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Shaping the Murine Macrophage Phenotype: IL-4 and Cyclic AMP Synergistically Activate the Arginase I Promoter

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Arginase I is a marker of murine M2 macrophages and is highly expressed in many inflammatory diseases. The basis for high arginase I expression in macrophages in vivo is incompletely understood but likely reflects integrated responses to combinations of stimuli. Our objective was to elucidate mechanisms involved in modulating arginase I induction by IL-4, the prototypical activator of M2 macrophages. IL-4 and 8-bromo-cAMP individually induce arginase I, but together they rapidly and synergistically induce arginase I mRNA, protein, and promoter activity in murine macrophage cells. Arginase I induction by IL-4 requires binding of the transcription factors STAT6 and C/EBPβ to the IL-4 response element of the arginase I gene. Chromatin immunoprecipitation showed that the synergistic response involves binding of both transcription factors to the IL-4 response element at levels significantly greater than in response to IL-4 alone. The results suggest that C/EBPβ is a limiting factor for the level of STAT6 bound to the IL-4 response element. The enhanced binding in the synergistic response was not due to increased expression of either STAT6 or C/EBPβ but was correlated primarily with increased nuclear abundance of C/EBPβ. Our findings also suggest that induction of arginase I expression is stochastic; that is, differences in induction reflect differences in probability of transcriptional activation and not simply differences in rate of transcription. Results of the present study also may be useful for understanding mechanisms underlying regulated expression of other genes in macrophages and other myeloid-derived cells in health and disease. The Journal of Immunology, 2013, 191: 000–000.

Macrophages are cells of the innate immune system that play a variety of roles, ranging from inflammation and host defense against pathogens to resolution of inflammation and wound healing. The specific functional roles of macrophages are critically dependent on the stimuli that activate them. Based primarily on their different responses to IFN-γ versus IL-4 or IL-13, macrophages are classified as classically activated or alternatively activated macrophages, respectively (1–4). It also was recognized that increased arginine metabolism is one of the most distinctive features of activated macrophages and that activated macrophages exhibit two distinct categories of increased arginine metabolism. Thus, activated murine macrophages characterized by elevated expression of the inducible isoform of NO synthase (iNOS or NOS2), which catalyzes conversion of arginine to NO and citrulline, were classified as M1 macrophages, whereas activated macrophages characterized by elevated expression of arginase I, which catalyzes hydrolysis of arginine to ornithine and urea, were classified as M2 macrophages (5); this classification scheme is used in this study. M1 macrophages include classically activated macrophages and M2 macrophages include alternatively activated macrophages. Although iNOS and arginase I can be expressed in many cell types in addition to macrophages, this study concerns only arginase I expression in macrophages. The molecular mechanisms that regulate expression of iNOS or arginase I in M1 or M2 macrophages remain to be fully elucidated. These broad classification schemes have been useful for generally describing activated macrophage phenotypes under a variety of physiologic and pathophysiologic conditions. However, both schemes are oversimplified, as macrophages can exhibit quantitative and qualitative variations in phenotype that are not adequately described by the classically and alternatively activated or M1 and M2 categories, including circumstances in which both iNOS and arginase I may be expressed to varying degrees (e.g., Refs. 6–10). Thus, macrophages classified as M2 due to lack of NO production may not necessarily lack iNOS expression but may fail to produce detectable NO due to substrate competition by arginase I. Part of the problem in characterizing different sets of macrophages arises from the fact that activated macrophages are often classified on the basis of their responses to single cytokines or other agents in cell culture, whereas macrophages in vivo are never exposed to only a single cytokine or other stimulus. Consequently, the phenotypes of cultured macrophages may not precisely reproduce macrophage phenotypes in vivo.

With this background in mind, the objective of this study is to elucidate mechanisms involved in modulating the macrophage phenotype by using IL-4 as stimulus and arginase I as a signature marker of the IL-4 response. Potential candidates for modulators of
the IL-4 response are signaling molecules that are present at sites of inflammation or wound healing. Examples of such molecules include catecholamines, PGs, and adenosine. Because arginase I expression also can be induced in macrophages by catecholamines (11), PGs (12), and adenosine (13), each of which can elicit increases in intracellular cAMP, these agents may well modulate induction of arginase I by IL-4 in vivo. Indeed, synergistic effects on IL-4–dependent induction of arginase I have been demonstrated for the β-adrenergic agonist isoproterenol (14), adenosine (13), and the phosphodiesterase 4 inhibitor rolipram and the cAMP analog 8-bromo-cAMP (8-Br-cAMP) (15). 8-Br-cAMP also synergistically enhanced arginase I induction by IL-13 (16). Previous studies have identified several DNA elements and transcription factors involved in induction of arginase I transcription by IL-4 in macrophages (17–21). In particular, IL-4 induces binding of the transcription factors STAT6 and C/EBPβ to the IL-4 response element of the arginase I promoter (17, 18). However, the interaction between cAMP and IL-4 at the level of arginase I transcription has not been defined. This interaction was the subject of the present study.

