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Altered IL-12 Signaling Pathways Contribute to the Deficient IFN-γ Production by T Splenocytes from Tumor-bearing Mice

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Abstract. *IFN-γ is a crucial cytokine produced by T and NK cells. Previous work from our laboratory has reported that in T cells of BALB/c mice bearing the D1-DMBA-3 mammary tumor, IFN-γ production is down-regulated, due to decreased expression of IL-12 by macrophages of tumor bearers. IL-12 is the main inducer of IFN-γ production in T and NK cells. To exert its function, IL-12 interacts with its receptor (IL-12R), activating a JAK/STAT signaling pathway. Our investigation suggests that there is also a deficiency in the response to IL-12 by T cells from tumor hosts. The present work reports the results of RT-PCR experiments in the study of the IL-12R expression on T cells from normal and tumor bearers. Data showed a deficient expression of the IL-12Rβ2 chain on T cells from tumor hosts. Gene expression arrays on IL-12-activated T cells from normal and tumor bearers confirmed the RT-PCR results, and also showed decreased expression of IL-18Rα in tumor bearers' T cells. Arrays also showed down-regulated expression of JAK2, STAT 1, 3, 4 and IRF-1. Finally, increased expression of SOCS 1,3,4,5 and 7, as well of Protein inhibitor of activated STATs (Pias) 1 and y was also observed in tumor bearers' T cells.*

IFN-γ is a cytokine produced by T and NK cells with crucial immunoregulatory and antiviral functions (1). Among its different immune regulatory biological activities, IFN-γ induces the expression of MHC II antigens on macrophages, T and B cells, as well as many tumor cells (2). IFN-γ is also critically involved in macrophage activation, by inducing the production of reactive oxygen species and the secretion of hydrogen peroxide by these cells, thus stimulating the intracellular killing of parasites by macrophages (3). IFN-γ is also responsible, together with LPS, for the induction of the enzyme nitric oxide synthase (iNOS) on macrophages (4); iNOS participates in the production of nitric oxide, a highly reactive gaseous molecule that plays a role in the antiviral, antimicrobial, antiparasitic and antitumoral activities of IFN-γ. The activation of the tumoricidal capacity of macrophages is a central function of IFN-γ on these cells, since tumor cell lysis by activated macrophages contributes to the mechanism of natural anti-tumor resistance (5). IFN-γ also stimulates the cytolytic activity of NK cells, effector cells that are in the first line of defense against tumor cells and some infectious agents (6). Because of the variety of relevant roles that this cytokine exhibits, its expression is tightly regulated, and is considered critical in the assurance of the immunological defenses of the host, in particular during tumor development. For that reason, it is not surprising to discover tumor-induced diminished expression of IFN-γ in a tumor host, as a mechanism developed by the neoplasia that contributes to tumor tolerance. Indeed, it has been reported that IFN-γ production is greatly depressed in cancer patients (7).

Previous work from our laboratory reported that, in BALB/c mice bearing the D1-DMBA-3 mammary adenocarcinoma, IFN-γ production is down-regulated in peritoneal as well as in splenic T cells (8). Our laboratory also demonstrated that this alteration, which is mainly present in $CD4^+$ but not in $CD8^+$ T cells, is not due to a shift from a Th1 to a Th2 phenotype, but instead is specifically related to a decreased IL-12 production by macrophages of tumor-bearing mice (9). IL-12, a pro-inflammatory cytokine produced by macrophages and dendritic cells, is the main inducer of IFN-γ production in T and NK cells, and plays a fundamental role in the differentiation of naïve T cells to Th1 cells, inducing the production of IFN-γ and IL-2 in them (10). To exert its function, IL-12 interacts with its cellular receptor (IL-12R) (11), inducing a JAK/STAT signaling pathway that leads to the expression of different genes, such as IFN-γ. Previous work from our laboratory has suggested the existence of a deficiency in the response to IL-12 by T cells from tumor hosts (9). The present work reports the results of RT-PCR experiments

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performed to study the gene expression pattern of IL-12R in T splenocytes from normal and tumor-bearing BALB/c mice. The finding of a deficient expression of the IL-12Rβ2 gene in T cells from tumor bearers prompted us to explore the gene expression of T splenocytes from normal and tumor-bearing mice, upon *ex vivo* stimulation with IL-12 by microarray analyses. The data from these experiments confirmed our RT-PCR results, and, moreover, showed deregulations in the expression patterns of genes relevant to the IL-12 signaling pathway in T cells from tumor bearers. DNA microarray technology enables simultaneous analysis of numerous genes, radically changing many areas of biology and medicine. Using defined pathway-specific cDNA arrays, we identified particular expression profiles of pro-inflammatory cytokines and receptors, together with signaling intermediates in T cells from normal and tumor-bearing mice. This has enabled us to highlight the genes that are particularly involved in the altered IFN-γ-mediated immune response observed in tumor hosts.

Materials and Methods

Mice and tumors. Eight to 12-week-old BALB/c mice, maintained by brother-sister matings in our facilities, were used in all experiments. The D1-DMBA-3 tumor is a transplantable mammary adenocarcinoma derived from a nonviral, noncarcinogen-induced, preneoplastic alveolar nodule treated with 7,12-dimethylbenzanthracene (12). This tumor is routinely transplanted in BALB/c mice by *s.c.* injection of $1x10^6$ tumor cells. Tumor is apparent 8 days after implantation, and mice begin to die after 4 weeks. Large tumor-bearing mice (4 weeks after tumor implantation) were used in all experiments. Our institutional animal care and use committee approved the animal experiments that were performed in this work.

