human CD4 T cells, C-MAF expression is regulated by IL-2R–mediated STAT5 activation, independently of TCR signaling. We elucidate upstream regions of the C-MAF gene containing epigenetic modifications corresponding to transcriptional enhancer regions in undifferentiated and fully differentiated Th1 and Th2 cells and reveal that these are stably maintained irrespective of the differentiation state. We show that IL-2 induces vivo STAT5 binding to IFN-γ activation site (GAS) motifs located within and around these regions of stable epigenetic modifications, and we characterize their role in regulating IL-2–induced C-MAF transcription. Furthermore, we show that blockade of IL-2R has a profound inhibitory effect on IL-4 production during in vitro Th2 differentiation. These findings indicate that IL-2 plays an important role in the initiation of Th2 differentiation in human T cells and support the notion that IL-2R–directed therapies may have utility in the treatment of allergic disorders (15).

Materials and Methods

PBMCs and CD4 T cell isolation, culture, and treatment

PMBCs were isolated from buffy coats from healthy donors (purchased from National Blood Service, Tooting, U.K.) by density gradient centrifugation using LSM 1077 (PAA Laboratories). CD4 T cells were purified from PMBCs by positive selection with human CD4 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of CD4 T cells ranged from 93 to 96% by FACS analysis using FITC-conjugated anti-CD3 and PE-conjugated anti-CD4 mAbs (both from Dako) on a FACS Calibur instrument (BD Biosciences). Cells were maintained in culture medium of RPMI 1640 supplemented with 10% FBS (both from PAA Laboratories) and 2 mM l-glutamine and penicillin/streptomycin (both from Sigma-Aldrich, Dorset, U.K.). When indicated, CD4 T cells were stimulated with 2 μg/ml PHA-L (EY Laboratories, San Mateo, CA) for 72 h and rested in culture medium for 16 h prior to activation with 100 U/ml IL-2 (Roche Diagnostics). CD4 T cells were activated via the TCR by stimulation with plate-bound anti-CD3 (UCHT1; 1 μg/ml) and soluble anti-CD28 Ab (2 μg/ml; both from Ancell) with or without the JAK3 inhibitor R333 (5 μM; a gift from Rigel Pharmaceuticals, South San Francisco, CA); IL-6 and RGD systems) was used at 10 ng/ml for stimulation experiments. CD4 T cells were inhibited by treatment with anti-human IL-2 Ab (10 μg/ml; BioSource) or humanized anti-Tac (HAT, or daclizumab; 10 μg/ml; provided by Hoffmann-La Roche, Nutley, NJ).

Chromatin immunoprecipitation cloning and chromatin immunoprecipitation sequencing

Chromatin immunoprecipitation (ChIP) cloning experiments were performed using 1.5 × 10^6 CD4 T cells stimulated with IL-2 for 20 min followed by a 10-min crosslink with formaldehyde (0.37%) at 37°C, as previously described (25). The sonicated chromatin was immunoprecipitated for an hour using μMACS protein A microbeads (Miltenyi Biotec) and anti-Stat5a or anti-Stat5b monoclonal or control mouse IgG Abs (all from Zymed Laboratories). Purified chromatin was blunt-end repaired using T4 DNA polymerase (New England Biolabs), ligated to linkers, PCR amplified, and cloned into TOPO vector (Invitrogen) and sequenced (Cogenics).

For identification of the sequences enriched in regions associated with specific histone phenotypes (ChIP assay coupled with massive parallel sequencing, or ChIP-seq experiments), ChIP was performed on naive chromatin from ex vivo differentiated human Th2 cells (26). Chromatin was prepared by using the protocol of Feil and colleagues (http://www.epigenome-noe.net/researchtools/protocol.php?protid=2) with some minor modifications. Briefly, nuclei were treated with micrococcal nuclease (10 U/μl, 7 min) and mono- and dinituclosem chromatin was recovered. Chromatin quality was assessed by agarose gel electrophoresis and semi-quantitated using a nanodrop. Chromatin (20 μg) was immunoprecipitated with 3 μg anti-H3K4me3 (ab8580) or anti-IgG control and magnetic protein G beads (Active Motif). Prior to sequencing, the diversity of sequences enriched by ChIP was assessed by comparing quantitative PCR (qPCR) of input DNA with anti-IgG or anti-H3K4me3 immunoprecipitated DNA using multiple probe sets (ChIP-qPCR). ChIP libraries were prepared from amplified DNA (17 cycles) from two biological replicates of Th2 cells (Illumina) according to the manufacturer’s protocol. DNA from separate experiments was sequenced on GAII genome analyzers (genomics core facility at the National Institute for Health Research at Guy’s and St. Thomas’ NHS Foundation Trust/King’s College London Comprehensive Biomedical Research Centre, King’s College London). Detection of regions of enriched H3K4me3 relative to input DNA was performed by using MACS and SICER programs. Output was converted to browser extensible data files displaying the number of tags in 200 bp windows and visualized within the University of California at Santa Cruz genome browser.