We therefore tested the hypotheses that enhanced expression of arginase I by costimulation involves increases in 1) expression of STAT6 and/or C/EBPβ, 2) cellular responsiveness to IL-4 as reflected by tyrosine phosphorylation of STAT6, 3) nuclear localization of STAT6 and/or C/EBPβ, or 4) binding of these transcription factors to the IL-4 response element of the arginase I gene. The results showed that synergistic induction of arginase I involves a coordinated increase in binding of both STAT6 and C/EBPβ to the IL-4 response element of the arginase I gene. Furthermore, the results are consistent with a stochastic model in which the probability of arginase I induction by IL-4 is enhanced by cAMP.

Materials and Methods

Cell culture

The RAW 264.7 murine macrophage cell line (American Type Culture Collection) was cultured in DMEM (Lonza) supplemented with 10% defined FBS (HyClone), 4.1 mM l-glutamine, 169 U/ml penicillin, 169 μg/ml streptomycin sulfate, and 15 mM HEPES (pH 7.5) at 37˚C in 5% CO2. Cells were stimulated by addition of 5 ng/ml recombinant mouse IL-4 (R&D Systems) and/or 0.5 mM 8-Br-cAMP (Sigma-Aldrich). Bone marrow–derived macrophages (BMDMs) were generated as described (22). Briefly, bone marrow was flushed from femurs and tibia of BALB/c mice, centrifuged to remove tissue debris, and cells were resuspended and plated in differentiating culture medium (DMEM plus 10% FBS plus M-CSF, 25 ng/ml). After at least 7 d and several media changes, BMDMs were plated in DMEM containing 10% FBS plus 15 mM HEPES (pH 7.5) and treated with 5 ng/ml recombinant murine IL-4 and/or 0.5 mM 8-Br-cAMP for 8 h. Experiments using mice were approved by the University of Pittsburgh Animal Care and Use Committee.

Isolation and analysis of RNA

RNA was isolated and converted to cDNA as described (15). Two-step quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) with Quantitect Primer Assays (Qiagen) for murine arginase I (QT00134288) and 18S rRNA (QT01036875) in the ABI Prism 7900HT sequence detection system (Applied Biosystems). The comparative threshold cycle (Ct) method (23) was employed to determine relative abundance of arginase I mRNA by using 18S rRNA as an internal reference.

Transient transfection and luciferase activity assays

The −3291/+76 wild-type and mutant arginase I promoter fragments (18) were cloned into the promoterless firefly luciferase reporter plasmid pGL4.10 (Promega). Arginase I promoter constructs (0.5 μg) plus 2 ng

Chromatin immunoprecipitation

RAW 264.7 cells were processed essentially as described in the protocol for a chromatin immunoprecipitation (ChIP) assay kit (Millipore, catalog no. 17-295). Briefly, cells were fixed in 1% formaldehyde in 5 mM HEPES (pH 8.0), 10 mM NaCl, and 0.1 mM EDTA for 10 min at room temperature, followed by addition of 125 mM glycerol to quench fixation. Cells were suspended in PBS containing protease inhibitor mixture (Complete Mini, EDTA-free; Roche) and 2 mM Pefabloc SC (Roche) and collected by centrifugation at 1200 × g for 10 min at 4˚C. The pelleted cells were resuspended in 1 ml L1 lysis buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 1% IGEPAL CA-630, 1% Igepal, 1% sodium deoxycholate, 1 mM EDTA, 2 mM Pefabloc SC), incubated on ice for 30 min, and then centrifuged at 2400 × g for 10 min at 4˚C. The supernatant was removed and the nuclear pellet was lysed in 1 ml SDS-lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS, protease inhibitor mixture, 2 mM Pefabloc SC). Chromatin was sheared to a fragment size of 200–1000 bp using a Sonicator 3000 Ultrasonic Cell Disruptor fitted with a micro tip (Misonix). Sonication was at setting 2 for a total of 4 min in cycles of 0.5–min bursts, followed by 1 min on ice. Chromatin samples were stored at −80˚C until use.

