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GENE EXPRESSION ANALYSIS IN INTERLEUKIN-12-INDUCED SUPPRESSION OF MOUSE MAMMARY CARCINOMA

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Abstract

Interleukin-12 (IL-12) has potent antitumor activities *via* natural killer cells and cytotoxic T lymphocytes. However, the molecular mechanisms whereby IL-12 induces tumoricidal activities are poorly understood. Here, we report the genome-wide analysis of gene expression in a primary murine mammary carcinoma model that resembles human breast cancer, following the therapeutic application of recombinant IL-12, which restricted tumor growth and metastasis. IL-12 was able to curtail neovascularization in the tumor as well as enhance the number of tumor-infiltrating lymphocytes. Comprehensive examination of global gene expression revealed IL-12-induced molecular changes associated with tumor regression and reduced lung metastasis, thus providing a high-resolution snapshot of a host response against a developing malignancy and a rich source of potential targets for therapeutic intervention of breast cancer.

Keywords

Interleukin-12; Interferon-γ; mammary carcinoma; tumor-infiltrating lymphocytes; metastasis; angiogenesis

Tumors often possess a number of potential recognition sites for immunologic effector cells, which, in theory, could make them susceptible to immune surveillance. Nevertheless, most of such tumors grow progressively in their natural hosts or syngeneic recipients, without being controlled effectively by the immune system. The lack of apparent immunogenicity of tumors *in situ* might be due to special properties of the tumor cells, *e.g.*, lack of costimulatory molecules, downregulation of MHC molecules, or production of immunosuppressive factors[1,2] or due to intrinsic tolerance mechanisms of the immune system.[3]

Two principal types of cells are immunologically potent tumor-killing effectors: NK and CTL. A major activator of both cell types is Interleukin-12 (IL-12). NK cells kill tumors by intrinsic and nonspecific mechanisms hinged on a lack of MHC molecules on the surface of the tumor. CTL, on the other hand, recognizes specific antigenic peptides that are derived from the tumor and presented to them by antigen-presenting cells such as dendritic cells (DCs). In recent years, immunotherapy has been rekindled that attempts to either mark the tumor by upregulating the surface antigens for enhanced interaction with immune effector cells[2,4] or by directly activating DC, T and NK cells for their heightened "scouting" capacity and increased cytolytic potency. IL-12 is a factor belonging to the latter class.[5] IL-12 has powerful anti-tumor and anti-metastatic activities against many murine tumors as well as human tumors.[6] Recent

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encouraging developments in clinical applications of IL-12 for human T cell lymphoma,[7,8] B cell non-Hodgkin lymphoma,[9] melanoma[10–14] and renal carcinoma[15] and SIV-infection model in rhesus macaques[16] strongly underscore the importance of understanding the cellular and molecular mechanisms of IL-12mediated anti-tumor responses. The potent anti-malignancy activities of IL-12 are thought to be mediated through similar mechanisms that are used by IL-12 against infectious agents, *i.e.*, *via* the activation of NK cells for the bulk nonantigen specific clearance, and activation of CTL and CD4 for tumor-specific elimination and long-term immunity; 4T1 is a tumor cell line isolated from a single spontaneously arising mammary tumor from a BALB/BfC3H mouse (MMTV+).[17] It is an excellent model system for breast cancer research because its tumor development is well characterized both oncologically and immunologically. The 4T1 tumor closely mimics human breast cancer in its anatomical site (mammary gland), immunogenicity, growth characteristics and metastatic properties.[18] 4T1 tumor spontaneously metastasizes to a variety of target organs including the lung, heart, bone, brain and liver through primarily a hematogenous route.[19] 4T1 is also poorly immunogenic in that immunization with irradiated 4T1 cells provides only slight delays in tumor growth against wild-type tumor, not sufficient to protect the animal.[20] IL-12 administration to 4T1-bearing mice resulted in a substantial reduction in tumor size and in spontaneous metastases in the lungs of 4T1 tumor-bearing mice and significantly prolonged their survival time.[21] Tumor-draining lymph node cells obtained from 4T1 tumor-bearing mice treated with IL-12 cDNA exhibit increased natural killer (NK) activity and produced enhanced levels of IFN-γ, suggesting the involvement of both NK cells and IFN-γ in this effect.[21] These properties make this mammary tumor model an excellent system in which to investigate the cellular and molecular changes throughout the malignant development.

To investigate the molecular mechanisms whereby IL-12 exerts its potent anti-tumor activities, we carried out a comprehensive analysis of gene expression in the 4T1 primary tumor following IL-12 treatment *in vivo*.

