Autocrine IL-4 Gene Regulation at Late Phases of TCR Activation in Differentiated Th2 Cells

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IL-4 is a multifunctional cytokine whose secretion displays important immunomodulatory functions. Its expression is regulated at the level of transcription, and one of the main factors involved is NFAT. The IL-4-induced transcription factor Stat6 is required for the development of naive T cells into Th2 phenotype, capable of secreting IL-4. However, IL-4 production by differentiated Th2 cells is IL-4 independent; thus, it remains unclear whether Stat6 plays any role in the IL-4 expression by mature Th2 cells. We have analyzed in the Th2 clone D10.G4.1 the nuclear proteins able to bind the regulatory element P1 of the IL-4 promoter. Gel-shift assays show NFAT1 as the most abundant nuclear protein that binds to P1 after ionomycin plus PMA activation, whereas Stat6 accounts for the bulk of the P1 binding in the presence of exogenous IL-4. Reporter experiments agree with an inhibitory effect of Stat6 on the NFAT1-induced transcriptional activity directed by the P1 element. CD3 signaling leads to an early induction of NFAT1-P1 complexes correlating with a strong induction of the IL-4 gene. In later phases of CD3 activation, P1 is also bound by Stat6 and a fall in the IL-4 mRNA levels takes place. These two late events during CD3 activation were found to be sensible in experiments conducted with an anti-IL-4 Ab. These results suggest that IL-4 endogenously produced by Th2 cells under TCR triggering modulates its own expression through Stat6.

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ester PMA (I+P). Among the transcription factors previously reported to bind P1, we found that only NFAT1 and Stat6 interact with P1 element in stimulated Th2 cells. Early phases of activation through TCR showed the induction of NFAT-P1 complexes, while in later phases Stat6 also interacted with the P1 site. A decrease in the levels of IL-4 mRNA during TCR activation correlated with the presence of the Stat6-P1 complex. Both late events were sensitive to an anti-IL-4 Ab, indicating the involvement of the IL-4 endogenously produced by D10.G4.1 cells. In addition, the NFAT1-dependent luciferase activity driven by the P1 element was inhibited by IL-4 or Stat6 cDNA cotransfection. Elimination of the Stat6 site in the P1 element strongly increased the response to NFAT1. These data together indicate a possible mechanism involving Stat6 for the autoregulation of IL-4 gene expression in TCR-activated Th2 cells.

Materials and Methods

Cells and stimulation conditions

The murine Th2 clone D10.G4.1 is a cell line specific for conalbumin in the I-A^b class II MHC context (31). Cells were maintained in Click’s medium supplemented with 10% heat-inactivated FCS and stimulated every 2 wk at 10^6 cells/ml in the presence of Concanavalin A (Con-A, 100 mg/ml) or anti-TCR Ab (3D3) (31), leading to identical results (data not shown). CD3 Ab (YCD3-1) were also conducted by stimulation with an anti-CD3 mAb (clone 145-2C11, used as a control of load, kindly provided by Dr. F. Varas (Centro de Investigaciones Energeticas, Medioambientales y Tecnologicas, Madrid, Spain).

Results

Different cell stimuli induce different patterns of P1-protein complexes in Th2 cells

To establish whether in Th2 cells different cell stimuli could induce different proteins able to bind the P1 NFAT site of the IL-4 promoter, we used the murine clone D10.G4.1. Fig. 1A shows a mobility shift assay performed with nuclear proteins obtained after different periods of cell culture in the presence of activating stimuli. Taking into account the mobility and the relative intensity of the DNA-protein complexes detected, different patterns of P1 complexes can be observed after different stimulations. The complexes detected were named I and II. The combination of I+P, used as a control of NFAT induction, strongly induced complex II, although in few assays a slight complex I was observed (data not shown). Cells treated with IL-4 or anti-CD3 Ab showed complexes I and II. However, complex I was detected in cell samples treated with IL-4 during 1 h, while in the presence of anti-CD3 it was undetectable until after 4 h of treatment. To determine whether there were earlier differences for the presence of P1 complexes among the different stimulations, we conducted kinetics experiments at shorter times of cell activation. As shown in Fig. 1B, complex I was already present at a time as short as 10 min of IL-4 stimulation. From this time on, a slight band corresponding to complex II was also detected. Short stimulation periods with anti-CD3 revealed that complex II was present in the nucleus after 20 min of activation, while complex I could not be detected at any time <4 h. The strongest and earliest induction of complex II was obtained by activation with I+P. All the experiments performed with the anti-CD3 Ab (YCD3-1) were also conducted by stimulation with an anti-TCR Ab (3D3) (31), leading to identical results (data not shown).