Chromatin immunoprecipitated chromatin samples dilute 10- to 20-fold in ChIP dilution buffer (167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1]) were precleared by incubation with salmon sperm DNA/protein G slurry (Millipore) preblocked with 1% BSA) for 1 h at 4˚C with rotation. Aliquots of each precleared input chromatin sample were used to calculate immunoprecipitation as percentage of input. Precleared chromatin samples were incubated with Ab to STAT6 (sc-981 X; Santa Cruz Biotechnology) or C/EBPβ (sc-150 X; Santa Cruz Biotechnology) overnight at 4˚C with rotation. Next, the samples were incubated with salmon sperm DNA/protein G slurry at 4˚C for 1 h with rotation and briefly centrifuged, followed by washes of the pelleted immune complexes with low salt immune complex buffer (150 mM NaCl in buffer A: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1]), protease inhibitor mixture, 2 mM Pefabloc SC), high salt immune complex buffer (500 mM NaCl in buffer A), LiCl immune complex buffer (250 mM LiCl, 1% IGEPAL CA-630, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]), protease inhibitor mixture, 2 mM Pefabloc SC), and finally with 10 mM Tris-HCl (pH 8.1) phosphate buffer. Aliquots of each chromatin sample without Ab also were incubated with the salmon sperm DNA/protein G slurry and subjected to washes as controls for non-specific binding.

The immunoprecipitated chromatin samples and reserved input chromatin solutions were incubated in 100 μl proteinase K/SDS buffer (0.5% SDS, 200 μg/ml proteinase K, in 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) for 3 h at 55˚C. Crosslinks were reversed by incubating the samples at 65˚C overnight, and DNA was purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions.

Purified DNA samples from input chromatin and immunoprecipitated chromatin fractions were analyzed by quantitative real-time PCR by using SYBR Green PCR Master Mix (Applied Biosystems) in the ABI Prism 7900HT (Applied Biosystems). Primer pairs were selected to amplify a 195-bp fragment containing the IL-4 response element (forward, 5′-ACGGAGGGTTTGAGCGACGAGAG-3′, reverse, 5′-ACCCTCAACCAAA-GTGGCACAACCTACGTA-3′) and a 150-bp fragment containing the transcription start site (forward, 5′-GATGGGAGGTTCTGTTGAC-3′, reverse, 5′-GGCGGGTCAGGCTCCT-3′) of the murine arginase I gene for each sample. The latter fragment served as a negative control for specificity of the immunoprecipitations. ChIP results were calculated as percentage of input DNA and normalized to the percentage in chromatin immunoprecipitates from cells treated with IL-4 plus 8-Br-cAMP for 4 h.

Immunoblotting of whole-cell and nuclear lysates

RAW 264.7 cells were extracted with Laemmli buffer (62.5 mM Tris-HCl [pH 6.8], 100 mM DTT, 2% SDS, 10% glycerol), and extracts were
electrophoresed on 12% polyacrylamide-SDS gels. Immunoblots were probed for arginase I and $\alpha$-tubulin as described (15).

Nuclear extracts were prepared essentially as described (24). Briefly, cells were washed with ice-cold PBS containing 1 mM NaF and 1 mM Na$_2$VO$_4$, pelleted in a microcentrifuge, washed in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM NaF, 1 mM DTT, 1 mM Na$_2$VO$_4$, complete protease inhibitor mixture, 2 mM Pefabloc SC), and resuspended in buffer A plus 0.1% (v/v) IGE PAL CA-630 on ice for 10 min. Nuclei were collected by centrifugation, lysed in Laemmli buffer, heated 3–5 min at 100°C, and sonicated 10–20 s to reduce viscosity. Protein concentrations were determined using a Bio-Rad protein assay.

Whole-cell or nuclear lysate proteins (20–30 µg) were electrophoresed on SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Membranes were blocked using either Odyssey blocking buffer (LI-COR Biosciences) or 5% nonfat milk in TBS. Immunoblots of whole-cell lysates were probed with Abs to the $\alpha$-chain of the IL-4R (IL-4R$\alpha$) (1:500; R&D Systems, catalog no. AF530) and $\alpha$-tubulin (1:2000; Santa Cruz Biotechnology, catalog no. sc-981-G) and $\alpha$-tubulin, or with Abs to C/EBP$\beta$ (1:2000; Santa Cruz Biotechnology, catalog no. sc-11762) and TATA binding protein (TBP) (1:2000; Novus Biologicals, catalog no. NB500-700) in Odyssey blocking buffer containing 0.1% Tween 20 or with Abs to C/EBP$\beta$ (1:2000) and TBP (1:2000) in 5% nonfat milk-TBST. For quantification, immunoblots were developed using IRDye-labeled secondary Abs from LI-COR Biosciences. Depending on the primary Abs used, immunoblots were incubated with IRDye 680LT donkey anti-rabbit IgG (926-6802), IRDye 800CW donkey anti-goat IgG (926-6802), or IRDye 800CW donkey anti-mouse IgG (926-6802), or IRDye 800CW donkey anti-rabbit IgG (926-32213). Signal intensities of STAT6, C/EBP$\beta$, and IL-4R$\alpha$ in immunoblots of whole-cell lysates were quantified using the Odyssey infrared scanner and software and normalized to signal intensity of $\alpha$-tubulin, used as internal loading control. Signal intensities of p-STAT6 and C/EBP$\beta$ in immunoblots of nuclear lysates were quantified and normalized to signal intensity of TBP, which was used as internal loading control.

