American Journal of Pathology, Vol. 150, No. 5, May 1997

Tumor Therapy with an Antibody-Targeted Superantigen Generates a Dichotomy between Local and Systemic Immune Responses

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Repeated injections of a fusion protein containing the superantigen staphylococcal enterotoxin A (SEA) combined with a Fab fragment of a tumor-specific antibody is a highly efficient immunotherapy for mice expressing lung melanoma micrometastasis. In the present study, the systemic and local immune responses generated by this therapy were analyzed at a cellular level. Two distinct but coupled immune reactions occurred after repeated therapy. Tumor necrosis factor and macrophage inflammatory protein-1α and -1β were immediately synthesized, in the absence of T lymphocytes, at the local tumor site in the lung. This was followed by the induction of VCAM-1 adhesion molecule expression on pulmonary vascular endothelial cells. Concurrently, the early response in the spleen was characterized by the induction of selective T cells producing interleukin (IL)-2. The primed and expanded SEA-reactive Vβ3- and Vβ11-expressing T lymphocytes accumulated to the tumor area only after Fab-SEA therapy and were not present in the lung when SEA, Fab fragment, or recombinant IL-2 was injected. The tumor-infiltrating T cells produced large amounts of interferon-γ, but no IL-2 or Th2 type of lymphokines were detected at the tumor site in the Fab-SEA-targeted antitumor immune response. These results emphasize the necessity to investigate several sites of antigen presentation to elucidate the effects of immunotherapy. (Am J Pathol 1997, 150: 1607–1618)

Superantigens are bacterial and viral proteins that share the ability to activate a large number of T lymphocytes. Bacterial superantigens bind to major histocompatibility complex (MHC) class II molecules as unprocessed proteins and subsequently interact with T cells expressing particular T cell receptor Vβ chains.[1–3] Superantigens are efficient inducers of cytokine production and T cell cytotoxicity.[4–8] To target superantigenicity to the site of a tumor, we have genetically fused the Fab region of a tumor-reactive monoclonal antibody (MAb) with the superantigen staphylococcal enterotoxin A (Fab-SEA).[9–11]

The effectiveness of this therapy has been demonstrated in syngeneic and xenogeneic animal models.[10–12] The poorly immunogenic B16 melanoma was transfected with the human colon carcinoma antigen C215 and used to evaluate the effects of C215 Fab-SEA treatment in a syngeneic lung metastasis model.[11] Treatment with C215 Fab-SEA fusion protein eradicated 90% of lung tumors in mice carrying established B16-C215 melanoma metastasis. The treatment required three to four repeated injections of Fab-SEA for biological effects and was demonstrated to be dependent on $CD4^+$ and $CD8^+$ T cells. It was also evident that the therapy was dependent on the combination of the Fab fragment and SEA, whereas each alone did not induce any significant therapeutic effect.[9]

Supported by grants from Pharmacia and Upjohn, the Swedish National Cancer Institute (grants 2490 and 2766), the Swedish Medical Research Council (grants 09082 and 10850), and von Kantzow's Foundation.

Accepted for publication January 2, 1997.

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In the present study, we have used immunohistochemistry to investigate the cellular and cytokine responses during repeated Fab-SEA tumor therapy and to document the local mechanism of action *in vivo*. The regional distribution of the fusion protein, the local production of chemokines, and the production of pro-inflammatory cytokines were compared in the tumor area with the responses in a tumor-free primary lymphoid organ. The study illustrated that there were two separate but coupled immune reactions that occurred after therapy and that each reaction contributed significantly to the overall anti-tumor response.

Materials and Methods

Animals

C57BL/6 mice were obtained from Brommice, Ry, Denmark, and kept under pathogen-free conditions. The mice were 8 to 12 weeks of age when used in this study.

Cells and Transfection

The expression vector pKGE839 containing the GA733–2 cDNA (encoding the C215 antigen) was transfected into B16 melanoma cells as described previously.[9] The metastasizing clone 7.B6 was selected from the B16-C215 cells, and 1.5×10^5 of these cells were injected intravenously (i.v.) into mice 18 days before Fab-SEA therapy.

Cloning of C215Fab-SEA

The construction and expression of C215 Fab-SEA was performed as previously described.[9] The fusion protein was expressed in *Escherichia coli* K-12 UL635 and purified on a protein G Sepharose column (Pharmacia LKB Biotechnology), and fractions containing C215 Fab-SEA were passed through a PD-10 column (Pharmacia LKB Biotechnology). The protein was >95% pure as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis. The protein (10 μg/animal) was injected i.v. into tumor-bearing mice at daily intervals consecutively for 4 days. Mice were injected with phosphate-buffered saline (PBS) as a negative control.

Antibodies

Cytokine-specific MAbs used were anti-tumor necrosis factor (TNF) XT22, anti-interferon (IFN)-γ XMG1.2, anti-interleukin (IL)-2 S4B6, anti-IL-4 11B11, and anti-IL-6 20F3 (all from PharMingen, San Diego, CA) and anti-IL-10 16E3 (provided by Dr. John Abrams, DNAX Research Institute, Palo Alto, CA).

Surface specific MAbs used were anti-CD4, anti-CD8, anti-Mac1, anti-Vβ3, and anti-Vβ11 (all from PharMingen), anti-NLDC (dendritic cell marker; BioSource), and anti-Vβ8 KJ16.[13]

Immunohistochemistry

Cryopreserved tissue sections were cut 8 μm thick and fixed with 2% formaldehyde in PBS for 20 minutes at 20°C. Slides were then washed with Earle's balanced salt solution (Gibco BRL, Gaithersburg, MD) and stored at −20°C for future use. Intracellular cytokine tissue staining was done by the saponin procedure as previously described[14] with slight modifications. Briefly, the sections were blocked with avidin (Vector Laboratories, Burlingame, CA) for 4 hours in 0.1% saponin. The sections were then incubated with biotin (Vector) in 0.1% saponin for 15 minutes. The sections were incubated overnight with anti-cytokine MAb at 2 μg/ml with 0.1% saponin. Sections were then incubated with biotinylated mouse-absorbed rabbit anti-rat 1:500 (Vector Labs) for 30 minutes followed by an incubation for 30 minutes with ExtraAvidin AP (1:2870; Sigma Chemical Co., St. Louis, MO). The substrate used was filtered New Fuchsin Red (DAKO, Glostrup, Denmark) for 10 minutes.