Analysis of the P1 complex composition

The recognition specificity of the P1 sequence by the nuclear proteins contained in complexes I and II was demonstrated by competition assays. Each of these complexes was efficiently competed by an excess of cold P1 oligonucleotide but not by an unrelated DNA sequence (Fig. 2). In Th lymphocytes, the most abundant NFAT proteins are NFAT1 and NFAT2 (41, 42). We performed supershift experiments to identify which of the P1 complexes detected in D10.G4.1 contained these NFAT transcription factors.
against these proteins, the interaction of Stat6 with NFAT1 or
NFAT2 in complex I could not be excluded. This interaction might be
masking the anti-NFAT Abs recognition site. Alternatively, Stat6 could be
complexed with another member of the NFAT family. To investigate these possibilities we analyzed whether com-
plex I induction shared two features of the induction of con-
ventional NFAT proteins, which are CsA susceptibility and Chx
resistance (13). Complex II induced by I+P behaved as expected
for NFAT proteins (Fig. 4A), whereas complex I induced by IL-4
showed a clear resistance to CsA, suggesting the absence of NFAT
proteins in this complex. However, complex I induced by anti-
CD3 was inhibited by CsA, indicating a calcineurin dependence
for its formation. Chx experiments showed that induction of com-
plex I by TCR signaling (Fig. 4) is dependent on endogenously
produced IL-4 in Th2 cells, which is transcriptionally regulated by NFAT. Because one of the
main effectors of IL-4 is the transcription factor Stat6 (25, 27, 43),
the IL-4 newly synthesized after TCR stimulation could be, in an
autocrine manner, the inductor of complex I containing Stat6. To
correlate the induction of complex I containing Stat6 with the
expression of internal IL-4, we performed transfection experiments in
D10.G4.1 cells showing that 2xP1 construct increases 13-fold transcriptional activity com-
pared to that conferred by the empty vector (Fig. 5). Basal
activity was not affected by IL-4, but it was induced 36-fold by I+P
stimulation and up to 54-fold when NFAT1 was cotransfected,
according to a positive transactivation of P1 by NFAT1. However,
cotransfection of Stat6 lowered both the basal levels driven by P1
and those induced by I+P stimulation (Fig. 5B). In addition, IL-4

**FIGURE 1.** Different cell stimuli in D10.G4.1 cells leads to distinct ki-
netics of induction of P1 binding proteins. Cultures were stimulated by
I+P, IL-4, or anti-CD3 Ab (YCD3-1). Samples were taken at long (A) or
short (B) time intervals of stimulation and subjected to nuclear protein
extraction. EMSA were conducted using 5' labeled P1 double stranded-
oligonucleotide as indicated in Materials and Methods. I and II indicate the
position of the complexes detected. Exposure times of x-ray film were 2 h
for I+P stimulation and 24 h for anti-CD3 or IL-4 stimulations.

**FIGURE 2.** Complex II contains NFAT1. Binding reactions were performed with the P1 probe and nuclear proteins from D10.G4.1 cells treated
with the stimuli indicated. For competition assays 50-fold molar excess of
cold P1 or nonrelated (nr) oligonucleotide was used. *, Samples in which anti-NFAT1 Ab was included; †, an unspecific band produced by the anti-
NFAT1 assayed.
strengthened the inhibition produced by Stat6. To further address whether the effects observed with NFAT1 and Stat6 were exerted through their respective recognition sequences in the P1 element, derivatives of 2xP1 lacking NFAT or Stat6 sites were generated (mut-NFAT and mut-Stat6, respectively). The resulting sequence in each of these mutants is shown in Fig. 6A. EMSAs performed with oligonucleotides containing the same mutated sequences showed the abrogation of the formation of complex II in mut-NFAT and complex I in mut-Stat6. Fig. 6B depicts fold induction luciferase activity referred to the value obtained for each plasmid in basal conditions (one for each case). In contrast to results obtained for wild-type plasmid, transient transfections of its derivatives showed that basal luciferase activity conferred by the mutants was not significantly modifed by Stat6 cDNA cotransfection. These findings were as expected, because mut-Stat6 lacks the Stat6 binding site and the basal activity conferred by mut-NFAT is near to the obtained with the empty vector. Fig. 6C shows that stimulation or NFAT1 cDNA cotransfection were not able to induce luciferase activity in mut-NFAT transfections. In contrast, mut-Stat6 strikingly showed a stronger response to NFAT1 than the wild-type plasmid. All these findings evidence a negative effect of the Stat6 site on the NFAT-dependent transcriptional activity directed by P1 and suggest the involvement of IL-4 in an autoregulatory mechanism to modulate its own expression in Th2 cells.