Preparation of cell extracts and Western blotting

Nuclear and total cell extracts were prepared as described previously (27). Samples were resolved on 8% SDS-PAGE gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Western blots were performed using pan-STAT5 (Invitrogen), anti–phospho-STAT5 Y694 (New England Biolabs), c-maf polyclonal Ab (M-153; Santa Cruz Bio-technology), and anti-lamin B or anti-tubulin Abs (both from Invitrogen) and developed using ECL Plus chemiluminescence reagent (GE Healthcare, Buckinghamshire, U.K.).

RNA isolation and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). RNA (250 ng) was reverse-transcribed to cDNA using RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) following the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed in triplicate using 2× Precision SYBR Green MasterMix (PrimerDesign, Southampton, U.K.) in a total reaction volume of 20 μl with the ABI Prism 7900 HT sequence detection system (Applied Biosystems), although ChIP-qPCR reactions were performed in duplicate. The human PPIA or 18S rRNA genes were used as reference controls for the various experiments. Cycle threshold values were exported with the sequence detector SDS 2.3 software (Applied Biosystems). Gene-specific primers for PP1A, 18S rRNA, C-MAF, CD25, and IL2 were obtained from PrimerDesign. The sequences of all other primers are given in Supplemental Fig. 3.

Luciferase reporter assay

HEK293T cells were cultured and transfected to reconstitute IL-2R signaling using the calcium phosphate method, as previously described (28). Trimerized GAS1–4 sequences were synthesized (MWG Biotec), cloned into pG4.23 vector (Promega), and verified by sequencing (Cogenics). Luciferase assays were performed by using the Dual-Glo luciferase kit (Promega) according to the manufacturer’s instructions. Firefly luciferase activities were standardized to the corresponding Renilla luciferase activities for each sample to control for transfection efficiencies.

Th2 skewing and intracellular cytokine staining

Fresh cord blood units were obtained from the Programa Concordia Banc de Sang i Teixits (Barcelona, Spain) with prior consent of patients and ethical committee approval. Cord blood units were diluted 1:1 with RPMI 1640 (Lonza) supplemented with 0.6% trisodium citrate and 40 nM 2-ME. Cord blood mononuclear cells were separated by density gradient centrifugation using Ficoll-Paque Premium (GE Healthcare UK). CD4 T cells were isolated by negative selection using a Miltenyi Biotec isolation kit. Mean ± SD positivity for CD45RA on isolated CD4 cells was 93 ± 5% (n = 3). Cord blood CD4 cells (0.5–1 million) were activated with IL-2 as follows: anti-CD3 (10 μg/ml), anti-CD28 (2 μg/ml; both BD Biosciences), and IL-2 (30 U/ml) without or with HAT (10 μg/ml). Th2 skewing was performed by previously described (26) cord blood cells (200,000 cells) were stimulated with anti-CD3 (10 μg/ml), anti-CD28 (2 μg/ml), IL-2 (30 U/ml), IL-4 (12.5 μg/ml; NBS Biologics), anti–IFN-γ and anti–IL-10 (both 5 μg/ml; BD Biosciences) without or with HAT (10 μg/ml).
Intracellular staining for IL-4 and IFN-γ was carried out according to standard methods. Briefly, cultured cells were restimulated with PMA (50 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich) for 4.5 h at 37°C in the presence of brefeldin A (3 μg/ml; eBioscience) in 1 ml culture medium. Washed cells were fixed with 4% PFA, blocked with 50% FCS (v/v in PBS), permeabilized with 0.1% saponin (w/v in 1% FCS in PBS), and stained for 30 min in the dark at 4°C with mAbs against IL-4 (BD Biosciences) and IFN-γ (Caltag Laboratories) or appropriate isotype controls (mouse IgG1-PE [BD Biosciences] and mouse IgG1-allophycocyanin [Caltag Laboratories]) at recommended concentrations. Within 4 h staining, data from live cells, based on forward/side scatter profiles, were acquired on a FACSCalibur flow cytometer (CellQuest software; BD Biosciences) and analyzed with FlowJo (Tree Star, Ashland, OR).

Data analysis
Data analysis used Microsoft Excel and GraphPad Prism v4 (GraphPad Software). Statistical analysis was performed by using paired parametric and nonparametric tests as appropriate. A p value <0.05 was considered to be statistically significant.

