SII-2

JAK Kinases and STAT Proteins IN IL-12 Receptor Signaling

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IL-12 is composed of two chains (p35 and p40) and secreted mostly by dendritic cells and phagocytes (1,2). But a number of human B cell lines and normal human peripheral blood mononuclear cells produced excess amount of uncomplexed p40 but not p35 (3,4) It has been demonstrated that the disulfide-linked heterodimer is necessary to obtain biological activities. Recent studies have revealed that a functional IL-12R complex is composed of two b-type cytokine receptor subunits (5). Cytokine receptors lacking intrinsic tyrosine kinase activity induce rapid tyrosine phosphorylation of signaling proteins through association with members of the Janus(JAK) 3 family of protein tyrosine kinases (6,7). Like other cytokine receptors, the stimulation of IL-12R with IL-12 was shown to result in the activation of two JAK family members, JAK2 and TYK2 (8). Activation of JAKs leads to the tyrosine phosphorylation of a family of STATs that are important in the regulation of gene expression by cytokine receptors (9,10). Accordingly, IL-12 was found to induce the tyrosine phosphorylation of two STAT family members, STAT3 and STAT4, defining the components of the JAK-STAT signaling pathway activated through IL-12R (11,12). While simultaneous activation of TYK2 and JAK2 is induced in IL-12R signaling, the relative requirements for these two JAKs in the phosphorylation of STATs and the expression of various IL-12 bioactivities remain to be investigated. In the present study, We investigated, using an IL-12-responsive T cell clone, the requirements for the components of the JAK-STAT signaling pathway in the two IL-12 bioactivities, T cell proliferation and IFN-g production. 2D6 could be maintained with either IL-12 (2D6-12) or IL-2 (2D6-2). 2D6-12 and 2D6-2 lines exhibited comparable levels of proliferation, but high and low levels of IFN-g production, respectively, in response to IL-12. In contrast to phosphorylation of TYK2 and STAT4 in 2D6 IL-12 , the phosphorylation levels were only marginal in 2D6 IL-2 . The reduced STAT4 activation was due largely to a decrease in the amount of STAT4 protein. The two 2D6 lines capable of proliferating in response to IL-12 exhibited comparable levels of

JAK2 activation and STAT5 phosphorylation. The phosphorylation of STAT5 associated with JAK2 was found to be induced in the absence of JAK3 activation. These results indicate that TYK2 activation is associated with STAT4 phosphorylation leading to IFN-g induction, while JAK2 activation correlates with STAT5 phosphorylation and cellular proliferation.

1. **Tyrosine phosphorylation of TYK2/JAK2 and STAT3/STAT4 induced in 2D6-12 cells following IL-12 stimulation**

Fig. 1A shown that TYK2 and JAK2 from IL-12-stimulated 2D6 cells were phosphorylated on tyrosine residues, whereas those from cells unstimulated or stimulated with IL-2 were not. Fig. 1B shows that IL-12, but not IL-2, induces tyrosine phosphorylation of STAT3 and STAT4. Together, the results indicate that IL-12 activates the thus far described components of the JAK-STAT signaling pathway in 2D6 cells.

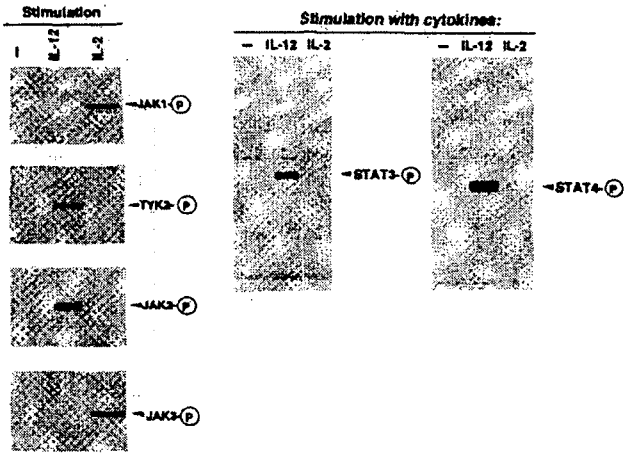

Figure 1. Tyrosine phosphorylation of TYK2, JAK2, STAT3, and STAT4 in 2D6 cells following IL-12 stimulation.