For detection of surface markers, the above procedure was slightly altered. Sections were incubated before fixation with 3.0 μg/ml primary MAb in 0.15% NaN_3 at 4°C for 30 minutes. After washing in Earle's balanced salt solution, the sections were fixed with 2% formaldehyde for 10 minutes. The protocol was then the same as described above but without the use of saponin.

For simultaneous detection of intracellular cytokines and surface cell markers, the staining procedure was performed as follows. Sections were incubated before fixation with 3.0 μg/ml of a surface marker detecting MAb in 0.15% NaN_3 at 4°C for 30 minutes and were subsequently fixed with 2% formaldehyde for 10 minutes. The surface markers were detected after indirect staining technique using the New Fuchsin Red as the developing substrate. In between the surface and the intracellular staining, biotin dissolved in 0.1% saponin Earle's balanced salt solution was incubated for 30 minutes. The intracellular cytokine MAb was then incubated overnight and the described above procedure was used with Blue AP (Vector) as the developing substrate.

All staining was performed in at least three separate experiments in three different animals and standard deviations were calculated.

Specificity of Cytokine Staining

To test the specificity of the immunohistochemistry, relevant recombinant cytokines were used to block specific cytokine staining. The cytokine-specific MAbs (2 μg/ml) were incubated overnight at 4°C with the corresponding recombinant cytokine at a protein concentration of 20 μg/ml. These supernatants were then added as primary steps and the staining was carried out as described above.

Computer-Aided Image Analysis

All of the cytokines detected and cellular infiltration were examined with a Polyvar 2 microscope (Reichert-Jung, Vienna, Austria) equipped with a 3 CCD color camera (Sony Corp., Tokyo, Japan) and a PC-based Quantimet 600 image analysis system (Leica Cambridge, Cambridge, UK) as previously described.[15] Briefly, each microscope field was quantified using specific color detection of single pixels in an acquired image. The computer was then able to quantify the stained signal by using positive pixels to measure how much cytokine or how many immunological cells were present for a given area. A summary was then created for the entire tissue section, which was made up of many different microscope fields (50 to 75) due to the high magnification (×250). These detection values were then used for assessing all cytokines, cell phenotypes, and controls.

Cytokine Levels in Serum after C215 Fab-SEA Treatment

Cytokine protein levels in the serum were measured by specific enzyme-linked immunosorbent assay according to instructions from the manufacturer (Holland Biotechnology, Leiden, The Netherlands).

Immunological Significance of C215 Fab-SEA

To elucidate the significance of the immune response generated by C215 Fab-SEA, similar tumor-bearing animals were injected i.v. with C215 Fab-SEA (10 μg/animal), SEA (3.5 μg/animal), Fab fragment (6.4 μg/animal), and recombinant (r)IL-2 (20,000 U/animal) at daily intervals. The immune reactions were characterized and compared after the primary and final fourth injections in both the spleen as well as the tumor-bearing lung tissue.

Statistical Method

The standard nonpaired two-tailed Student's *t*-test was used to evaluate and compare the therapeutic observations and the immunological results obtained from image analysis.

Results

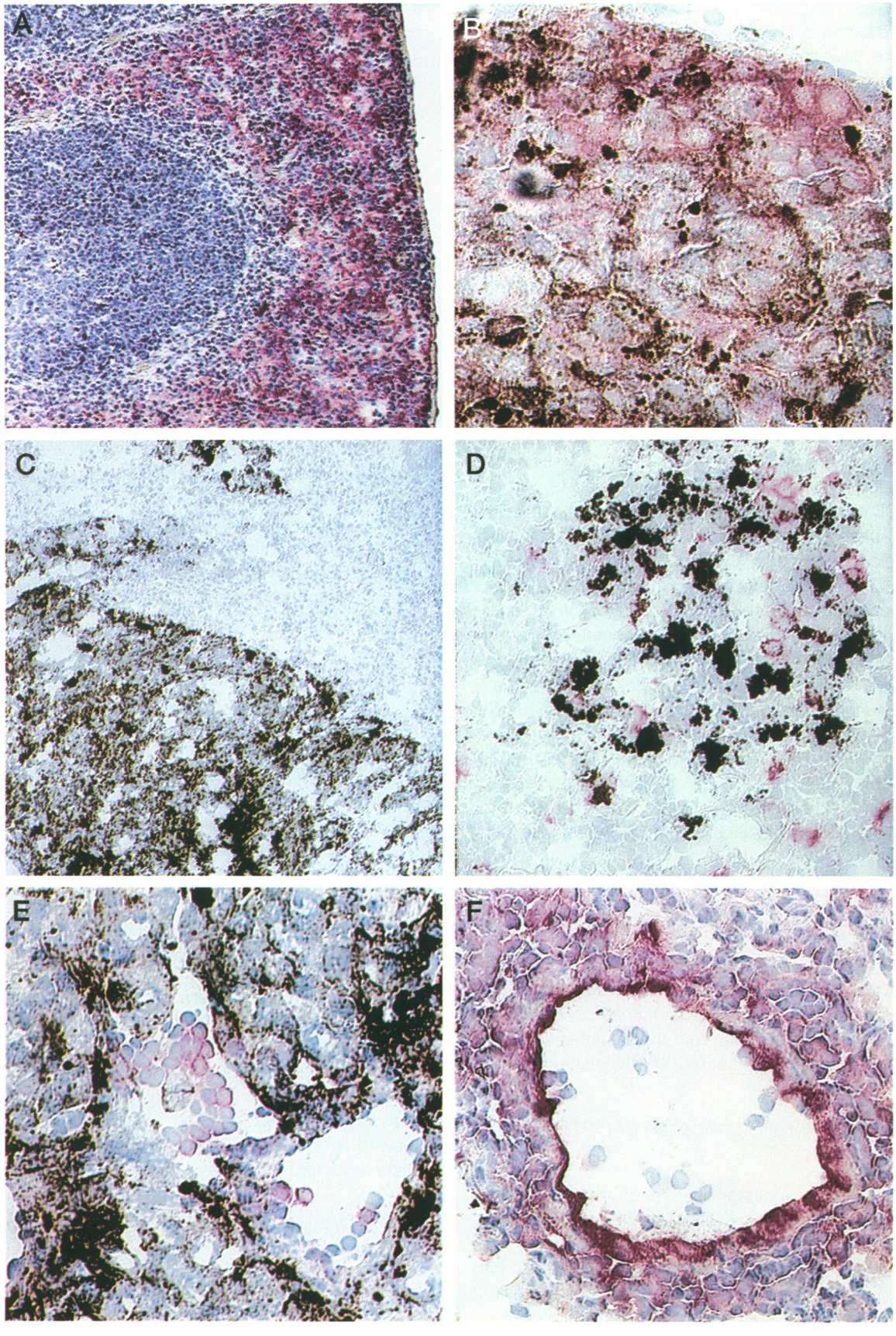
Murine B16-F10 C215 transfected melanoma cells (B16-C215) form disseminated disease after i.v. injection and establish macroscopically detectable micrometastasis within 4 to 5 days after inoculation.[11] To study the immune response after C215 Fab-SEA therapy, immunohistochemistry and computer-aided image analysis were used to quantify the interaction between cell infiltration and cytokine production in tumor and spleen tissue sections. These results were then compared with the immune response generated by SEA, Fab fragment, and rIL-2 to elucidate the significance of the C215 Fab-SEA therapeutic immune reaction.