MATERIAL AND METHODS

Mice

Female BALB/c mice (6–8 week old) were purchased from the Jackson Laboratory (Bar Harbor, Maine). All mice were housed at the Weill Medical College of Cornell University Animal Facilities in accordance with the Principles of Animal Care (NIH publication number 85–23, revised 1985). Mice bearing 4T1 tumors were all sacrificed no later than day 28 due to the morbidity caused by large tumor size and strong metastasis.

Tumor implantation, size measurement, lung metastasis assay

4T1 mammary carcinoma cells (1×10^5) were injected subcutaneously into the abdominal mammary gland area of recipient mice in 0.1 ml of a single-cell suspension in phosphate-buffered saline (PBS) on day 0. The dose of tumor implantation was empirically determined to give rise to tumors of ~10 mm in diameter in untreated wild-type mice in 28 days. Primary tumors were measured using electronic calipers every other day. Reported measurements are the square root of the product of 2 perpendicular diameters. Numbers of metastatic cells in lung were determined by the clonogenic assay.[18] In brief, lungs were removed from each mouse, finely minced and digested in 5 ml of enzyme cocktail containing 1× PBS and 1 mg/ml collagenase type IV for 2 hr at 37°C on a platform rocker. After incubation, samples were filtered through 70 μm nylon cell strainers and washed twice with PBS. Resulting cells were resuspended and plated serially diluted in 10 cm tissue culture dishes in medium RPMI1640 containing 60 μM thioguanine for clonogenic growth. 6-Thioguanine-resistant tumor cells formed foci within 10–14 days, at which time they were fixed with methanol and stained with 0.03% methylene blue for counting.

IL-12 treatment

Recombinant murine IL-12 was provided by Genetics Institute (Cambridge, MA). IL-12 treatment was given by intraperitoneal injection at 1 μg per mouse every other day starting on day 7 until the end of each experiment unless otherwise described. This regimen of IL-12 was well tolerated with no signs of overt toxicity.

Histopathology

On the day of sacrifice, primary tumors were removed, fixed, sectioned and stained with H&E, and examined by light microscopy for their architecture, evidence of lymphocyte infiltrating and angiogenesis.

Microarray experiment

The high-density oligonucleotide microarray system of Affymetrix (Santa Clara, CA), murine Genome U74A Array version 2 containing 12,488 genes was used. Total RNA was isolated from freshly isolated 4T1 tumors of all surviving mice on day 23. RNA samples of each mouse within each experiment group were pooled from 7–10 mice. Ten micrograms of total RNA was used to synthesize cDNA using Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA) with a primer containing oligo (dT) and T7 RNA polymerase promoter sequences. Double-stranded cDNA was then purified by phase lock gel (Eppendorf, Westbury, NY) with phenol/chloroform extraction. The purified cDNA was used as a template to generate biotinylated cRNA using the Bioarray High Yield RNA Transcript Labeling Kit (Enzo Biochem, Farmingdale, NY) and then biotinylated cRNAs were fragmented and hybridized to Affymetrix Test 3 chips (Affymetrix, Inc., Santa Clara, CA). All RNA samples passed quality control (ratio of 3′ to 5′ < 3) and then the samples were hybridized to the Murine Genome Array U74Av2 array, which contains 12,488 well-substantiated mouse genes. After overnight hybridization, the arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) on the GeneChip Fluidics Station (Affymetrix) and scanned according to the standard Affymetrix protocol.

Microarray data collection and analysis

Affymetrix GeneChip 5.0 was used as the image acquisition software for the U74Av2 chips. The signal, which represents the intensity of each gene, was extracted from the image. The target intensity value from each chip was scaled to 250. Data normalization, log transformation, and statistical analysis were performed with GeneSpring software (Silicon Genetics, Redwood City, CA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Primers used for PCR were 1. ASM-like phosphodiesterase 3a (PDE3a): sense-TGGCTGGGTGAAGAAGCTAT, anti-sense- GGAGATAGCCCACTGGAACA; 2. Serum amyloid A 2 (SAA2): sense- GCGAGCCTACACTGACATGA, anti-sense-GGCAGTCCAGGAGGTCTGTA; 3. spi2 proteinase inhibitor (spi2/eb1): sense-ACATTGATGGTGCTGGTGAA, anti-sense- AGTGCAGGACAGCTCCTCAT; 4. Immunoresponsive gene 1 (Irg1): sense- CGTGAGAAAGCACCTTGTGA, anti-sense-CTGTGGAAGGATGGGACAGT; 5. sex-limited protein alpha-gamma chain Slp(w7) : sense-TGCCTTCCGTCTCTTTGAGT; antisense, ACATTTGTTCCGAAGGCATC; 6. IL-12Rβ2: sense- AATTCAGTACCGACGCTCTCA, anti-sense- ATCAGGGGCTCAGGCTCTTCA; 7. hypoxanthineguanine phosphoribosyl transferase (HPRT): sense-GTTGGATACAGGCCAGACTTTGTTG, anti-sense-GAGGGTAGGCTGGCCTATGGCT; 8. RANTES: sense- CCCTCACCATCATCCTCACT, anti-sense- CTTCTTCTCTGGGTTGGCAC; 9. MIG: sense-AGAACTCAGCTCTGCCATGAAGTC, anti-sense-

CTAGGCAGGTTTGATCTCCGTTCT; 10. MCP2: sense-GATCTACGCAGTGCTTCTTTGCCT, anti-sense GACATACCCTGCTTGGTCTGGAAA and 11. SDF-1α: sense- GCTCTGCATCAGTGACGGTA, anti-sense-TGGGCTGTTGTGCTTACTTG.