Results
IL-2–activated STAT5 binds to the C-MAF locus in vivo in CD4 T cells
IL-2 is one of the earliest cytokines produced by activated T cells and mediates its actions primarily through the activation of STAT5 proteins. A STAT5-ChIP assay was performed using chromatin from freshly isolated CD4 T cells to identify in vivo IL-2–activated STAT5 gene targets. The immunoprecipitated chromatin yielded a number of distinct clones based on sequencing. One clone mapped to chromosome 16 at −152,916 to −153,096 upstream of the C-MAF gene and contained a consensus GAS motif (Fig. 1A). Because IL-2 and STAT5 play significant roles in gene regulation during Th2 cell differentiation, we examined whether IL-2–induced STAT5 also regulates C-MAF gene expression.

In vivo binding of STAT5 was assessed in cross-linked chromatin fragments derived from either IL-2–stimulated or untreated freshly isolated CD4 T cells by immunoprecipitation with anti-STAT5 Abs in a ChIP assay. DNA was isolated from precipitated chromatin and assayed for the presence of the upstream C-MAF sequence by using PCR with specific primers that detect the aforementioned cloned fragment. IL-2 treatment induced binding of STAT5a and STAT5b to this sequence (Fig. 1B). As expected, this sequence was not enriched in the IL-2–treated sample by ChIP performed with control IgG (Fig. 1B). Thus, there was specific IL-2–induced binding of STAT5 proteins to a sequence upstream of the C-MAF transcriptional start site (TSS). Because this sequence is located at a long distance upstream of the TSS of the C-MAF gene, we next determined whether IL-2–induced STAT5 binds to other 5′ upstream regions that were closer to the C-MAF promoter. We postulated that transcriptionally relevant binding sites would be located within cell type-specific DNase1 hypersensitive (DHS) sites (29, 30).

The genome-wide DHS study of CD4 T cells identified three DHS sites (DHS1–3) in the C-MAF 5′-upstream region (Fig. 1C) (30). DHS1 encompasses a broad region containing three peaks located between 78,189,000 and 78,194,500 nucleotides (−2,388 bp

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**FIGURE 1.** STAT5 binding sites in the 5′-upstream region of C-MAF were located within or adjacent to architectural regions of chromatin in resting and Th2-differentiated CD4 T cells. A, Sequence of the cloned fragment obtained from STAT5-Chip assay. Bold type indicates a consensus GAS motif; italic type indicates a putative imperfect GAS motif, positioned at an optimal distance to facilitate tetrameric STAT5 binding. B, STAT5 binding to the cloned sequence. ChIP was performed on untreated or IL-2–stimulated fresh CD4 T cells by using anti-STAT5a– or anti-STAT5b–specific Abs, or a negative control IgG Ab. Input is a sample of total chromatin taken before immunoprecipitation. C, Chromatin architecture of the C-MAF 5′-upstream regulatory region and positions of associated GAS motifs. Custom Track View (hg 16 build) is shown of DHS sites (DHS1–3), histone modifications H3K4me1, H3K4me3, and H2A.Z in resting CD4 T cells, H3K4me3 in Th1 and Th2 cells, and positions of CpG islands in this region of chromosome 16 upstream of the TSS of the C-MAF gene. The positions of consensus GAS motifs (GAS1–5) associated with the three DHSs are also indicated.
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upstream of TSS). DHS2 is between 78,232,000 and 78,233,000 nucleotides (−39,888 bp upstream of TSS) and DHS3 is between 78,361,500 and 78,362,500 nucleotides (−169,388 bp upstream of TSS). Analysis of the sequences within and around the three DHS sites revealed the presence of five consensus GAS motifs. GAS1 and GAS5 were within the DHS1–3 peaks, whereas GAS2–4 were nearby (Fig. 1C). Analysis of the sequence of these GAS motifs revealed strong evolutionary conservation of GAS1 and GAS2, suggesting that these may be important regulatory sequences, whereas GAS3–5 were less well conserved (Supplemental Fig. 1).

To understand whether these GAS motifs coincided with transcriptionally active chromatin during T cell differentiation, we undertook ChIP-seq analysis of fully differentiated Th1 and Th2 cells using an anti-H3K4me3 Ab and compared sites of enrichment with previously published genome-wide CD4 ChIP-seq data for H3K4me1, H3K4me3, and H2AZ enrichment (10). These studies revealed that GAS1, 2, 4, and 5 were located within regions enriched for monomethylated histone H3K4, whereas GAS1, 2, and 4 were also coincident with regions enriched for H3K4me3 in both Th1 and Th2 cells (Fig. 1C). All of the GAS motifs coincided with regions enriched for binding of the histone H2 variant H2AZ. Thus, despite being located a long distance upstream of the C-MAF TSS, the distal GAS motifs mapped to regions containing chromatin modifications consistent with potential enhancer function.

