Signatures Associated with Rejection or Recurrence in HER-2/*neu*–Positive Mammary Tumors

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Abstract

We have previously shown T-cell–mediated rejection of the neu-overexpressing mammary carcinoma cells (MMC) in wild-type FVB mice. However, following rejection of primary tumors, a fraction of animals experienced a recurrence of a neu antigen-negative variant (ANV) of MMC (tumor evasion model) after a long latency period. In the present study, we determined that T cells derived from wild-type FVB mice can specifically recognize MMC by secreting IFN-γ and can induce apoptosis of MMC *in vitro*. Neu transgenic (FVBN202) mice develop spontaneous tumors and cannot reject it (tumor tolerance model). To dissect the mechanisms associated with rejection or tolerance of MMC tumors, we compared transcriptional patterns within the tumor microenvironment of MMC undergoing rejection with those that resisted it either because of tumor evasion/antigen loss recurrence (ANV tumors) or because of intrinsic tolerance mechanisms displayed by the transgenic mice. Gene profiling confirmed that immune rejection is primarily mediated through activation of IFN-stimulated genes and T-cell effector mechanisms. The tumor evasion model showed combined activation of Th1 and Th2 with a deviation toward Th2 and humoral immune responses that failed to achieve rejection likely because of lack of target antigen. Interestingly, the tumor tolerance model instead displayed immune suppression pathways through activation of regulatory mechanisms that included in particular the overexpression of interleukin-10 (IL-10), IL-10 receptor, and suppressor of cytokine signaling (SOCS)-1 and SOCS-3. These data provide a road map for the identification of novel biomarkers of immune responsiveness in clinical trials. [Cancer Res 2008;68(7):2436–46]

Introduction

Challenges in the immune therapy of cancers include a limited understanding of the requirements for tumor rejection and prevention of recurrences after successful therapy. Evaluation of T-cell responses in human tumors based predominantly on the metastatic melanoma model has clearly shown that the tumor-bearing status primes systemic immune responses against tumor-associated antigens, which, however, are insufficient to induce tumor rejection (1, 2). Moreover, the experience gathered through the induction of tumor antigen–specific T cells by vaccines has shown that the frequency of tumor antigen–specific T cells in the circulation (3, 4) or in the tumor microenvironment (5, 6) does not directly correlate with successful rejection or prevention of recurrence (7). Similarly, patients with preexisting immune responses against HER-2/*neu* are not protected from the development of HER-2/*neu*–expressing breast cancers (8). Although several and contrasting reasons have been proposed to explain this paradox, two lines of thoughts summarize these hypotheses: either tolerogenic and/or immune-suppressive properties of tumors may hamper T-cell function (9–11) or characteristics of the tumor microenvironment could induce tumor escape and evade the antitumor function of an otherwise effector T cells (12, 13).

In spite of this paradoxical coexistence of tumor-specific T cells and their target antigen-bearing cancer cells, recent observations in cancer patients suggest that T cells control tumor growth and mediate its rejection. Galon et al. and others (14–16) observed that T cells modulate the growth of human colon cancer and T-cell infiltration of primary lesions may forecast a better prognosis. In addition, these authors observed that tumor-infiltrating T cells in cancers with good prognosis displayed transcriptional signatures typical of activated T cells, such as the expression of IFN-stimulated genes (ISG), IFN-γ itself, and cytotoxic molecules, particularly granzyme B (15). Similar observations were reported by others in human ovarian carcinoma (17). These important observations derived from human tissues provide novel prognostic markers but cannot address the causality of the association between T-cell infiltration and natural history of cancer. Recent reports based on adoptive transfer of tumor-specific T cells suggest a cause-effect relationship between the administration of T cells and tumor rejection (18). However, the complexity of the therapy associated with adoptive transfer of T cells, which includes immune ablation and systemic administration of interleukin (IL)-2, prevents a clear interpretation of this causality.

We, therefore, adopted an experimental model that could address the paradoxical relationship between adaptive immune responses against cancer antigens and rejection or persistence of antigen-bearing cancers with the intent of comparing functional signatures between the experimental model and previous human observation that could shed mechanistic information on this relationship and potentially provide novel predictive or prognostic biomarkers to be tested in the clinical settings. In this study, we compared transcriptional patterns of mammary tumors undergoing rejection

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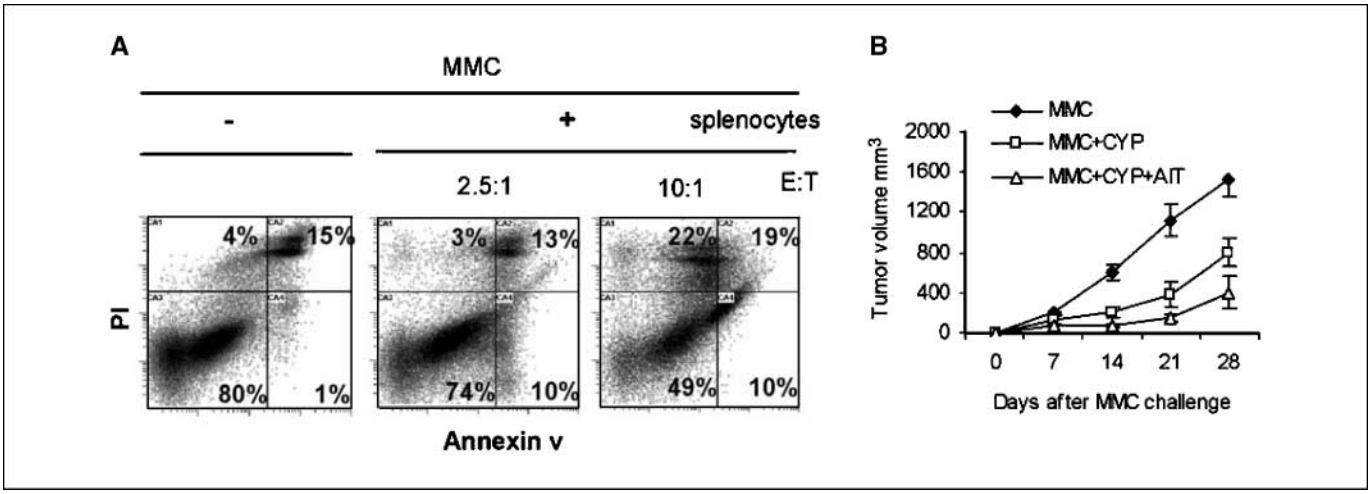

Figure 1. T cells derived from wild-type FVB mice will induce apoptosis in MMC *in vitro* but fail to reject MMC in FVBN202 mice following adoptive immunotherapy. *A,* flow cytometry analysis of MMC after 24 h of culture with splenocytes of FVB mice following three-color staining. Gated neu-positive cells were analyzed for the detection of Annexin V^+ and PI^+ apoptotic cells. Data are representative of quadruplicate experiments. *B,* donor T cells were enriched from the spleen of FVB mice using nylon wool column following the rejection of MMC. FVBN202 mice ($n = 4$) were injected with cyclophosphamide (*CYP*) followed by inoculation with MMC (4×10^6 cells per mouse) and tail vein injection of donor T cells. Control groups were challenged with MMC in the presence or absence of cyclophosphamide treatment. Tumor growth was monitored twice weekly. *AIT,* adoptive immunotherapy.

with that of related tumors that evaded immune recognition through antigen loss (evasion model) or resided in tolerized transgenic mice (tolerogenic model). For this purpose, we used FVB mice that reject neu-overexpressing mammary carcinomas (MMC) because of the presence of a potent neu-specific T-cell response. Although MMCs are consistently rejected after a few weeks, occasionally MMCs recur and in such instance they resist further immune pressure by invariably loosing HER-2/neu expression (tumor evasion model; refs. 19, 20). Moreover, FVBN202 mice that constitutively express high levels of HER-2/neu fail to reject MMC because they cannot mount effective antitumor T-cell responses (tolerogenic model). Thus, we compared the tumor microenvironment at salient moments of immune response/evasion/tolerance to gain, in this previously well-characterized model (19, 20), insights about the immune mechanisms leading to tumor rejection and their failure in conditions of tumor evasion or systemic tolerance. Interestingly, the tolerance model, which was expected to show tolerance, displayed immune suppression pathways through activation of regulatory mechanisms that included in particular the overexpression of IL-10, IL-10 receptor, and suppressor of cytokine signaling (SOCS)-1 and SOCS-3.

