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## T<sub>H</sub>2 Cytokines and Allergic Challenge Induce Ym1 Expression in Macrophages by a STAT6-dependent Mechanism\*

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**The diverse functions of macrophages as participants in innate and acquired immune responses are regulated by the specific milieu of environmental factors, cytokines, and other signaling molecules that are encountered at sites of inflammation. Microarray analysis of the transcriptional response of mouse peritoneal macrophages to the T<sub>H</sub>2 cytokine interleukin-4 (IL-4) identified Ym1 and arginase as the most highly up-regulated genes, exhibiting more than 68- and 88-fold induction, respectively. Molecular characterization of the Ym1 promoter in transfected epithelial and macrophage cell lines revealed the presence of multiple signal transducers and activators of transcription 6 (STAT6) response elements that function in a combinatorial manner to mediate transcriptional responses to IL-4. The participation of STAT6 as an obligate component of protein complexes binding to these sites was established by analysis of nuclear extracts derived from STAT6-deficient macrophages. Macrophage expression of Ym1 was highly induced *in vivo* by an IL-4- and STAT6-dependent mechanism during the evolution of allergic peritonitis, supporting the biological relevance of the IL-4-dependent pathway characterized *ex vivo* in peritoneal macrophages. These studies establish Ym1 as a highly inducible STAT6-dependent transcript in T<sub>H</sub>2-biased inflammation and define Cis-active elements in the Ym1 promoter that are required for this transcriptional response.**

The macrophage plays an important role bridging between innate and acquired immune function. It is capable both of responding to nonspecific stimuli, such as bacterial lipopolysaccharides (LPS)<sup>1</sup> and activated complement factors, but can also

engage processes of the acquired immune response such as antibody-dependent opsonization and subsequent antigen presentation to T-cells (1, 2).

Macrophage function can be influenced profoundly by cytokines released from T-cells and other immune cells. These cytokines have been divided largely along two axes: T<sub>H</sub>1 cytokines, dominated by INF- $\gamma$  and T<sub>H</sub>2 cytokines, dominated by IL-4 (3). Macrophage stimulation by T<sub>H</sub>1 cytokines results in free-radical release and increased cytokine secretion, implicated as essential signaling components of a successful response to infection by intracellular bacteria and viruses (4–6). In contrast, efficient defense against extracellular pathogens and parasites requires a different response, characterized by the release of T<sub>H</sub>2 cytokines (7). These mediate B-cell class switching to IgE antibodies, macrophage opsonization of soluble antigens, and activation of mast cells, basophils, and eosinophils. These two cytokine axes have been proposed to be mutually antagonistic, both in the commitment of T-cells toward a T<sub>H</sub>1 or T<sub>H</sub>2 lineage and in their influence on effector cells such as the macrophage.

In parallel with the separation of T-cells into either T<sub>H</sub>1- or T<sub>H</sub>2-biased cells, a number of groups have proposed recently that the exposure of macrophages to a specific set of cytokines also biases them toward either an M-1 phenotype (activated macrophages) or an M-2 phenotype (alternatively activated macrophages) (8–10). Macrophage exposure to IL-4 substantially blunts subsequent LPS- or INF- $\gamma$ -stimulated production of cytokines (IL-6, IL-8, IL-12, and tumor necrosis factor- $\alpha$ ), receptors (CD14, Fc $\gamma$ I, Fc $\gamma$ II, and Fc $\gamma$ III), and superoxide radicals (11–14). At the same time, macrophage exposure to IL-4 results in the increased expression of scavenger receptors (the mannose receptor, the scavenger receptor type I, and CD163), CD13 (the aminopeptidase, capable of inactivating inflammatory mediators), and CD23 (FceRII), thus blunting the cytotoxic effects of the macrophage and shifting its opsonizing focus from IgG antibodies to IgE while increasing its phagocytic capabilities (9, 15).

Allergic challenge of BALB/c mice with ovalbumin following sensitization has been shown to result in the recruitment of macrophages into an environment enriched in IL-4 and IL-5 (16–18). This allergic response provides a useful model of macrophages activated alternatively because of the relative abundance of macrophages, the cytokine milieu they are recruited into, and their high expression of arginase I, a marker of macrophages activated alternatively (19). In addition, this model of allergic response has been used to characterize general allergic phenomenon as they may apply to human pathophysiology of allergy and asthma.

INF- $\gamma$  and LPS induce large and profound transcriptional changes in the macrophage (13, 20, 21). In contrast, relatively

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF533510.

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<sup>1</sup> The abbreviations used are: LPS, lipopolysaccharides; IL, interleukin; INF, interferon; STAT, signal transducers and activators of transcription; RT, reverse transcriptase; TK, thymidine kinase; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; BAL, bronchial alveolar lavage.

few genes have been characterized as direct targets of IL-4 stimulation. The development of large scale expression arrays allows near global glimpses of changes in gene expression profiles during cell proliferation, division, and in response to environmental signals. We applied this technology to probe the transcriptional response of murine macrophages to the quintessential  $T_H2$  cytokine, IL-4. Ym1 was one of the most highly induced IL-4 target genes, exhibiting a level of induction of 70-fold or greater in multiple macrophage populations. Studies of the Ym1 promoter suggest that the binding of STAT6 to multiple sites within a 600-bp region up-stream of the transcriptional start site is required for the IL-4 response in BEAS-2B cells and RAW macrophages. Ym1 was also highly induced in macrophages recruited to the peritoneal cavity during allergic peritonitis in an IL-4- and STAT6-dependent manner. Collectively, these findings establish molecular mechanisms underlying the transcriptional response of the most highly induced IL-4 target gene identified to date in macrophages.

#### MATERIALS AND METHODS

**Cell Culture**—Thioglycollate-elicited macrophages were isolated by peritoneal lavage 3 days following peritoneal injection of 2.5 ml of 3% thioglycollate (Difco). Cells were plated in 10% RPMI and washed after 5 h. Bone marrow-derived macrophages were generated by culturing total bone marrow in macrophage colony stimulating factor provided by 30% L-cell-conditioned media for 7 days in RPMI + 10% fetal bovine serum (22). ECoM-M monocytic leukemia cells were cultured as described previously (23). RAW 267.4 macrophages and BAES-2B bovine airway epithelial cells were grown in 10% fetal bovine serum and RPMI (Invitrogen).

**Expression Array Profiling**—Total RNA was purified with Trizol (Invitrogen) and RNeasy columns (Qiagen). cRNA was generated from 10  $\mu$ g of total RNA using Superscript (Invitrogen) and the high yield RNA transcription labeling kit (Enzo). Fragmented cRNA was hybridized to Affymetrix arrays according to manufacturer's instruction. Data was analyzed with Microarray Suite (Affymetrix) and GeneSpring (Silicongenetics).