Statistical tests

Tumor growth and metastasis data to be compared were first subjected to normality test. Where the samples studied were normally distributed, statistical comparisons were performed using the Student's *t*-test. Where the samples deviated from normality, a nonparametric, Mann Whitney Rank-Sum test was used for comparisons. Statistical analyses were performed using SigmaStat software. For all experiments, the mean and the SD are depicted.

RESULTS

IL-12 induces anti-tumor activities in vivo

To assess the effects of IL-12 in tumor regression and metastasis, 4T1 mammary carcinoma was initiated by *s.c.* injection of 4T1 cells into mice on the syngeneic BALB/c background. Recombinant IL-12 was given *i.p.* starting on day 7 post tumor injection to both types of mice when the primary tumor had grown to ~ 4 mm in diameter. The timing of IL-12 administration was based on potential therapeutic considerations to mimic clinical situations in which breast cancer patients do not get therapy until the presence of malignant growth in the breast has been identified by mammogram or other means. As shown in Figure 1*a*, by day 23, there were significant differences in tumor growth in mice with or without IL-12 treatment. The pattern of lung metastasis in these mice in response to IL-12 treatment was very similar to that of the primary tumor growth (Fig. 1*b*). There was a positive correlation between primary tumor size and the number of metastatic cells in the lung, *i.e.*, the larger the tumor, the greater the lung metastases (Fig. 1*c*,*d*). IL-12 treatment prevented 4T1 lung metastasis from crossing the lethal threshold of 10^4 metastatic tumor cells per lung[22] in all 10 mice while in nontreated mice (1*d*), 90% of the mice bore lung metastases in excess of 10^4 tumor cells (Fig. 1*c*). IL-12 treatment also reduced the death rate resulting from the tumor from 47% in nontreated mice to 13% in 6 separate studies over the 28-day experimental period.

IL-12 enhances lymphocyte infiltration and reduces neovascularization in the tumor

We examined microscopically tumor sections with or without IL-12 treatment (Fig. 2). One of the readily recognizable features of these tumor sections is the large amount of neovascularization in PBS-treated mice (Fig. 2*a*,*c*, indicated by red arrows). IL-12 treatment strongly blocked the formation of these vessels (Fig. 2*b*,*d*), confirming the well-established role of IL-12 in inhibition of neoangiogenesis.[23] In addition, PBS-treated tumors had few infiltrating T lymphocytes (Fig 2*a*,*c*). IL-12 treatment dramatically increased the number of tumor-infiltrating lymphocytes (TILs) (Fig. 2*b*,*d*, indicated by white arrows). These results are confirmatory for the anti-angiogenic activity of IL-12 and its ability to induce TILs.[24]

Genome-wide analysis of gene expression in primary tumors

To obtain a detailed appreciation of the molecular events taking place in 4T1 tumors exposed *in vivo* to IL-12, we performed global gene expression analysis of the 2 groups of tumors on day 23 using the Affymetrix oligonucleotide microarray system (Murine Genome U74A Array version 2 containing 12,488 genes). To reduce variations between individual mice, RNA samples were pooled from all mice within each group for the microarray analysis. We applied this technology to the search of genes that undergo altered expression in 4T1 tumor cells *in vivo* following IL-12 therapy in an attempt to identify the downstream targets of IL-12. RNA samples were prepared from the 2 experimental groups shown in Figure 1 for comparison of

differential gene expression. This microarray experiment (10 mice from each group) yielded a large amount of data, which was processed through the GeneSpring software for i) data normalization (bias correction), ii) data transformation (to ensure normal distribution) and (iii) gene filtering to identify specific genes that are expressed differentially by using appropriate statistic tools. The group of PBS-treated wild-type mice was set as the baseline (with an expression value of 1.0) to which the IL-12-treated group was compared (Fig. 3). Most of the genes exhibit no altered expression. The vast majority of the genes were either present equally (red dots) or absent (yellow dots) in both samples. A small number of genes manifested changes in expression to varying degrees (blue dots) as indicated by the distances they move away from the central line. This microarray experiment was repeated once with high similar results.