The TSS and the 5′-upstream region of the c-maf gene are depicted with the aforementioned sequences and positions of DHS-associated GAS motifs (GAS1–5), as well as a negative control GAS6, which is a random, consensus GAS motif (Fig. 2A). To determine whether IL-2 induces in vivo binding of STAT5 to each of the GAS motifs, we performed a ChIP analysis with anti-STAT5α or control Ab on unstimulated or IL-2–stimulated, PHA-activated human CD4 T cells. The immunoprecipitated chromatin was analyzed for the specific enrichment of GAS1–6 motifs by qRT-PCR. STAT5α bound to GAS1–4 in a specific IL-2–inducible manner, but not to GAS5 or the negative control GAS6 (Fig. 2B). The nonspecific IgG control did not enrich chromatin with GAS1–6 sequences in IL-2–stimulated cells by ChIP (Fig. 2B). Thus, IL-2 induced STAT5 binding to multiple sites in the C-MAF promoter at varying distances 5′ to the TSS, and these sites coincided with markers of nucleosomal instability and potential enhancer function. We consistently noted that the lowest level of STAT5 binding was to GAS3, suggesting that this GAS motif alone is insufficient for optimum STAT5 binding. Comparison of the GAS3 sequence (TTCTTTGAA) with the optimal STAT5 binding site [TTC(T/C) Ng(A/G)AAG] reveals an imperfect match and may suggest a need for cooperative tetrameric STAT5 binding to this region. In this regard, we noted that there is an imperfect GAS motif spaced seven nucleotides away from GAS3 (indicated in italic type in Fig. 1A), which may facilitate tetrameric STAT5 binding (28, 31).

To elucidate the STAT5-dependent enhancer activity of these GAS motifs, we assessed the ability of transfected STAT5b to activate constructs containing GAS motifs 1–4 in luciferase reporter assays performed in an IL-2R reconstitution system in HEK293T cells (28). These studies revealed that GAS1–, GAS2–, and GAS4–driven luciferase reporter constructs increased IL-2–induced, STAT5b-dependent transcriptional activation (Fig. 2C). A similar pattern of transactivation of these reporter constructs was observed with STAT5a (data not shown). The GAS3–driven luciferase reporter construct was not transactivated by STAT5b, consistent with poor binding observed at this GAS motif (Fig. 2B). Thus, IL-2–induced specific STAT5 binding and transcriptional activation from promoter proximal (GAS1, GAS2) and distal (GAS4) GAS motifs within the C-MAF 5′-upstream region.

**IL-2 specifically induces C-MAF gene expression**

The role of IL-2 in C-MAF gene expression was elucidated by comparing C-MAF mRNA levels from freshly isolated and PHA-treated CD4 T cells that were untreated or treated with IL-2 for 0.5–12 h. C-MAF mRNA expression in these samples was analyzed by qPCR. IL-2 significantly induced C-MAF mRNA expression in both freshly isolated (Fig. 3A) and PHA-activated CD4 T cells (Fig. 3B) between 2 and 6 h after stimulation, and as early as 30 min after stimulation. To determine whether IL-2 induction of C-MAF gene transcription was associated with a concomitant increase in protein expression, we prepared nuclear extracts from preactivated CD4 T cells that were stimulated with IL-2 for the indicated times and examined levels of C-MAF, phosphorylated STAT5 (pY-STAT5), total STAT5, and the loading control lamin B1 by immunoblot analysis. As shown in Fig. 3C, C-MAF protein expression was clearly increased by 6 h after IL-2 treatment and followed the peak increase in tyrosine phosphorylated STAT5 protein in the nucleus at 30 min. Of note, we observed the sustained presence of IL-2–activated STAT5 in the nucleus for the duration of the experiment (24 h) (Fig. 3C). Taken together,

**FIGURE 2.** IL-2–induced STAT5 binding and the regulatory effects of GAS motifs in the C-MAF 5′-upstream region. A, Schematic outline of the chromosomal locations (bp) of GAS1–5 and a negative control GAS motif (GAS6) relative to the C-MAF TSS on the left. B, Enrichment of chromatin from fresh CD4 T cells by ChIP with anti-STAT5α as compared with control IgG. Sequences were analyzed by qRT-PCR for the different GAS motifs indicated in A. Data are expressed as fold enrichment for IL-2–induced STAT5α binding for each site. Results are representative of three independent experiments from three different donors. C, IL-2–induced STAT5b-dependent transcriptional activation of GAS1–, GAS2–, and GAS4–driven luciferase constructs in HEK293T cells reconstituted with IL-2Rβ, γc, JAK3, and STAT5b by transfection. Pooled results from three independent experiments are shown. *p < 0.05.
C-MAF is induced without TCR stimulation by IL-2 alone in freshly isolated, as well as PHA-activated, CD4 T cells. Because the cloned sequence from the STAT5-ChIP maps to a 5′ region distal to the TSS of the C-MAF gene, we wanted to determine whether IL-2–induced STAT5 may regulate either of the flanking genes, namely WWOX (∼380 kb 3′ of C-MAF) or DNCL2B (∼940 kb 5′ of C-MAF). We therefore performed qPCR analysis to examine the IL-2–induced expression of these two genes in CD4 T cells. Neither WWOX nor DNCL2B was induced by IL-2 stimulation (Fig. 3D). Thus, IL-2–induced binding of STAT5 to upstream sequences specifically activates the C-MAF gene.