Reagents. RPMI 1640 containing 10% FCS, 2mM L-glutamine and 100 U/ml of penicillin and 100 μg/ml of streptomycin (all from Hyclone, Logan, UT, USA) was used as complete culture medium in all experiments. Con A and PMA were from Sigma-Aldrich (St. Louis, MO, USA), and murine IL-12 was purchased from PrepoTech (Rocky Hill, NJ, USA).

T cell purification and culture. Spleens were removed from normal and 4-week tumor-bearing mice and compressed in sterile Teflon tissue homogenizers. The resulting single cell suspension was pelleted, subjected to hypotonic shock for red cell removal, washed, resuspended in RPMI 1640 with 5% FCS, placed in 150-mm tissue culture dishes and incubated at 37˚C, 5% CO_2 for 1 h to remove adherent cells. The nonadherent T lymphocyte population was obtained by passage through nylon wool columns (13). The T cells obtained by this method were greater than 90% pure populations as assayed by immunofluoresence. Twenty million T cells were cultured in RPMI complete medium with or without Con A (5μg/ml), PMA (25 ng/ml) or murine IL-12 (100 ng/ml) for 3h at 37˚C in 5% CO_2 in 150-mm tissue culture dishes.

RNA extraction. After culture, the cells were pelleted and treated with RNAwizTM reagent (Ambion, Austin, TX, USA). Total RNA was isolated according to the manufacturer's instructions.

Reverse transcriptase (RT) - PCR. RT-PCR reactions were performed using the Gene Amp RNA PCR Kit (Perkin Elmer, Branchburg, NJ, USA) following the manufacturer's instructions. One microgram of total RNA from T cells was reverse-transcribed into first strand complementary DNA (cDNA), using randomer hexamers as primers for the cDNA synthesis, in a 20-μl reaction. The total volume of the reverse transcription (RT) reactions was used for the amplification of murine IL-12R β1 and β2 cDNAs. The primer sets for amplification reactions were, for the IL-12Rβ1 chain: sense primer: 5' -CCA GCA CAG GAA CCA CAC A- 3'; antisense primer: 5'- CAG AGA CGC GAA AAT GAT G-3'. For the IL-12Rβ2 chain sense primer: 5' -AAT TCA GTA CCG ACG CTC TCA -3'; antisense primer: 5' - ATC AGG GGC TCA GGC TCT TCA -3' (14). Parallel reactions with the same amounts of total RNA were run to amplify murine G3PDH cDNA as an internal control in all the experiments. For this purpose, we used the murine G3PDH primer set from Clontech Labs, Inc. (Palo Alto, CA, USA): sense primer: 5' -TGA AGG TCG GTG TGA ACG GAT TTG GC- 3'; antisense primer: 5' -CAT GTA GGC CAT GAG GTC CAC CAC- 3'. Amplifications were performed on a programmable heater for 40 cycles under the following conditions: denaturation 1 min at 94˚C, annealing at 55˚C for 1 min and extension at 72˚C for 2 min (14). Taq gold polymerase was used in all the cases, which is activated by a hot start of 10 min at 94˚C before the beginning of the amplification cycles. All the PCR products were subjected to electrophoresis in a 1% agarose gel in 0.5-x TBE buffer containing 0.5 mg/ml ethidium bromide (Sigma). The bands were visualized by UV illumination. The sizes of the expected PCR bands are as follow: 532 bp (IL-12Rβ1), 399 bp (IL-12Rβ2) and 983 bp (G3PDH).

cDNA arrays. The relative mRNA expression of inflammatory cytokines and their receptors, as well as of members of the JAK/STAT signaling pathway, were analyzed by microarray technology. Samples of total RNAs from untreated and IL-12-treated splenic T cells from normal and tumor-bearing mice were examined using the Mouse Inflammatory Cytokines & Receptors Gene Array (MM-015) and the Mouse JAK/STAT Signaling Gene Array (MM-039) (GEArray Q Series, SuperArray Inc., Bethesda, MD, USA). Q Series gene expression arrays contain up to 96 cDNA fragments from genes associated with a specific biological pathway. Detailed information about these cDNA arrays, including the mechanism, description of gene probes, experiment protocol, data analysis method and data analysis software can be obtained at the supplier's website (www.superarray.com). Briefly, 2.5 μg of total RNA from each sample was used for probe synthesis, in which experimental RNA is converted into a labeled cDNA probe, using SuperArray's AmpoLabeling-LPR (linear polymerase reaction) Kit (L-03) for chemiluminescent detection, together with Biotin-16-dUTP (Roche Diagnostics, Indianapolis, IN, USA) for probe labeling. Amplified and biotinilated cDNA samples were hybridized overnight to the different gene-specific probes spotted on the GEArray membranes. After washing, the arrays were incubated with alkaline phosphatase-conjugated streptavidin (AP) 1/8000 (SuperArray's Chemiluminescent Detection Kit, D-01). The array image was developed with CDP-Star chemiluminescent substrate and recorded with X-ray film. The image was scanned with a Scan Jet 5370C scanner (Hewlett Packard), analyzed and converted to digital data with the use of Super Array's GEArray Expression Analysis Suite software. This software enables comparisons of gene expression levels between pairs of different