According to this hypothesis, TCR activation was expected to produce an early induction of IL-4 gene transcription and a later negative control mediated by the IL-4-induced Stat6. To test this, determining the kinetics of the transcriptional activity directed by 2xP1 construct in CD3-stimulated cells was ruled out. Because luciferase codified by pGL3 plasmids has a long half-life, the presence of the protein initially produced could mask a later inhibition of transcriptional activity. Thus, to know whether the negative effect exerted by Stat6 through the P1 element correlated with a
down-regulation of IL-4 expression, we directly analyzed the levels of IL-4 transcripts after stimulation with I+P, IL-4, or anti-CD3. Results depicted in Fig. 7 show that I+P stimulation induced a strong increase in the mRNA levels, which was maintained for at least 24 h, while exogenous IL-4 did not stimulate the production of IL-4 transcripts. This supports the results of the luciferase experiments. Cells activated by CD3 ligation showed a transitory induction in the IL-4 mRNA levels (maximum, I+P 1.5 h) followed by a later decrease at times at which Stat6 binding to P1 is detected by mobility shift assay (Fig. 1). Thus, the presence of nuclear P1-NFAT1 complexes correlates with IL-4 gene induction, while the presence of P1-Stat6 complexes coincides with a down-regulation of this gene.

To know whether neutralizing the autocrinally produced IL-4 had any effect on the decrease of IL-4 mRNA at late phases of anti-CD3 stimulation, we analyzed the IL-4 mRNA levels produced in the presence of 11B11. Results depicted in Fig. 8 showed that, although some reduction in the levels of transcripts is yet observed in the presence of anti-IL-4, the decrease in the steady-state levels of IL-4 message was significantly affected in these culture conditions, supporting the autoregulatory role of IL-4 on its own expression.

**Discussion**

Several reports conclude that NFAT1 and NFAT2 are responsible for the NFAT DNA binding activity in mature T lymphocytes (8, 11, 42, 44, 45). Although there are some discrepancies about the