**Northern Blot Analysis**—RNA analysis by Northern blotting followed the procedure of Ausubel (24). 5–10  $\mu$ g of total RNA were separated by gel electrophoresis and transferred to nitrocellulose (Supercharge; Schleicher & Schuell). Prior to hybridization, membranes were UV cross-linked (Stratagene) and stained with methylene blue (MRC). Probes were generated by RT-PCR using the following primers followed by random priming labeling (Invitrogen) and hybridization with Quick-Hyb (Stratagene): Arginase I, GAAACAGAGTATGACGTGAGAG, AGGTGGTTTAAGTGTAGTCAGTC; I $\beta$ 30, TTCTGCTTCTGCTGTCCCACT and ACTCCATGATACTCTCTGTGAC; Hspa5, GTGAGGTAGA-AAGGCTAAGAG and GAAGAAACTTTTCCAGTTGC; tissue inhibitor of metalloproteinase, ATCTGGCATCCTCTTGTGCTA and GATCTCCAAGTGCACAAGCCTA; Ym1, CTGTGTACTCACCTGATCTATG and GAAAGAACCCTGAAGTCATCC; Scya9, TTCTGCTTCTGCTGTTCCACT and ACTCCATGATACTCTGTGAC.

**Western Blot Analysis**—Macrophages were washed with ice-cold phosphate-buffered saline, and the cell pellet was resuspended in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM EGTA, protease inhibitors (Roche Molecular Biochemicals)) and incubated for 10 min at 4 °C. Cell debris were spun down for 10 min at 14,000  $\times$  g, and supernatants were collected. The total protein content was quantified, and 50  $\mu$ g of total protein was separated on a 10% NuPAGE Bis-Tris gel (Invitrogen) and transferred onto a nitrocellulose membrane. The membrane was probed with an anti-Ym1 antibody (1:500) kindly provided by Shioko Kimura (NCI, National Institutes of Health). Immune complexes were revealed by a peroxidase-conjugated anti-rabbit IgG and visualized by chemiluminescence (Pierce).

**RT-PCR**—cDNA was generated from 1  $\mu$ g of total RNA using Superscript (Invitrogen) and amplified with the following primers: Ym1, CTGGAATTGGTGCCCTACA and CAAGCATGGTGGTTTTACAGGA or Ym2, CAGAACCCTGACATTCATTA and ATGGTCTTCCAGTAGGTAATA. PCR was performed for 25 cycles with Mastermix Taq (Qiagen) at an annealing temperature of 58 °C and visualized by gel electrophoresis. *Bst*YI digestion was performed on cDNA amplified with primers redundant for Ym1 and Ym2: CCTACCAGCTGATGTGCTACTA and GAAAGAACCCTGAAGTCATCC using *Pfu* (Stratagene) and for 35 cycles with an annealing temperature of 58 °C.

**Primer Extension**—Protocol followed that of Ausubel (24) with the substitution of Superscript (Invitrogen) for avian myeloblastosis virus reverse transcriptase. In brief, 10  $\mu$ g of total RNA from control and IL-4-treated ECoM-M macrophages were hybridized at 65 °C for 90 min to end-labeled primers GGAAGATCCCAGCTGTACGTTCCAGAAG or GCACATCAGCTGGTAGGAAGATCCAG. Reverse transcription was performed for 1 h at 42 °C with Superscript in the appropriate buffer. RNA was degraded with RNaseH (Invitrogen), and cDNA was isolated by phenol-chloroform extraction, separated on a 9% acrylamide/7 M urea sequencing gel, and visualized by autoradiography.

**EMSA**—Nuclear extracts were generated following Nonidet P-40 cell lysis as described previously (25). Nuclear extracts (5  $\mu$ g) were incubated for 10 min on ice with bovine serum albumin (2  $\mu$ g), poly(dI-dC) (2  $\mu$ g) in 10 mM Hepes, pH 8, 2.5 mM EDTA, pH 8, 0.5 mM sodium orthovanadate, 1 mM dithiothreitol, and 10% glycerol followed by 30 min on ice with appropriate end-labeled, annealed oligonucleotides as follows: A, GATCCGGCAAGTTCTTTGGAAGTCTTA and GATCTAAGAGTTCCAAAGAAGTTCGCG; B, GATCTACTCTTTCTGAAGAATGTGTG and GATCCACACCATTCTTCAGAAAGAAGTA; C, GATCCAATGTCTTCCATGGAATCAA and GATCTTGATTCCATGGAAGACATGTG; D, GATCCTATGTTTCTAAGAAGTGGGTA and GATCTACCCACTTCTTAGAAACATAG. Samples were separated in a gel consisting of 5% polyacrylamide, 0.5 $\times$  TBE, and 5% glycerol at 4 °C, 300 V. Gel was pre-run for 1 h at 4 °C, 300 V.

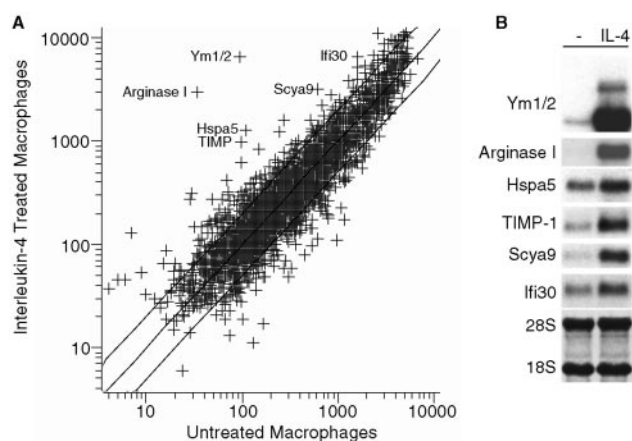
**Promoter Studies**—Promoter fragments were amplified from the genomic clone 5b (26) using Pfx (Stratagene), cloned into TOPOII-Blunt (Invitrogen) and transferred to the BNXH luciferase or minimal TK-luciferase reporter (27) by *Bam*HI/*Hind*III digestion and subsequent ligation. Promoter constructs used in deletion studies were amplified from genomic clone 5A, a generous gift from Robert B. Kirkpatrick (GlaxoSmithKline) (26), using the following primers: CTTAGGATCCC-AATATCCATGA TTAAGGATCCAGTGTCCAAGAC, ATGTGGATCCG-ATTTGTCTAGG, ATAGGGACTCTGACTGAACTGG, TCGCCTAAG-CTTCAGATTGC, CACCAGATCTGTCAAGATAGC, CCTAGACAGA-TCTTATTACATAG, GGGCAGATCTCTGGACATTG, GCTTGGATC-CAGGCAAGTTC. Oligonucleotides described for EMSA analysis of the C site were used to generate the multimerized C reporter. BEAS-2B cells were transfected with LipofectAMINE (Invitrogen) and treated overnight with human IL-4 (10 ng/ml) (Endogen). Extracts were harvested and assayed as described previously (27). The expression vector for human STAT6 was a generous gift of William LaRoche (NCI, National Institutes of Health) (28, 29). RAW 264.7 cells were transfected with 5  $\mu$ g of Ym-1/luciferase promoter construct DNA and 3  $\mu$ g of a murine STAT6 expression vector (gift of Yoshihiro Ohmori). 1  $\mu$ g of  $\beta$ -galactosidase expression vector was also co-transfected as a control for transfection efficiency. Ten million cells were electroporated in 0.4-cm electrode gap cuvettes using a Bio-Rad Gene Pulser II and pulsing at 280 V, 1070 microfarad. Immediately after pulsing, cells were recovered into pre-warmed RPMI supplemented with 20% fetal calf serum, antibiotics, and L-glutamine. The medium was replaced 1 h later with standard 10% fetal calf serum RPMI in the presence or absence of 20 ng/ml recombinant murine IL-4, and cells were harvested 16 h later for analysis of luciferase activity. Luciferase activity was normalized to control transfected  $\beta$ -galactosidase enzyme activity.