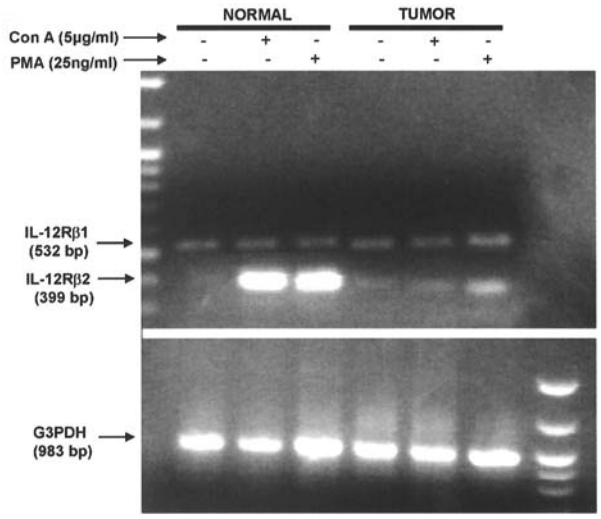

Figure 1. *Gene expression of the IL-12Rβ2 chain is down-regulated in T splenocytes from tumor bearers but not in normal mice. The figure shows a 1% agarose gel with amplified bands from an RT-PCR experiment performed on total RNA isolated from T cells of normal and tumor-bearing mice, and represents one of three independent experiments with similar results.*

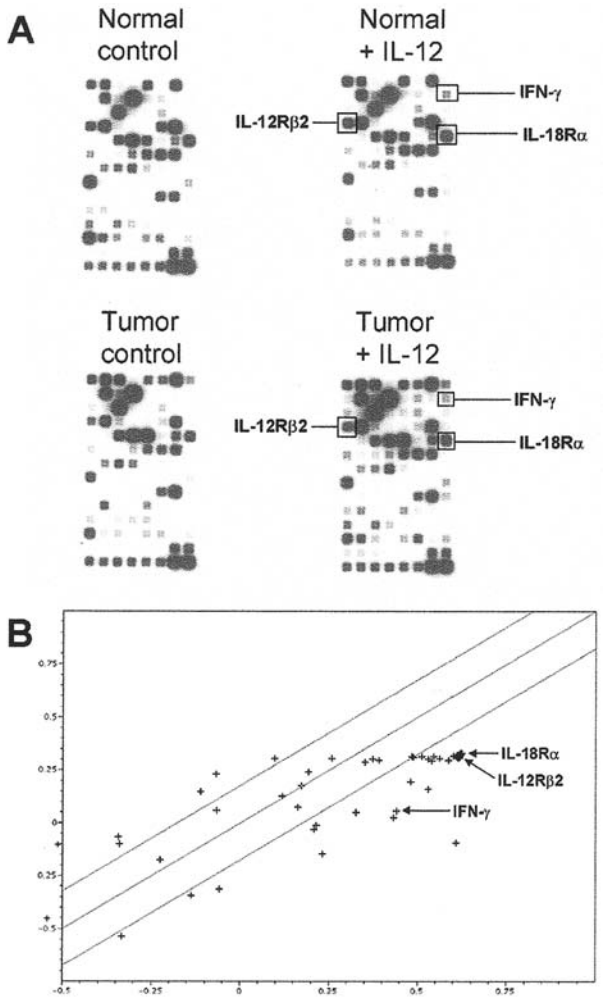

Figure 2. *A representative Mouse Inflammatory Cytokines and Receptors Gene Array. T cells from the spleens of normal and tumor-bearing mice were isolated and cultured with or without IL-12 (100ng/ml) for 3 h, after which total RNA was isolated and processed. A) Genes that showed significant changes in their expression levels in any of the analyzed combinations are indicated in the figure, which shows the results of one of three independent experiments with similar outcome. B) Corresponding scatter plot diagram of IL-12-mediated gene expression for the Mouse Inflammatory Cytokines and Receptors Array, representing gene expression in splenic T cells from normal and tumor-bearing mice after treatment with IL-12. The horizontal axis represents genes from IL-12-treated normal animals, and the vertical axis represents the genes from IL-12-treated tumor bearers. Between the diagonal lines are the genes that show equal expression in normal and tumor-bearing animals; outside and at the left of those diagonals are the genes overexpressed in IL-12-treated tumor bearers, whereas outside and at the right of the diagonals are the genes that are underexpressed in IL-12-treated tumor bearers. Each cross represents the expression of an individual gene in the expression array, and arrows point to genes relevant to our study.*

experimental groups. The genes that are overexpressed and underexpressed for each pair of groups, as well as the fold change in gene expression for all the genes that were significantly changed in the comparison, are displayed on an Excel spreadsheet and as a scatter gram. The signal from the expression of each different gene is assessed and normalized automatically by the program. Importantly, normalization was not performed with the use of housekeeping genes such as β-actin or other control genes, but instead using the interquartile adjustment. A more than 1.5-fold increase in signal intensity between groups was considered significant by the software.