Statistics

Results are expressed as means ± SEM. Data were analyzed by Student t test for pairwise comparisons and by ANOVA with a Bonferroni posttest for multiple comparisons, using GraphPad Prism 5.0 (GraphPad Software). Significance was defined as $p < 0.05$.

Results

**IL-4 and 8-Br-cAMP synergistically induce arginase I expression**

To minimize the possibility of potentially confounding secondary responses that may occur by 16–24 h of treatment, responses of arginase I mRNA to IL-4, 8-Br-cAMP, or IL-4 plus 8-Br-cAMP were determined at various times up to 8 h (Fig. 1A). Levels of arginase I mRNA were consistently elevated by IL-4 and IL-4 plus 8-Br-cAMP, but there was no statistically significant difference in induction by these two treatments at 1 h (Fig. 1A, inset). In contrast, induction in response to 8-Br-cAMP at 1 h was slight and observed in only one of three experiments. There were marked differences in response to the various treatments by 2 h. Induction of arginase I mRNA by IL-4 at 2 h was approximately twice as great as at 1 h, and a modest induction in response to 8-Br-cAMP was now consistently observed (Fig. 1A, inset). Notably, a clear synergistic response to IL-4 plus 8-Br-cAMP, which was already greater than the maximal response to IL-4 alone at 8 h, was first observed at 2 h, coincident with the first consistent response to 8-Br-cAMP. The synergistic response continued to dramatically increase over the remainder of the 8-h period, even as levels of arginase I mRNA continued to increase in a nearly linear fashion in response to IL-4 and 8-Br-cAMP. By 8 h, responses of arginase I mRNA to IL-4 and 8-Br-cAMP were indistinguishable.

Effects of IL-4 and 8-Br-cAMP on arginase I expression were evaluated also in BMDMs from BALB/c mice, the strain from which RAW 264.7 cells were derived. Overall, the profile of arginase I mRNA to IL-4 and 8-Br-cAMP were indistinguishable.

**Statistics**

Results of TBP, which was used as internal loading control.

**Signal intensities of STAT6, C/EBP$\beta$, and IL-4R$\alpha$ in immunoblots of whole-cell lysates were quantified using the Odyssey infrared scanner and software and normalized to signal intensity of $\alpha$-tubulin, used as internal loading control. Signal intensities of p-STAT6 and C/EBP$\beta$ in immunoblots of nuclear lysates were quantified and normalized to signal intensity of TBP, which was used as internal loading control.

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Results are expressed as means ± SEM. Data were analyzed by Student t test for pairwise comparisons and by ANOVA with a Bonferroni posttest for multiple comparisons, using GraphPad Prism 5.0 (GraphPad Software). Significance was defined as $p < 0.05$.

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**Figure 1.** Synergistic induction of arginase I mRNA by IL-4 plus 8-Br-cAMP in RAW 264.7 cells (A) and BALB/c BMDMs (B). (A) Time course of arginase I mRNA induction in RAW 264.7 cells. An expanded scale to show 1 and 2 h values is presented in the inset. The 0 h time point represents untreated controls. (B) Induction of arginase I mRNA in BALB/c BMDMs after 8 h treatment with the indicated stimuli. Arginase I mRNA levels in each experiment were normalized to the value in cells treated with IL-4 for 8 h (arbitrarily set to 100) and are expressed as means ± SEM for three independent experiments (A) or mean and range for two independent experiments (B). cAMP, 8-Br-cAMP.

Arginase I response in BMDMs was very similar to that observed in RAW 264.7 cells, that is, robust induction by IL-4 or 8-Br-cAMP alone, with a strongly synergistic response to the combination of both agents (Fig. 1B). These results strongly suggest that the mechanisms involved in the synergistic induction of arginase I are, at least qualitatively, the same in both BMDMs and RAW 264.7 cells.

Synergistic induction of arginase I was clearly apparent also at the protein level (Fig. 2). Whereas arginase I protein was undetectable in untreated RAW 264.7 cells and only barely detectable in cells treated with either IL-4 or 8-Br-cAMP for 10 h, levels of arginase I protein in cells treated with IL-4 plus 8-Br-cAMP for 10 h already had increased greatly. By 22 h, arginase I was clearly induced in all treated cells, with very high levels of arginase I in cells treated with IL-4 plus 8-Br-cAMP. Remarkably, arginase I levels in cells treated with IL-4 plus 8-Br-cAMP for 22 h were nearly equivalent to levels present in adult liver (Fig. 2), which is normally the site of highest arginase I expression in the body.