Localization and Kinetics of Fab-SEA Accumulation in the Spleen and in the Lung Tissue

An anti-SEA MAb was used in indirect immunohistochemistry to analyze the accumulation and distribution of the Fab-SEA protein in the tissues. The fusion protein was detected within 1 to 2 hours in the lung and the spleen after i.v. injection. In the spleen, which did not contain detectable B16 melanoma cells, Fab-SEA showed a peak accumulation 1 hour after injection (Figure 1A) and gradually declined to undetectable levels after 8 hours. The fusion protein was primarily located to cells in the red pulp regions and was never observed in the B cell follicles. In the lung tissue, Fab-SEA was detected in the local vicinity of the tumor cells (Figure 1B). The kinetic pattern of the accumulation of Fab-SEA was similar to that seen in the spleen. Repeated injections of Fab-SEA showed a similar distribution compared with those observed after the initial injection.

Cellular Infiltration in the Lung Tumor Tissue after Repeated Fab-SEA Treatments

In untreated tumor-bearing mice, the cellular infiltration in the lung was characterized by a few scattered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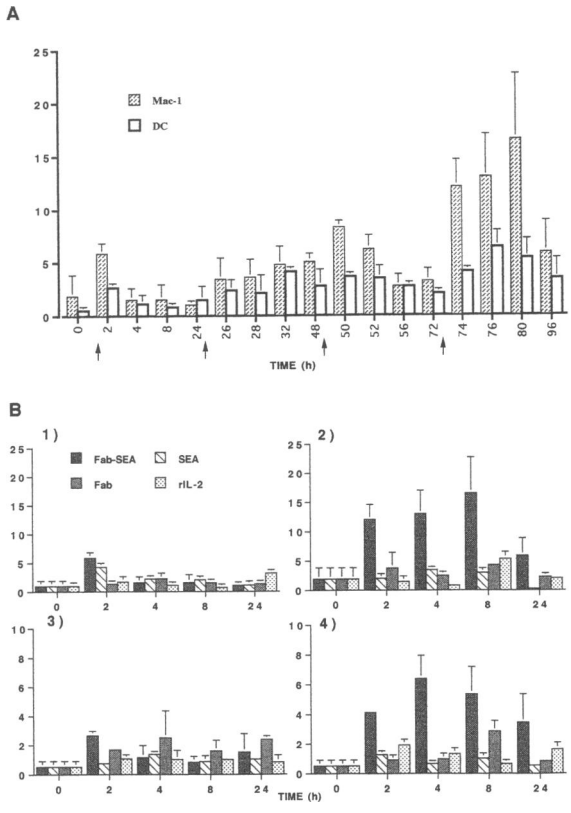


Figure 2. A: *Kinetics of the infiltration of macrophages and dendritic cells in the lung tumor area as detected by immunohistochemistry and quantified by computerized image analysis after repeated daily* (arrow) *Fab-SEA immunizations. The y axis illustrates the positive area occupied by the infiltrating dendritic cells and macrophages as calculated as a percentage of the total tissue area.* B: *Significant infiltration of macrophages* (quadrants 1 *and* 2) *and dendritic cells* (quadrants 3 *and* 4) *into the tumor area after Fab-SEA injections were compared with SEA, Fab fragment, or rIL-2 after primary injection* (quadrants 1 *and* 3) *or fourth injection* (quadrants 2 *and* 4).

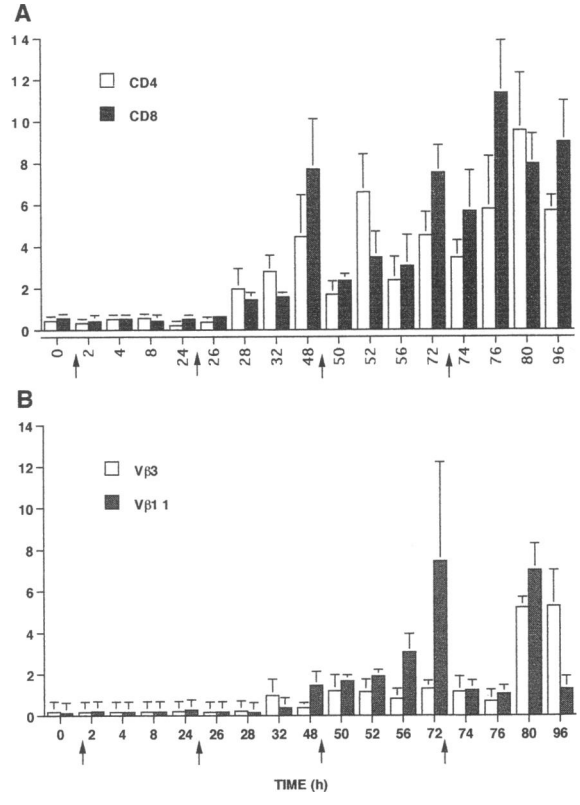


Figure 3. *The kinetics of infiltration of CD4$^+$ and CD8$^+$ T cells* (A) *and of Vβ3 and Vβ11 T cells* (B) *in the lung tumor area were detected by immunohistochemistry and quantified by computerized image analysis after repeated daily Fab-SEA immunizations* (*indicated by the* arrows). *The y axis illustrates the positive area occupied by the infiltrating T cells as calculated as a percentage of the total tissue area.*

macrophages and dendritic cells within the tumor areas (Figure 1, C and D). After the first injection of Fab-SEA, there was a significant increase in macrophage and dendritic cell infiltration but no T cell recruitment to the lung tissue. After each subsequent injection, there was an influx of numerous macrophages and dendritic cells in the tumor-containing lung tissue (Figure 2A). After the fourth injection, C215 Fab-SEA significantly ($P < 0.05$) induced a much stronger infiltration of both macrophages and dendritic cells when compared with SEA, Fab fragment, or rIL-2 (Figure 2B).