Results

T cells from tumor-bearing mice exhibit a diminished expression of the IL-12Rβ2 gene. Earlier work from our laboratory found evidence for a deficient response to IL-12 in T splenocytes isolated from tumor hosts. The response to IL-12 is known to be mediated through the activity of IL-12R, a heterodimeric protein comprised of two different chains, β1 and β2 (11). In order to analyze whether a reduced expression of the IL-12 R might explain the impaired response to IL-12 in T cells from tumor hosts, we evaluated the gene expression levels of IL-12R β1 and β2 chains in T cells from normal and tumor-bearing mice. Thus, RT-PCR experiments were performed using total RNA from T splenocytes of normal and tumor-bearing mice, without activation, and upon activation with Con A and PMA, two mitogens that non-specifically activate T cells. Figure 1 shows the results of one such experiment, which demonstrates that the expression of the IL-12Rβ2 gene is strongly induced upon activation by these two

Table I. *IL-12 stimulation alters gene expression patterns of selected inflammatory cytokines and their receptors in T cells from normal and tumor-bearing mice.*

Genes in T cells from tumor bearers that are underexpressed after IL-12 treatment as compared to those from IL-12-treated normal mice	
Gene	Fold
IFN-γ	-2.40
IL-12Rβ2	-2.00
IL-18Rα	-1.95
Genes down-regulated by IL-12 in T cells from tumor bearers	
IL-18Rα	-1.64

mitogens, but significantly down-regulated in T cells from tumor bearers. Conversely, the IL-12Rβ1 chain is constitutively expressed, not modulated by these two mitogens, and shows no difference between T cells from normal and tumor hosts.

T splenocytes from tumor hosts exhibit underexpression of the genes for IFN-γ, IL-12Rβ2 and IL-18Rα upon stimulation with IL-12. The gene expression patterns of inflammatory cytokines and their receptors were analyzed in T splenocytes from tumor-bearing and normal animals after IL-12 activation, using the Mouse Inflammatory Cytokines and Receptors Gene Array (Figure 2A). Figure 2B shows the corresponding scatter plot diagram of the results generated by the GEArray Expression Analysis Suite software. IL-12 stimulation modulated the expression of different genes. Genes relevant to the IFN-γ/IL-12 signaling pathway *i.e.*, IFN-γ, IL-12Rβ2 and IL-18Rα were significantly underexpressed in tumor bearers as compared to normal animals (Table I). In fact, IL-12 stimulation actually caused a decrease in the expression of the IL-18Rα gene in the splenic T cells from the tumor bearers.

IL-12 induces decreased gene expression of JAK 2, STAT 1, 3 and 4 and IRF-1 in T cells from tumor hosts. To analyze whether the signaling pathways downstream of the IL-12R were affected at the level of gene expression in T cells from tumor bearers, we used the Mouse JAK/STAT Signaling Pathway Gene Array. We compared T cells from tumor bearers with and without IL-12 stimulation (Figure 3A), and focused on the expression patterns of genes that were relevant to the IL-12R signaling pathway. Although it is difficult to see in the raw data, after stimulation with IL-12 T cells from tumor bearers exhibit decreased gene expression of the kinase JAK2 and the transcription factors

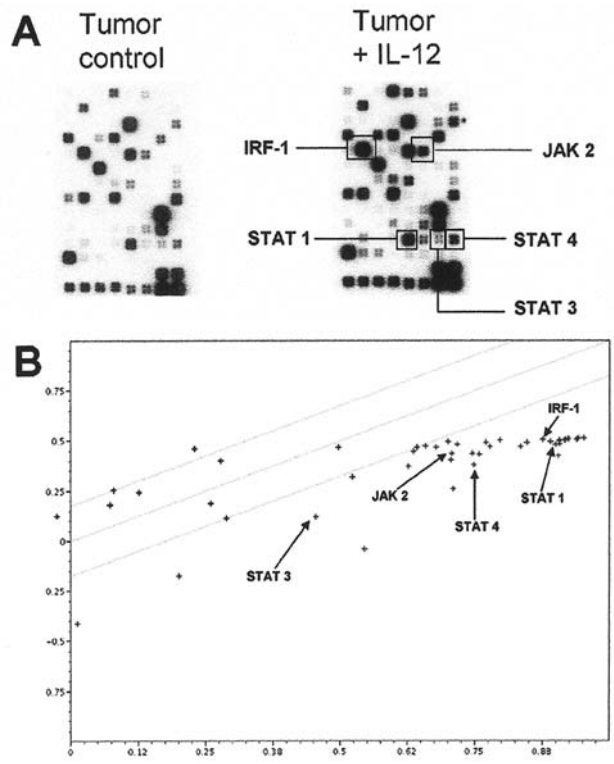

Figure 3. *A representative Mouse JAK/STAT Signaling Pathway Gene Array. T splenocytes from tumor-bearing mice were isolated and cultured with or without IL-12 (100ng/ml) for 3 h, after which total RNA was isolated and processed. A) Genes that showed significant changes in their expression levels are indicated in the figure. The array shows the results of one of three independent experiments with similar outcome. B) Corresponding scatter plot of IL-12-mediated gene expression for the Mouse JAK/STAT Signaling Pathway Gene Array, representing gene expression in IL-12-treated and non-treated splenic T cells from tumor-bearing animals. The horizontal axis represents genes from untreated tumor bearers, and the vertical axis represents genes from the IL-12-treated tumor bearers. Between the diagonal lines are genes that show equal expression in both groups; outside and at the left of those diagonals are the genes up-regulated in IL-12-treated tumor bearers, whereas outside and at the right of the diagonals are the genes that are down-regulated in IL-12-treated tumor bearers. Each cross represents the expression of an individual gene in the expression array, and arrows point to genes relevant to our study.*

Table II. *JAK/STAT pathways pertinent to IL-12 signaling in T cells from tumor bearers: relevant genes are down-regulated after IL-12 activation.*

Genes down-regulated by IL-12 in T cells from tumor bearers	
Gene	Fold
IRF-1	-2.32
JAK2	-1.85
STAT 1	-2.60
STAT 3	-2.15
STAT 4	-2.32