Genes that exhibit altered expression following IL-12 in vivo treatment

To further examine the details of the genes differentially expressed in the 2 groups of 4T1 tumor in an effort to understand the molecular mechanisms involved in IL-12-mediated anti-tumor activities, we categorized these genes using additional stringencies, *i.e.*, by choosing only those genes that were expressed 2-fold or higher in IL-12-treated tumors with statistically significant change *p* values ($p < 0.05$) and present calls. By these criteria, 52 genes were found to be induced in IL-12-treated tumor by at least 2 fold to a detectable level (called present), compared to PBS-treated tumor (Table I*a*), and 17 genes were inhibited by at least 2-fold (Table I*b*).

It is clear from a glance at the gene list in Table I*a* that IL-12 *in vivo* treatment induced a whole host of genes involved in many aspects of its antitumor activities: metabolism, cell cycle, proliferation, apoptosis, metastasis, antigen presentation, chemotaxis, immune response *etc*. in the form of the altered expression of genes that encode surface, intracellular and extracellular molecules. Many of the genes have well-defined functions; others are poorly or not at all characterized (EST clones). Of the 45 induced genes with known functions, 23 are likely derived primarily frominfiltrating lymphocytes and phagocytes such as IFN-γ-induced proteins, chemokines, complement factors, Fas ligand, CD8, CD3, Ly-6E.1, MHC class II antigens, iNOS *etc*., likely representing an IL-12-induced, concerted response against the developing mammary tumor. The remaining 22 genes are probably expressed by the tumor cells or the stroma.

In contrast to IL-12-induced genes, the list of IL-12-inhibited genes (Table I*b*) were predominantly expressed in cells of nonhematopoietic origin.

Verification of differential gene expression in vitro *and* in vivo

We randomly selected 3 genes from this list [ASM-like phosphodiesterase 3a (PDE3a), spi2 proteinase inhibitor (spi2/eb1) and sex-limited protein alpha-gamma chain (Slpw7)] and performed RT-PCR to verify their status of differential mRNA expression between PBS-treated and IL-12-treated tumors (Fig. 4*a*). All 3 genes exhibited significantly differential expression between PBS and IL-12 treatments. The expression pattern was then compared to 4T1 cells treated *in vitro* with IL-12 for 24, 48 and 72 hr. In contrast to 4T1 tumor cells treated *in vivo* with IL-12, the expression of PDE3a, Spi2 and Slp was not detectable *in vitro* in IL-12-treated or PBS-treated samples (data not shown). These results suggest that the induction of many genes shown in Table I may require additional factors other than IL-12. These factors are available *in vivo* presumably provided by other cells but not *in vitro* in isolated 4T1 cells.

Several chemokines were notably stimulated by the IL-12 treatment. This microarray data was also confirmed by RT-PCR for regulated upon activation of normal T cells expressed and secreted (RANTES), stroma cell-derived factor-1α (SDF-1α), monokine induced by γ

interferon (MIG, CXCL9) and monocyte chemoattractant protein-2 (MCP-2, CCL8) (Fig. 4*b*).

We also examined the expression of IL-12Rβ2 mRNA expression by RT-PCR in the primary 4T1 tumors with or without the IL-12 treatment (Fig. 4*c*). It appears that IL-12Rβ2 was expressed in tumors tissues without exogenous IL-12, and the IL-12 treatment significantly enhanced its expression. These results suggest that 4T1 tumors are able to respond to IL-12 directly because they express the critical IL-12Rβ2 chain.[25] However, presently it's not clear whether it is the 4T1 tumor cells themselves or the TILs that express IL-12R.

DISCUSSION

IL-12 has potent antitumor activities with strong clinical relevance to cancer therapy. However, the molecular mechanisms that underline these activities of IL-12 are poorly understood, presenting an obstacle to its clinical applications. The current work constitutes an important step towards comprehensively identifying the key factors involved in host response induced by IL-12 against a developing malignancy.

The approach that we took to examine the global gene expression profile in the whole tumor with its stroma and lymphocytic infiltrates, as opposed to looking at certain purified populations of cells within the tumor, provides us with the ability to view all of the transcriptional changes (limited by the number of genes on the microarray and by the detection limit) effected by IL-12, thus permitting a more comprehensive analysis of the interactions between malignant cells and its microenvironment without potential perturbations to the various cell populations embodied in the tumor due to *in vitro* manipulation. The counter argument is that this "wholesome" approach would not allow the identification of specific cell types that may play a more disproportionate role in IL-12-mediated anti-tumor responses than other cells. One of the ways to circumvent this dilemma is to analyze whole tumor mass in mice that are genetically deficient in producing certain immune cell types.