Materials and Methods

Mice. Wild-type FVB (The Jackson Laboratory) and FVBN202 female mice (Charles River Laboratories) were used throughout these studies. FVBN202 is the rat neu transgenic mouse model in which 100% of females develop spontaneous mammary tumors by 6 to 10 mo of age, with many features similar to human breast cancer. These mice express an unactivated rat *neu* transgene under the regulation of the mouse mammary tumor virus promoter (21). Because of the overexpression of rat neu protein, FVBN202 mice are expected to tolerate the neu antigen as self-protein and in cases where there might be a weak neu-specific immune response before the appearance of spontaneous mammary tumors are still well tolerated (22, 23). On the other hand, rat neu protein is seen as non–self-antigen by the immune system of wild-type FVB mice, resulting in aggressive rejection of primary MMC (19, 24). The studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Tumor cell lines. The MMC cell line was established from a spontaneous tumor harvested from FVBN202 mice as previously described (11, 15). Tumors were sliced into pieces and treated with 0.25% trypsin at 4°C for 12 to 16 h. Cells were then incubated at 37°C for 30 min, washed, and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; refs. 19, 20). The cells were analyzed for the expression of rat neu protein before use. Expression of rat neu protein was also analyzed before each experiment and antigen-negative variants (ANV) were reported accordingly (see Results).

***In vivo* tumor challenge.** Female FVB or FVBN202 mice were inoculated s.c. with MMC (4×10^6 to 5×10^6 cells per mouse). Animals were inspected twice every week for the development of tumors. Masses were measured with calipers along the two perpendicular diameters. Tumor volume was calculated by the following formula: $V = (L \times W^2) / 2$, where L is the length and W is the width. Mice were sacrificed before a tumor mass exceeded 2,000 mm^3.

IFN-γ ELISA. Secretion of MMC-specific IFN-γ by lymphocytes was detected by coculture of lymphocytes (4×10^6 cells) with irradiated MMC or ANV (15,000 rads) at 10:1 E:T ratios in complete medium (RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin) for 24 h. Supernatants were then collected and subjected to IFN-γ ELISA assay using a Mouse IFN-γ ELISA Set (BD PharMingen) according to the manufacturer's protocol. Results were reported as the mean values of duplicate ELISA wells.

Flow cytometry. A three-color staining flow cytometry analysis of the mammary tumor cells (10^6 per tube) was carried out using mouse anti-neu (Ab-4) antibody (Calbiochem), control Ig, FITC-conjugated anti-mouse Ig (Biolegend), phycoerythrin (PE)-conjugated Annexin V, and propidium iodide (PI; BD PharMingen) at the concentrations recommended by the manufacturer. Cells were finally added with Annexin V buffer and analyzed at 50,000 counts with the Beckman Coulter EPICS XL within 30 min.

Microarray performance and statistical analysis. Total RNA from tumors was extracted after homogenization using Trizol reagent according to the manufacturer's instructions. The quality of secondarily amplified RNA was tested with the Agilent Bioanalyzer 2000 (Agilent Technologies) and amplified into antisense RNA (aRNA) as previously described (25, 26). Confidence about array quality was determined as previously described (27). Mouse reference RNA was prepared by homogenization of the following mouse tissues (lung, heart, muscle, kidneys, and spleen), and RNA was pooled from four mice. Pooled reference and test aRNA were isolated and amplified in identical conditions during the same amplification/hybridiza-