**IL-4 and 8-Br-cAMP synergistically activate the arginase I promoter**

To determine whether the synergistic induction of arginase I mRNA reflected synergistic activation of the arginase I gene, we examined responses of RAW 264.7 cells transfected with plasmids containing the luciferase reporter gene driven by the wild-type murine arginase I promoter or by the arginase I promoter containing mutations in the STAT6 and C/EBP$\beta$ binding sites that are required for the re-
response to IL-4 (17, 18). Confirming previous results (17, 18), the wild-type arginase I promoter was induced by IL-4 but the response was abolished by mutations in either the STAT6 or C/EBPβ binding site (Fig. 3). The wild-type arginase I promoter was modestly induced also by 8-Br-cAMP, and the response was diminished by mutation of the C/EBPβ binding site but not by mutation of the STAT6 site. Although a previous study showed that C/EBPβ was required for induction of arginase I by cAMP (21), to the best of our knowledge this is the first report that this specific C/EBPβ binding site may be involved in the cAMP response. As in the case of arginase I mRNA, there was a dramatic synergistic activation of the arginase I promoter by the combination of IL-4 and 8-Br-cAMP, and ∼85% of the synergistic response was abolished by mutations in either the STAT6 or C/EBPβ site (Fig. 3). The basis for the residual response to IL-4 plus 8-Br-cAMP in the mutants is unclear but a modest induction was seen also in truncated versions of the arginase I promoter that completely lack the IL-4 response element (results not shown), indicating the presence of cryptic response elements within the promoter and/or plasmid vector that are activated only when both stimuli are present.

IL-4 and 8-Br-cAMP synergistically enhance binding of STAT6 and C/EBPβ to the IL-4 response element of the murine arginase I promoter

We used ChIP to conduct a detailed analysis of the binding of STAT6 and C/EBPβ to the promoter of the murine arginase I gene in response to IL-4, 8-Br-cAMP, and IL-4 plus 8-Br-cAMP. Importantly, note that specificity of the ChIP results for the IL-4 response element was confirmed by the very low abundance of chromatin containing the arginase I transcription start site (which lacks binding sites for either STAT6 or C/EBPβ) in the STAT6 or C/EBPβ immunoprecipitates (Fig. 4B, 4D, 4F). No specific binding of STAT6 at the IL-4 response element was detectable at any time point when IL-4 was absent. Comparison of enrichments for the IL-4 response element and transcription start site indicates that there is a modest basal level of C/EBPβ specifically bound to the response element in untreated cells. In agreement with previous ChIP analyses (17), IL-4 alone induced binding of both STAT6 and C/EBPβ to the IL-4 response element (Fig. 4A). Binding of STAT6 was elevated to an equivalent degree at 1 and 4 h of IL-4 treatment but had declined somewhat by 8 h. Binding of C/EBPβ was modestly elevated at 1 and 4 h of IL-4 treatment and had returned to control levels by 8 h (Fig. 4A).

Stimulation with 8-Br-cAMP alone resulted in greatly increased binding of C/EBPβ to the IL-4 response element at 4 and 8 h but not at 1 h (Fig. 4C), consistent with the delayed induction of arginase I mRNA (Fig. 1A). Strikingly, the level of C/EBPβ bound in response to 8-Br-cAMP at 4 and 8 h greatly exceeded the level of C/EBPβ bound in response to IL-4 at any time point (p < 0.05). As expected, treatment with 8-Br-cAMP alone did not elicit STAT6 binding.

Binding of STAT6 and C/EBPβ after 1 h of treatment with IL-4 plus 8-Br-cAMP did not differ significantly from binding of these factors following 1 h of treatment with IL-4 alone (Fig. 4E). Importantly, note that levels of bound STAT6 and C/EBPβ were normalized to the values in cells treated with with IL-4 plus 8-Br-cAMP for 4 h, thus allowing direct comparison of the extent of changes in binding of these transcription factors. Thus, it is apparent not only that binding of both factors at 4 and 8 h following treatment with IL-4 plus 8-Br-cAMP was significantly greater (p < 0.05) than in response to IL-4 alone, but also that binding of STAT6 and C/EBPβ increased in a coordinate fashion, with a profile that matched the profile of C/EBPβ binding in response to 8-Br-cAMP alone. STAT6 binding at 4 and 8 h of IL-4 plus 8-Br-cAMP treatment was 4- and 10-fold greater, respectively, than at equivalent times of treatment with IL-4 alone, and C/EBPβ binding at 4 and 8 h of treatment with either 8-Br-cAMP or IL-4 was ∼11-fold greater than in cells treated with IL-4 alone at the same time points.