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Elimination of Stat6 site in the P1 element abrogates inhibition mediated by Stat6 and increases transcriptional activity dependent on NFAT1. A, Abrogation of the formation of complex II and I with mut-NFAT and mut-Stat6 oligonucleotides, respectively. After 4 h of anti-CD3 stimulation, nuclear extracts were obtained and EMSA was performed with wild-type P1 (wt), mut-NFAT, and mut-Stat6 probes. The sequence of each element is shown at the top. The changed and deleted nucleotides are indicated in lower case letters and hyphens, respectively. B and C, Luciferase activity driven by wild-type (wt) P1, mut-NFAT, or mut-Stat6. Triplicate samples of 10^5 D10.G4.1 cells were electroporated with 5 μg of wild-type (wt), mut-NFAT, or mut-Stat6. For coexpression of Stat6 (B) or NFAT1 (C), 25 μg of plasmid containing the respective cDNA were used. Samples without cDNA were completed with 25 μg of carrier plasmid pBluescript. All the samples were cotransfected with the pRL-TK plasmid codifying Renilla luciferase. Transfected cells were subjected to the culture conditions indicated. After 18 h firefly luciferase activity was determined and normalized to Renilla luciferase values. Results for each construct are indicated in fold activity referred to the value obtained with each one in basal conditions.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** IL-4 mRNA levels in D10.G4.1 cells are strongly induced by I+P, not affected by exogenous IL-4, and first increased and then decreased by anti-CD3 ligation. Total RNA (5 μg) from cells stimulated during the indicated times were subjected to Northern analysis by hybridization with a specific DNA probe for IL-4. Membranes were rehybridized with a DNA probe for 7s RNA as a control of load.
predominance of each one, they are probably due to the different stimulation conditions, activation status, or NFAT-regulated elements analyzed in each study. Our results show that the predominant NFAT protein bound to the P1 site in mature Th2 cells is NFAT1. The supershift assays of Fig. 2 showed that nearly whole complex II is removed by an anti-NFAT1-specific Ab. Thus, the presence of NFAT2, if any, would be at trace levels. This result is according to the reported by Cron et al. (8) for primed T cells and by Adachi et al. (42), showing that NFAT1 becomes the dominant NFAT protein in mature activated CD4+ T cells. However, a study in the defined Th1 clone Ar-5 showed that, after CD3 engagement, both NFAT1 and NFAT2 bind to the distal NFAT site of the IL-2 promoter (11). This apparent discrepancy with our results is likely to have came not from the different Th phenotype but from the different NFAT sites analyzed in each study. The P1 site of the IL-4 promoter used here seems to be more selective in the NFAT elements analyzed in each study. Our results show that the predominant NFAT protein bound to the P1 site in mature Th2 cells is NFAT1. The supershift assays of Fig. 2 showed that nearly whole complex II is removed by an anti-NFAT1-specific Ab. Thus, the presence of NFAT2, if any, would be at trace levels. This result is according to the reported by Cron et al. (8) for primed T cells and by Adachi et al. (42), showing that NFAT1 becomes the dominant NFAT protein in mature activated CD4+ T cells. However, a study in the defined Th1 clone Ar-5 showed that, after CD3 engagement, both NFAT1 and NFAT2 bind to the distal NFAT site of the IL-2 promoter (11). This apparent discrepancy with our results is likely to have came not from the different Th phenotype but from the different NFAT sites analyzed in each study. The P1 site of the IL-4 promoter used here seems to be more selective in the NFAT proteins it binds than the distal NFAT site of the IL-2 promoter (22). Among these proteins, only Stat6 was found to bind P1 in murine Th2 cells under the stimulations assayed in the present work. The absence of NF-xB proteins bound to P1 was not surprising because it has been reported that the binding of this factor to the murine P1 sequence is weaker than the interaction with the human P1 counterpart (23). Furthermore, the strength of the P1-NF-xB interaction and the resulting activity seem to be dependent on determined cell stimulations (24). In addition, Oct and HMG I(Y) complexes with the P1 element are well established in some cell lines as Jurkat or EL4 (21, 22), but to our knowledge none of these interactions has been shown to be relevant for the expression of IL-4 in defined Th2 cells. The diversity of transcription factors recognizing the P1 element depending on the cell type, stimuli, or differentiation stage might constitute a strict IL-4 gene regulation to enable distinct responses to particular extracellular environments. Even more complexity could be added by the multiple cis-acting elements involved in the IL-4 expression, whose integrated action would be responsible for the definitive IL-4 gene activity, modulated for each specific situation.

The binding of AP-1 proteins to the P1 site of the IL-4 promoter was first reported by Rooney et al. (6). Moreover, Li et al. (9) found that JunB, a member of the AP-1 family, is involved in the regulation of IL-4 expression through the P1 site during Th cell differentiation. Nevertheless, Rinco´n and Flavell (45) reported other gel-shift assays in which they did not found AP-1 complexes with the P1 element, discussing the special experimental conditions required to detect them. This could also explain the absence of AP-1 complexes indicated by the inability of AP-1 oligonucleotides to compete the P1 complexes detected in our analysis, which was observed for oligonucleotides containing the AP-1 sequences of the metallothionein II A promoter (Fig. 3A), the TRE IL-2 enhancer, or a perfect consensus for AP-1 recognition (Santa Cruz Biotechnology) (data not shown). Purified recombinant Stat6 was previously reported to bind the P1 site of the human IL-4 promoter (16). In this work we show the first demonstration of endogenous Stat6 interacting with the murine P1 element in normal Th2 cells activated by anti-CD3 or IL-4.
addition. The presence of Stat6 in complex I was evidenced by the results of competition and supershift assays depicted in Fig. 3. CsA resistance of complex I induction indicates that any conventional NFAT protein is included in it (Fig. 4); thus, Stat6 and NFAT do not complex together, but they independently interact with the P1 site. A number of facts points out the involvement of the endogenously produced IL-4 in the Stat6 induction after TCR Th2 cell activation. First, 4-h stimulation with anti-CD3 is necessary to detect nuclear Stat6 in D10.G4.1 cells (Fig. 1). Second, the induction of Stat6 by the TCR signal is dependent on protein synthesis (Fig. 4A). Third, blockage of the IL-4 signal abolished the anti-CD3-mediated Stat6 activation (Fig. 4C). All these results suggest that the lag period in the Stat6 induction after CD3 ligation corresponds to the time needed for the cells to produce IL-4, which would be directly responsible for the Stat6 activation after TCR stimulation.