**TCR plus CD28-mediated activation of C-MAF transcription is dependent on IL-2R signaling**

In the above studies, exogenous IL-2 alone efficiently stimulated C-MAF expression in the absence of TCR stimulation. Thus, we next examined whether TCR activation of C-MAF was dependent on IL-2 signaling by using clinically relevant inhibitors of the IL-2 receptor (HAT) or signaling molecule JAK3.

HAT (daclizumab) binds to the α-chain (CD25) of the high-affinity IL-2R, inhibiting binding of IL-2 to the receptor complex and causing efficient blockade of JAK3/STAT5 activation (15). CD4 T cells were isolated fromuffy coats and stimulated with anti-CD3 plus anti-CD28 in the presence or absence of HAT for the indicated times. As shown in Fig. 4A, C-MAF expression is significantly induced at 17 h after stimulation, and HAT blocked this induction. Thus, the anti-CD3 plus anti-CD28–stimulated early activation of C-MAF is dependent on IL-2R signaling.

Because IL-2 induction of C-MAF during T cell activation may specifically involve activation of the JAK3/STAT5 pathway or may use additional IL-2–dependent pathways, the effects of a potent JAK3 inhibitor R333 (32) during TCR activation was examined. Fresh CD4 T cells were purified and stimulated with anti-CD3 plus anti-CD28 in the presence or absence of R333, and total cell extracts and RNA were prepared for further analysis. The effect of R333 on activation of STAT5 was investigated by Western blot analysis of total cell extracts using phospho-STAT5 antibodies. STAT5 activation (pY-STAT5) was detectable as early as 4 h, increased by 17 h, and was blocked by R333 (Fig. 4B). Thus, the JAK3-specific inhibitor R333 efficiently inhibited STAT5 activation.

RNA samples were analyzed by real-time PCR for C-MAF, IL2, and CD25 expression. As noted in Fig. 4A, anti-CD3 plus anti-CD28 activation of freshly isolated CD4 T cells increased C-MAF transcription at 17 h after stimulation. JAK3 inhibitor R333 blocked the induction of C-MAF transcription (Fig. 4C). The increase in CD25 and IL2 expression at 17 h posttreatment, following peak IL2 expression at 6–8 h (data not shown), was also significantly reduced by R333 (Fig. 4C). Taken together, these data indicated that the induction of C-MAF transcription during T cell activation was dependent on IL-2 signaling and JAK3/STAT5 activation in CD4 T cells.

**IL-2 and IL-6 functionally synergize to potently induce C-MAF gene expression**

Because both IL-2 and IL-6 can independently activate C-MAF expression and subsequently promote Th2 differentiation, we
examined whether these cytokines functionally cooperate in the regulation of C-MAF. Purified CD4 T cells were stimulated as above with anti-CD3 plus anti-CD28 or in the copresence of IL-2 and/or IL-6. As expected, C-MAF transcription was induced by anti-CD3 plus anti-CD28 stimulation, and addition of either cytokine alone modestly increased this induction. However, the presence of both IL-2 and IL-6 significantly increased C-MAF transcription in a synergistic manner (Fig. 5A). The presence of an inhibitory Ab to IL-2 or CD25 or the JAK3 inhibitor R333 blocked the synergy, indicating the increase was dependent on IL-2/IL-2R signaling.

To understand whether this synergistic effect extended to induction of GATA3, the signature transcription factor of Th2 cells, we quantified GATA3 message in purified CD4 T cells stimulated as above with anti-CD3 plus anti-CD28 or in the copresence of IL-2 and/or IL-6. In contrast to C-MAF, we did not observe any further increase in anti-CD3 plus anti-CD28–induced GATA3 mRNA expression by IL-2 or the combination of IL-2 plus IL-6.