**Ovalbumin Sensitization and Allergic Challenge**—Mice were sensitized with subcutaneous injections of 100  $\mu$ g of ovalbumin (Sigma) absorbed in 0.1 ml of aluminum hydroxide (Inject Alum; Pierce) and 0.1 ml of PBS on days 1 and 7. Efficiency of sensitization was assessed by blood IgE levels and by splenocyte IL-4 production following *in vitro* ovalbumin challenge. On day 14, mice were challenged with intraperitoneal injection of 10  $\mu$ g of ovalbumin in 0.2 ml of PBS. Cells were harvested at indicated time points.  $\alpha$ IL-4 (11B.11) (NCI, National Institutes of Health) was injected 1 h prior to and 4 h after ovalbumin challenge. BALB/c, C57Bl/6, STAT6 knock-out, and WBB6F1/J mice were purchased from Harlan and Jackson Laboratories.

**Adoptive Transfer**—Splenocytes were isolated, and B-cells, macrophages, monocytes, and dendritic cells were depleted by magnetic cell sorting using major histocompatibility complex II (Ia) microbeads (Miltenyi Biotec) according to the manufacturer's instructions.  $10 \times 10^7$  cells were washed with PBS, resuspended in 500  $\mu$ l of PBS, and injected intravenously into naive mice. 24 h after adoptive transfer, mice were challenged as described above.

#### RESULTS

**Expression Profiling of Macrophage IL-4 Stimulation**—To assess genome-wide effects of IL-4 on macrophage physiology, we characterized the transcriptional response of thioglycollate-



**FIG. 1. Expression array profiling of the response of thioglycolate-elicited macrophages to IL-4.** *A*, scatter plot of cRNA generated from control TG-M $\phi$  and TG-M $\phi$  treated with IL-4 (10 ng/ml) for 24 h and hybridized to Affymetrix Mu11kA and B arrays. *B*, Northern blot analysis of the expression of selected genes induced by IL-4 in the microarray experiment.

elicited macrophages (TG-M $\phi$ ) to IL-4 treatment using Affymetrix Mu11kA and Mu11kB microarrays. Together these arrays contain a total of 13179 targets, of which 4215 (~32%) were scored as being present in at least one of the two treatment groups (Fig. 1*A*). Arginase I, mannose receptor, and TIMP-2, which have been demonstrated previously to be regulated by IL-4 (30, 31), were induced and served as positive controls (data not shown). In addition, several additional genes were induced, including the glucose-regulated heat shock protein Hspa5 (GenBank<sup>TM</sup> accession number AA086684, Unigene accession number Mm.918), the tissue inhibitor of metalloproteinase TIMP-1 (NM\_011593, Mm.8245), the cytokine Scya9 or MIP-1 $\gamma$  (U19482, Mm.2271), and the interferon- $\gamma$ -inducible lysosomal thiol reductase Ifi30 (AA106931, Mm.30241) (Fig. 1*B*). A more complete description of the global transcriptional response of macrophages to IL-4 will be presented elsewhere.<sup>2</sup>

Two genes were up-regulated dramatically in both the array experiment and subsequent confirmation, arginase I (U51805, Mm.154144) and *Ym1* (M94584, Mm.4571) (Fig. 1). The up-regulation of arginase I in response to IL-4 has been described previously and suggested to be an important marker of macrophage bias toward an M-2 phenotype (19), where it competes with the inducible nitric-oxide synthase for a common substrate (32). In contrast, relatively little is known about the transcriptional regulation of *Ym1*, a member of the chitinase family that is sufficiently similar to *Ym2* at the nucleotide sequence level to make it difficult to differentiate the two by microarray and Northern blot techniques.

**Macrophage Regulation of *Ym1/2* by IL-4**—*Ym1/2* expression is up-regulated in response to IL-4 stimulation in multiple populations of murine macrophages including bone marrow-derived macrophages (BM-M $\phi$ ), TG-M $\phi$ , and the immortalized monocytic cell line ECoM-M (23) (Fig. 2, *A* and *B*) (data not shown). Induction of *Ym1/2* in primary macrophages was found to be STAT6-dependent as *Ym1/2* expression did not respond to IL-4 in TG-M $\phi$  derived from STAT6-deficient mice (Fig. 2*B*). In contrast, three separate clones of the macrophage cell line RAW 264.7 failed to up-regulate *Ym1/2*, suggesting a lack of an essential signaling component in these cells (data not shown). Although multiple populations of primary murine macrophages robustly up-regulate *Ym1/2* expression in response to IL-4 stimulation, the mixed splenocyte population, consisting

largely of T-cells and B-cells, with a smaller population of monocytes, displayed a blunted response, suggesting that the response to direct stimulation with IL-4 may be largely monocyte/macrophage-specific (data not shown).

We examined the responsiveness of *Ym1/2* expression to a large panel of cytokines and macrophage stimuli. In addition to IL-4, IL-13 was capable of strongly inducing *Ym1/2* RNA and protein expression and did so nearly equally (Fig. 2, *C* and *D*). Both of these cytokines bind to the  $\gamma$ -common chain of the IL-4 receptor resulting in STAT6 activation (33). In contrast, IL-5, IL-6, IL-7, IL-10, IL-15, granulocyte macrophage colony stimulating factor, macrophage colony stimulating factor, granulocyte macrophage colony stimulating factor, INF- $\gamma$ , tumor necrosis factor- $\alpha$ , 13-hydroxyoctadecadienoic acid, 15-deoxy- $\Delta^{12,14}$ PGJ<sub>2</sub>, and LPS failed to induce *Ym1/2* expression (Fig. 2*D*) (data not shown). Thus, *Ym1/2* induction appeared, as arginase I, to be a restricted response to T<sub>H</sub>2-biased cytokines. Because arginase I was described to be induced synergistically by IL-4 and IL-10 in bone marrow-differentiated macrophages and dendritic cells (19), we tested the effect of the combination of these cytokines on *Ym1* expression in TG-M $\phi$ . Although IL-4-induced *Ym1* RNA expression seemed to be increased slightly further by IL-10 (Fig. 2*F*), no synergy between these two cytokines was observed at the protein level (Fig. 2*E*).