Exogenous IL-4 did not increase the basal levels of P1-driven transcriptional activity (Fig. 5) or IL-4 mRNA expression (Fig. 7). This is according to previous reports showing that IL-4 production in differentiated Th2 cells is not IL-4 dependent (28). In contrast, the results from the transient transfections depicted in Fig. 5 indicate that overexpression of Stat6 inhibits both basal and I-F-induced transcriptional activity of P1. Additionally, elimination of the Stat6 recognition site allows a higher transcriptional activity of the P1 element in response to NFAT1 (Fig. 6). These findings support a negative role of the Stat6 site on P1 transcriptional activity. However, the final expression of IL-4 has to be considered to be regulated by multiple cis-acting elements; therefore, the activity promoted by P1 could or could not be definitive in the final IL-4 gene activity. In Fig. 7, Northern analysis shows a clear increase in the levels of the IL-4 mRNA in the early stages of anti-CD3 stimulation, coinciding with the early NFAT1 binding to P1 (Fig. 1). Afterward, a decrease in the levels of IL-4 mRNA takes place, correlating with the recognition of the P1 element by Stat6 in the gel-shift assays. In addition, the endogenously produced IL-4 is, at least in part, responsible for the late decrease of IL-4 message under CD3 stimulation, as inferred from the results obtained in the presence of 11B11 Ab (Fig. 8). However, 11B11 could not completely revert the fall in IL-4 mRNA levels. Therefore, it could not be excluded that other causes may also be involved, such as the reduction in nuclear NFAT1 levels that takes place during late phases of TCR triggering in differentiated Th cells (Ref. 11 and our unpublished observations). Nevertheless, this possibility alone could not fully explain the results shown here with 11B11 (Fig. 8) or the luciferase data (Figs. 5 and 6). Therefore, we proposed that, in effector Th2 cells activated by TCR, induction of Stat6 binding to P1 could be part of an autoregulatory mechanism of IL-4 to modulate its own expression. A model for the sequence of the events that could take place is presented in Fig. 9. In early phases of TCR activation, NFAT1 would drive the up-regulation of the IL-4 gene expression to carry out the effector Th2 functions. In late phases, when the levels of this IL were enough to autocriminally induce Stat6, this factor would participate in lowering the transcriptional rate of IL-4 by competition with NFAT1 in the P1 site occupancy. The relative quantities of each factor would determine its probability to bind P1, without necessarily implying an advantage of any of them in affinity by P1. In conditions of strong NFAT1 activation, such as I-F stimulation (Fig. 1), the balance between available NFAT1 and Stat6 would be displaced toward NFAT-P1 interaction. This could explain the low probability of complex I formation compared to complex II under this nonphysiological stimulation.

In addition to the composed NFAT-Stat6 P1 element, two more Stat6 binding sites overlapping P2 and P4 NFAT elements were identified within the human IL-4 promoter, and competition between both transcription factors for binding to these sites has been proposed (16). Although previous reports showed that Stat6 positively regulates the transcriptional activity driven by P2 when it is linked to heterologous promoters (29, 30), Huang et al. (28) showed that multimerization of the Stat6 site present in the P2 element strongly inhibited the activity of the IL-4 minimal promoter. Thus, the inhibitory effect of Stat6 along activation by TCR in defined Th2 cells could be also exerted through P2 and P4 sites. Nevertheless, according to our results Stat6 does not inhibit completely the IL-4 expression, because the levels of IL-4 mRNA after 24 h of CD3 ligation do not decrease to the basal levels in nonactivated cells (Figs. 7 and 8). Thus, after the first period of high activity of the IL-4 gene, probably a balance is established between Stat6 and NFAT binding to P1 site to allow a moderate production of IL-4 until the extinction of the extracellular stimulus.

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