CD4$^+$ and CD8$^+$ T cells did not infiltrate the lung tissue until 28 hours after the first Fab-SEA injection (Figure 3A). After the third and fourth booster, both CD4$^+$ and CD8$^+$ T cells were highly prominent, encompassing up to 25% of the tissue area together (Figure 3A). In contrast, control experiments with SEA, Fab fragment, or rIL-2 induced only a transient presence of T cells (Figure 4). The superantigen SEA is known to selectively activate and expand Vβ3- and Vβ11-expressing T cells but not Vβ8 T cells.[16,17] Homing Vβ3- and Vβ11-expressing T cells were identified in the lung tumor area 32 hours after the first injection (Figures 1E and 3B). Vβ8 T cells were never detected in the lung of studied animals (data not shown). When SEA, Fab fragment, or rIL-2 was

Figure 1. *All of the photos illustrate immunohistochemically stained cryopreserved sections of lung and spleen tissue. The indirect immunohistochemical staining was developed in red with the alkaline phosphatase substrate red fuchsin and counterstained in blue with hematoxylin. The melanoma cells were easily detectable in the lung sections by their black or brown melanin content.* A: *The Fab-SEA (red) accumulated in the red pulp in the spleen 2 hours after immunization (magnification, ×100).* B: *Fab-SEA appeared in red on the surface of the brown melanoma cells (×500) in the lung parenchyma.* C: *No dendritic cells (NLDC-145$^+$) could be demonstrated in the lung tissue before Fab-SEA immunization (×100).* D: *Accumulation of dendritic cells (red) in the lung tumor area 32 hours after initiation of the therapy. The dendritic cells in the photo represent 1.8% of the total area (×400).* E: *Vβ3 T cells (red) infiltrating the lung tumor area 48 hours into therapy (×400).* F: *VCAM-1 surface expression (red) maximally expressed on vascular endothelial cells in the pulmonary tissue 48 hours into therapy (×300).*

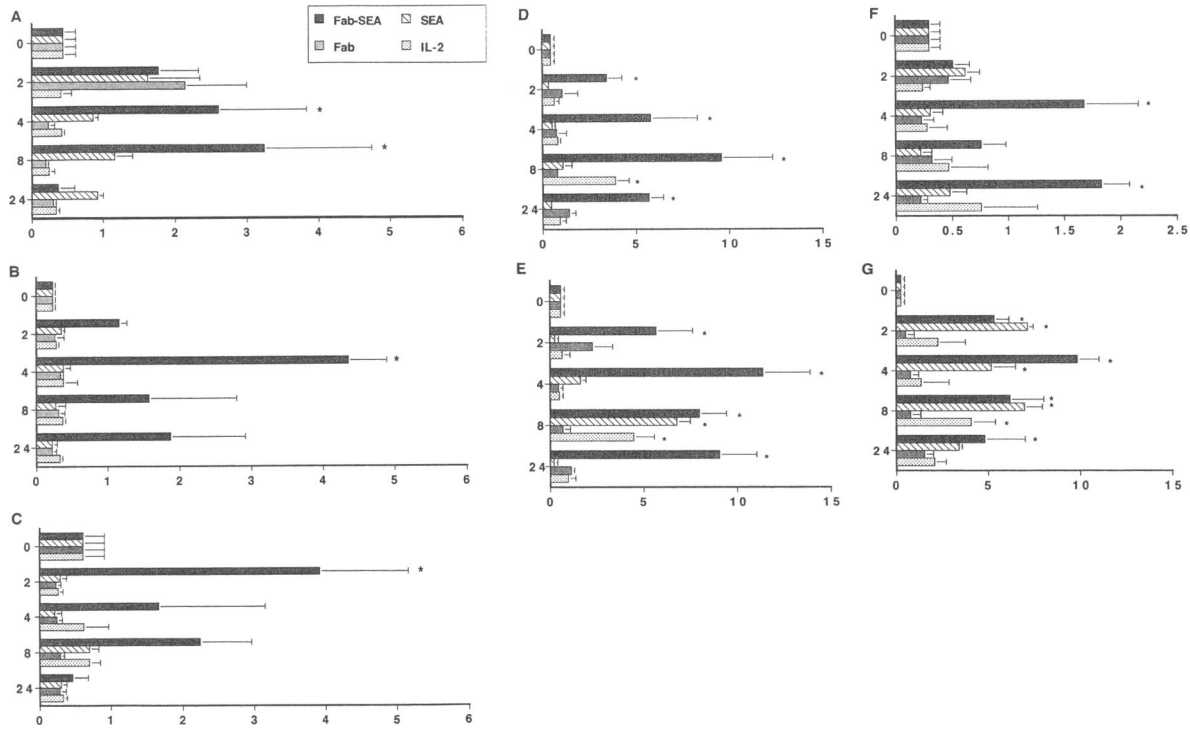


Figure 4. *Cytokine production and cell infiltration studied in the lung of tumor-bearing animals after treatment. The values were expressed on the* x *axis as a percentage of the total studied lung tumor area showing specific immunoreactivity as assessed by computerized image analysis technology over time in hours on the* y *axis. Effects of Fab-SEA, SEA, Fab-fragment, or rIL-2 were compared. The results were obtained from experiments with three different animals;* $P < 0.05$ *versus PBS control animals.* **A** *to* **C**: *TNF* (**A**), *MIP-1α* (**B**), *and MIP-1β* (**C**) *production in the lung of tumor-bearing mice after the first injection of Fab-SEA.* **D** *to* **G**: *CD4* (**D**), *CD8* (**E**), *TNF* (**F**), *and IFN-γ* (**G**) *immunoreactivity in the lung of tumor-bearing animals after the fourth injection of Fab-SEA.*

injected, there was never any selective Vβ T cell infiltration in the lung tissue.