IL-4 and 8-Br-cAMP enhance nuclear abundance of p-STAT6 and C/EBPβ

One possible explanation for the profile of STAT6 binding to the IL-4 response element (Fig. 4) is that it simply reflects nuclear
Treatment with IL-4 plus 8-Br-cAMP enhances coordinate recruitment of STAT6 and C/EBPβ to the IL-4 response element of the arginase I gene. RAW 264.7 cells were stimulated with IL-4 (A, B), 8-Br-cAMP (C, D), or IL-4 plus 8-Br-cAMP (E, F). At the times indicated, cells were fixed and analyzed by ChIP. Purified input and immunoprecipitated DNA were subjected to real-time PCR with primer pairs to amplify either the arginase I IL-4 response element (A, C, E) or the arginase I transcription start site (B, D, F), which served as control for specificity of immunoprecipitation. Results were calculated as percentage of input and expressed relative to STAT6 or C/EBPβ enrichment at the IL-4 response element in cells treated with IL-4 plus 8-Br-cAMP for 4 h (arbitrarily set to 100; mean ± SEM values of percentage input for STAT6 and C/EBPβ immunoprecipitates in cells treated with IL-4 plus 8-Br-cAMP for 4 h were 0.98 ± 0.18 and 6.10 ± 2.13%, respectively). Results are shown for five independent experiments, except for three independent experiments for STAT6 ChIP in cells treated with 8-Br-cAMP, cAMP, 8-Br-cAMP. *p < 0.05 versus untreated control cells, †p < 0.05 versus both untreated control cells and cells treated at 1 h, ‡p < 0.05 versus cells treated at 8 h.

For all treatment conditions. However, there was no change in this ratio relative to that of untreated cells for any condition (Fig 5B). Thus, any differences in nuclear p-STAT6 abundance in Fig. 5A were not due to differences in STAT6 expression.

To evaluate the additional possibility that the differences in levels of p-STAT6 between IL-4–treated and IL-4 plus 8-Br-cAMP–treated cells at 4 and 8 h might reflect differences in JAK-STAT activation due to differences in levels of the IL-4 receptor, we measured levels of IL-4Rα. We found no significant difference in IL-4Rα levels under any of the experimental conditions (Fig. 6). However, we cannot exclude the possibility that 8-Br-cAMP treatment resulted in some posttranslational modification of IL-4Rα or downstream signaling components that altered the response to IL-4.

The increased C/EBPβ binding to the arginase I promoter in cells treated with 8-Br-cAMP and IL-4 plus 8-Br-cAMP could reflect increases in expression of C/EBPβ and/or increased nuclear localization and DNA binding activity of C/EBPβ. IL-4 alone did not significantly alter nuclear abundance of C/EBPβ at any time, nor was there any change in abundance at 30 or 60 min following treatment with 8-Br-cAMP or IL-4 plus 8-Br-cAMP (Fig. 7A). However, treatment with either 8-Br-cAMP or IL-4 plus 8-Br-cAMP resulted in a nearly 2-fold increase in nuclear abundance of C/EBPβ at 4 and 8 h, which correspond to the times of large increases in C/EBPβ bound to the IL-4 response element. Similar to results obtained for total cellular STAT6, levels of C/EBPβ in whole-cell lysates of treated cells at 4 and 8 h were identical to
nuclear localization or expression of C/EBP effects of 8-Br-cAMP, IL-4 alone had no detectable effect on cells (arbitrarily set to 100).

Thus, 8-Br-cAMP either alone or in combination with IL-4 increased nuclear localization of C/EBPβ and levels of C/EBPβ bound to the IL-4 response element without altering total C/EBPβ expression. These results are consistent with a previous report that elevated cAMP resulted in increased nuclear localization of C/EBPβ without affecting its expression (25). In contrast with the effects of 8-Br-cAMP, IL-4 alone had no detectable effect on nuclear localization or expression of C/EBPβ (Fig. 7).

Discussion

Macrophages are comprised of a diverse set of immune cell populations whose phenotypes reflect integrated responses to a wide variety of cytokines and other stimuli present in inflammation, infection, and the microenvironment within different tissues. This complexity represents a challenge for understanding how specific cell signaling molecules and attendant molecular mechanisms result in the various phenotypes found in vivo. The fact that arginase I is expressed in macrophages and information regarding regulation of its transcription in macrophages is growing (17–21, 26, 27) suggests that it would be a useful model for elucidating molecular mechanisms underlying responses to combinations of cytokines and other stimuli in macrophage subtypes. IL-4, the prototypical inducer of the macrophage M2 phenotype, also induces arginase I in murine macrophages. However, the highest levels of arginase I expression in primary macrophages or RAW 264.7 macrophage cells are not elicited in response to IL-4 alone but occur via synergistic responses to combinations of IL-4 and IL-10 (17, 28) or agents that result in elevated levels of intracellular cAMP (13–15). These observations are consistent with the notion that elucidation of arginase I induction by IL-4 alone will likely provide an incomplete picture of mechanisms involved in arginase I expression in vivo.