FIGURE 4. TCR-induced C-MAF expression is dependent on IL-2 signaling via the JAK3/STAT5 pathway. A, CD4 T cells were stimulated by anti-CD3 plus anti-CD28 treatment in the presence or absence of HAT (10 μg/ml), and mRNA was prepared at various time points after stimulation and analyzed by qRT-PCR for C-MAF gene expression using PPIA as reference control. C-MAF mRNA levels are presented as fold increase over those of unstimulated cells. Results are from three independent donors. *p < 0.05. B and C, CD4 T cells were stimulated with anti-CD3 plus anti-CD28 in the presence or absence of the JAK3 inhibitor R333 (5 μM) for the indicated times. B, STAT5 is activated in a JAK3-dependent manner following TCR stimulation. Whole cell extracts were prepared from cells after treatment. The activation of STAT5 (pY-STAT5) was determined by Western blot analysis using anti–phosphotyrosine-STAT5 and pan-STAT5 Abs and, as a loading control, anti-tubulin Ab. The lack of pSTAT5 signal at 6 h was due to the loss of sample during preparation, as indicated by the anti-tubulin Western blot. C, Activation of C-MAF transcription by TCR stimulation requires JAK3 activity. qRT-PCR analysis was performed to detect the mRNA expression of C-MAF, CD25, and IL2 genes using PPIA as the reference gene. mRNA expression of each of the genes is presented as fold increase over unstimulated cells. The results show pooled data from four independent experiments. *p < 0.05.

FIGURE 5. IL-2 and IL-6 synergized to potently induce transcription of C-MAF, but not GATA3. CD4 T cells were stimulated with anti-CD3/anti-CD28 for 17 h in the presence of the indicated cytokines (IL-2, IL-6) and/or inhibitors. qRT-PCR was performed to assess mRNA expression of C-MAF and GATA3 (A) and IL4 genes (B). Expression of each of the genes is presented as fold increase over unstimulated cells. In A and B, n = 5. *p < 0.05.
Thus, C-MAF transcription is specifically activated by IL-2 signaling during early CD4 T cell activation.

**IL-4 expression is dependent on IL-2 signaling during T cell activation**

The key Th2 cytokine gene *IL4* is directly regulated by c-maf (19). We wanted to clarify the role of IL-2 signaling in *IL4* activation during T cell activation. CD4 T cells were stimulated with anti-CD3 plus anti-CD28 and IL-2 and IL-6 in the presence or absence of the inhibitors anti–IL-2, HAT, or R333. As expected, *IL4* expression is potently induced by the activating stimuli (Fig. 5B). However, the addition of anti–IL-2, HAT, or R333 significantly reduced *IL4* gene expression, indicating that IL-2R signaling is important for activation of *IL4* transcription during T cell activation (Fig. 5B).

**IL-2 signaling is required for IL-4 production during Th2 cell differentiation**

To determine whether IL-2 signaling was required for IL-4 protein expression during in vitro Th2 cell polarization, we purified naive CD4 T cells from cord blood samples from three donors and performed in vitro differentiation experiments under Th2 polarizing conditions or TCR (anti-CD3 plus anti-CD28) plus IL-2 stimulation in the presence or absence of the IL-2R–blocking HAT Ab. Fourteen days after treatment, cells were analyzed for cytokine production by flow cytometry. A representative plot is depicted in Fig. 6A, and the pooled results from all three donors are presented in Fig. 6B. As expected, anti-CD3 plus anti-CD28 activation of naive T cells induced the production of both key Th1 (IFN-γ) and Th2 cytokines (IL-4) (26). HAT treatment significantly reduced the percentage of IFN-γ and IL-4 expressing cells, consistent with previous studies using PBMCs (15). After 14 d under Th2 conditions, CD4 T cells produced predominantly IL-4, not IFN-γ (Fig. 6A). In the presence of HAT, IL-4 was significantly inhibited by ∼90% in all three donors (Fig. 6). Furthermore, when exogenous IL-2 was omitted in the skewing experiments, the cells were unable to polarize to IL-4–producing cells (Supplemental Fig. 2). Thus, these results indicated that IL-2 signaling plays an important role in the in vitro Th2 differentiation of naive CD4 T cells.

**Discussion**

The pleiotropic actions of IL-2, one of the earliest cytokines produced following T cell activation, are central to the propagation of the ensuing immune response (33). During human T cell activation, functional blockade of the IL-2R with daclizumab blocks STAT5 activation and inhibits Th1 and Th2 cytokine production (15). We thus hypothesized that in humans IL-2/STAT5 may also play a critical role in the differentiation of both these lineages. In fully differentiated murine Th2 cells, target loci for Stat5 binding have recently been identified by genome-wide ChIP-seq analysis. These include motifs in the promoters of known key regulators of Th2 differentiation in the mouse, such as *il4r*, *gata3* and c-maf (17). Because previous studies have revealed differences between
Th1/Th2 differentiation in humans and mice, we sought to establish the role of IL-2/STAT5 in human Th2 differentiation (26). In this study, we show that IL-2 signaling plays a significant role in initiating C-MAF and IL4 transcription during early T cell activation and thus subsequent IL-4 production during differentiation of human Th2-skewed human CD4 T cells.