Induction of Adhesion Molecules in the Lung Tissue

The induction of various adhesion molecules on the endothelium of lung vessels was analyzed to characterize adhesive pathways involved in the recruitment of superantigen-activated T cells to the lung. A constitutive expression of ICAM-1 was detected on the surface of vascular endothelial cells in tumor-containing lung tissue. The ICAM-1 expression was unchanged during Fab-SEA therapy. However, the adhesion molecule VCAM-1, which was absent in untreated animals, was induced in endothelial cells in the lung tumor area 8 hours after exposure to the superantigen therapy (Figure 1F).

Cytokine Production at a Single-Cell Level in the Spleen after Repeated Fab-SEA Stimulation

Melanoma metastasis was not detected in the spleen in B16-melanoma-inoculated mice (data not shown). Despite this fact, TNF-producing cells were detected before Fab-SEA injection at relatively low levels (Figure 5, A and B).

Previous *in vivo* studies have shown that cytokines are produced very rapidly after superantigen stimulation. The primary injection of Fab-SEA generated TNF production that lasted until 24 hours (Figures 5A and 6). Repeated injections of Fab-SEA caused a significant increase of TNF synthesis in the spleen (Figures 5A and 7A). This increase was also detected in control experiments with SEA, Fab fragment, and rIL-2. However, Fab-SEA generated a significantly stronger production of TNF after the fourth injection when compared with controls ($P < 0.001$; Figure 6). A substantial induction of IL-2-producing cells in the spleen was seen 2 hours after Fab-SEA injection, and further enhanced levels were seen 24 to 32 hours after the first injection (Figures 5B and 7C). A minor IFN-γ production response was noted after the primary injection and was further augmented after 24 hours (Figure 5A). The third injection produced an accelerated response with induction of high numbers of IFN-γ-producing cells already 2 hours after the injection. Maximal production of IFN-γ occurred 8 hours after the fourth injection, indicating

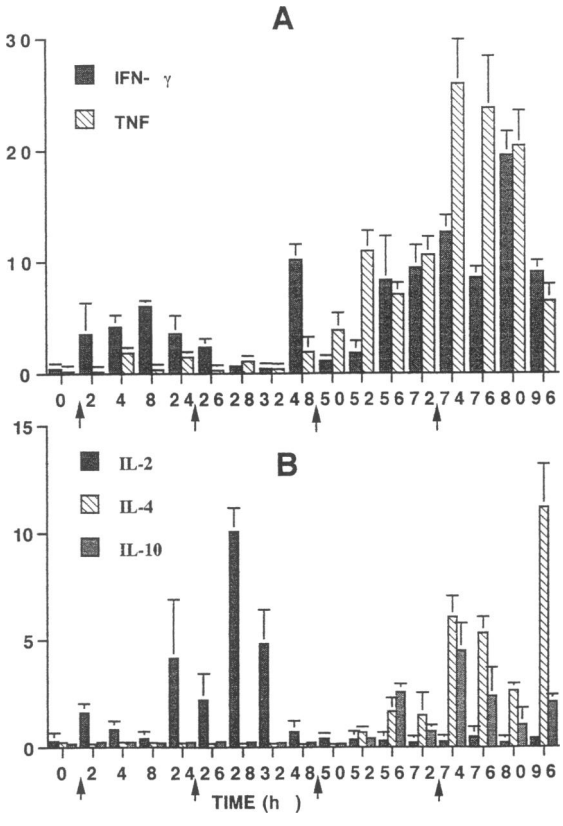


Figure 5. *Kinetics for the appearance of IFN-γ and TNF* (**A**) *and of IL-2-, IL-4-, and IL-10-producing cells* (**B**) *in the spleen detected by immunohistochemistry and quantified by computerized image analysis after repeated daily Fab-SEA immunizations (represented by* **arrows**). *The* y *axis is the positive area occupied by the cytokine-producing cells as calculated as a percentage of the total tissue area.*

a strong and rapid memory response from the primed T cells that had been most likely expanded by IL-2. SEA, Fab fragment, and rIL-2 also induced IFN-γ production but to a significantly ($P < 0.001$) lesser extent than Fab-SEA (Figure 6).

It has been postulated that repeated superantigen stimulation might shift the cytokine production to a Th2 phenotype of response (IL-10 and IL-4).[18,19] The number of IL-4-producing cells increased after each injection of Fab-SEA, but the frequency remained lower compared with that of IFN-γ-producing cells (Figure 5B). IL-10-synthesizing cells were first detected after the second injection and peaked 2 hours after the fourth injection (Figures 5B and 7E).

Cytokine Production at a Single-Cell Level in the Lung Tissue after Repeated Fab-SEA Stimulation

TNF-producing cells were present at low levels before Fab-SEA therapy (Figure 8) whereas IFN-γ-, IL-2-, IL-4-, or IL-10-positive cells were not detected (data not shown). TNF-, macrophage inflammatory protein (MIP)-1α-, and MIP-1β-producing cells appeared in the lung very rapidly after the first Fab-SEA injection at a time point when no T cells could be detected in the tumor area (Figures 4, 7B, and 8). MIP-1α and MIP-1β production were detectable only after Fab-SEA injections and were not observed in control experiments with SEA, Fab fragment, and rIL-2 (Figure 4). However, in contrast to the results seen in the spleen, TNF production in the lung decreased after each immunization. This was a general phenomenon and was found in Fab-SEA as well as control experiments (Figure 4). The appearance of IFN-γ-producing cells coincided with the initiation of T cells infiltrating the lung tissue (Figures 3, 7D, and 8). In addition, IFN-γ production in the lung increased after each Fab-SEA injection and reached very high levels after the fourth stimulation (Figure 8). Two-color staining demonstrated that $CD4^+$ T cells were the dominant producer cells of IFN-γ (Figure 7F). SEA injection induced IFN-γ production to the same extent as Fab-SEA, whereas Fab fragment or rIL-2 administration produced significantly lower levels (Figures 4 and 8). The majority of IFN-γ synthesis in the lung tissue after SEA or rIL-2 injection occurred at time points at which only low numbers of $CD4^+$ or $CD8^+$ T cells could be detected (Figure 4). IL-2- or IL-10-producing cells were never detected at any studied time point in the lung tumor area after Fab-SEA or control injections.