2. **Both 2D6-12 and 2D6-2 express IL-12 receptor and exhibit comparable levels of proliferation in response to IL-12**

Fig. 2 shows that comparable levels of IL-12R were detected on 2D6-12 and

2D6-2 by flow cytometry analysis. We next compared the IL-12 responsiveness of two 2D6 lines, 2D6-12 and 2D6-2, in proliferation assays. Fig. 3 shows that these two lines similarly in response to IL-12, which is accordant with the data for comparable levels of IL-12R expression on both lines.

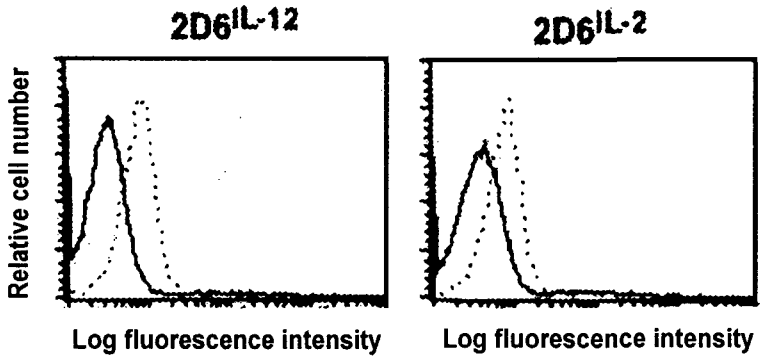

Figure 2. 2D6-12 and 2D6-2 express comparable levels of IL-12R.

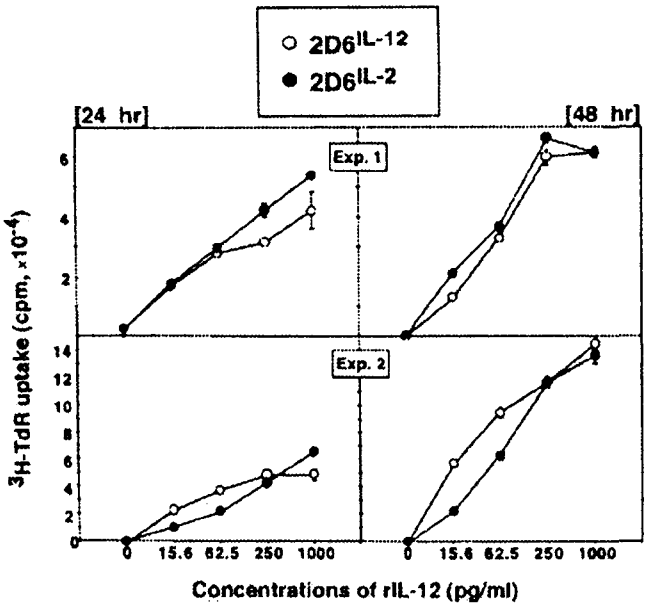

Figure 3. Comparable levels of proliferation of 2D6-12 and 2D6-2 cells following IL-12 stimulation.

3. Comparison of tyrosine phosphorylation of JAK kinases and STAT proteins between 2D6-12 and 2D6-2 stimulated with IL-12

As shown in Fig. 4A, IL-12 stimulation caused increased tyrosine phosphorylation of JAK2 protein in both 2D6 lines. In contrast, the phosphorylation of TYK2 was induced in 2D6-12 cells, but was hardly detectable in 2D6-2 cells. we also compared the activation of STAT3 and STAT4 between 2D6-12 and 2D6-2 following IL-12 stimulation. As shown in Fig. 4B, the phosphorylation of STAT3 and STAT4 was again observed in 2D6-12 cells stimulated with IL-12. In contrast, the phosphorylation of STAT4 in 2D6-2 was very weak compared with that in 2D6-12. We examined the DNA-binding activity of activated STAT4 by the EMSA. Nuclear extracts were prepared from 2D6-12 and 2D6-2 unstimulated or stimulated with IL-12 and examined for binding to an oligonucleotide probe corresponding to consensus binding site for STAT4 (13). As shown in Fig. 5A, nuclear extracts from IL-12-stimulated 2D6-12 cells contained proteins that bound to the STAT4-related sequence. The IL-12-induced DNA-protein complex was only marginally observed for extracts from IL-12-stimulated 2D6-2 or unstimulated 2D6-12 or 2D6-2. Together, these observations demonstrate that STAT4 activation and STAT4 DNA-binding activity are induced only in 2D6-12 following IL-12 stimulation.