The present study therefore examined the response of the arginase I gene to IL-4, with and without a cAMP analog, representing the second messenger elicited in response to catecholamines, PGs, and adenosine. The transcription factors STAT6 and C/EBPβ were chosen as a major focus of this project because they are both involved...
The Journal of Immunology

in the induction of arginase I by IL-4, and their transcriptional activities are known to be activated in response to IL-4 or cAMP, respectively. Note, too, that arginase I is induced by IL-4, IL-13, and PGs also in myeloid-derived suppressor cells (29–32), indicating that the results obtained in this study will be relevant to elucidating regulation of gene expression also in those cells.

Basal arginase I expression is negligible in unstimulated RAW 264.7 cells but was induced ~100-fold by either IL-4 or 8-Br-cAMP within 8 h. Synergistic induction of arginase I mRNA and protein by IL-4 plus 8-Br-cAMP was rapid and remarkably robust, with arginase I mRNA reaching a level at 8 h that was an additional 200-fold greater than the response to IL-4 or 8-Br-cAMP alone. A similar pattern of arginase I induction was found also in BMDMs derived from BALB/c mice, that is, strong induction by either IL-4 or 8-Br-cAMP and a very strong synergistic response to IL-4 + 8-Br-cAMP, indicating that the synergistic response is not merely a peculiarity of the RAW 264.7 cell line. Mutational analysis showed that the STAT6 and C/EBPβ binding sites in the promoter/enhancer region of the arginase I gene that are required for the response to IL-4 are required also for most of the synergistic response to IL-4 plus 8-Br-cAMP.

Given the requirements for STAT6 and C/EBPβ binding sites for induction of arginase I transcription, we used ChIP to evaluate the effects of the various treatments on binding of STAT6 and C/EBPβ at the IL-4 response element. As reported previously (17), IL-4 resulted in recruitment of both STAT6 and C/EBPβ to the IL-4 response element. There was a modest level of C/EBPβ specifically bound to the response element even in untreated cells, suggesting that this represents a basal level of “active” C/EBPβ present in nuclei of RAW 264.7 cells. Whereas IL-4 modestly increased recruitment of C/EBPβ to the IL-4 response element, treatment with 8-Br-cAMP alone elicited binding of C/EBPβ to the IL-4 response element that was several-fold greater than the binding elicited by IL-4. However, arginase I expression was much lower in cells treated with 8-Br-cAMP alone than in cells treated with IL-4 plus 8-Br-cAMP, despite the fact that the level of C/EBPβ bound to the IL-4 response element was the same in both cases. This may indicate that the ability of C/EBPβ to recruit or stabilize interactions with transcriptional coactivators or chromatin remodeling enzymes is significantly lower in the absence of STAT6 binding than in its presence.

Whereas binding of C/EBPβ to the response element does not require STAT6, results of the present and previous studies (17, 18, 21) suggest that binding of STAT6 to the IL-4 response element in intact cells may be dependent on binding of C/EBPβ to the response element. DNA binding and transactivation activity of C/EBPβ are regulated by a variety of mechanisms, including phosphorylation at a variety of sites (33). Several studies have shown that DNA binding of C/EBPβ is intrinsically repressed to a significant degree (34–36), suggesting that DNA binding of C/EBPβ in cells treated with IL-4 represents a basal level of activated C/EBPβ. This is consistent with our observation that some C/EBPβ is specifically bound to the IL-4 response element even in untreated control cells. The enhanced DNA binding of C/EBPβ in cells treated with 8-Br-cAMP or IL-4 plus 8-Br-cAMP correlated with increased nuclear abundance of C/EBPβ, without any corresponding increase in total cellular C/EBPβ content. This is consistent with previous reports that cAMP-dependent phosphorylation of C/EBPβ induces its translocation into the nucleus without altering its overall level of expression (25, 37). In cells treated with IL-4 plus 8-Br-cAMP, the enhanced DNA binding of C/EBPβ may itself be sufficient to account for the enhanced DNA binding of STAT6, either via direct interactions between C/EBPβ and STAT6 or between C/EBPβ and transcription cofactors or chromatin remodeling enzymes to facilitate or stabilize STAT6 binding. Because STAT6 can be phosphorylated at sites other than Tyr641 (38–40) and also acetylated (41) and methylated (42), we cannot exclude the possibility that enhanced binding of STAT6 to the IL-4 response element in cells treated with IL-4 plus 8-Br-cAMP may involve posttranslational modifications in addition to phosphorylation at Tyr641. Interestingly, a recent study found that deacetylation of C/EBPβ is required for IL-4–dependent induction of murine macrophages and that trichostatin A, an inhibitor of histone deacetylases, inhibited a subset of IL-4–inducible genes, including arginase I (43). The potential roles of such modifications are topics for future studies, the results of which may lead to strategies for modulating macrophage arginase I expression in disease. In any case, our results demonstrate that binding of STAT6 and C/EBPβ to the IL-4 response element of the arginase I gene can be regulated independently and that maximal binding occurs in response to the combination of IL-4 and 8-Br-cAMP, coincident with maximal expression of arginase I mRNA and protein.