Serum Cytokine Levels after Repeated Fab-SEA Stimulation

The analysis of cytokine levels in the serum correlated to the recorded pattern of cytokine synthesis displayed in the spleen tissue rather than to the pattern seen in the lung tumor tissue. Serum IL-2 peaked at 2 hours after the first injection and declined after the second Fab-SEA stimulation (Table 1). Serum IFN-γ showed maximal concentration after the third injection of Fab-SEA and then decreased to low levels. Serum IL-10 levels peaked after the fourth injection of Fab-SEA. IL-4, on the other hand, was not detected in the serum at any time point during the therapy (data not shown).

Discussion

Successful superantigen-targeted tumor therapy has been shown to depend on at least three important factors. First, the therapy must direct the superantigenic response to the tumor cells. Next, a large

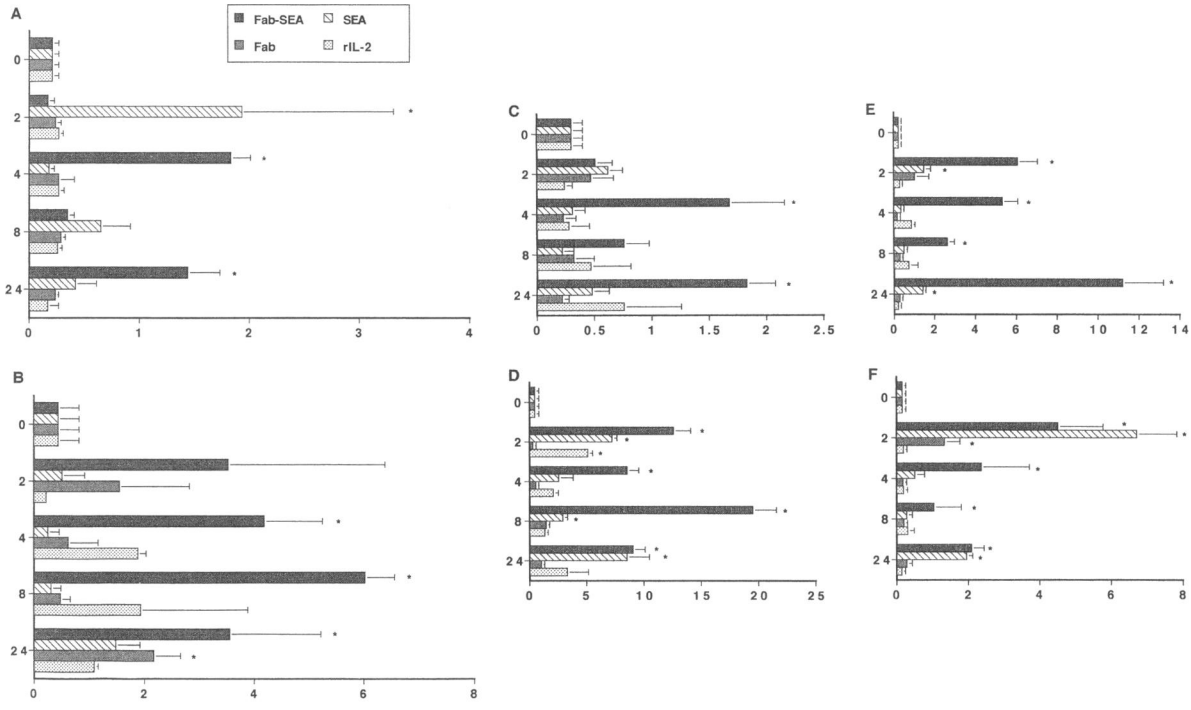


Figure 6. *Cytokine production in the spleen. The values were expressed on the* x *axis as a percentage of the total studied area showing specific immunoreactivity as assessed by computerized image analysis technology over time in hours on the* y *axis. Effects of Fab-SEA, SEA, Fab-fragment, or rIL-2 were compared. The results were obtained from experiments with three different animals;* $P < 0.05$ versus *PBS control animals.* **A** *and* **B**: *TNF* (**A**) *and IFN-γ* (**B**) *production in the spleen of tumor-bearing mice after the first injection of Fab-SEA.* **C** *to* **F**: *TNF* (**C**), *IFN-γ* (**D**), *IL-4* (**E**), *and IL-10* (**F**) *immunoreactivity in the spleen of tumor-bearing animals after the fourth injection of Fab-SEA.*

number of immunocompetent cells need to be recruited to the tumor site. Finally, these cells must be locally and repeatedly activated to induce tumor cell death. All of these factors have previously been demonstrated to occur *in vivo* after Fab-SEA therapy.[10–12] Previously, it has been shown that the therapy is dependent on multiple injections, T cells, and both parts of the fusion protein.[9,11] However, the local immune responses induced by repeated Fab-SEA injection therapy have not been characterized. In the present report, the immune reactions have been investigated in the spleen and lung tumor area as well as systemically.

The Fab-SEA fusion protein was detected within an hour after injection in the spleen as well as on the melanoma tumor cells in the lung tissue (Figure 1, A and B). In the spleen, the Fab-SEA was seen mainly on cells in the red pulp but was absent in the B-cell-enriched regions. In the lung, Fab-SEA was detected solely on the melanoma tumor cells. These differences might be attributed to the amount of MHC class-II-positive cells in each region. In the red pulp, 75% of the cells are MHC class II positive whereas only 10% of the cells in the lung express the MHC class II molecule on their cell surface.[20] Since the affinity of the C215 Fab-SEA protein for the tumor antigen is known to be 100 times higher than for the SEA MHC class II binding site,[9] this could have accounted for the observed distribution.

It is known that TNF is produced within minutes after primary superantigen stimulation, which is then followed by IL-2 and IFN-γ production.[14,21,22] TNF

Table 1. *Accumulation of Serum Levels of IL-2, IFN-γ, IL-10, and TNF as Detected by ELISA Assessments after Repeated Daily Fab-SEA Immunizations*

Treatment	Number of treatments	IL-2	IFN-γ	IL-10	TNF-α
PBS		0.01 ± 0.004	0.01 ± 0.003	0.01 ± 0.004	0.02 ± 0.004
C215 Fab-SEA	1	31.3 ± 3.443	0.8 ± 0.056	0.01 ± 0.003	0.26 ± 0.013
C215 Fab-SEA	2	19.7 ± 2.364	7.3 ± 0.876	0.02 ± 0.003	0.73 ± 0.08
C215 Fab-SEA	3	6.5 ± 0.585	3.4 ± 0.374	0.2 ± 0.03	0.39 ± 0.035
C215 Fab-SEA	4	7.8 ± 1.092	0.08 ± 0.013	0.24 ± 0.042	0.24 ± 0.061