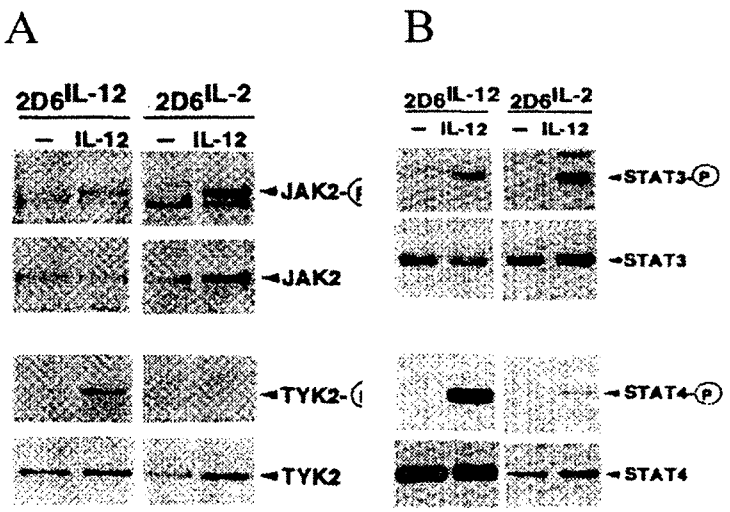

Figure 4. Tyrosine phosphorylation JAK2, TYK2, STAT3, and STAT4 in.2D6-12 and 2D6-2 following IL-12 stimulation.

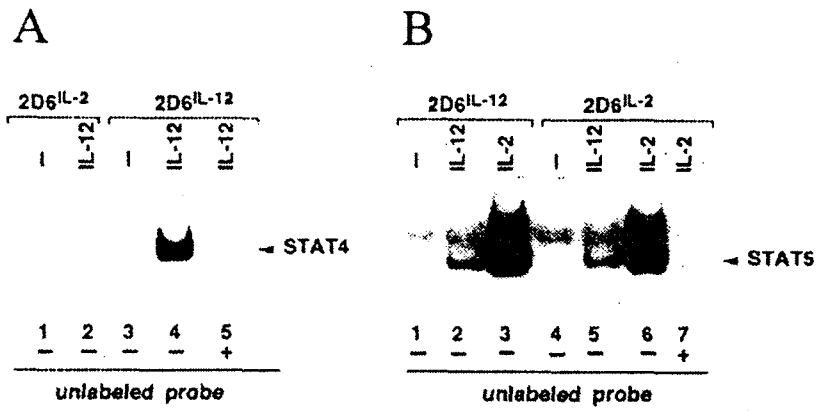

Figure 5. The binding of nuclear extracts from IL-12- or IL-2-stimulated 2D6-12 or 2D6-2 cells to the STAT4- or STAT5-related sequence.

4. IFN-g production of 2D6-12 and 2D6-2 cells following IL-12 stimulation

We next compared IL-12-stimulated IFN-g production between 2D6-12 and 2D6-2 lines. 2D6-12 and 2D6-2 were starved of each cytokine used for maintenance and then stimulated IL-12. Fig. 6 shows that 2D6-12 produce IFN-g in IL-12 dose-dependent manner. In contrast, 2D6-2 exhibited apparently reduced levels of IFN-g production.

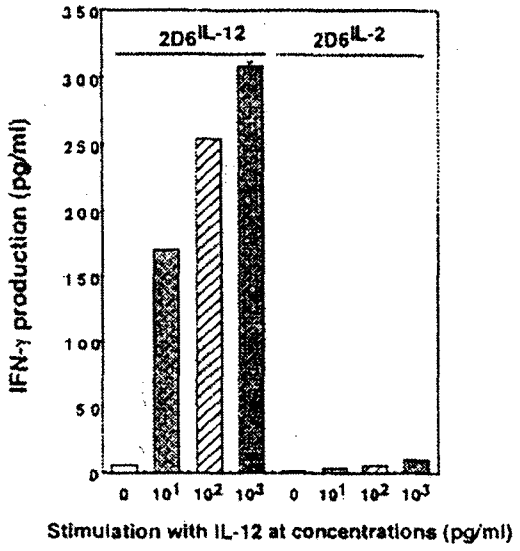

Figure 6. Differential capacities of 2D6-12 and 2D6-2 to produce IFN-g response to IL-12