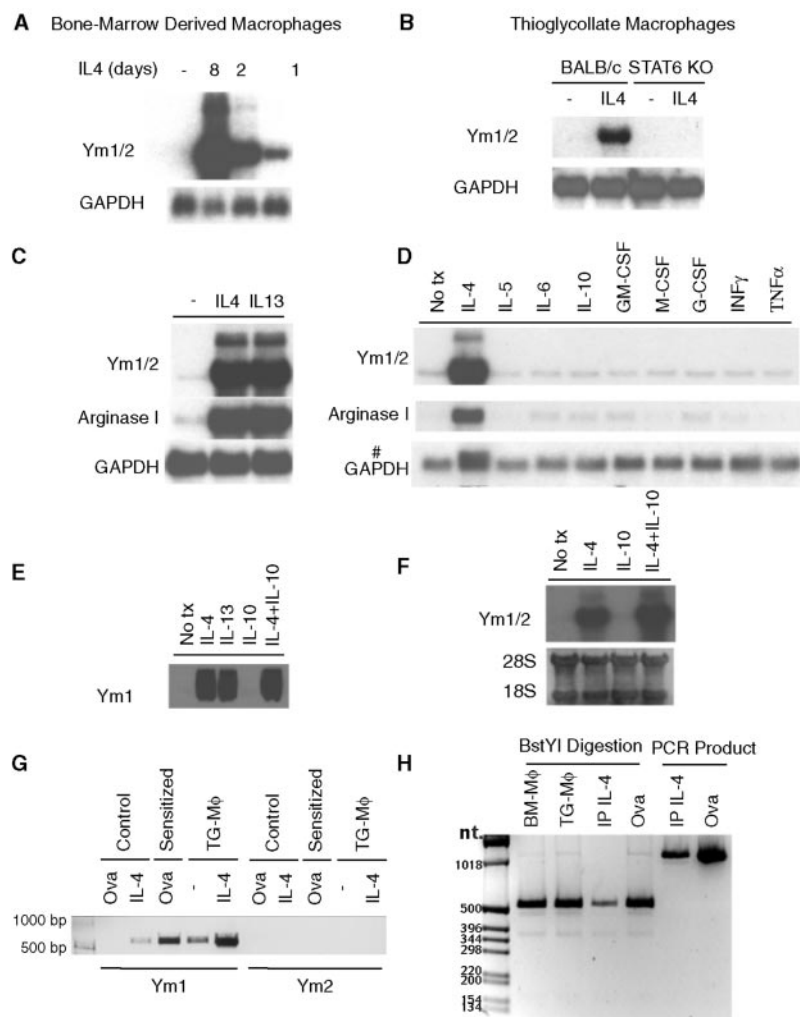
*Ym1* is related closely to *Ym2*, another member of the murine chitinase family (26). *Ym2* shares 95% RNA sequence identity with *Ym1* (34). Using RT-PCR and primers specific for *Ym1* or *Ym2*, we identified the IL-4-induced macrophage transcript as *Ym1* (Fig. 2*G*). In addition, *Ym1* and *Ym2* can be distinguished based on three restriction sites: *ScaI*, *BglII*, and *BstYI*. *ScaI* specifically digests *Ym1*, *BglII* specifically digests *Ym2*, and *BstYI* digests *Ym1* in two fragments of nearly equal size and *Ym2* in three fragments. We amplified a 1054-bp product with primers common to *Ym1* and *Ym2* from the same RT reactions and found that digestion with *BstYI* yielded a single band of ~520 bp (Fig. 2*H*). *BglII* digestion did not alter the size of the fragment, and *ScaI* digestion yielded two fragments, one 880 bp, and the other 270 bp (data not shown). This digestion pattern further confirms that the IL-4-induced macrophage transcript was *Ym1*.

**Characterization of *Ym1* Promoter**—To characterize the promoter and enhancer structure of *Ym1*, we identified a trace sequence (krv64h11.b1) from the mouse genome project containing the first exon of *Ym1* (26) (Fig. 3*A*). Using primers specific for exon 1 and exon 3, we confirmed that this promoter sequence was located immediately up-stream of exon 1 and 3.4 kb up-stream of exon 3 in both the Lambda FIXII clone 5A (26) and in the Celera database (mCG10749) (data not shown). This is important, because exons 1–3 of *Ym2* differ from exons 1–3 of *Ym1* only by nine nucleotides, but intron 1 of *Ym2* (mCG63439) is 4.3 kb, allowing one to distinguish between promoters of *Ym1* and *Ym2* by their position relative to exon 3. Using primer extension analysis, we identified the mRNA start site and found it to be 20 nucleotides up-stream of the ATG located in exon 1 (Fig. 3) (data not shown). The trace krv64h11.b1 and clone 5A sequence matched the information in the Celera database, with the exception of the deletion of a single T, 29 nucleotides up-stream of the mRNA start site.

Previous work has characterized the canonical binding site for STAT proteins as the sequence TTCNXGAA (35). STAT6 homodimers generally prefer binding to sites containing a four-nucleotide spacer ( $N_4$ ), whereas STAT1 homodimers generally prefer a three nucleotide spacer ( $N_3$ ) (Fig. 3*C*). We identified four such sites in the 600 nucleotides up-stream of the transcriptional start site and only one in the next 9400 nucleotides up-stream. Two of these (A and C) are canonical  $N_4$  sites, whereas the most immediate site up-stream of the start site, D,

<sup>2</sup> Welch *et al.*, manuscript in preparation.

**FIG. 2. IL-4 and IL-13 induction of M $\phi$  Ym1 expression.** Northern blot analysis of Ym1/2 expression in BM-M $\phi$  (A) and TG-M $\phi$  (B) derived from BALB/c- and STAT6-deficient mice. M $\phi$  were treated as indicated or for 24 h with IL-4 (10 ng/ml). C, D, and F, Northern blot analysis of TG-M $\phi$  treated for 24 h as indicated. # indicates unstripped arginase I probe. E, Western blot analysis of Ym1 expression in TG-M $\phi$  from BalB/c mice. M $\phi$  were treated for 24 h with IL-4 (10 ng/ml), IL-13 (10 ng/ml), and IL-10 (10 ng/ml). G, RT-PCR of samples in D. Lanes 1 and 2, Fig. 5A, lanes 2, 3, and 8 amplified with gene-specific primers. H, BstY1 digestion of PCR product generated with primers degenerate for Ym1 and Ym2. cDNA was generated from total RNA samples represented in A, lane 3; D, lane 2; Fig. 5A, lanes 3 and 8.



is an N<sub>3</sub> site (Fig. 3C). The B site contains two overlapping sites, one an N<sub>5</sub> and the other an N<sub>4</sub>.

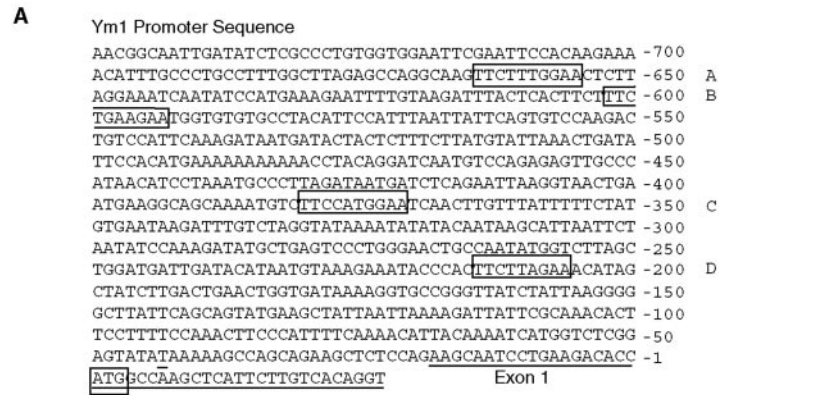
The A, C, and D sites all bound IL-4-inducible complexes with similar mobility when incubated with nuclear extracts prepared from BM-M $\phi$  as assessed by EMSA analysis, with strongest binding to the A site (Fig. 4A). These complexes were absent in BM-M $\phi$  from STAT6-deficient mice, indicating that they contain STAT6 and require its presence for DNA binding. In contrast, nuclear extracts from the ECoM-M cell line induced IL-4-dependent shifts on the A and C probes but bound a complex with different mobility on the D probe that was lost after IL-4 activation (Fig. 4B). We were unable to identify this complex by supershift analysis with antibodies against STAT1 $\alpha$ , STAT2, STAT5a, and STAT5b. Unlike the A and C sites, the D site is an N<sub>3</sub> site. This differential binding of complexes in different cell types, one induced and one lost during IL-4 stimulation, suggests that the D site may be an important negative regulator or modulator of basal Ym1 expression in some cell types. The B site was not retarded in the presence of nuclear extracts from cells under either condition, suggesting that the overlapping sequences prevent binding of STAT6 to this site (data not shown).