The results were calculated from experiments with three different animals.

production is known to play a major role in the initiation of an inflammatory response.[23] The present study showed a similar rapid and coordinated induction of a panel of cytokines in response to Fab-SEA injections. Splenic TNF-producing cells were detected shortly after Fab-SEA, SEA, and Fab fragment i.v. injections (Figures 5A, 6, and 7A). Simultaneously, TNF-producing cells were rapidly detected in the lung area despite the lack of T cells present at this time point (Figures 4, 7B, and 8). This would suggest that MHC class II^+ macrophages in the tumor area were stimulated by superantigens without a need for T cell help. Indeed, it has previously been demonstrated that superantigens can directly induce TNF gene transcription in monocytes through cross-linking of MHC class II molecules.[24,25]

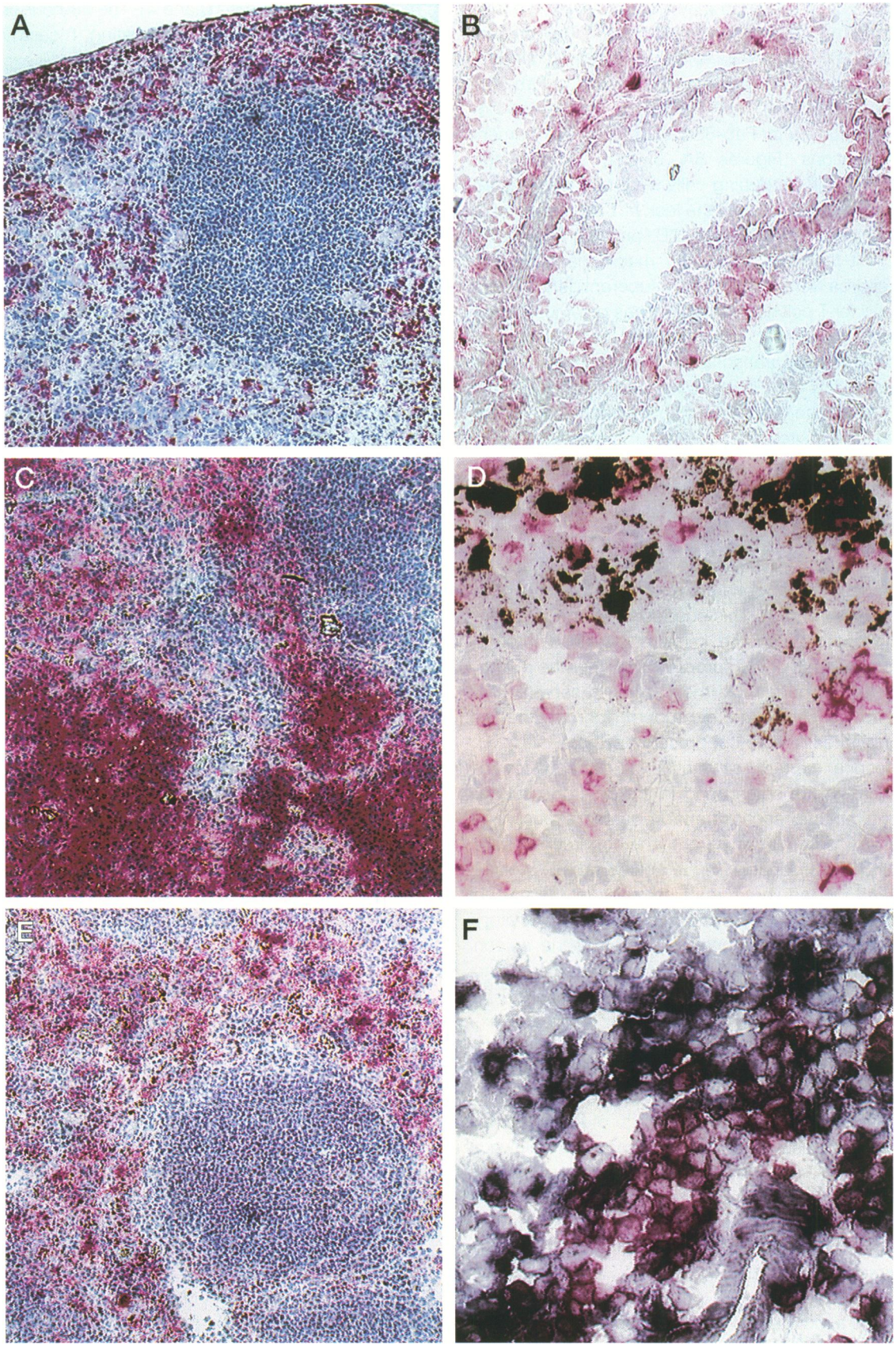
The immune responses differed significantly at the two studied sites after repeated injections of Fab-SEA. In the spleen, TNF production increased after repeated Fab-SEA, SEA, or rIL-2 injections (Figures 5A and 6). In contrast, the production of TNF in the lung tissue decreased after each Fab-SEA, SEA, or Fab fragment stimulation, despite the abundant presence of macrophages and dendritic cells (Figures 2A, 4, and 8). This suggests that the microenvironment dictated the immune response through tight regulation of TNF production. Either the pulmonary macrophages lost their ability to secrete TNF and to be stimulated by superantigen or the responding cells were not present anymore.

Despite certain similarities of immediate responses in the spleen and in the lung, the subsequent immune reactions after repeated exposure to Fab-SEA were quite different. The congruent results of cytokine production in the spleen to the cytokine detected in the serum may indicate that the spleen contributed to the systemic release of inflammatory modulators. The immune responses in the spleen included numerous IL-2-producing cells whereas no IL-2 production was recorded in the lung tissue. This suggests that resting T cells were primed and expanded in lymphoid organs and were then recruited as effector T cells to the inflammatory response in the lung tumor area. The lack of IL-2 and IL-4 production in the lung tissue during any mode of tested stimulations suggests that no expansion of T cells occurred at this site. However, we cannot exclude that other T cell growth factors such as IL-15 or IL-7 were produced locally to compensate for the lack of IL-2 production. When IL-2 production in the spleen ceased after Fab-SEA stimulation, SEA-reactive $V\beta3$- and $V\beta11$-expressing T cells strongly accumulated near the tumor cells in the lung tissue, which was not observed after control injections with SEA, Fab fragment, or rIL-2 (Figures 1B and 4). The 48-hour delay in the recruitment of tumor-infiltrating T cells indicated that initial production of IL-2 and T cell expansion preceded the homing to peripheral inflammatory sites. At this time point, IFN-γ was also detected in the area of melanoma metastasis in the lung tissue (Figure 7D). In fact, the number of IFN-γ-producing cells in the lung increased after each Fab-SEA injection.