5. IL-12 induces tyrosine phosphorylation of STAT5 in two 2D6 lines and Con A blasts

STAT5 is known to be phosphorylated following IL-2 stimulation during IL-2-dependent growth promotion (7, 14, 15). We finally investigated whether STAT5 is phosphorylated in 2D6-12 and/or 2D6-2 following stimulation with either IL-2 or IL-12. Fig. 7A and Fig. 8 show that IL-2 induce high levels of STAT5 phosphorylation in among 2D6-12, 2D6-2 and Con A blasts. Although the activation of JAK3 was not observed in among 2D6-12, 2D6-2, and Con A blasts following IL-12 stimulation (Fig. 1A and Fig. 8, respectively), phosphorylation of STAT5 was also induced by stimulation with IL-12 (upper panel of Fig. 7A and Fig. 8). The STAT5 DNA-binding activity of nuclear extracts from IL-12- or IL-2-stimulated 2D6-12 or 2D6-2 cells was examined in the EMSA (Fig. 5B). High levels of gel-shift bands were observed for extracts from both lines of 2D6 cells following IL-2 stimulation. The band were also generated by those from IL-12-stimulated 2D6-12 and 2D6-2, although the levels were apparently lower than those of IL-2-stimulated 2D6 cells. Thus, the phosphorylation levels of STAT5 correlate with the STAT5 DNA-binding activity in IL-12/IL-2-stimulated 2D6-12 or 2D6-2. More importantly, JAK2 was found to be associated with STAT5, as shown by anti-JAK2 immunoblotting for anti-STAT5 immunoprecipitates (Fig. 7B and Fig. 9). Thus, the results show that JAK2 is associated with STAT5 in among 2D6-12, 2D6-2, and Con A blasts and that following IL-12 stimulation, comparable levels of STAT5 phosphorylation are induced along with JAK2 activation (Fig. 4A).

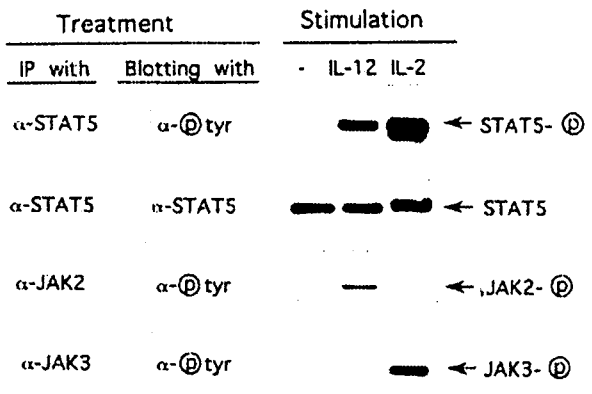

Figure 7.Tyrosine phosphorylation of STAT5 in 2D6-12 and 2D6-2 cells and association of STAT5 with JAK2.

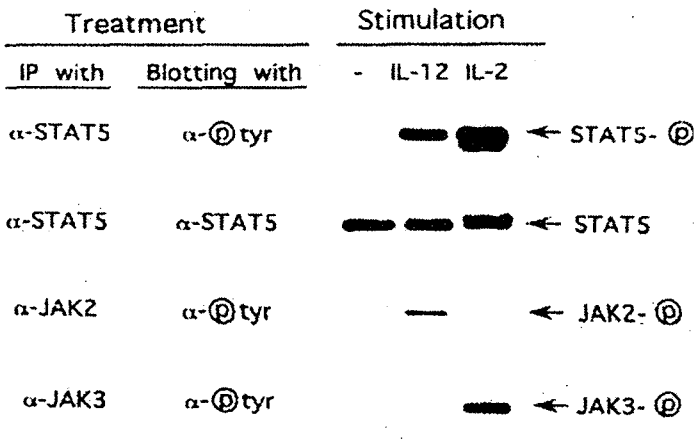

Figure 8. Phosphorylation of STAT5 in Con A blasts following stimulation with IL-2 or IL-12.

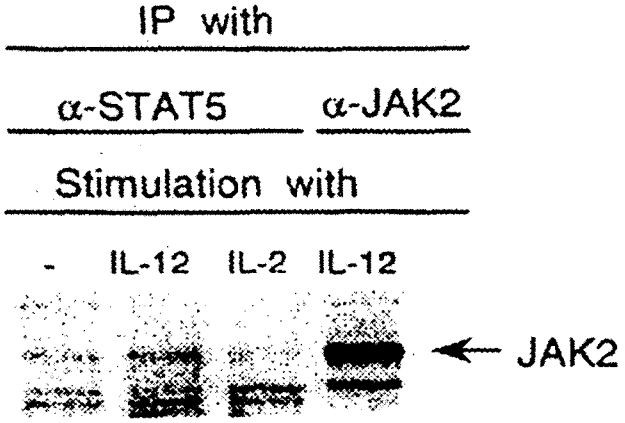

Figure 9. Association of STAT5 with JAK2 in Con A blasts.

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