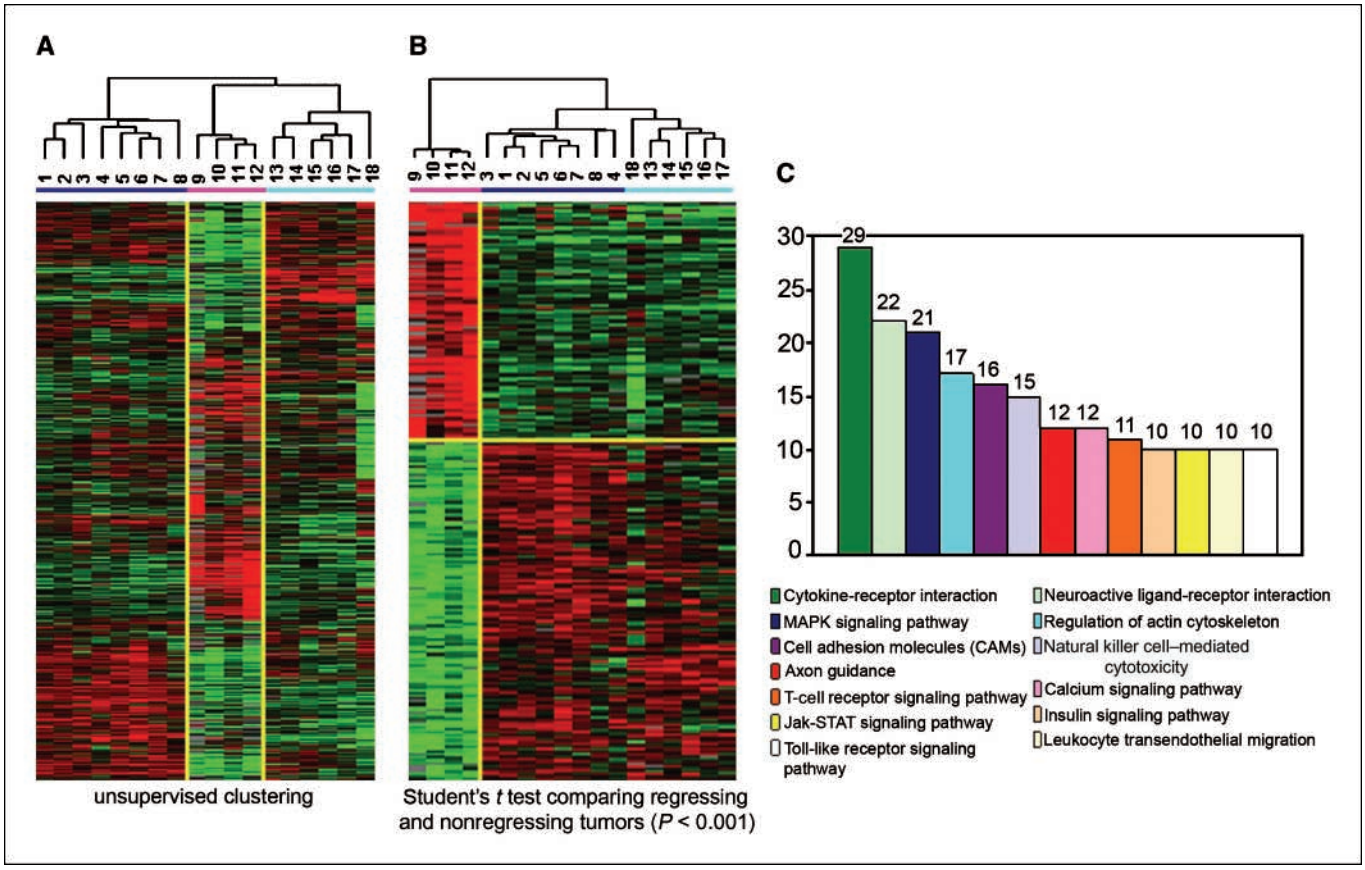

Figure 2. Gene expression profiling and gene oncology pathway analyses in tumor regressing and tumor nonregressing groups. *A,* unsupervised cluster visualization of genes differentially expressed among regressing tumors (*pink bar*) and nonregressing tumors (*blue bar*, evasion model; *turquoise bar*, tolerogenic model). MMC tumors were harvested 10 d after challenge and hybridized to 36K oligo mouse arrays. Genes (11,256) with at least 3-fold ratio change and 80% presence call among all samples were projected using log2 intensity. *B,* supervised cluster analysis ($P < 0.001$, Student's t test; fold change, >3) comparing regressing tumors (*pink bar*) and nonregressing tumors (*blue bar*, evasion model; *turquoise bar*, tolerogenic model). Differentially expressed genes (2,449) have been selected for further analysis. *C,* Gene Ontology databank was queried to assign genes to functional categories and up-regulated genes within the tumor regression group to functional categories.

tion procedure to avoid possible interexperimental biases. Both reference and test aRNA were directly labeled using ULS aRNA Fluorescent Labeling kit (Kreatech) with Cy3 for reference and Cy5 for test samples.

Whole-genome mouse 36K oligo arrays were printed in the Infectious Disease and Immunogenetics Section of Transfusion Medicine, Clinical Center, NIH (Bethesda, MD) using oligos purchased from Operon. The Operon Array Ready Oligo Set version 4.0 contains 35,852 longmer probes representing 25,000 genes and ~38,000 gene transcripts and also includes 380 controls. The design is based on the Ensembl Mouse Database release 26.33b.1, Mouse Genome Sequencing Project, National Center for Biotechnology Information RefSeq, Riken full-length cDNA clone sequence, and other Genbank sequence. The microarray is composed of 48 blocks and one spot is printed per probe per slide. Hybridization was carried out in a water bath at 42°C for 18 to 24 h and the arrays were then washed and scanned on a GenePix 4000 scanner at variable photomultiplier tube to obtain optimized signal intensities with minimum (<1% spots) intensity saturation.

Resulting data files were uploaded to the mAdb databank[8] and further analyzed using BRBArrayTools developed by the Biometric Research Branch, National Cancer Institute (28)[9] and Cluster and TreeView software (29). The global gene expression profiling consisted of 18 experimental samples. Subsequent filtering (80% gene presence across all experiments and at least 3-fold ratio change) selected 11,256 genes for further analysis. Gene ratios were average corrected across experimental samples and displayed according to uncentered correlation algorithm (30).

Statistical analysis. Rate of tumor growth was compared statistically by unpaired Student's t test. Unsupervised analysis was performed for class confirmation using the BRBArrayTools and Stanford Cluster program (30). Class comparison was performed using parametric unpaired Student's t test or three-way ANOVA to identify differentially expressed genes among tumor-bearing, tumor rejection, and relapse groups using different significance cutoff levels as demanded by the statistical power of each comparison. Statistical significance and adjustments for multiple test comparisons were based on univariate and multivariate permutation test as previously described (31, 32).

Results

T-cell–mediated rejection of MMC and relapse of ANV in wild-type FVB mouse. Wild-type FVB mice are capable of rejecting MMC within 3 weeks because of specific recognition of rat neu protein by their T cells as opposed to their transgenic counterparts, FVBN202, which tolerate rat neu protein and fail to reject MMC (19, 24). To determine whether aggressive rejection of primary MMC by T cells may lead to relapse-free survival in wild-type FVB mice, we performed follow-up studies. Animals (n = 15) were challenged with MMC by s.c. inoculation at the right groin.

[8] http://nciarray.nci.nih.gov
[9] http://linus.nci.nih.gov/BRB-ArrayTools.html

Animals were then monitored for tumor growth twice weekly. All mice rejected MMC within 3 weeks after the challenge (Supplementary Fig. S1). However, a fraction of these animals (8 of 15 mice) developed recurrent tumors at the site of inoculation. These relapsed tumors had lost neu expression under immune pressure (19, 24). Relapse-free groups were maintained as breeding colonies and did not show any relapse during their life span. Splenocytes of FVB mice secreted IFN-γ in the presence of MMC only (2,200 pg/mL), whereas no appreciable IFN-γ was detected when lymphocytes were stimulated with ANV (110 pg/mL). No IFN-γ was secreted by splenocytes or tumor cells alone (data not shown).

T cells derived from wild-type FVB mice will induce apoptosis in MMC. To determine whether neu-specific recognition of MMC by T cells may induce apoptosis in MMC, *in vitro* studies were performed. Splenocytes of naive FVB mice were stimulated with irradiated MMC for 24 h followed by 3-day expansion in the presence of IL-2 (20 units/mL). Lymphocytes were then cocultured with MMC (E:T ratio of 2.5:1 and 10:1) for 48 h in the presence of IL-2 (20 units/mL). Control wells were seeded with MMC or splenocytes alone in the presence of IL-2. Cells (floaters and adherents) were collected and subjected to a three-color flow cytometry analysis using mouse anti-rat neu antibody (Ab-4), PE-conjugated anti-mouse Ig, control Ig, Annexin V, and PI. Gated neu-positive cells were analyzed for the detection of Annexin V^+ and PI^+ apoptotic cells. As shown in Fig. 1*A*, 80% of MMC were Annexin V^- and PI^- in the absence of lymphocytes, whereas only 49% of MMC were Annexin V^- and PI^- in the presence of lymphocytes at 10:1 E:T ratio. At a lower E:T ratio (2.5:1), there was a slight dropping in the number of viable MMC (from 80% to 74%) but marked increase in the number of early apoptotic cells (Annexin V^+/PI^-) from 1% to 10%. At a higher E:T ratio (10:1), early (Annexin V^+/PI^-) or late (Annexin V^+/PI^+) apoptotic cells and necrotic cells (Annexin V^-/PI^+) were markedly increased.

Adoptive immunotherapy of FVBN202 mice using T cells derived from wild-type FVB donors failed to reject MMC. To determine whether T cells of FVB mice with neu-specific and antitumor activity may protect FVBN202 mice against MMC challenge, adoptive immunotherapy was performed. Using nylon wool column, T cells were enriched from the spleen of FVB donor mice following the rejection of MMC. FVBN202 recipient mice were injected i.p. with cyclophosphamide (100 μg/g) to deplete endogenous T cells. After 24 h, animals were challenged with MMC tumors (4×10^6 cells per mouse). Four to 5 h after tumor challenge, donor T cells were transferred into FVBN202 mice (6×10^7 cells per mouse) by tail vein injections. Control FVBN202 mice were challenged with MMC in the presence or absence of cyclophosphamide treatment. Animals were then monitored for tumor