A prominent feature among the genes induced by IL-12 *in vivo* is the production of chemokines: RANTES, SDF-1α, MIG, MCP-2, N-formylpeptide receptor-like 2. The production of these chemotactic factors may be responsible for the influx of TILs observed in 4T1 tumors treated with IL-12 (Fig. 2*d*).

Some of the 22 IL-12-induced genes apparently of nonhematopoietic origins are recognized to be associated with tumor growth and/or metastasis. Take adipsin and lipoprotein ligase for example. Induction of adipocyte-specific gene expression including these 2 genes in the rat mammary carcinoma is correlated with tumor regression by the retinoid X receptor-ligand LGD1069 (targretin).[26] Phosphodiesterase (PDE) 3a and 3b were detected in human neoplastic submandibular gland intercalated duct HSG cells. The PDE3-specific inhibitor, cilostamide, inhibited the growth of HSG cells. PDE3 thus appears to be a potential target for antiproliferative therapies.[27] Cellular repressor of E1A-stimulated gene(CREG) is a secreted glycoprotein during differentiation of embryonic stem cells and is able to enhance differentiation of the human embryonal carcinoma cell line NTERA-2 characterized by changes in morphology, altered patterns of gene expression, reduced proliferative potential and a loss of tumorigenicity.[28] Therefore, induction of the expression of these growth-inhibiting genes by IL-12 may impede the succession of the mammary tumor.

One of the interesting novel genes that were induced by IL-12 treatment is onzin whose function is currently unknown. Human onzin was identified as a differentially expressed gene in a subtractive hybridization screening between IL-3/CD40L-activated plasmacytoid DCs (tester) and CD40-activated monocyte-derived DCs (driver). This novel gene, also called C-15, encodes a putative cytokine because its deduced polypeptide is composed of 112 aa (115 aa

for mouse C-15), including a 23-aa signal peptide which allows its secretion. Onzin/C-15 is expressed in different hematopoietic cells of T, B, monocyte, granulocyte lineages.[29] Human C-15 contains 16 cysteines, 15 of which are conserved in the mouse homologue. So it could also be a member of the defensin-like molecules given that defensins are a family of small peptides with 3 or 4 intramolecular cysteine disulfide bonds.[30] Onzin/C-15 was also identified as the most strongly stimulated gene in a microarray analysis of draining auricular lymph node tissue sampled at 48 hr following exposure to the potent contact allergen 2,4-dinitrofluorobezene (DNFB).[31] Expression of onzin/C-15 has been reported to be inhibited by *c-myc* in the myeloid cell line 32D,[32] suggestive of a role in cell cycling. Taken together, onzin/C-15 could be an important player in IL-12-mediated activation and migration of DCs and lymphocytes to confer its anti-tumor activity.

The most remarkable feature among the 17 genes whose mRNA expression was inhibited by the IL-12 treatment *in vivo* is the dominance of genes involved in extracellular matrix (ECM) remodeling and cell adhesion functions. Structural changes in ECM are necessary for cell migration during normal and pathologic tissue remodeling and neoplastic cell invasion. The matrix metalloproteinases (MMPs) and their inhibitors are critical modulators of ECM composition and are thus important in neoplastic cell progression, invasion and metastasis.[33–36] Strong expression of MMP-3 and -10 was found in childhood astrocytomas,[37] in lung adenocarcinomas,[38] especially in the ECM adjacent to blood vessels. Expression of MMPs and their inhibitors correlates with invasion and metastasis in squamous cell carcinoma of the head and neck.[39] EGP314 is a panepithelial glycoprotein.[40] Transfection of a low metastasizing fibrosarcoma, pheochromoblastoma and adenocarcinoma with the rat ortholog of human and mouse EGP314, D5.7A facilitated tumor metastasis *via* the lymphatic system or hematogeneously, depending on the origin of the tumor.[41] Particularly after proteolytic cleavage, D5.7A exhibited enhanced cell-cell adhesion and provided a proliferative signal upon crosslinking.[41] PEG1/MEST is an imprinted gene involved in the growth of the fetus and/or placenta.[42] Pedersen *et al*.[43] reported that monoallelic PEG1 expression was observed in normal breast tissues, indicating the presence of a functional imprint. However, they noted a loss of imprinting in all informative invasive breast carcinomas examined, possibly involving a novel mechanism of promoter switch that causes biallelic PEG1expression.[44] Thus, inhibition of the expression of these tumor/metastasis-promoting genes by IL-12 may directly blunt the progression of the malignancy.

In summary, the comprehensive analysis of gene expression in the 4T1 tumor model following IL-12 treatment uncovers many candidates that may be involved in IL-12-regulated immune responses against tumor progression, or in direct modification of the cancer cell, its stroma, and the microenvironment such that the growth and metastasis of the tumor is retarded. It also provides a rich pool of prospective targets desired for the development of therapeutic agents to control breast cancer.