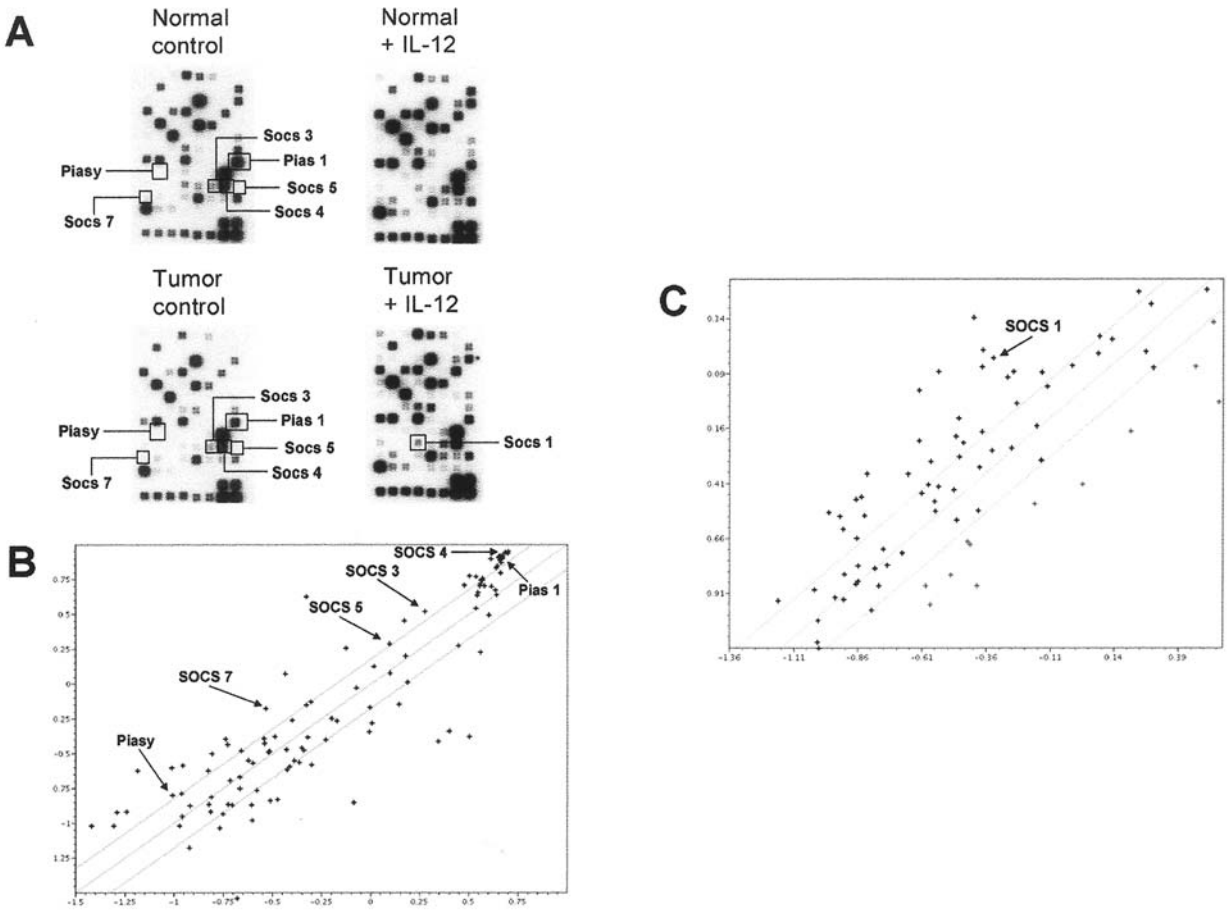

Figure 4. *A representative Mouse JAK/STAT Signaling Pathway Gene Array. T splenocytes from tumor-bearing mice were isolated and cultured with or without IL-12 (100ng/ml) for 3 h, after which total RNA was isolated and processed. A) Indicated in the figure are genes that displayed significant changes in their expression levels. The array shows the results of one of three independent experiments with similar outcome. B) Corresponding scatter plot diagram of IL-12-mediated gene expression for the Mouse JAK/STAT Signaling Pathway Gene Array, representing gene expression in untreated splenic T cells from normal animals and tumor-bearing animals. The horizontal axis represents genes from untreated normal mice, and the vertical axis represents genes from the untreated tumor bearers. Between the diagonal lines are genes that show equal expression in both groups; outside and at the left of those diagonals are the genes overexpressed in untreated tumor bearers, whereas outside and at the right of the diagonals are the genes that are underexpressed in untreated tumor bearers, as compared to untreated normal mice. Each cross represents the expression of an individual gene in the expression array, and arrows point to genes relevant to our study. C) Scatter plot diagram of IL-12-mediated gene expression for the Mouse JAK/STAT Signaling Pathway Gene Array, representing gene expression in T cells from tumor-bearing mice cultured with or without IL-12. The horizontal axis represents genes from untreated tumor bearers, and the vertical axis represents genes from the IL-12-treated tumor bearers. Between the diagonal lines are genes that show equal expression in both groups; outside and at the left of those diagonals are the genes up-regulated in IL-12-treated tumor bearers, whereas outside and at the right of the diagonals are the genes that are down-regulated in IL-12-treated tumor bearers. Each cross represents the expression of an individual gene in the expression array, and the arrow points to the gene relevant to our study.*

IRF-1, STAT 1, 3 and 4, as compared with the unstimulated tumor T cells. Table II lists these results numerically. This analysis, as well as the corresponding scatter plot diagram in Figure 3B, were created by the GE Array Expression Analysis Suite software.