Table 1. Differentially expressed ISGs, chemokines, and their receptors

		Mouse	Human		Rejection	Controls
CXC chemokines and receptors						
Cxcl2	Chemokine (C-X-C motif) ligand 2	*GRO; MIP-2;KC?*	*GROξ;MGSA-ξ*	CXCR2	14.54	0.44
Cxcl1	Chemokine (C-X-C motif) ligand 1	*GRO; MIP-2;KC?*	*GROa;MGSA-ξ*	CXCR2>1	5.40	0.70
Cxcl11	Chemokine (C-X-C motif) ligand 11	*I-TAC*	*I-TAC*	CXCR3	3.93	0.68
CC chemokines and receptors						
Ccl1	Chemokine (C-C motif) ligand 1	*TCA-2;P500*	*I-309*	CCR8	2.15	0.80
Ccl4	Chemokine (C-C motif) ligand 4	*MIP-1ξ*	*MIP-1ξ*	CCR5	5.06	0.63
Ccl5	Chemokine (C-C motif) ligand 5	*RANTES*	*RANTES*	CCR1;3,5	5.42	0.62
Ccl5	Chemokine (C-C motif) ligand 5	*RANTES*	*RANTES*	CCR1;3,5	3.96	0.67
Ccl6	Chemokine (C-C motif) ligand 6	*C10;MRP-1*	Unknown	Unknown	3.79	0.68
Ccl8	Chemokine (C-C motif) ligand 8	*MCP-2*	*MCP-2*	CCR3;5	5.94	0.60
Ccl9	Chemokine (C-C motif) ligand 9	*MRP-2;CCF18;MIP-1ξ*	Unknown	CCR1	6.72	0.58
Ccl11	Small chemokine (C-C motif) ligand 11	*Eotaxin*	*Eotaxin*	CCR3	4.34	0.64
Ccl22	Chemokine (C-C motif) ligand 22	*ABCD-1*	*MDC/STCP-1*	CCR4	4.08	0.74
Ccrl2	Chemokine (C-C motif) receptor-like 2			CCL2, 7, 12, 13, 16	6.58	0.62
Ccr10	Chemokine (C-C motif) receptor 10			CCL27, 28	2.22	0.83
Cklf	Chemokine-like factor				2.86	0.74
Darc	Duffy blood group, chemokine receptor				2.83	0.86
Cklf	Chemokine-like factor, transcript variant 1				2.82	0.74
ISGs						
Ifna2	IFN-α2				2.91	0.80
Ifng	IFN-γ				2.89	0.72
Ifi202b	IFN-activated gene 202B				6.05	0.60
Ifi27	IFN, α-inducible protein 27				4.68	0.64
Ifi204	IFN-activated gene 204				4.14	0.67
Ifitm1	IFN-induced transmembrane protein 1				3.73	0.75
Irf6	IRF6				3.59	0.76
Ifit1	IFN-induced protein with tetratricopeptide repeats 1				3.33	0.77
Irf4	IRF4				2.97	0.73
Mx1	Myxovirus (influenza virus) resistance 1				2.63	0.76
Stat2	STAT2				2.35	0.78
Stat6	STAT6				2.14	0.80
Irf2bp1	IRF2 binding protein 1				0.29	1.42

Table 2. Differentially expressed cytokines and signaling molecules

Genes up-regulated in the rejection model				Genes down-regulated in the rejection model			
Symbol	Description	Reject	Control	Symbol	Description	Reject	Control
ILs and receptors							
Il1a	IL-1α	2.71	0.81				
Il1b	IL-1β	8.91	0.54				
Il1f9	IL-1 family, member 9	1.97	0.82				
Il5	IL-5	1.64	0.87				
Il7	IL-7	3.11	0.84				
Il17f	IL-17F	3.86	0.68				
Il31	IL-31	2.16	0.80				
		1.00	1.00				
Il1rap	IL-1 receptor accessory protein, transcript variant 2	3.56	0.70				
Il2rg	IL-2 receptor, γ chain	1.75	0.85				
Il7r	IL-7 receptor	2.46	0.88				
Il23r	IL-23 receptor	7.38	0.63				
Il17rb	IL-17 receptor B	4.46	0.65				
		1.00	1.00				
Cytotoxic and proapoptotic molecules		1.00	1.00				
Gzmb	Granzyme B	1.90	0.83				
Gzmb	Granzyme B	1.57	0.88				
Ctla2a	CTL-associated protein 2α	3.60	0.69				
Klra9	Killer cell lectin-like receptor subfamily A, member 9	1.95	0.87				
Klrd1	Killer cell lectin-like receptor, subfamily D, member 1	7.36	0.57				
Fasl	Fas ligand [tumor necrosis factor (TNF) superfamily, member 6]	2.78	0.75	*Tnfaip1*	TNF, α-induced protein 1	0.51	1.21
Tnfsf11	TNF (ligand) superfamily, member 11	2.56	0.80				
Tnfrsf1b	TNF receptor superfamily, member 1b	2.21	0.83	*Tnfrsf12a*	TNF receptor superfamily, member 12a	0.14	1.52
Tnfrsf4	TNF receptor superfamily, member 4	2.77	0.73	*Ngfrap1*	Nerve growth factor receptor (TNFRSF16)-associated protein 1	0.38	1.15
TLRs and lymphocyte signaling							
Tlr4	TLR4	2.14	0.80				
Tlr6	TLR6	2.98	0.86				
Il4i1	IL-4 induced 1	3.34	0.77				
Alcam	Activated leukocyte cell adhesion molecule	2.12	0.81				
Bcl2a1c	B-cell leukemia/lymphoma 2–related protein A1c	3.41	0.70				
Itk	IL2-inducible T-cell kinase	2.63	0.76	*Ilf3*	IL enhancer binding factor 3, transcript variant 2	0.44	1.27
Ebf4	Early B-cell factor 4	6.32	0.75				
Ly6a	Lymphocyte antigen 6 complex, locus A	2.83	0.74				
Ly6c	Lymphocyte antigen 6 complex, locus C	3.18	0.72	*Ly6e*	Lymphocyte antigen 6 complex, locus E	0.22	1.53
Ly6f	Lymphocyte antigen 6 complex, locus F	3.62	0.67	*Ly6e*	lymphocyte antigen 6 complex, locus E	0.47	1.18
Ly6f	Lymphocyte antigen 6 complex, locus F	2.41	0.78	*Btla*	B and T lymphocyte-associated, transcript variant 2	0.38	1.32
Lck	Lymphocyte protein tyrosine kinase	2.26	0.79	*Bcap31*	B-cell receptor–associated protein 31	0.34	1.36
Tagap	T-cell activation Rho GTPase-activating protein	2.36	0.78	*Tiam2*	T-cell lymphoma invasion and metastasis 2	0.47	1.24
Tlx1	T-cell leukemia, homeobox 1	7.36	0.65	*Ikbkap*	Inhibitor of κ light polypeptide enhancer in B cells	0.44	1.27
Tcl1b1	T-cell leukemia/lymphoma 1B, 1	2.37	0.78				
Nkrf	NF-κB repressing factor	3.90	0.75				
Nkiras2	NF-κB inhibitor interacting Ras-like protein 2	2.48	0.82	*Irak1bp1*	IL-1 receptor–associated kinase 1 binding protein 1	0.33	1.26

(*Continued on the following page*)

Table 2. Differentially expressed cytokines and signaling molecules (Cont'd)