The lung epithelial cell line BEAS-2B was chosen initially for analysis of the Ym1 promoter because of its responsiveness to IL-4 upon expression of STAT6 (36). The Ym1 promoter (−664 to +27) was induced consistently 10-fold by IL-4 treatment when these cells were co-transfected with a STAT6 expression vector (Fig. 4C). IL-4 induction was not observed in the absence

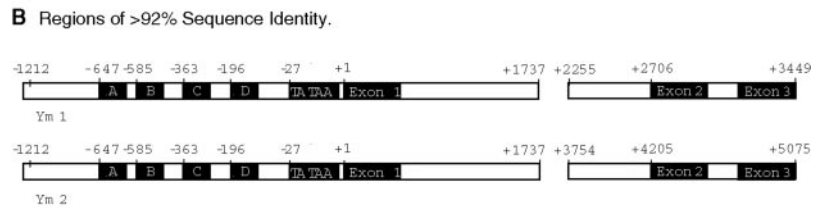
of cotransfected STAT6 (data not shown). Constructs lacking the A site were no longer responsive to IL-4 stimulation (D1, −630 to +27; D2, −550 to +27; D3, −326 to +27; D4, −176 to +27) (Fig. 4C). Although the B and C sites were not sufficient for responses to IL-4 in the context of the YM1 proximal promoter, the multimerized C site (3xC-TK) was as responsive to IL-4 as the multimerized STAT6 site from the human 12/15-lipoxygenase promoter (4xSTAT6-TK) (36) (Fig. 4C). A 3′-deletion series of the YM1 promoter was tested for the ability to enhance the activity of the TK promoter in an IL-4-dependent manner. All constructs transferred modest IL-4 induction, with deletion of the D site resulting in increased basal activity.

We next evaluated IL-4 induction of the Ym1 promoter in RAW 264.7 cells. These studies revealed a very similar profile of activity for the 5′-deletion series, with removal of the A site abolishing IL-4 responsiveness (Fig. 4D). The D2, D3, and D4 deletions were also not inducible by IL-4 (data not shown). In contrast to the findings in BEAS-2B cells, the presence of the D site did not appear to have an inhibitory effect on basal expression or IL-4 induction in RAW macrophages, and the responses of all of the 3′-deletions to IL-4 induction were more robust (Fig. 4D).

Using the Celera database we compared the promoter sequences of Ym1 and Ym2. Despite the fact that Ym1 and Ym2 expression show distinct and largely non-overlapping patterns of basal expression (26), the first 1200 nucleotides located upstream of exon 1 are 92% identical to the sequence up-stream of Ym2 exon 1, with preservation of the STAT binding sites (Fig.



**FIG. 3. Ym1 sequence up-stream of exon 1.** A, promoter sequence of Ym1. Boxes indicate canonical STAT binding sites and translational start site. Underlined sequences indicate exon 1 and differences between the trace sequence *krv64h11.b1* and the Celera database (*mCG10749*). B, regions of genomic sequence identity shared between Ym1 and Ym2. C, differences in STAT binding site sequences in Ym1 and Ym2 promoter elements. ND, not determined.



**C** Comparison of Ym STAT Binding Sites.

Site	Sequence	STAT Binding
Ym1-A	TTCTTTGGAA	STAT6
Ym2-A	TTCTTTGGAA	STAT6
Ym1-B	TTCTTTCTGAAGAA	None observed
Ym2-B	TTCTTTCTTAAGAA	ND
Ym1-C	TTCCATGGAA	STAT6
Ym2-C	TTCCACAGAA	ND
Ym1-D	TTCTTAGAA	STAT6, ?
Ym2-D	TTCTTAGAA	STAT6, ?
Consensus N <sub>4</sub>	TTCNNNNGAA	STAT6
Consensus N <sub>3</sub>	TTCNNNNGAA	STAT1, STAT3 STAT5, STAT6

3B). The A and D sites were identical in the Ym1 and Ym2 promoter, the C site had two nucleotide changes in the N<sub>4</sub> region (Ym1, TTCCATGGAA; Ym2, TTCCACAGAA), and the B site N<sub>4</sub> remained intact with a G → T alteration potentially abrogating the effect of the overlapping N<sub>5</sub> site (Ym1, TTCTTCTGAAGAA; Ym2, TTCTTTCTTAAGAA) (Fig. 3C).

Not only are the sequences up-stream of exon 1 conserved, but the first 1700 nucleotides down-stream of exon 1 are also 93% identical. Although STAT binding sites were common in the up-stream sequences, the conserved sequence down-stream of exon 1 contained only two sites. An unshared insertion of 500 nucleotides (Ym1) or 2000 nucleotides (Ym2) followed the region of downstream homology. Then, 500 nucleotides up-stream of exon 2, homology resumed and continued through the end of exon 3 (Fig. 3B). This high degree of genomic similarity suggests that Ym1 and Ym2 were generated by a recent gene duplication event.