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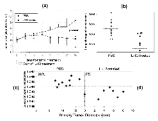

Figure 1.

IL-12 induces anti-tumor activities *in vivo*. (*a*) 4T1 tumor cells were injected *s.c.* in the abdominal mammary gland with 0.1 ml of a single-cell suspension containing 10^5 tumor cells. Tumor growth was monitored every 2–3 days and tumor size in diameter (mm) was measured with an electronic caliper. Each data point is comprised of 10 mice. Error bars represent standard deviation. IL-12 treatment started on Day 7 and was injected *i.p.* every 2 days until day 21. (*b*) Lung metastasis was measured at the end of this experiment (day 23) by the 6-thiogaunine clonogenicity assay. The dashed horizontal lines represent the mean of each group. (*c*,*d*) The same data shown in (*a*,*b*) are plotted on X and Y axis to show the correlation between the size of the primary tumors and their ability to metastasize to the lung.

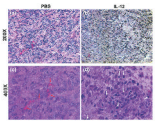

Figure 2.
Angiogenesis and lymphocyte infiltration in primary 4T1 tumors. Parafin-embedded tumor sections were stained with H&E and microscopically examined for blood vessels and tumor infiltrating lymphocytes at 200× (*a*,*b*), and 400× (*c*,*d*) magnification. Shown for each group is 1 representative slide of 3 tumor samples randomly picked from the 2 experimental groups. Red arrows, blood vessels; white arrows, TILs.

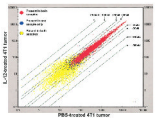

Figure 3.

Affymetrix microarray analysis Total RNA samples were extracted from 4T1 tumors excised out of all mice treated or not with rmIL-12 as shown in Figures 1 and 2, and mixed equally within each group to eliminate bias in sampling. The RNA was converted into biotinylated cRNA by *in vitro* transcription. Microarray hybridization was performed and data graphically represented by scatter plot of the data points. The baseline data points (PBS-treated tumor) are plotted along the X-axis (horizontal), and those of the experimental group (IL-12-treated tumor) along the Y-axis (vertical). The green lines indicate signal intensities above (+) or below (−) the center line, which is set as 1. This microarray experiment was independently repeated with similar results.

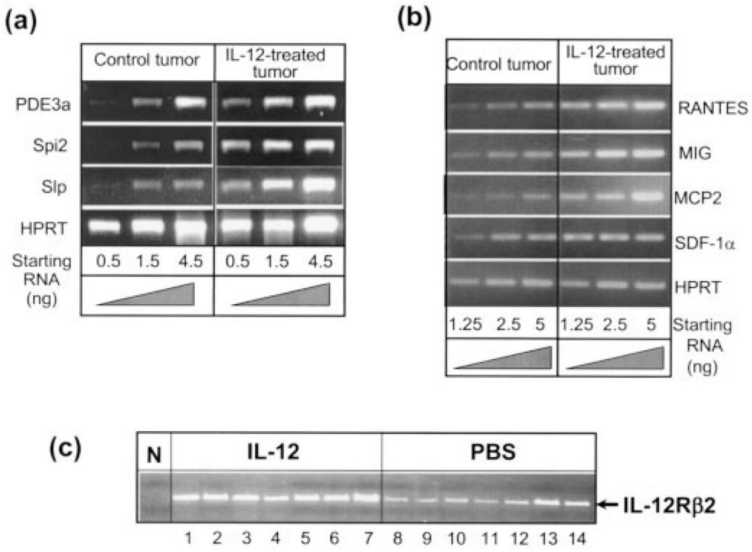

Figure 4.

Confirmation of differential mRNA expression by RT-PCR. (*a*) Reverse transcription (RT) was performed with the same total RNA samples used for the microarray experiment followed by PCR amplification using appropriately designed primers for each cDNA. Thirty-four cycles of amplification were carried out with serially diluted cDNA samples. The equivalent corresponding starting amount of total RNA each serial dilution represents is indicated at the bottom. (*b*) RT-PCR was performed as described in (*a*) with RNA samples taken from 4T1 cells treated or not *in vivo* with rIL-12 for RANTES, SDF-1α, MIG and MCP-2. (*c*) RT-PCR was performed to examine the mRNA expression of IL-12Rβ2 in 4T1 tumors exposed to IL-12 or not *in vivo.* Each lane represents an individual tumor tissue isolated from one mouse. Lanes 1–7, tumors from IL-12-treated mice; lanes 8 –14, tumors from PBS-treated mice. N, negative control (PCR without cDNA added).