Genes up-regulated in the rejection model				Genes down-regulated in the rejection model			
Symbol	Description	Reject	Control	Symbol	Description	Reject	Control
Nfkbiz	NF-κB light polypeptide gene enhancer in B-cell inhibitor, ζ	3.83	0.68	*Irak1*	IL-1 receptor–associated kinase 1	0.31	1.40
Nfat5	Nuclear factor of activated T cells 5, transcript variant b	3.34	0.71				
FC-type receptors							
Lilrb4	Leukocyte immunoglobulin-like receptor, subfamily B, member 4	3.60	0.74				
Mgl1	Macrophage galactose *N*-acetyl-galactosamine–specific lectin 1	6.23	0.59				
Mgl2	Macrophage galactose *N*-acetyl-galactosamine–specific lectin 2	2.25	0.79				
Msr1	Macrophage scavenger receptor 1	2.78	0.80				
Immunoglobulins							
Igh-6	Immunoglobulin heavy chain 6	7.43	0.56				
Igh-6	Immunoglobulin heavy chain 6 (heavy chain of IgM)	2.39	0.78				
Igj	Immunoglobulin joining chain	2.86	0.74				
Igk-V28	Immunoglobulin κ chain variable 28	2.82	0.79				
Igl-V1	Immunoglobulin λ chain, variable 1	3.69	0.76				
Igl-V1	Immunoglobulin λ chain, variable 1	2.03	0.82				
Igkv4-90	Immunoglobulin light chain variable region	1.82	0.84				

growth. As shown in Fig. 1*B*, cyclophosphamide treatment of animals resulted in retardation of tumor growth in FVBN202 mice as expected. Student's *t* test analysis on days 14, 21, and 28 after challenge showed significant differences between these two groups ($P = 0.005$, 0.007, and 0.01, respectively). Adoptive transfer of neu-specific effector T cells from MMC-sensitized FVB mice into cyclophosphamide-treated FVBN202 groups did not significantly inhibit tumor growth compared with cyclophosphamide-treated control groups ($P > 0.05$). Adoptive transfer of neu-specific effector T cells from untreated FVB mice into cyclophosphamide-treated FVBN202 groups showed similar trend of tumor growth (data not shown). These experiments suggest that T-cell responses associated with MMC rejection in wild-type FVB mice (19) may represent an epiphenomenon with no true cause-effect relationship or that FVBN202 mice retain tolerogenic properties in spite of cyclophosphamide treatment that can hamper the function of potentially effective anticancer T-cell responses. We favor the second hypothesis based on our previous depletion experiments that showed the requirement of endogenous effector T cells of FVB mice for rejection of MMC tumors (19).

Genetic signatures defining rejection or tolerance of MMC tumors. To ascertain whether the presence of neu-specific effector T cells may trigger a cascade of events that may determine success or failure in tumor rejection, wild-type FVB and FVBN202 mice were inoculated with MMC. Historically, all FVB mice reject MMC; however, a fraction of mice develop a latent tumor relapse. In contrast, FVBN202 mice fail to reject transplanted MMC. Ten days after the tumor challenge, transplanted MMC tumors were excised and RNAs were extracted from both FVB and FVBN202 carrier mice based on the presumption that the biology of the former would be representative of active tumor rejection and that of the latter would be representative of tumor tolerance. Thus, the timing of tumor harvest was chosen to capture transcriptional signatures associated with the active phase of the tumor rejection process in wild-type FVB mice in comparison with the corresponding tolerance of spontaneous mammary tumors in the FVBN202 mice. We speculated that this comparison would allow distinguishing whether tolerance was due to inhibition of T-cell function within the tumor microenvironment of spontaneous mammary tumors or to a complete absence of such responses. To enhance the robustness of the comparison, a similar analysis was performed extracting total RNA from spontaneous tumor in FVBN202 mice. In addition, RNA was extracted from MMC tumors in wild-type FVB mice that experienced tumor recurrence following the initial rejection of MMC. This second analysis allowed the comparison of mechanisms of tumor evasion in the absence of known tolerogenic effects. Microarray analyses were then performed on the amplified RNA (aRNA) extracted from these tumors using 36K oligo mouse arrays. Hence, genes considered as differentially expressed in the study groups could represent either MMC tumor cells or host cells infiltrating the tumor site. Probes with missing values >80% or a change <3-fold were excluded from further analysis. Unsupervised clustering showed outstanding differences among the three experimental groups (Fig. 2*A*). Genes of spontaneous mammary tumors (samples 13, 15, 16, and 17) clustered closely to those of transplanted MMC (samples 14 and 18) excised from FVBN202 mice, suggesting that the biology of MMC tumors remains comparable between these two experimental models of tolerance. Global transcriptional patterns associated with tumor relapse (samples 1–8) were instead clearly different from those of spontaneous mammary tumors or MMC transplanted in tolerant FVBN202 mice, suggesting that a completely different biological process was at the basis of tumor evasion through loss of target antigen expression. Finally, MMC tumors undergoing rejection

(samples 9–12) were clearly separated from either kind of nonregressing tumors.

Biomarkers of rejection. Our first class comparison searched for differences between the four tumor samples undergoing rejection and the rest of the MMC tumors whether belonging to the tolerogenic or the evasion process. This approach followed the exclusion principle whereby factors determining the occurrence of a phenomenon should be discernible from unrelated ones independent of the causes preventing its occurrence. An unpaired Student's *t* test with a cutoff set at $P < 0.001$ identified 2,449 genes differentially expressed between regressing and nonregressing tumors (permutation $P = 0$), of which 1,003 genes were up-regulated in regressing tumors clearly distinguishing the two categories (Fig. 2*B*). Of those, a large number were associated with immune regulatory functions. Gene Ontology databank was queried to assign genes to functional categories and up-regulated pathways were ranked according to the number of genes identified by the study belonging to each category (Fig. 2*C*). The top categories of genes that were up-regulated in primary rejected MMC tumors were cytokine-cytokine interaction, mitogen-activated protein kinase signaling, cell adhesion–related transcripts and axon guidance, T-cell receptor, Janus-activated kinase-signal transducer and activator of transcription (STAT), and Toll-like receptor (TLR) signaling pathways. Natural killer cell–mediated cytotoxicity and calcium signaling pathways were also enriched in up-regulated genes. In contrast, very little evidence of immune activation could be observed in either category of nonregressing tumors, suggesting that lack of immune rejection is due to absent or severely hampered immune responses in the tumor microenvironment independent of the mechanisms leading to this resistance.

To better describe the immunologic pathways associated with tumor regression, we organized genes with immune function into three categories, including chemokines, IFN-α2, IFN-γ, and ISGs (Table 1), and cytokines and signaling molecules (Table 2). From this analysis, it became clear that T-cell infiltration into tumors was associated with activation of various pathways leading to the expression of IFN-α, IFN-γ, and several ISGs, including *IFN regulatory factor 4* (*IRF4*), *IRF6*, and *STAT2*. In addition, several cytotoxic molecules were overexpressed, including calgranulin A, calgranulin B, and granzyme B, all of them representing classic markers of effector T-cell activation in humans (10) and in mice (33). Thus, tumor rejection in this model clearly recapitulates patterns observed in various human studies in which expression of ISGs is associated with the activation of cytotoxic mechanisms among which granzyme B seems to play a central role.

Is there a difference between signatures of immune evasion and immune tolerance? As shown in Table 3, the high expression of IL-10 and the IL-10 receptor-β chain concordant with IRF1 in the tolerogenic model strongly suggests the presence of regulatory mechanism within the microenvironment of MMC-bearing FVBN202 mice. Preferential expression of SOCS-1 and SOCS-3 in the microenvironment of MMC tumors of FVBN202 mice also strongly suggests a marked activation of regulatory functions present in the tolerized host (Table 3).