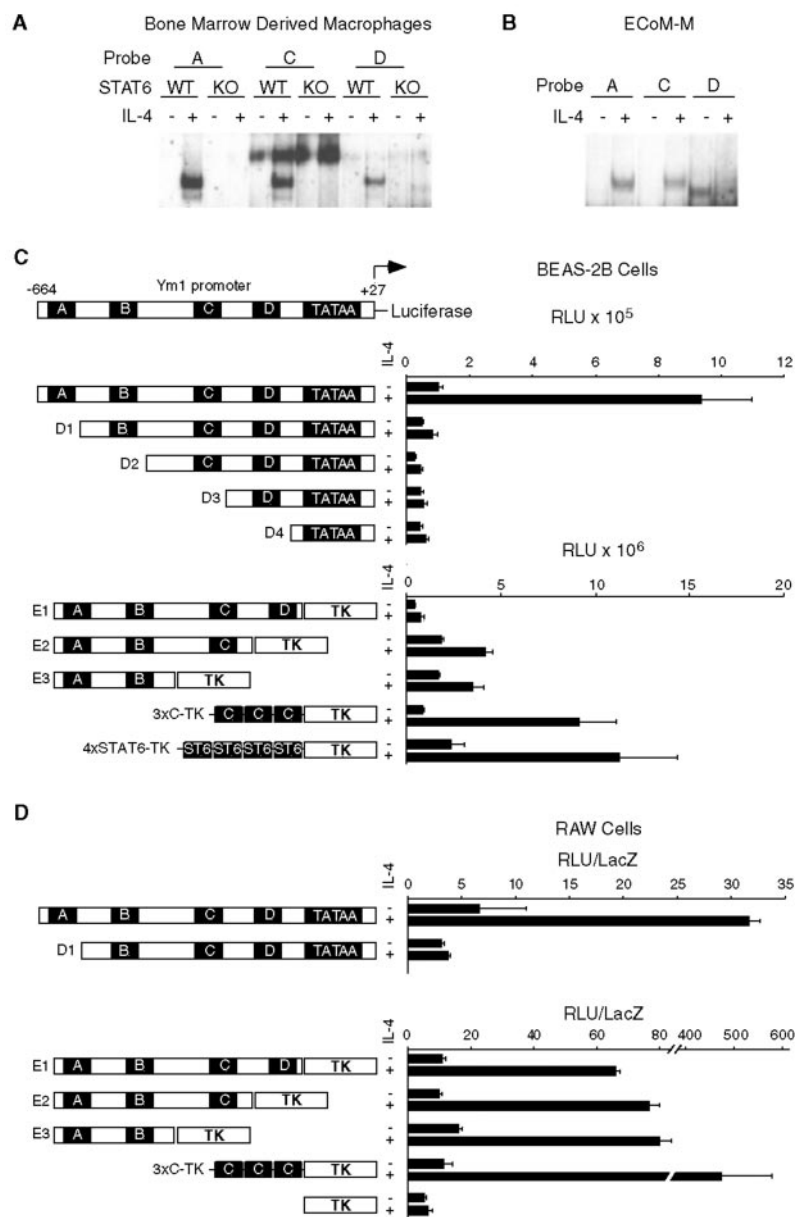
**Allergic Expression of Ym1**—To investigate the role of the IL-4-STAT6 pathway in regulation of Ym1 expression *in vivo*, we examined the possibility that Ym1 might be expressed in a model of murine allergic peritonitis where the release of both IL-4 and IL-5 precedes a peritoneal eosinophilia (16–18). We found that T<sub>H</sub>2-biased BALB/c mice sensitized subcutaneously with ovalbumin in aluminum hydroxide followed by an ovalbumin challenge (ova) dramatically up-regulated their peritoneal expression of Ym1 transcripts in comparison to mice not sensitized but challenged similarly (Fig. 5A). T<sub>H</sub>1-biased C57Bl/6 mice, which mount less robust responses to allergic challenge, induced Ym1 expression much less profoundly (data not

shown). This peritoneal response appears specific to the type of induced inflammation. Unlike the allergic peritonitis, thioglycollate-induced peritonitis results in minimal Ym1 expression (see Fig. 1B and Fig. 2B). By RT-PCR and subsequent digestion with *Bst*YI, *Sca*I, and *Bgl*II we also excluded up-regulation of Ym2 in this model (Fig. 2, G and H). The epitopes in ovalbumin required for induction of Ym1 appear to be conserved so sufficiently in bovine serum albumin that challenge with bovine serum albumin, but not lysozyme, also induced Ym1 expression (Fig 5, A and B). Using adhesion selection of peritoneal cells recovered 24 h after ova challenge, we found that the macrophage population was responsible for nearly all of the resulting peritoneal expression of Ym1 (Fig. 5B).

Previous studies have characterized Ym1 as an eosinophil chemokine (37). We were unable to observe expression of other eosinophil chemokines transcripts, chemokine C-C motif ligand 5 (Ccl5) and Eotaxin, under identical conditions (data not shown). The high level of Ym1 expression and lack of Ccl5 and Eotaxin transcripts suggests a potential role of Ym1 in this eosinophil recruitment.

Ym1 was characterized recently as a highly expressed neutrophil protein (38). We also observed Ym1 expression in bone marrow cells and that this expression decreased over a period of 7 days as the granulocytic cells died out, and the population matured into macrophages (data not shown). It is likely that the high expression of Ym1 observed 6 h after challenge (Fig. 5A) is the result of an early recruited neutrophil population. However, Ym1 expression remained high in peritoneal cells 24 and 48 h following challenge (Fig. 5A) (data not shown). By

**FIG. 4. STAT6 binding to Ym1 upstream elements.** A, EMSA of nuclear extracts from BM-M $\phi$  derived from BALB/c- and STAT6-deficient mice treated for 24 h with IL-4 (10 ng/ml). Probes A-D correspond to sequences described in the legend to Fig. 3. B, EMSA of nuclear extracts from differentiated ECoM-M cells treated for 24 h with IL-4 (10 ng/ml). Only the upper half of the gel exhibiting protein-DNA complexes is illustrated. C, promoter analysis of Ym1 up-stream elements in BEAS-2B cells. Cells were transfected with the indicated reporter constructs and STAT6-pcDNA3 expression vector (28, 29), treated for 24 h with human IL-4 (10 ng/ml) as indicated prior to analysis of luciferase activity. D, promoter analysis of Ym1 upstream elements in RAW 264.7 macrophages. Cells were transfected with the indicated promoter constructs and a STAT6 expression vector and treated for 16 h with murine IL-4 (20 ng/ml) prior to analysis of luciferase activity. Induction of the 3xC-TK construct (35-fold) was approximately twice that observed for the 4xSTAT6-TK promoter (16-fold) in this experiment (data not shown).



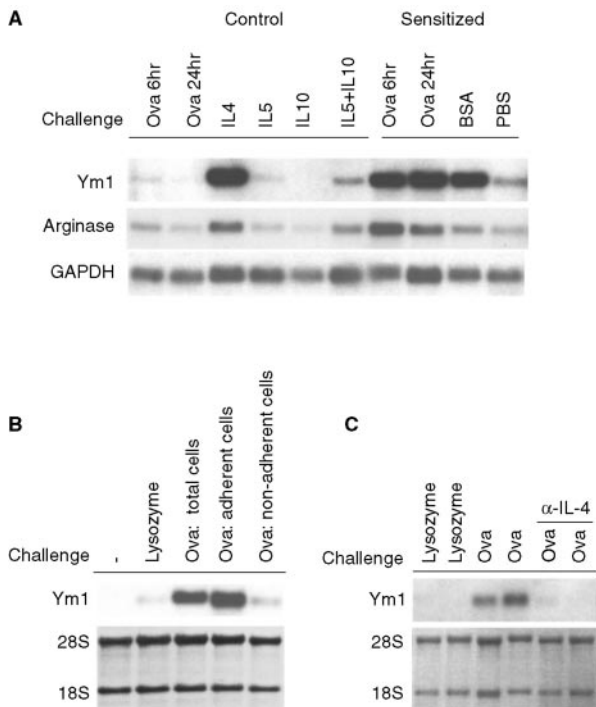
then, the population of peritoneal neutrophils has been replaced nearly completely by macrophages (16, 39, 40). This pattern of prolonged cellular expression following ova challenge, in addition to the adhesion selection, supports a role of macrophage-expressed Ym1 in the allergic response to ova peritoneal challenge.

Three lines of evidence support a role of IL-4 in the ova challenge increase of macrophage Ym1 expression. First, this up-regulation is not seen in STAT6-deficient mice both sensitized and challenged with ova or in STAT6-deficient mice sensitized and challenged subsequently after adoptive transfer of wild type splenocytes from sensitized animals (Fig. 6A). Second, injection of anti-IL-4 (11B.11) 1 h prior to and 4 h after ova challenge blocked subsequent Ym1 expression (Fig. 5A). Third, intraperitoneal injection of IL-4, but not IL-5, IL-10, or IL-13, resulted in up-regulation of Ym1 message (Fig. 5A) (data not shown).