Negative regulators of STATS are up-regulated in T cells from tumor bearers. Analysis of the JAK/STAT microarray experiments revealed that several suppressors of cytokine signaling (SOCS) were overexpressed in T cells from tumor bearers (Figure 4A). Only the T cells from tumor hosts were found to constitutively express SOCS 3, 4, 5 and 7, as well as Protein inhibitor of activated STATs (Pias) 1 and y (Figure 4B, Table III). Moreover, tumor bearers' T cells, but not those from normal animals, overexpressed SOCS 1 upon IL-12 activation (Figure 4C).

Table III. *Suppressors of cytokine signaling are up-regulated in T cells from tumor bearers.*

Genes constitutively overexpressed in T cells from tumor bearers as compared to T cells from normal mice	
Gene	Fold
Pias1 (Protein inhibitor of activated STAT 1)	+1.75
Piasy (Protein inhibitor of activated STAT PIASy)	+1.62
SOCS 3	+1.77
SOCS 4	+1.81
SOCS 5	+1.57
SOCS 7	+2.30
Genes up-regulated by IL-12 in T cells from tumor bearers	
SOCS 1	+3.45

Discussion

Previous work from our laboratory analyzed the functionality of IL-12R in IFN-γ-deficient T cells from tumor bearers. Experiments were performed in which increasing concentrations of IL-12 were added *ex vivo* to T cell cultures, in order to study the corresponding production of IFN-γ by normal animals and tumor hosts. It was found that, as opposed to T cells from normal mice, which produced higher amounts of IFN-γ as a response to increasing concentrations of IL-12, T cells from tumor bearers produced much less IFN-γ upon treatment with the same increasing concentrations of IL-12. In fact, the levels of IFN-γ produced by T cells from tumor bearers after activation with the highest dose of IL-12 corresponded to the levels produced by T cells from normal animals without IL-12 stimulation at all (9). These results suggested a deficiency in the response to IL-12 in T cells from tumor hosts.

The biological response to IL-12 is mediated through a specific receptor expressed on T and NK cells (IL-12R). Upon binding to this receptor, IL-12 induces the activity of the cytoplasmic tyrosine kinases JAK2 and TYK2 (15), which interact with and phosphorylate IL-12R, and are themselves phosphorylated. STAT transcriptions factors, specifically STAT1, STAT3 and STAT4 (although STAT4 plays the main role in the IL-12-mediated signal transduction), then bind to the phosphorylated receptor and are in turn phosphorylated by the activated JAKs. Phosphorylated STATs form dimers and move into the nucleus to initiate gene transcription, leading to the production of IFN-γ (16, 17). Two IL-12R subunits have been identified in mouse and humans, the IL-12Rβ1 and the IL-12Rβ2 chains (11). Different studies suggest that IL-12Rβ1 is primarily responsible for binding IL-12 in the mouse system, whereas IL-12Rβ2 is further required for IL-12 responsiveness (18). In contrast to IL-12Rβ1, which does not contain any cytoplasmic tyrosine residues, the cytoplasmic region of IL-12Rβ2 contains three tyrosine residues, suggesting an important role for the β2 subunit in IL-12 signal transduction. With the purpose of clarifying whether the insufficient production of IFN-γ observed in T cells from tumor bearers upon activation with IL-12 was due to a deficient expression of the IL-12R chains, we performed RT-PCR experiments on T splenocytes isolated from normal and tumor-bearing mice. Our results show that IL-12Rβ1 gene is constitutively expressed in both groups of mice, is not induced by either Con A or PMA, and has similar expression levels in normal animals and tumor hosts. The finding that the IL-12Rβ1 gene is constitutively expressed is in agreement with others, who have reported that the IL-12Rβ1 gene is constitutively expressed on both Th1 and Th2 cells (19), and also that RT-PCR analysis on Ba/F3 cells shows the presence of detectable levels of constitutive mouse IL-12Rβ1 mRNA (11). In contrast to β1, our results with the IL-12Rβ2 chain show that this gene is not constitutively expressed, and is instead highly inducible by either mitogen. Most importantly, our results show that the IL-12Rβ2 gene is strongly down-regulated in T cells from tumor hosts. Work from other investigators has shown that the IL-12Rβ2 chain is expressed in Th1 cells and lost upon differentiation to the Th2 phenotype (20). Following differentiation of CD4+ T cells *in vitro*, Th2 cells expressing IL-12Rβ1, but not IL-12Rβ2, fail to respond to IL-12. Also, IL-12Rβ2$^{-/-}$ mice have a severe defect in their ability to generate Th1 responses, as measured by their production of IFN-γ (18). Additionally, in contrast with the wild-type cells, STAT4 phosphorylation is not observed after treatment of IL-12Rβ2-deficient splenocytes with IL-12 (18). Taken together, the current information suggests that the control of IL-12Rβ2 expression may constitute an important mechanism for regulating IL-12 responsiveness. Our results would then suggest that, due to their decreased expression of the IL-12Rβ2 chain and deficient IFN-γ production, T splenocytes from D1-DMBA-3 tumor-bearing mice should express a Th2 phenotype. Interestingly, however, this is not the case. In contrast to what has been reported in other murine tumor models, in which decreased IFN-γ production in tumor hosts is accompanied by an increase in IL-4 and a decrease in IL-2 levels (21), previous work from our laboratory has shown that T cells from tumor bearers have no increase in the classic Th2 cytokines, IL-4, IL-6 and IL-10, and in addition, the Th1-type cytokine IL-2 is also unaltered (9). Thus, tumor bearers' T cells are deficient in their ability to produce IFN-γ not because they have experienced a shift towards a Th2 phenotype, but instead because they have a decreased production of IL-12 by macrophages from tumor hosts. Together with this deficiency, they also have a diminished expression of the IL-12Rβ2 gene, concurrently with