To further investigate whether similar mechanisms were involved in failure of tumor rejection in tolerance and evasion models, we characterized potential differences between the two models of immune resistance; we compared statistical differences between the tolerogenic and the evasion model comparing the two nonregressing groups by unpaired Student's *t* test using as a significance threshold a *P* value of <0.001. This analysis was performed on preselected genes that had been filtered for an at least 80% presence of data in the whole data set and a minimal fold increase of 3 in at least one experiment (Fig. 3). This analysis identified 1,369 genes differentially expressed by the two groups (multivariate permutation test $P = 0$), of which 462 were up-regulated in the tolerogenic model and 907 were up-regulated in the tumor evasion model (Fig. 3*A*). Several of these genes were specifically expressed by either group, although the expression of a few of them was shared by the regressing MMC tumors. Annotations and functional analysis based on Gene Ontology database showed that the predominant functional classes of genes transcriptionally active in one of the other type of nonresponding MMC tumors were not associated with classic activation of T-cell effector functions but rather were associated with more general metabolic processes (Fig. 3*B* and *C*). However, detailed analysis of transcripts associated with immunologic function (Table 3) defined dramatic differences between the two mechanisms of immune resistance.

Discussion

FVB mice reject primary MMC by T-cell–mediated neu-specific immune responses. However, a fraction of animals develop tumor relapse after a long latency. On the other hand, their transgenic counterparts, FVBN202, fail to mount effective neu-specific immune responses and develop tumors (19). Although FVBN202 mice seem to elicit weak immune responses against the neu protein within a certain window of time (22), the neu-expressing MMC tumors are still well tolerated and animals develop spontaneous mammary tumors. Despite the observation that T cells derived from FVB mice were capable of recognizing MMC and inducing apoptosis in these tumors *in vitro*, adoptive transfer of such effector T cells into FVBN202 mice failed to protect these animals against challenge with MMC.

It has been suggested that T cells play a significant role in determining the natural history of colon (14–16) and ovarian (17) cancer in humans. Transcriptional signatures have been identified that suggest not only T-cell localization but also activation through the expression of IFN-γ, ISGs, and cytotoxic effector molecules such as granzyme B (10). We have recently shown that rejection of basal cell cancer induced by the activation of TLR agonists also is mediated, at least in part, by localization and activation of CD8-expressing T cells with increased expression of cytotoxic molecules (34). Yet, a comprehensive experimental overview of the biological process associated with tumor rejection in its active phase has not been reported. Thus, our first class comparison searched for differences between the four tumor samples undergoing rejection and the rest of the MMC tumors whether belonging to the tolerogenic or the evasion process. Unlike nonregressing tumors (tolerance and evasion models), regressing tumors (rejection model) showed up-regulation of immune activation genes, suggesting that failure in tumor rejection is due to immune evasion or severely hampered immune responses in the tumor microenvironment. A particularly interesting observation was the relative low expression of ISGs, with the exception of Irf2bp1. Although the transcriptional patterns differentiating regressing from nonregressing tumors were striking and in many ways representative of previous observations in humans by our and other groups (35), differences among MMC tumors nonregressing in FVBN202 mice and those relapsing after regression in FVB mice were subtle. We have previously proposed that lack of regression of human tumors is primarily associated with indolent immune responses rather

Table 3. Manually selected genes with immunologic function based on supervised comparison of evasion and tolerogenic tumor models and tumor rejection model

Symbol	Description	Evasion	Tolerogenic	Rejection
Chemokines				
Ccl2	Chemokine (C-C motif) ligand 2	2.304	0.614	0.392
Ccl4	Chemokine (C-C motif) ligand 4	1.899	0.478	0.838
Ccl6	Chemokine (C-C motif) ligand 6	2.525	0.536	0.400
Ccr7	Chemokine (C-C motif) receptor 7	1.441	0.696	0.829
Cxcl10	Chemokine (C-X-C motif) ligand 10	2.341	0.781	0.264
Cxcl9	Chemokine (C-X-C motif) ligand 9	1.440	0.354	2.288
Xcl1	Chemokine (C motif) ligand 1	4.913	0.279	0.282
Cx3cl1	Chemokine (C-X3-C motif) ligand 1	5.120	0.393	0.155
ILs and signaling				
Il12b	IL-12b	1.768	0.866	0.397
Il13	IL-13	1.475	0.779	0.669
Il17d	IL-17D	1.690	0.674	0.633
Il23r	IL-23 receptor	2.048	0.457	0.772
Il2rg	IL-2 receptor, γ chain	1.798	0.742	0.484
Il4	IL-4	2.342	0.497	0.521
Il4i1	IL-4 induced 1	2.290	0.701	0.325
Il6	IL-6	1.105	0.504	2.291
Il7r	IL-7 receptor	1.218	0.439	3.063
Il9	IL-9	1.711	0.801	0.477
Tlr11	TLR11	2.656	0.435	0.540
Blnk	B-cell linker	1.457	0.749	0.726
Bok	Bcl-2–related ovarian killer protein	2.071	0.432	0.822
Vpreb3	Pre-B lymphocyte gene 3	2.712	0.148	2.398
Lcp2	Lymphocyte cytosolic protein 2	1.640	0.588	0.824
Ly6d	Lymphocyte antigen 6 complex, locus D	4.093	0.223	0.567
Nfkb1	NF-κB light chain gene enhancer 1, p105	1.737	0.785	0.477
Pias2	Protein inhibitor of activated STAT2	1.336	0.502	1.458
Pias3	Protein inhibitor of activated STAT3	1.763	0.828	0.427
Stat4	STAT4	1.579	0.685	0.630
Il10	IL-10	0.788	2.528	0.400
Il1r2	IL-1 receptor, type II	0.804	2.504	0.391
Il10rb	IL-10 receptor, β	0.422	2.839	1.174
Socs1	SOCS-1	0.566	4.683	0.308
Socs3	SOCS-3	0.621	1.751	1.120
Bak1	BCL2-antagonist/killer 1	0.663	2.698	0.514
Lsp1	Lymphocyte specific 1	0.609	1.468	1.514
Tank	TRAF family member-associated NF-κB activator	0.718	2.204	0.351
Tlr6	TLR6	0.480	1.141	3.559
ISGs				
Ifnb1	IFN-β1, fibroblast	1.388	0.644	1.003
Irf7	IRF7	1.500	0.677	0.406
Ifrd1	IFN-related developmental regulator 1	2.248	0.337	1.011
Ifnar1	IFN (α and β) receptor 1	1.884	0.855	0.356
Igtp	IFN-γ–induced GTPase	1.863	0.671	0.524
Irf1	IRF1	0.549	2.715	0.671
Irf3	IRF3	0.845	1.759	0.479
Irf6	IRF6	0.601	1.816	1.282
Ifngr1	IFN-γ receptor 1	0.582	4.515	0.308
Ifrg15	IFN-α–responsive gene	0.591	1.913	1.168

than dramatic changes in the tumor microenvironment enacted to counterbalance a powerful effector immune response (13, 31, 35). The MMC tolerance model allowed investigating this hypothesis at least in this restricted case. Spontaneous mammary tumors or transplanted MMC tumors in FVBN202 mice displayed immune-suppressive properties that were identified by transcriptional profiling through the activation of genes associated with regulatory function. This would occur only in case an indolent adaptive immune response occurred in these transgenic mice and was hampered at the tumor site by a mechanism of peripheral suppression. If, however, central tolerance was the reason for the lack of rejection, minimal changes should be observed in tolerogenic model similar to those detectable in the tumor evasion model where MMC tumors lost expression of HER-2/*neu* and become