Although both mast cells (41) and splenic T-cells have been demonstrated to release IL-4, neither of these cells appear responsible for the macrophage expression of Ym1 during ova-challenged allergic peritonitis. Neither intraperitoneal injection of the mast cell activating chemical 48/80 nor passive

sensitization with anti-2,4-dinitrophenol IgE followed by 2,4-dinitrophenol-bovine serum albumin challenge altered peritoneal cell Ym1 expression (data not shown). More importantly, mast cell-deficient mice responded with Ym1 expression increase similar to wild type mice (Fig. 6B). Adoptive transfer of T-cell-enriched splenocytes from sensitized mice to naive mice also did not alter the Ym1 expression of mice challenged subsequently (Fig. 6A). Likewise, exposing macrophages from control animals to splenocytes from sensitized animals in the presence of ovalbumin did not increase Ym1 expression (data not shown). These experiments suggest that the cell signaling the macrophage to increase Ym1 expression is likely neither a mast cell nor a T-cell found in the splenocyte compartment.

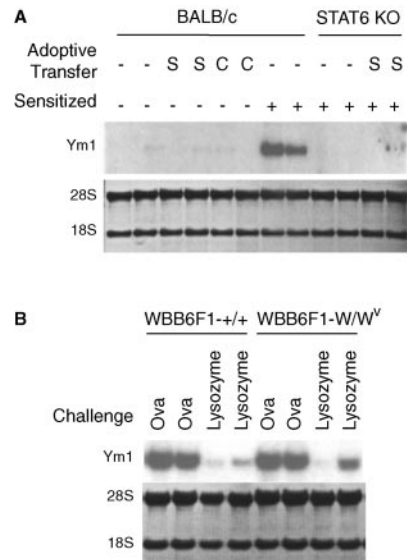
Although Ym1 has been demonstrated to induce eosinophil chemotaxis, Ym1 expression during allergic peritonitis does not appear necessary for general peritoneal recruitment of eosinophils. Although the adoptive transfer of splenocytes did not induce Ym1 expression, it did confer partial ability to recruit eosinophils to the general peritoneal cavity, suggesting that Ym1 may not be essential for eosinophil transepithelial recruitment (data not shown).



**FIG. 5. Ym1 expression during allergic peritonitis.** A, mice were sensitized by subcutaneous injection of ovalbumin in alum on days 1 and 7. On day 14, mice were challenged with 10  $\mu$ g of ova or 10 ng of cytokine in PBS. Peritoneal cells were harvested on day 15 and subjected to Northern blot analysis. B, mice were sensitized and challenged as before, and peritoneal cells were subjected to 5 h of adhesion selection to plastic tissue culture dishes prior to RNA extraction and Northern blot analysis. C, mice were injected with 1 mg of  $\alpha$ IL-4 (11B.11) 1 h prior to and 4 h after challenge. Peritoneal cells were harvested 24 h after ova challenge. Duplicate lanes represent separate mice.

DISCUSSION

**Regulation of Ym1 Expression by T<sub>H</sub>2 Cytokines and Allergic Challenge**—Ym1 has been characterized previously as a secreted, self-crystallizing member of the chitinase family that is expressed during peritoneal exposure to nematodes. Speculation as to the physiological and pathological function of Ym1 and the highly related Ym2 is difficult at this time. Although both Ym1 and Ym2 contain mutations in their active site that exist only in members of the chitinase family without chitinase activity, one group did observe chitinase activity (42), but others could not (37, 38). If Ym1 or Ym2 do exhibit chitinase activity, it is possible that they act as nonspecific immune agents. Chitin is a common element in organisms including parasites, fungi, and bacteria but does not occur in mammalian tissue (42), allowing for a selective anti-microbial activity of a chitinase. Alternatively, Ym1 may serve a role in the removal of chitin-containing antigens following invasions by such microorganisms (38). Ym1 has been characterized as an eosinophil chemokine (37). However, another group was unable to observe eosinophil chemotaxis in response to Ym2 (34). This may be because of subtle differences between Ym1 and Ym2, but this seems unlikely as the two are highly conserved, and the proposed CXC motif is preserved in both (37). If Ym1 or Ym2 does function as an eosinophil chemokine, this would indicate a mechanism of macrophage-eosinophil cross-talk via Ym1/2 expression unexplored previously and would suggest that such eosinophil cross-talk may be an important part of the macrophage response to T<sub>H</sub>2-type cytokines. Finally, analysis of Ym1 crystal structure indicated the presence of a  $\beta/\alpha$  TIM barrel similar to the lectin family of cell surface receptors. Indeed, Ym1 has also been shown to bind to oligosaccharides



**FIG. 6. Lack of regulation of Ym1 expression during allergic peritonitis by splenic T-cells and mast cells.** A, mice were sensitized on day 1 and 7. On day 14, indicated mice received  $10 \times 10^7$  splenocytes intravenously following negative selection for major histocompatibility complex II from sensitized (S) or control (C) animals. 24 h later, indicated mice were challenged. Peritoneal cells were harvested following an additional 24 h, and total RNA was subjected to Northern blot analysis. B, mast cell-deficient mice and littermate controls were sensitized, and indicated mice were challenged. 24 h later peritoneal cells were harvested and subjected to Northern blot analysis. Duplicate lanes represent individual mice.

with a free amine group, such as GlcN, found in many lectin receptors. This has lead to a final proposal that Ym1 might act in inflammatory resolution by masking lectin binding sites and preventing entry of new inflammatory cells to the site (43). However, this is clearly not an essential part of all inflammatory resolution as macrophages elicited during thioglycollate peritonitis express low levels of Ym1.

The present studies demonstrate that Ym1 is a highly induced IL-4 and IL-13 target gene in multiple macrophage populations. While this manuscript was in preparation, Raes *et al.* (44) also reported macrophage induction of a Ym1 or Ym2 transcript in response to the cytokines IL-4 and IL-13. The use of microarrays in the present studies to profile global transcriptional responses of macrophages to IL-4 indicated that the Ym1 or Ym2 transcript is one of the genes induced most dramatically in response to T<sub>H</sub>2 cytokine stimulation. We further identified this Ym transcript as Ym1 and demonstrated that this induction does not occur in macrophages derived from STAT6-deficient mice, indicating that Ym1 is a target of the IL-4/STAT6 signal transduction pathway.

In addition, we observed a striking induction of macrophage Ym1 expression during allergic challenge of ova-sensitized mice. The induction of peritoneal and alveolar eosinophilia by allergic challenge has been shown previously to elicit the release of IL-4 (7). Deletion of STAT6 (45, 46) or the blockade of both IL-4 and IL-13 activity (47, 48) has been observed to prevent eosinophil accumulation and airway hyper-reactivity in murine models of allergy. The high level of Ym1, in addition to arginase I, in macrophages during allergic challenge suggests that these are both distinct markers of macrophage response to T<sub>H</sub>2 cytokines and that they are likely to play an important role in allergic immune function.