TABLE I

GENES IN 4T1 TUMOR[1]

Genbank Accession	Signal ratio (log of 2)	Change p-value	Gene description	Gene function
a. Genes that show enhanced expression in 4T1 tumor following IL-12 treatment *in vivo*				
AF065947[3]	3.20	0	NOD/LtJ small inducible cytokine A5 (ScyA5) RANTES	Chemoattractant for Mϕ/DC, T/NK/basophil/ eosinophil
AF061569	2.60	0.00001	c-myc coding region determinant binding protein	Stabilizes c-myc mRNA
L12029	2.30	0.000001	Stromal cell derived factor 1-α	Chemoattractant
X06454[3]	2.20	0	Sex-limited protein Slp(w7) alpha-gamma chain	Complement-like
U34881[3]	2.10	0.000649	CD8 antigen, alpha chain	Cytotoxic and DC marker
K02782[3]	1.80	0.000008	Complement component C3 mRNA, alpha and beta subunits	Complement
X04673	1.80	0.000408	Adipsin	Complement D activity, activates alternative pathway
M34815[3]	1.80	0	Monokine induced by gamma interferon (MIG); CXCL9	Chemoattractant for activated T cells
AB023418[3]	1.80	0	monocyte chemoattractant protein-2 (MCP-2), CCL8	Chemoattractant for T/monocyte/basophil/ eosinophil
AL078630[2]	1.80	0.000388	Mouse genomic DNA sequence from clone 573K1 (EST)	Unknown
M63335	1.80	0.004863	lipoprotein lipase	Hydrolyzes triglycerides in lipid metablism
X04653[3]	1.70	0	Ly-6 alloantigen (Ly-6E.1)	T-lymphocyte activation
AF010254[3]	1.50	0.000001	Complement component 1 inhibitor	Inhibitor of C1 and several plasma serine proteases
X03505	1.50	0	Serum amyloid A 3	Acute stress response
M64085	1.50	0	spi2 proteinase inhibitor (spi2/eb1)	Inhibitor of serine proteases
AW124337[2]	1.40	0.000002	UI-M-BH2.1-apq-f-08-0-UI.s1 Mus musculus cDNA, 3 end	Homologous to microsomal glutathione S-transferase 1
AF061260[3]	1.40	0.000031	Immunosuperfamily protein BI2, nectin-like protein 2 (Necl2)	Cell-cell adhesion and transmembrane protein localization
U35330[3]	1.40	0	Histocompatibility 2, class II, locus Mb1	Antigen presentation to $CD4^+$/T cells
U06924[3]	1.40	0	Signal transducer and activator of transcription 1 (Stat1)	IFN-γ signaling
AF071180	1.40	0.000001	N-formylpeptide receptor-like 2 gene	Chemotactic for phagocytes (antibacterial

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Genbank Accession	Signal ratio (log of 2)	Change p-value	Gene description	Gene function
				and tissue repair)
X00958[3]	1.30	0	I-E (beta-b) gene	Antigen presentation to $CD4^+$ T cells
Y08135	1.30	0	Acid Sphingomyelinase-like phosphodiesterase 3a	Novel binding protein of tumor suppressor DBCCR1 (deleted in bladder cancer chromosome region 1)
AA790307[2]	1.30	0	vw17a10.r1 Mus musculus cDNA, 5′ end (Onzin)	Unknown
U53219	1.30	0	GTPase IGTP	Interferon induced GTP-binding protein
X78445	1.30	0.000001	Cytochrome P450	Detoxification by oxidation
X02339[3]	1.30	0.000115	CD3 antigen, delta polypeptide	T-lymphocyte marker
AI386093[3]	1.30	0.000008	Similar to protein tyrosine kinase ZAP-70	T-lymphocyte activation signaling
U88566	1.20	0.000001	Secreted frizzled related protein sFRP-1 (Sfrp1)	Inhibitor of Wnt, induces apoptosis
V00833[3]	1.20	0	E-alpha gene for immune response gene	Antigen presentation to $CD4^+$/T cells
U35323[3]	1.10	0	H2-M chain, H2-M2, H2-M1, low molecular weight protein 2 (Lmp2)	Antigen presentation to $CD4^+$ T cells
M21932[3]	1.10	0	MHC class II H2-I-A-beta gene (k haplotype)	Antigen presentation to $CD4^+$ T cells
M16238	1.10	0	Fibrinogen-like protein 2	Thrombosis, cytotoxic T-specific protein
D44464	1.10	0	Uridine phosphorylase	Catalyzes the reversible phosphorolysis of uridine to uracil
M68903[3]	1.10	0.000001	Integrin beta 7, Ly69	Lymphocyte homing and retention
AA914345	1.10	0.000001	Interferon-inducible GTPase	Interferon induced GTP-binding protein
U43428[3]	1.10	0.000018	Nitric oxide synthase 2, inducible, macrophage	Synthesis of nitric oxide
M55544	1.10	0	Guanylate nucleotide binding protein 1	GTPase
AF084524	1.10	0.000018	Cellular repressor of E1A-stimulated genes CREG	Inhibits cell growth and/or promotes cell differentiation
AW049768	1.10	0	Tubulointersititial nephritis antigen-related protein	Unknown
U12884	1.00	0	Vascular cell adhesion molecule-1 truncated form T-VCAM-1	Unknown
X52643[3]	1.00	0	Histocompatibility 2, class II antigen A, alpha	Antigen presentation to $CD4^+$ T cells
AI842828[2]	1.00	0.000002	EST UI-M-AP1-agi-h-10-0-UI.s1 Mus musculus cDNA (EST)	Unknown
M55181	1.00	0.000311	Preproenkephalin 2	Five-amino-acid neuropeptide with morphin-like activity