The function of c-maf as an essential transcription factor required for IL-4–driven Th2 lineage commitment is well established (19, 20). However, the molecular mechanisms that regulate C-MAF expression prior to specific lineage differentiation are not well understood. We show that during early T cell activation IL-2 increases C-MAF gene expression by inducing STAT5 binding to GAS motifs located in the 5′-upstream region. Of note, the STAT5 binding sites are not only located proximal to the promoter but also at a large distance upstream of the TSS, and they map within DHSs present in the genome of resting CD4 T cells (30). Moreover, these binding sites coincide with regions of enrichment of the histone marks H2AZ, H3K4me1, and H3K4me3, which indicate structural features such as enhancers and therefore imply regions of active chromatin. Thus, mechanistically, our studies indicated that the C-MAF locus is already in a transcriptionally permissive configuration in resting CD4 T cells. Furthermore, the H3K4me3 enrichment pattern for this locus in resting CD4 T cells (10) showed a similar pattern to that observed in Th1 and Th2 cells from our ChIP-seq analysis. Therefore, in different CD4 T cell lineages, similar regulatory regions appear to be involved in “priming” the C-MAF gene for cytokine-specific transcriptional activation. c-maf is also important for murine Th17 differentiation, where its expression is regulated by IL-6–activated Stat3 (21). Interestingly, a recent genome-wide ChIP-seq study of Stat3 binding sites in differentiated murine Th17 cells revealed that Stat3 binds to a site that corresponds to the GAS1 region identified in our study (34). Of note, the chromatin architecture in this region appears to be very similar between WT and Stat3−/− mice, suggesting that the c-maf chromatin architecture is unaffected by Th17 lineage differentiation. This finding is consistent with our present observation in human CD4 T cells that there are no major changes in the C-MAF 5′-upstream chromatin architecture between resting and Th1- and Th2-differentiated human CD4 T cells, and it supports the notion that the C-MAF locus is in an epigenetically active configuration irrespective of differentiation status and is primed for transcription before specific lineage differentiation.

Additionally, we provide evidence that IL-2 alone can activate C-MAF in freshly isolated T cells in the absence of TCR stimulation, which we have also confirmed in CD4+CD25− T cells (S. John, unpublished observations). Moreover, activation of C-MAF occurs early during T cell activation, following increases in IL-2 and CD25 transcription, and is blocked by functional blockade of the IL-2R signaling (Fig. 4). Therefore, IL-2–induced STAT5 is necessary for the expression of C-MAF during early T cell activation. This finding is consistent with the observation that van-deficient mice, which have impaired IL-2 production, also show lower expression of both c-maf and IL-4 and subsequent Th2 development (35, 36). Murine studies have shown that c-maf is also induced by IL-6 via Stat3 activation, leading to increased il4 expression and subsequent Th2 differentiation (24, 37). However, in contrast to IL-2, IL-6 activation of c-maf is dependent on TCR stimulation. Our data show that IL-2 and IL-6 can synergize to more potently induce human C-MAF gene expression, and that this synergy is significantly inhibited by blocking IL-2 or IL-2R. Thus, both IL-2–activated STAT5 and IL-6–activated STAT3 are important regulators of C-MAF gene expression.

The IL-2/IL-2R blockade studies indicate that during T cell activation, IL-2 signaling is not only required for C-MAF expression but also for early expression of IL4, indicating that early in differentiation IL-2 plays an important role in creating an environment that favors Th2 polarization. We confirmed this functionally by showing that blockade of high-affinity IL-2R with daclizumab significantly inhibited in vitro Th2 polarization of naive CD4 T cells, as evidenced by the significant decrease in IL-4 expression. Furthermore, there was no Th2 differentiation of naive T cells when IL-2 was omitted in the in vitro polarization experiments (Supplemental Fig. 2). Previously, murine studies have shown that ICOS–ICOSL interactions are important for c-maf and il2 expression and subsequent Th2 differentiation. These studies showed that ICOS can be induced in murine cells by IL-4, 2) enhances IL-2 but not IL-4 production following optimal stimulation through CD3 and CD28, and 3) is only important during suboptimal CD3/CD28 T cell stimulation or through CD3 plus IL-2 alone (38–40). Because our experimental set-up is APC-independent, we have not dissected the contribution of ICOS–ICOSL interactions in this study in the anti-CD3 plus anti-CD28/IL-2–mediated induction of human C-MAF and IL4. In this regard, it will be interesting to elucidate whether IL4 and C-MAF induction by IL-2 is only important during suboptimal CD4 T cell activation, similar to murine T cells (40).