The requirement for repeated stimulations to obtain optimal therapeutic effects[11] and sufficient local release of IFN-γ and TNF could be attributed to at least two factors. One essential factor was the necessity for priming, expansion, and differentiation of effector cells such as seen in the spleen. Moreover, as the Fab-SEA was detected only for 8 hours after each injection, repeated infusion might be needed to retain activated effector cells in peripheral inflamed tissue, such as in the tumor area.

T lymphocytes from mice exposed to superantigens have been demonstrated to be unresponsive or anergic to a second challenge of superantigens *in vitro*.[26,27] *In vivo*, it has been shown that after superantigen re-challenge there was an induction of $CD8^+$ cytotoxic T cells whereas the $CD4^+$ T cells were pushed into an anergic state.[28–30] In the present study, splenic T cells lost the ability to produce IL-2 after the third injection of Fab-SEA (Figure 5A), which could be interpreted as anergy. However, the splenic T cells did not lose the ability to produce IFN-γ (Figures 5A and 6). In contrast, once IL-2 production ceased, IFN-γ was produced much more rapidly and in higher quantities, which could be attributed to the increased number of memory effector cells. Thus, an induction of anergy selectively affecting IL-2 production in $CD4^+$ T cells seemed to occur. Indeed, this confirms earlier studies demonstrating induction of split anergy in the T cell compartment.[30] These data would support the notion that IL-2 production by T cells is tightly regulated and that IL-2-producing T cells might not be present at inflammatory target sites. The results in the current study strongly suggested that repeated Fab-SEA stimulation will cause migration of specific SEA-reactive $V\beta3$ and $V\beta11$ but not SEA nonresponding $V\beta8^+$ T cells from lymphoid organs to the inflammatory tumor site. In contrast, SEA, Fab fragment, or rIL-2 injections induced only unselective T cell migration to the lung tumor area. It is possible that MIP-1α and MIP-1β might be important factors that regulated the migration of specific T cells.

Uncovering the molecular mechanism that directed T cell migration to the tumor sites in the lung tissue is crucial for the development of T-cell-based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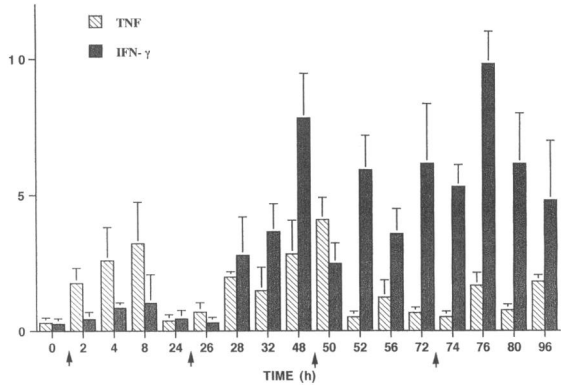

Figure 8. *Kinetics of IFN-γ- and TNF-producing cells in the lung tumor areas were detected by immunohistochemistry and quantified by computerized image analysis after repeated daily Fab-SEA immunizations. Each* arrow *indicates the time of daily immunization. The* y *axis illustrates the positive area occupied by the cytokine-producing cells as calculated as a percentage of the total tissue area.*

therapy. The results imply that T cells migrated to and accumulated in the lung as a consequence of the initial macrophage-derived TNF, MIP-1α, and MIP-1β production induced by Fab-SEA. TNF is known to rapidly induce adhesion molecules such as VCAM-1 on vascular endothelial cells.[31] VCAM-1 was clearly detectable on endothelial cells in the lung (Figure 1F) after repeated exposure to Fab-SEA, possibly as a result of the observed preceding local production of TNF. It has also been shown that MIP-1β will enhance the binding of T cells to VCAM-1.[32] Thus, a plausible scenario might be that VCAM-1 in conjunction with chemokines such as MIP-1α and MIP-1β, demonstrated to be locally produced in this study, recruited activated T cells to the tumor site. The growth of melanoma cells has been reported to be sensitive to a combination of IFN-γ and TNF,[33] and the transfected B16-C215 cells were suppressed *in vitro* by less than 10 U/ml rIFN-γ and TNF (data not shown). This suggests that the large production of IFN-γ in combination with TNF in the tumor tissue after Fab-SEA therapy may contribute to the anti-tumor effect.

In summary, the immune reaction in Fab-SEA tumor therapy was a multi-step process. It relied on an intricate network of cytokines that mobilized an innate and an adaptive immune response. Repeated Fab-SEA stimulations were needed to prime, expand, and differentiate effector T cells in lymphoid organs. Subsequently, these T cells were then recruited by chemotactic signals to the inflamed tumor area. These results indicate that MAb targeting of superantigens to selected locations can be used clinically to induce anti-tumor responses against weakly immunogenic tumors considered beyond reach for immunotherapy.

Acknowledgments

The skillful work of Ms. Kristina Behm with the animal experiments is greatly acknowledged. We acknowledge the gift of cytokine-specific antibodies from Dr. John Abrams (DNAX Research Institute, Palo Alto, CA) and PharMingen (San Diego, CA).

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Figure 7. *Cytokine-producing cells immunohistochemically stained in cryopreserved sections of lung and spleen tissue. The melanoma cells were detectable in the lung sections by their black or brown melanin content.* A: *Cells producing TNF (red) in the spleen 72 hours after therapy localized to the red pulp (magnification, ×100).* B: *TNF-producing cells (red) localized around vessels in the lung tumor area 2 hours after initiation of therapy (×200).* C: *The extensive production of IL-2 (red) in the spleen 28 hours into therapy (×100).* D: *Infiltrating IFN-γ-producing effector cells (red) located near the melanoma cells in the lung tissue 80 hours into therapy (×400).* E: *Accumulation of IL-10-producing cells (red) in the red pulp of the spleen 84 hours into therapy (×100).* F: *$CD4^+$ T cells (red) producing IFN-γ (purple) in the lung tumor area 80 hours into therapy (×400).*

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