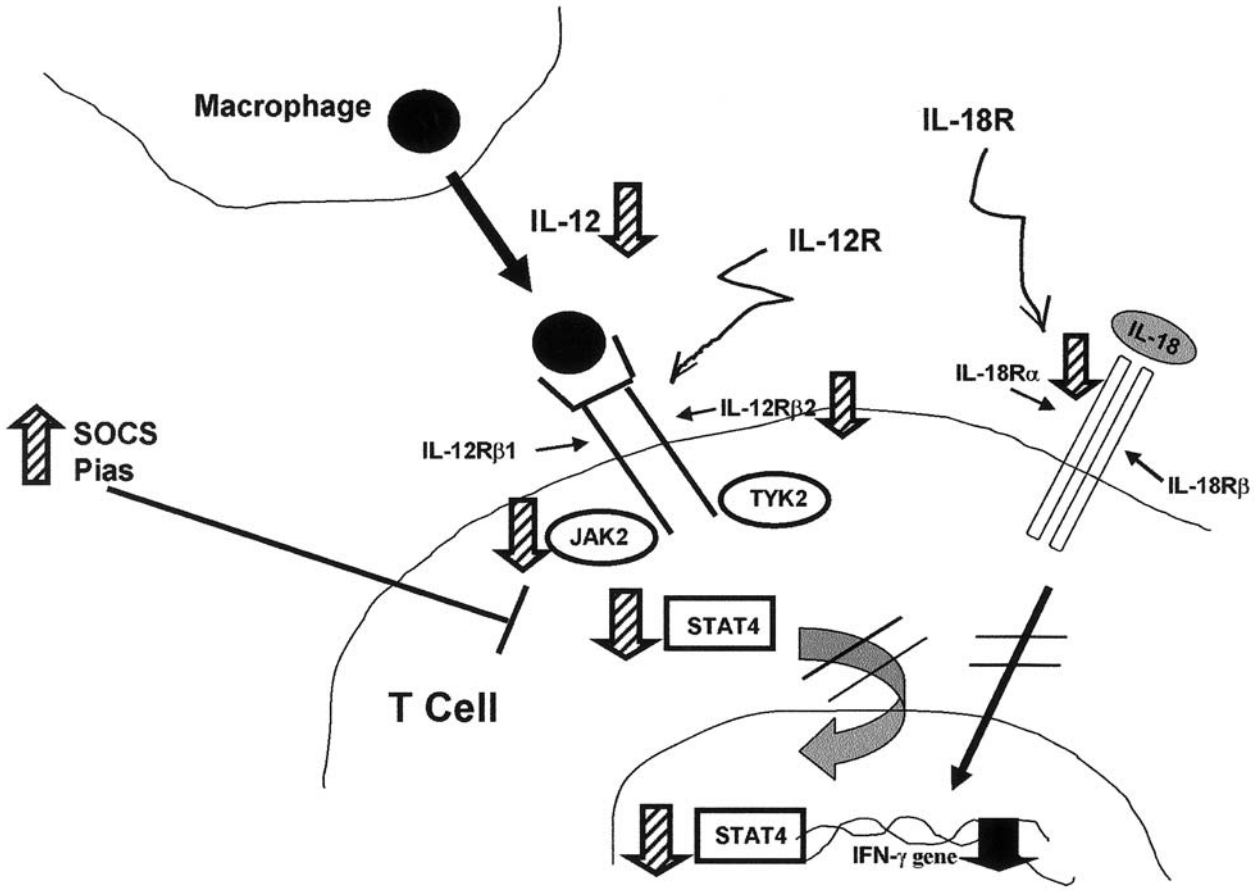

Figure 5. *Model proposed to explain the decreased production of IFN-γ by T cells from D1-DMBA-3 tumor-bearing mice.*

additional defects in other genes related to the IL-12R signaling pathways downstream to the IL-12R that we also describe here. Several recent studies have demonstrated the importance of IL-12R expression in mouse models and human diseases. IL-12R-deficient mice are immunodeficient (18), and the unresponsiveness in lepromatous patients is in part due to insufficient IL-12Rβ2 expression (19), providing additional evidence for the importance of IL-12R in situations in which immunodepression prevails, such as in tumor progression.

In order to simultaneously analyze the expression patterns of different inflammatory cytokines and their receptors upon IL-12 stimulation, we utilized cDNA microarray technology. In contrast to Con A and PMA, which did not enhance the expression of the IL-12Rβ1 gene, IL-12 did induce the expression of this gene, both in normal and tumor bearers, but there were no differences in its expression levels between these two groups. The modulation of IL-12Rβ1 by IL-12 was anticipated and has been previously reported (22). Moreover, our gene array results confirmed the decreased expression of both IFN-γ and IL-12Rβ2 in T cells from tumor bearers upon activation with IL-12, as compared to IL-12-treated normal animals. Interestingly, the gene for the cytokine IL-18 receptor α chain was down-regulated in T cells from tumor bearers upon IL-12 activation. IL-18 is a cytokine known to induce IFN-γ production, however, in contrast to IL-12, IL-18 plays important roles in both Th1 and Th2 responses, depending on its cytokine environment (23). IL-18 together with IL-12 stimulates IFN-γ production by Th1 and NK cells in a synergistic manner (24, 25). Although IL-18 does not induce *per se* the development of Th1 cells, it is essential for the effective induction and activation of Th1 cells by IL-12. When Th1 and Th2 cells are stimulated with IL-12 and IL-18, only the Th1 markedly augmented IFN-γ production in response to IL-18, suggesting that IL-18 responsiveness between Th1 and Th2 cells resulted from their differential expression of IL-18R (26). The receptor for IL-18 is composed of an α subunit (IL-18Rα) and a β subunit (IL-18Rα). Both IL-18Rα and IL-18Rβ belong to the IL-1R