Genbank Accession	Signal ratio (log of 2)	Change p-value	Gene description	Gene function
AJ007970[3]	1.00	0	GBP-2 protein	GTPase inducible by IFN-γ in macrophages
AJ007971	1.00	0	IIGP protein	IFN-γ-inducible GTPase
M16118[3]	1.00	0.000009	T-cell receptor insulin (A-chain) reactive alpha chain VJC	T-cell receptor
X75129	1.00	0	Xanthine dehydrogenase	Catalyzes oxidation of hypoxanthine, xanthine, and purine.
AA958903[2]	1.00	0.000001	EST: ua19f08.r1 Mus musculus cDNA (EST)	Unknown
AW047476[2]	1.00	0	EST: UI-M-BH1-all-g-04-0-UI.s1 Mus musculus cDNA (EST)	Unknown
D31788[3]	1.00	0.000001	BP-3 alloantigen, bone marrow stromal cell antigen 1 (BST-1)	CD157 with ADP-ribosyl cyclase and NAD glycohydrolase activities
AJ006474	1.00	0.000004	Carbonic anhydrase III	Protects proteins from oxidation catalyzed by iron-containing degradation products of haemoglobin and myoglobin
AV239653[2]	1.00	0.000006	EST: Mus musculus cDNA, 3′ end/clone = 4732435F04 (EST)	Unknown
b. Genes that show reduced expression in 4T1 tumor following IL-12 treatment				
AA592182	−1.8	0	Nephronectin	Extracellular matrix protein associated with integrin α8β1
AW047875[2]	−1.8	0	EST, UI-M-BH1-als-g-03-0-UI.s1	Unknown
Y13185	−1.7	0.000002	Matrix metalloproteinase 10	Modulator of extracellular matrix composition and structure
M76124	−1.6	0	EGP314, epithelial glycoprotein	Involved in metastasis
Y17793	−1.6	0.000063	Roundabout homolog 1, mouse homologue of human DUTT1	Lung development
M83219	−1.5	0	MRP14, myeloid related protein	Neutrophil and monocyte activation
U43327	−1.4	0	Lamc2, Laminin, gamma 2	Constituent of lamin-5, a major adhesion ligand of epithelial cells
AJ242625	−1.4	0.000001	Dmp-1, dentin matrix protein-1	Involved in matrix mineralization
M83218	−1.3	0	MRP8, intracellular calcium-binding protein	Neutrophil and monocyte activation
U88064	−1.3	0	Basonuclin	Increases transcription of the ribosomal RNA genes
AF087825	−1.3	0.000011	Claudin 7	A transmembrane protein that seals tight junctions
AA716963[2]	−1.2	0	EST, vu69f10.r1	Unknown
AF017994	−1.2	0.000008	Peg1/MEST	Imprinted gene of unknown function

Genbank Accession	Signal ratio (log of 2)	Change p-value	Gene description	Gene function
				expressed in mouse embryo
C76643[3]	−1.2	0.000002	EST, D15Ertd30e	Unknown
X53798[3]	−1.1	0.000002	MIP2, macrophage inflammatory protein-2	Chemotactic for monocytes/macrophages
AJ242912[3]	−1.1	0.000003	Decysin, disintegrin metalloprotease	Expressed in macrophages and dendritic cells
AV112006	−1.1	0.000025	Procollagen, type XV	Precursor of constituent of skin and bone

[1]The signal ratios given (in log of 2) have been normalized as follows the 50th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control, assuming that this was at least 10. The bottom tenth percentile was used as a test for correct background subtraction. This was never less than the negative of the synthetic positive control. Each gene was normalized to itself by making a synthetic positive control for that gene and dividing all measurements for that gene by this positive control, assuming it was at least 0.01. This synthetic control was the median of the gene's expression values over all the samples. Lastly, normalized values below 0 were set to 0. Change *p* values reflect statistical significance in differential expression between the 2 groups (IL-12-treated *vs*. PBS-treated).

[2]EST clones.

[3]Genes with primary immune cell origins.

The gene lists include only those that were reproduced in 2 separate experiments (72% reproducibility rate).