The greater abundance of nuclear p-STAT6 at 4 and 8 h in cells treated with IL-4 plus 8-Br-cAMP, relative to its abundance in cells treated with IL-4 alone, did not reflect a difference in level of IL-4 receptors, suggesting involvement of other mechanisms for the difference. For example, STAT6 has been found to cycle between the cytosol and nucleus but accumulates in the nucleus as p-STAT6 following treatment with IL-4 (44). The nuclear accumulation of p-STAT6 reflects a decreased rate of export from the nucleus and is dependent on its ability to bind DNA (44). Thus, another possible mechanism for increased nuclear abundance of p-STAT6 at 4 and 8 h in costimulated cells versus IL-4 alone is increased residence time of p-STAT6 on IL-4 response elements in costimulated cells due to increased abundance of activated C/EBPβ that stabilizes DNA binding of p-STAT6.

Although this study focused on binding of STAT6 and C/EBPβ, we recognize that other transcription factors such as PU.1, CBP, KLF4, and PPARα also have been reported to be involved in induction of arginase I by IL-4 (17, 19, 20), and their roles in the synergistic response remain to be determined. Nonetheless, results of our study provide important insights into regulation of the arginase I gene in macrophages. For example, the arginase I mRNA induction profiles and the ChIP results are consistent with a stochastic model in which activation of arginase I gene transcription by IL-4 and/or 8-Br-cAMP reflects the probability that transcriptionally active STAT6 and C/EBPβ bind to the IL-4 response element in each cell. According to the stochastic model, the increased arginase I expression in cells costimulated with IL-4 plus 8-Br-cAMP, relative to cells treated with IL-4 or 8-Br-cAMP alone, reflects an increase in the probability of transcriptional activation (thus resulting in an increase in the number of cells expressing arginase I) rather than an increase merely in the rate of arginase I transcription in each cell; for example, see Fiering et al. (45) and Hume (46) for discussion of stochastic regulation of transcription.

Note, too, that there are parallels between the IL-4 response element of the arginase I gene and the well-studied enhancer of the IFN-β gene, which is activated in response to viral infection (reviewed in Ref. 47). As in the case of the IL-4 response element (17, 18), the IFN-β enhancer has binding sites for multiple transcription factors (NF-κB, IFN regulatory factor (IRF)3/IRF7, and ATF-2/c-Jun), all of which must be present for synergistic activation of transcription (48). Virally induced expression of the IFN-β also is stochastic (49). Similar to the present results indicating that C/EBPβ is a limiting factor for activation of arginase I transcription by IL-4, the transcription factor IRF7 is limiting for virally induced activation of IFN-β transcription and providing additional...
IRF7 significantly enhanced activation of IFN-β transcription (49). We suggest that these apparent similarities indicate actual similarities in structure and function of these two enhancers and thus will be helpful in informing future studies of the regulation of arginase I transcription.

The apparent enhancing effect of activated C/EBPβ on STAT6 binding and induction of arginase I expression may be relevant to observations that arginase I is not induced by IL-4 alone in human monocytes or macrophages (50–52). This is somewhat surprising, as inspection of the human arginase I gene sequence indicates that there are at least four potential STAT6 binding sites within 6 kb of the promoter/enhancer region (S.M. Morris, Jr., unpublished observations). However, we showed previously that arginase activity (probably due to arginase I) was induced by IL-4 in human alveolar macrophages but only under conditions that also result in elevated intracellular cAMP (i.e., treatment with forskolin plus isobutylmethylxanthine) (15). This suggests that the role of cAMP, possibly via activation of C/EBPβ, is as a permissive, rather than an enhancing, factor in IL-4 induction of arginase I in human macrophages. Whether induction of arginase I in human macrophages requires interactions between STAT6 and C/EBPβ or other cAMP-regulated factors on the arginase I promoter remains to be determined.

Because M2 macrophages are more abundant and arginase I expression is greater in adipose tissue of lean versus obese individuals (53, 54) and in regressing versus progressing atherosclerotic plaques (55), there is considerable interest in elucidating mechanisms that enhance the M2 phenotype, and perhaps arginase I expression in particular, as potential therapeutic targets. Regarding adipose tissue macrophages in lean animals, note that the M2 phenotype is maintained by expression of IL-4 in adipose tissue macrophages in lean animals, note that the M2 phenotype is maintained by expression of IL-4 in adipose 

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Disclosures
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References
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