Although c-maf is important for Th2 differentiation, the critical master regulator of this lineage is GATA3. As in previous murine studies by others we did not find any evidence for the activation of human GATA3 transcription by IL-2 or IL-6 at early times after TCR activation. Similarly, previous murine studies showed that neutralization of IL-2 had no effect on gata3 expression (41). However, our data do not exclude the possibility that IL-2 may regulate GATA3 RNA and/or protein levels in differentiated Th2 cells. Hwang et al. (42) have reported that c-maf enhances cd25 expression in developing murine Th2 cells, which is consistent with our observation that blockade of IL-2R during anti-CD3 plus anti-CD28 stimulation of CD4 T cells reduces CD25 expression. Because our present studies reveal an important role for IL-2–induced STAT5 in the activation of C-MAF, we speculate C-MAF may play a role in the differentiation of peripheral human Tregs, whose maintenance and functional integrity are dependent on this pathway. We have observed that C-MAF transcription is induced by anti-CD3 plus anti-CD28 and/or IL-2 stimulation in peripheral human Tregs (B. Afzali and S. John, unpublished observations). Although the functional implications of this observation are presently unclear, it is suggestive of a role for C-MAF in Treg biology and is supported by the finding that the aryl hydrocarbon receptor can interact with c-maf to promote the differentiation of type 1 regulatory cells in mice (43).

In summary, we have identified and characterized the molecular mechanism by which IL-2–induced STAT5 regulates the Th2 lineage-promoting transcription factor C-MAF in a TCR-independent manner. Furthermore, IL-2 synergizes with IL-6 during T cell activation to significantly promote activation of C-MAF. Additionally, we show that the anti-CD3 plus anti-CD28–induced activation of the critical Th2 cytokine IL-4 is also dependent on IL-2 signaling. Finally, we demonstrate that IL-4 expression during in vitro Th2 differentiation is inhibited by functional IL-2R blockade. These findings help elucidate the function of IL-2/IL-2R in human Th2 differentiation and support the proposition that IL-2R–directed therapies, such as daclizumab, may have utility in the treatment of allergic disorders. Consistent with this suggestion, a recent controlled clinical trial showed improvement of pulmonary function in asthmatic patients treated with daclizumab (44).
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Disclosures
The authors have no financial conflicts of interest.

References
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Figure S1: Alignment of the conservation pattern for the five GAS sequences studied. The figure above shows the five GAS sequences studied and their sequence homologies using a screen capture of the UCSC genome browser. The base pair numbers and the genomic assembly from the UCSC genome browser (hg 16 build) for humans is indicated on the top in each case.
Figure S2. Cytokine expression by skewed cord blood CD4 T cells. A, representative intracellular stains, from one experiment out of three, of cytokine expression by cord blood CD4 T cells activated in vitro under TH2 skewing conditions without IL-2 ± HAT. B, cumulative data from 3 experiments showing mean percentage ± s.d. of cells positive for each cytokine
IL-4-R: 5' GAAAGGCTGACAGGAAAC 3'
IL-4-R: 5' CTCCGTGTTGCTCTTGCAC 3'
WWOX-P: 5' ATACGACGCGACACACAC 3'
WWOX-R: 5' GCGGGTTTCTGACACCTAT 3'
DNS3-2R: 5' CCTGAGCCGAGAPTCCG 3'
DNS3-2R: 5' ACATATTCTCATATAACCCCTT 3'
GaS1_forw 5' TTAAGTGCAGCTTATATAAAGTGTT 3'
GaS1_rev 5' GGGGGAGCCCATTTGGAAGT 3'
GaS2_forw 5' TACACGCGTTAAGTAATATCATACAAATC 3'
GaS2_rev 5' TCACAAGTGGCAGACGACTG 3'
GaS3_forw 5' CTCTCTCTCTCTCTCTCTCTAC 3'
GaS3_rev 5' GCACAATATAGTATGTTACTG 3'
GaS4_forw 5' ATACGACGCGACACACACAC 3'
GaS4_rev 5' TGGGGAGCCCATTTGGAAGT 3'
GaS5_forw 5' CCATATCTCGAAGTTTGGG 3'
GaS5_rev 5' AAGACGACGCGACACACACAC 3'
GaS6_forw 5' CTCTCTCTCTCTCTCTCTCTAC 3'
GaS6_rev 5' ACCACCGAAGACGACTG 3'

For PCR analysis of the cloned cDNA/PTP fragment (chr16: 78,345,828-78,345,868),
the following primer pair was used: 5'-CTCTCTCTCTCTCTCTCTCA,
R:5'-GAGACGACGCTGTGACCCATA, which amplifies a 139bp fragment. All
primers were obtained from MWG Operon.

Figure S3: List of primer sequences used in this study