superfamily (27, 28). Investigations into the mechanism of the synergism have revealed that IL-12 up-regulates expression of the IL-18R on cells producing IFN-γ (24).

Interestingly, in our tumor-bearing T cells, we observed the existence of an IL-12- induced down-regulation of IL-18R. Down-regulation of IL-18R has been previously described in cancer patients (29), and attributed to a drastic decrease of NK cells and CTL, which constitutively and highly express IL-18R. In our tumor-bearing mice, the IL-12-induced mechanism of IL-18R expression seems to be disrupted, and the reverse situation occurs. Interestingly, macrophages and NK cells derived from B6-*mi*/*mi* mice, which have an impaired IFN-γ production, also showed decreased expression levels of IL-12Rβ2 and IL-18Rα (30). On the other hand, it is known that IL-18 exhibits significant antitumor effects in BALB/c mice challenged *i.p.* with a syngeneic sarcoma tumor (31). The fact that T cells from the BALB/c tumor-bearing mice have a decreased gene expression of the receptor for this antitumor cytokine IL-18, certainly facilitates the development of a state of tumor progression and tolerance, with a deficient production of IFN-γ.

Not only did IL-12Rβ2 and IL-18Rα result in the IFN-γ deficiency in T cells from tumor hosts; the analysis of our results with the JAK/STAT array showed that signaling intermediates downstream of the membrane IL-12R are also altered in tumor hosts. Upon IL-12 activation, T splenocytes from tumor-bearing mice have a diminished expression of the JAK2 kinase, as well as of transcription factors STAT1, STAT 3, STAT 4 and IRF-1. As mentioned previously, on binding to its receptor, IL-12 activates the receptor-associated kinases JAK 2 and TYK 2. STAT 4 is then phosphorylated by these tyrosine kinases, homodimerizes *via* its SH2 domain, translocating into the nucleus where it can recognize traditional STAT target sequences in IL-12 responsive genes in Th1 cells (32), leading to the production of IFN-γ (16, 20, 33). However, little is known about the exact mechanism by which STAT 4 activation leads to Th1 differentiation, including the target genes of STAT 4. The IFN-inducible factor-1 (IRF-1) is considered to be a STAT 4 target gene, since STAT 4 can bind to and transactivate the IRF-1 promoter (34). Interestingly, IRF-1-deficient mice were defective in their Th1 responses. By inducing inadequate gene expression levels of JAK2, STAT 4 and IRF-1, T cells from tumor bearers are impaired in their ability to properly operate in the production of IFN-γ.

The control of immune homeostasis requires the existence of negative regulators of such a critical signaling pathway as the JAK/STAT system is. Three major classes of negative regulators of the JAK/STAT system have been described so far: phosphatases, Pias proteins (Protein inhibitors of activated STATs) and SOCS proteins (Suppressor of Cytokine Signaling) (35). Both phosphatases and Pias proteins are constitutively expressed. Pias recognize STAT dimers and block their transcriptional activity, whereas SOCS are induced by STAT functions, and hence act like classic feedback inhibitors, through pathways that are currently being clarified (36-38). The diverse mechanisms that control IL-12 signaling remain to be elucidated in their entirety. One possibility is that inhibitory molecules such as the SOCS proteins may regulate IL-12 signaling. SOCS1 has been shown to be a key inhibitor of IFN-γ signaling (39). Recent experiments, in which responses of SOCS $1^{-/-}$ and STAT $4^{-/-}$ mice to IL-12 were assessed, revealed that, in addition to IFN-γ, SOCS 1 regulates the response to IL-12 (40, 41). In general terms, as negative regulators of the STATs, both Pias and SOCS would most likely be overexpressed if the STAT functions were to be depressed. This is exactly the situation that we observed in T cells from tumor bearers. Interestingly, in unstimulated T cells from tumor hosts, not only Pias 1 and y, but also several SOCS, such as 3, 4, 5 and 7, showed an enhanced expression, as compared to the situation in unstimulated T cells from normal mice. To note, SOCS 1, which among the different members of the SOCS family is the one purportedly involved in the regulation of IL-12 signaling, was overexpressed upon IL-12 activation in T cells from tumor bearers.

Taking all these facts into consideration, we conclude that the impaired expression of IFN-γ by T cells from mammary tumor-bearing mice is the overall result of several alterations (Figure 5). Previous data from our laboratory indicated the role played by an impaired IL-12 production by macrophages from tumor bearers. We have now shown that, concomitantly, other deficiencies develop in T cells from tumor animals, such as diminished expressions of IL-12Rβ2 and IL-18Rα. Importantly, the existence of insufficient positive signals (JAK/STATs), together with an overexpression of negative control elements (SOCS, Pias) in the IL-12 signaling pathways of T cells from tumor hosts, are contributing factors to the altered immunological defenses against the developing tumor.

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