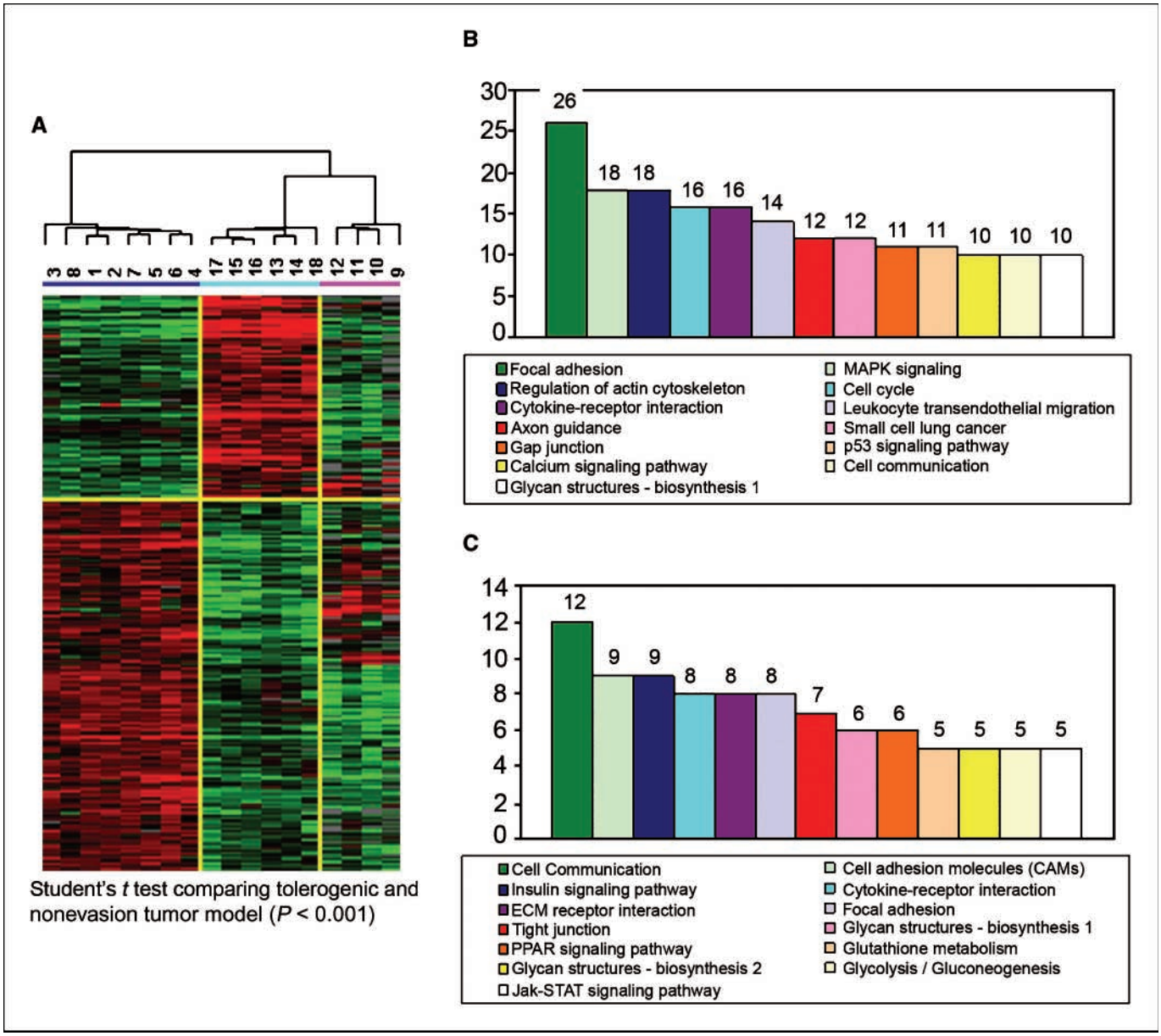

Figure 3. Gene expression profiling and gene oncology pathway analyses in tolerance and evasion models. *A,* supervised cluster analysis ($P < 0.001$, Student's *t* test; fold change, >3) comparing evasion (*blue bar*) and tolerogenic group (*turquoise bar*). Differentially expressed genes (1,326) have been visualized also including tumor regression samples (*pink bar*). Gene ontology pathway analysis projecting either up-regulated pathways in evasion group (*B*, 854 genes) or tolerogenic tumor models (*C*, 475 genes).

irrelevant targets for HER-2/*neu*–specific T-cell responses. The presence of regulatory mechanism within the microenvironment of MMC-bearing FVBN202 mice was associated with increased IL-10 as well as increased expression of SOCS-1 and SOCS-3. It has recently been shown that myeloid-derived suppressor cells (MDCS) induce macrophages to secrete IL-10 and suppress antitumor immune responses (36). Importantly, it was shown that high levels of MDCS in neu transgenic mice would suppress antitumor immune responses against tumors (37). IL-10 is increasingly recognized to be strongly associated with regulatory T-cell (38) and M2-type tumor-associated macrophage function (39), and its expression is mediated in the context of chronic inflammatory stimuli by the overexpression of IRF1. SOCS-1 inhibits type I IFN response, CD40 expression in macrophages, and TLR signaling (40–42). Expression of SOCS-3 in dendritic cells converts them into tolerogenic dendritic cells and supports Th2 differentiation (43). Importantly, tumors that express SOCS-3 show IFN-γ resistance (44).

Unlike tolerance model, recurrence model revealed expression of Igtp, suggesting the involvement of IFN-γ in this model (Table 3). This observation is consistent with our previous findings on the role of IFN-γ in neu loss and tumor recurrence (19). MMC tumors evading immune recognition had undergone a process of complex immune editing that resulted not only in the loss of the HER-2/neu target antigen but also in the up-regulation of various Th2-type cytokines, such as IL-4 and IL-13 (45), and the corresponding transcription factor IRF7 overexpression predominantly associated to a deviation from cellular Th1 to Th2 and humoral type immune

responses (46). In addition, the microenvironment of recurrent tumors was characterized by the coordinate expression of STAT4, IL-12b, IL-23 receptor, and IL-17; this cascade has been associated with the development of Th17-type immune responses that play a dominant role in autoimmune inflammation (47, 48) and T-cell–dependent cancer rejection (49, 50). Because both humoral and cellular immune responses are potentially involved in the rejection of HER-2/neu–expressing tumors (51), these data suggest that a cognitive and active immune response is still attempting to eradicate MMC tumors that may still express subliminal levels of the target antigen. However, the overall balance between host and cancer cells favors, in the end, tumor cell growth because the expression of HER-2/neu, the primary target of both cellular and humoral responses, is critically reduced.

Altogether, these observations suggest that neu antigen loss and subsequent immunologic evasion from cellular Th1 to Th2 and humoral type immune response is a major mechanism in evasion model, whereas peripheral suppression, such as sustained IL-10, SOCS-1, and SOCS-3 expression, is a major player in tolerance model. This conclusion provides a satisfactory explanation for the lack of rejection of MMC tumors in FVBN202 mice receiving adoptively transferred HER-2/neu–specific T cells. In this case, effective T-cell responses exclude central tolerance or peripheral ignorance as the only mechanism potentially hampering their effector function at the tumor site, suggesting that other regulatory mechanisms such as peripheral suppression could be responsible for inactivation of donor effector T cells. High levels of MDSC in neu transgenic mice support this possibility, and the role and mechanisms of MDSC in suppression of adoptively transferred neu-specific T cells remain to be determined in FVBN202 mice.