Webb *et al.* (34) observed recently striking induction of Ym2 in a model of allergic eosinophilia similar to the one we applied. They observed, after intraperitoneal sensitization with ovalbumin, that repeated aerosol challenge of ovalbumin led to eosin-



nophilia and cellular expression of both Ym1 and Ym2 in bronchial alveolar lavage (BAL) fluid, with Ym2 as the dominant expressed Ym member. They also found the increase in Ym expression, and release depended on the IL-4R $\alpha$  subunit and on the release of IL-4 or IL-13. In contrast, we observed a striking STAT6-dependent macrophage induction of Ym1, but not Ym2, following subcutaneous sensitization and peritoneal challenge with ovalbumin. We also observed some subtle differences in the regulation of these allergic-induced Ym sequences. First, peritoneal Ym1 expression could be blocked by injection of anti-IL-4. In contrast, BAL Ym induction could only be abrogated with the blockade of both IL-4 and IL-13. Second, intraperitoneal injection of IL-4, but not IL-13, was sufficient to induce Ym1 expression, whereas expression of Ym protein in BAL cells could be induced with exposure to IL-13 alone. Third, adoptive transfer of T-cell-enriched splenocytes was insufficient to induce Ym1 expression following ova challenge in a naive mouse, whereas depletion of CD4<sup>+</sup> T-cells with the GK1.5 antibody abolished Ym expression in BAL cells. This final difference suggests that either the cell population signaling macrophage Ym1 expression during peritoneal ova challenge is different from the population that signals cellular expression of BAL Ym, or that it is a common population of CD4<sup>+</sup> T-cells, which exists outside the splenic population.

Peritoneal Ym1 expression has also been described in mice infected with either *Mesocestoides corti* (37) or *Brugia malayi* (49). In contrast to the allergic induction of Ym1 or Ym2, *M. corti* induction of Ym1 required CD4-CD8<sup>+</sup> T-cells. Furthermore, deletion of IL-4 or IL-5 alone was insufficient to inhibit *B. malayi*-induced Ym1 expression. As in the lung allergic response, this would support a more important physiologic role for IL-13 than would be inferred from experiments involving peritoneal allergic challenge.

**Mechanisms Responsible for IL-4-dependent Activation of Ym1**—To characterize the molecular mechanisms underlying the transcriptional response of Ym1 to IL-4, we analyzed the Ym1 promoter and identified *Cis*-active elements that are essential for IL-4 induction. The Ym1 promoter exhibits a canonical TATAA box 30 nucleotides up-stream of the site of transcriptional initiation and thus represents a conventional *Pol*II promoter. In addition, multiple potential STAT binding sites were identified within a 600-bp region of 5'-flanking information that was sufficient to mediate induction by IL-4 in transient transfection assays. Although the A, B, and C sites within this region match the TTCN<sub>4</sub>GAA consensus sequence identified previously for STAT6, only the A and C sites bound STAT6-containing complexes with high affinity in EMSA experiments. The obligate participation of STAT6 in protein complexes binding to the A and C sites was demonstrated unambiguously by the lack of binding activity in nuclear extracts derived from STAT6-deficient macrophages. The B site contains two overlapping STAT6 consensus sequences, such that ambiguity in selection of half-sites by STAT6 dimers could potentially inhibit high affinity binding. Alternatively, the flanking or spacer sequences in the B site may inhibit STAT6 binding by more general mechanisms, *i.e.* unfavorable base-specific contacts with residues in the STAT6 DNA binding domain.

The combination of A site with elements surrounding the TATAA box was essential for IL-4-dependent activation of the Ym1 promoter in both BEAS-2B cells and RAW 264.7 macrophages. Removal of the A site abolished IL-4 responsiveness, whereas transfer of the A-C or A-B regions to the basal TK promoter resulted in much weaker responses to IL-4 than the Ym1 promoter itself. Although not essential in these assays, insertion of three copies of the C site up-stream of the basal TK

promoter was sufficient to transfer IL-4-dependent induction better than that observed for the Ym1 promoter itself. These findings suggest that the binding of activated STAT6 to the A and C sites results in combinatorial interactions with additional factors that together mediate robust transcriptional response of the Ym1 promoter to IL-4. It will be of interest in future studies to identify factors that cooperate with STAT6 in this manner.

The promoter regions of Ym1 and Ym2 are highly related, with near complete conservation of the STAT6 binding sites observed. Despite remarkable sequence conservation, differential basal expression of Ym1 and Ym2 expression have been demonstrated by two groups (7, 50). Both groups found basal Ym1 expression highest in spleen and lung with lower expression in thymus, intestine, and kidney, whereas Ym2 expression was found highest in stomach with lower levels in thymus and kidney. The high degree of genomic similarity indicates that Ym1 and Ym2 were likely generated by a duplication event. The conservation of STAT6 sites likely accounts for the similarly striking induction of Ym1 and Ym2 expression in T<sub>H</sub>2-type environments but cannot account for either the spatial segregation of peritoneal Ym1 and lung Ym2 expression during allergic challenge or for the distinct and largely non-overlapping patterns of basal expression. Intriguingly, the presence of the D site inhibited basal expression of Ym1-TK fusion genes in BEAS-2B cells, but not in RAW 264.7 macrophages, suggesting a role of this element in cell-specific control of Ym1 expression. Consistent with this, we observed a distinct complex bound to the D site in the ECoM-M cells that was not observed in the BM-M $\phi$ . Sherman (41) identified an IL-4-inducible complex bound to the canonical STAT6 element up-stream of the IL-4 promoter, but which had a faster mobility in mast cells compared with B-cells and acted as a repressor. This complex was absent in mast cell extracts from STAT6-deficient mice, suggesting that it comprises a novel STAT6 isoform. Although differences in tissue-specific expression of Ym1 and Ym2 are likely to be accounted for or by essential enhancer sequences located outside the conserved genomic regions, it is possible that a novel STAT6 isoform or other cell type-specific factors binding to the identical D sites in their respective promoters act to restrict Ym1 and Ym2 expression to appropriate cell types.

In conclusion, we have shown a striking effect of IL-4 on macrophage transcription programs exemplified by the induction of arginase I and Ym1. We characterized the promoter sequence of Ym1 and identified three new functional STAT6 binding sites, demonstrated the requirement of STAT6 activity for IL-4-stimulated induction, and identified the 5'-most site, the A site, as essential for IL-4 responsiveness. Furthermore, we found that peritoneal macrophages in an allergic, T<sub>H</sub>2-type environment express high levels of Ym1, suggesting an important function for the chitinase family members in T<sub>H</sub>2-biased immune response. Ym1 is related closely to Ym2, which is also highly induced during a similar lung allergic challenge. However, the near identity of the proximal and distal sequences surrounding exon 1 of Ym1 and Ym2 can account only for their common responses to STAT6 signaling during ovalbumin-induced allergic eosinophilia but cannot account for their distinct separation in tissue specificity. These data indicate that the chitinase family members Ym1 and Ym2, in addition to arginase I, are highly induced during alternative macrophage activation, are likely to play important roles in T<sub>H</sub>2-biased immune responses, and may be used as markers of macrophage response to T<sub>H</sub>2 stimulation.

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## **T<sub>H</sub>2 Cytokines and Allergic Challenge Induce Ym1 Expression in Macrophages by a STAT6-dependent Mechanism**

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