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References

1. Marincola FM, Rivoltini L, Salgaller ML, Player M, Rosenberg SA. Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence for *in vivo* priming by tumor cells. J Immunother 1996;19:266–77.
2. D'Souza S, Rimoldi D, Lienard D, Lejeune F, Cerottini JC, Romero P. Circulating Melan-A/Mart-1 specific cytolytic T lymphocyte precursors in HLA-A2$^+$ melanoma patients have a memory phenotype. Int J Cancer 1998;78:699–706.
3. Lee K-H, Wang E, Nielsen M-B, et al. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to *in vitro* stimulation but does not lead to tumor regression. J Immunol 1999;163:6292–300.
4. Marincola FM. A balanced review of the status of T cell-based therapy against cancer. J Transl Med 2005;3:16.
5. Panelli MC, Riker A, Kammula US, et al. Expansion of tumor-T cell pairs from fine needle aspirates of melanoma metastases. J Immunol 2000;164:495–504.
6. Zippelius A, Batard P, Rubio-Godoy V, et al. Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. Cancer Res 2004;64:2865–73.
7. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8$^+$ T cells in patients with melanoma. J Immunol 2005;175:6169–76.
8. Disis ML, Calenoff E, McLaughlin G, et al. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res 1994;54:16–20.
9. Lee PP, Yee C, Savage PA, et al. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med 1999;5:677–85.
10. Monsurro V, Wang E, Yamano Y, et al. Quiescent phenotype of tumor-specific CD8$^+$ T cells following immunization. Blood 2004;104:1970–8.
11. Nagorsen D, Voigt S, Berg E, Stein H, Thiel E, Loddenkemper C. Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. J Transl Med 2007;5:62.
12. Marincola FM, Jaffe EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T cell recognition: molecular mechanisms and functional significance. Adv Immunol 2000;74:181–273.
13. Marincola FM, Wang E, Herlyn M, Seliger B, Ferrone S. Tumors as elusive targets of T cell-based active immunotherapy. Trends Immunol 2003;24:335–42.
14. Pages F, Berger A, Camus M, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005;353:2654–66.
15. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4.
16. Galon J, Fridman WH, Pages F. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. Cancer Res 2007;67:1883–6.
17. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003;348:203–13.
18. Dudley ME, Wunderlich JR, Yang JC, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol 2005;23:2346–57.
19. Kmieciak M, Knutson KL, Dumur CI, Manjili MH. HER-2/neu antigen loss and relapse of mammary carcinoma are actively induced by T cell-mediated anti-tumor immune responses. Eur J Immunol 2007;37:675–85.
20. Manjili MH, Arnouk H, Knutson KL, et al. Emergence of immune escape variant of mammary tumors that has distinct proteomic profile and a reduced ability to induce "danger signals." Breast Cancer Res Treat 2006;96:233–41.
21. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A 1992;89:10578–82.
22. Takeuchi N, Hiraoka S, Zhou XY, et al. Anti-HER-2/neu immune responses are induced before the development of clinical tumors but declined following tumorigenesis in HER-2/neu transgenic mice. Cancer Res 2004;64:7588–95.
23. Kmieciak M, Morales JK, Morales J, Grimes M, Manjili MH. Danger signal and nonself entity of tumor antigen are both required for eliciting effective immune responses against HER-2/neu positive mammary carcinoma: implications for vaccine design. Cancer Immunol Immunother. Epub 2008 Feb 16.
24. Knutson KL, Almand B, Dang Y, Disis ML. Neu antigen-negative variants can be generated after neu-specific antibody therapy in neu transgenic mice. Cancer Res 2004;64:1146–51.
25. Wang E, Miller L, Ohnmacht GA, Liu E, Marincola FM. High fidelity mRNA amplification for gene profiling using cDNA microarrays. Nat Biotechnol 2000;17:457–9.
26. Wang E. RNA amplification for successful gene profiling analysis. J Transl Med 2005;3:28.
27. Jin P, Zhao Y, Ngalame Y, et al. Selection and validation of endogenous reference genes using a high throughput approach. BMC Genomics 2004;5:55.
28. Rubinfeld B, Robbins P, el Gamil M, Albert I, Porfiri E, Polakis P. Stabilization of β-catenin by genetic defects in melanoma cell lines. Science 1997;275:1790–2.
29. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998;95:14863–8.
30. Ross DT, Scherf U, Eisen MB, et al. Systematic variation in gene expression patterns in human cancer cell lines. Nat Genet 2000;24:227–35.
31. Wang E, Miller LD, Ohnmacht GA, et al. Prospective molecular profiling of subcutaneous melanoma metastases suggests classifiers of immune responsiveness. Cancer Res 2002;62:3581–6.
32. Basil CF, Zhao Y, Zavaglia K, et al. Common cancer biomarkers. Cancer Res 2006;66:2953–61.
33. Kaech SM, Hemby S, Kersh E, Ahmed R. Molecular and functional profiling of memory CD8 T cell differentiation. Cell 2002;111:837–51.
34. Panelli MC, Stashower M, Slade HB, et al. Sequential gene profiling of basal cell carcinomas treated with imiquimod in a placebo-controlled study defines the requirements for tissue rejection. Genome Biol 2006;8:R8.
35. Mantovani A, Romero P, Palucka AK, Marincola FM. Tumor immunity: effector response to tumor and the influence of the microenvironment. Lancet. Epub 2008 Feb 12.
36. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. J Immunol 2007;179:977–83.
37. Melani C, Chiodoni C, Forni G, Colombo MP. Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity. Blood 2003;102:2138–45.
38. Wu K, Bi Y, Sun K, Wang C. IL-10-producing type 1 regulatory T cells and allergy. Cell Mol Immunol 2007;4:269–75.

39. Biswas SK, Gangi L, Paul S, et al. A distinct and unique transcriptional programme expressed by tumor-associated macrophages: defective NF-κB and enhanced IRF-3/STAT1 activation. Blood 2006;107:2112–22.

40. Fenner JE, Starr R, Cornish AL, et al. Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. Nat Immunol 2006;7:33–9.

41. Mansell A, Smith R, Doyle SL, et al. Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. Nat Immunol 2006;7:148–55.

42. Qin H, Wilson CA, Lee SJ, Benveniste EN. IFN-β-induced SOCS-1 negatively regulates CD40 gene expression in macrophages and microglia. FASEB J 2006;20: 985–7.

43. Li Y, Chu N, Rostami A, Zhang GX. Dendritic cells transduced with SOCS-3 exhibit a tolerogenic/DC2 phenotype that directs type 2 Th cell differentiation *in vitro* and *in vivo*. J Immunol 2006;177:1679–88.

44. Fojtova M, Boudny V, Kovarik A, et al. Development of IFN-γ resistance is associated with attenuation of SOCS genes induction and constitutive expression of SOCS 3 in melanoma cells. Br J Cancer 2007;97:231–7.

45. Kroemer G, Hirsch F, Gonzalez-Garcia A, Martinez C. Differential involvement of Th1 and Th2 cytokines in autoimmune diseases. Autoimmunity 1996;24:25–33.

46. Sasaki S, Amara RR, Yeow WS, Pitha PM, Robinson HL. Regulation of DNA-raised immune responses by cotransfected interferon regulatory factors. J Virol 2002; 76:6652–9.

47. Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nat Rev Immunol 2005;5:521–31.

48. Afzali B, Lombardi G, Lechler RI, Lord GM. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. Clin Exp Immunol 2007;148:32–46.

49. Hao JS, Shan BE. Immune enhancement and anti-tumour activity of IL-23. Cancer Immunol Immunother 2006;55:1426–31.

50. Shan BE, Hao JS, Li QX, Tagawa M. Antitumor activity and immune enhancement of murine interleukin-23 expressed in murine colon carcinoma cells. Cell Mol Immunol 2006;3:47–52.

51. Fulton A, Miller F, Weise A, Wei WZ. Prospects of controlling breast cancer metastasis by immune intervention. Breast Dis 2006;26:115–27.