Interleukin 2-Antibody and Tumor Necrosis Factor-Antibody Fusion Proteins Induce Different Antitumor Immune Responses in Vivo

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ABSTRACT

Two fusion proteins, composed of interleukin 2 (IL-2) or tumor necrosis factor (TNF) coupled to an antibody [fusion protein (FuP); IL-2-FuP or TNF-FuP], were capable of retarding growth of a human malignant melanoma in the severe combined immunodeficient mouse depending on the concomitant application of human peripheral blood leukocytes. Here we have analyzed the mechanisms that determine the therapeutic effect. Tumor-bearing severe combined immunodeficient mice received once per week an i.v. injection of HLA-matched peripheral blood leukocytes and twice per week i.v. or intratumoral injections of FuPs. Leukocyte recovery and their activation state were monitored. The number of draining lymph node cells (LNCs) and tumor-infiltrating leukocytes increased continuously, and the yield of draining LNCs was improved significantly when the FuPs were applied locally. In IL-2-FuP-treated mice, the majority of draining LNCs and tumor-infiltrating lymphocytes expressed T-cell activation markers and IL-2, thus being classified as T helper type 1 cells. These cells displayed strong proliferative activity and initiated activation of lymphokine-activated killer cells and CTLs. TNF-FuP supported activation of CTLs and of monocytes as revealed by TNF expression and cytostatic activity. Neither the antibody, nor IL-2, nor TNF, nor the mixture of antibody and cytokines exhibited the full-fledged activational potency of the FuPs. Notably, activation of immune effector mechanisms was much stronger when the FuPs were applied intratumorally. This is the first report to show that FuPs are efficient immuno-stimulants in vivo for native leukocytes. Although IL-2-FuP induced a T helper type 1 response with recruitment of LAK and CTL, TNF-FuP efficiently recruited and activated monocytes and, in a less pronounced manner, CTLs.

INTRODUCTION

Although many tumors express tumor-associated antigens that allow for in vitro induction of an immune response (1–3), tumor immunotherapy still records disappointingly low success (4–8), which is partly attributable to the missing second signal upon the first encounter of lymphocytes with the tumor cell (9–11). Several concepts have been proposed to circumvent this obstacle. Earlier approaches based on the systemic supply of cytokines have met only limited success because of the side effects (12–15). Great efforts have been devoted to vaccination with tumor cells transfected with cDNAs of costimulatory molecules, MHC class II antigens, or cytokines (16–21). Alternatively, concepts for targeting effector cells and concomitantly providing a local stimulatory support have been proposed (22, 23), which are based either on (sets) of bispecific antibodies that bind the tumor cell, the TCR/CD3 complex and a costimulatory molecule (24–28) or on FuPs,3 which consist of an antibody recognizing a surface molecule of the tumor cell and either a superantigen (29–34) or an appropriate cytokine (35). In the latter case, it is expected that the local availability of a cytokine obviates the requirement for a costimulatory signal (35).

In most experimental models described thus far, IL-2-FuP have been used. IL-2-FuP has been reported to induce the activation of CTLs (35–40), of LAK cells (40–43), of TH cells (44) and under appropriate circumstances, of a memory response (39). Apart from T lymphocytes, activation of neutrophils, monocytes, and B cells has also been described (45, 46). The therapeutic efficacy of TNF-FuP has been explored to a lesser extent, which may be attributable to the severe side effects observed after systemic application of TNF (47–49). Yet, without question, the proinflammatory cytokine TNF can be a potent drug in tumor immunotherapy (49, 50). It has been described to activate the cytotoxic potential of γ/δ T cells (51), to recruit CD8+ cells into the tumor (52), to synergize with IFN-α and IFN-γ (53), to initiate up-regulation of adhesion molecules thereby facilitating leukocyte rolling (54), to initiate NO synthesis by neutrophils and monocytes (55), and to restore and/or increase apoptotic programs with bystander killing (56). Furthermore, retroviral infection with a TNF fusion protein has

3 The abbreviations used are: FuP, fusion protein; SCID, severe combined immunodeficient; DC, dendritic cell; EGFR, epidermal growth factor receptor; GvH, graft versus host; IL, interleukin; i.t., intratumoral; LAK, lymphokine-activated killer cell; LNC, lymph node cell; mAb, monoclonal antibody; Mb, monocytes; PBL, peripheral blood leukocyte; SC, spleen cell; TH, helper T cell; TIL, tumor-infiltrating leukocyte; TNF, tumor necrosis factor; NK, natural killer.

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been described to initiate a protective immune response, mainly mediated by CD8+ cells (57).

The therapeutic concept of cytokine fusion proteins relies on targeting leukocytes to the tumor cell and concomitantly supporting their activation. In view of this sequence of events, it was intriguing to test the local, i.e., i.t., supply of FuPs to avoid exhaustive stimulation of circulating lymphoid cells prior to the targeting event. As described in a previous report (58), the systemic application of FuPs initiated most effectively a lethal GVHD disease in the “humanized” SCID mouse, which sheds light on the detrimental interaction with circulating lymphoid cells. This problem could be partially circumvented by an i.t. application, which also led to a significant improvement of the therapeutic efficacy. Finally, IL-2-FuP as well as TNF-FuP displayed therapeutic activity only in mice that received human PBLs, which clearly establishes the immunostimulatory role as opposed to, e.g., a direct cytostatic role of both FuPs. In fact, by the local application of IL-2-FuP, a potent TH response could be induced that activated initiation of CTL and recruitment of LAK. TNF-FuP, although less efficient, also initiated a T-cell response, mainly of CD8+ CTls, and had a strong effect on Mφ activation.

MATERIALS AND METHODS

Mice and Tumors. SCID mice (H-2d) were bred at the central animal facilities of the German Cancer Research Center. Animals were kept under specific pathogen-free conditions and were fed standard diet and water ad libitum. Animals were used for experiments at the ages of 11–12 weeks.

The melanoma lines BLM (59), BLM-gp100 (BLM cells transfected with gp100 cDNA; Ref. 60), A375, and the murine lymphoma line YAC were cultured in RPMI 1640, supplemented with antibiotics, l-glutamine, and 10% FCS (RPMI-s). Confluent cultures were split after detachment of cells by 0.1% trypsin. The BLM lines are HLA-A2.1+, and the A375 line is HLA-A2.1+. All three lines express the EGFR at a medium level.

Blood Samples. Heparinized peripheral blood was collected from healthy donors or was obtained from the blood bank. Leukocytes were separated by Ficoll-Hypaque gradient centrifugation. Cells were either used immediately or were frozen in aliquots.

Generation of IL-2-FuP and TNF-FuP. The generation of both FuPs has been described in detail in a previous report (58). In brief, two vectors were constructed. One vector (pcDNA3.1/huCH3-IL-2 and pCneo/huCH3-TNF) coded for the anti-EGFR-heavy chain (humanized variable sequence and constant region of the human IgG1 gene; Ref. 61) fused to the IL-2 gene and the TNF gene, respectively; the second vector (pCDhfr-huL) coded for the humanized light chain of the antibody with the human κ constant region gene (61). The cDNA encoding human IL-2 and human TNF was from British Biotechnology, Ltd. The hamster kidney cell line BHK21 (a subclone of CCL-10, obtained from the American Type Culture Collection) was transfected by the calcium phosphate precipitation method, and stable transfectants were selected using 1 μg/ml Gentamicin and 200 μg/ml MTX. Best producers were selected. Fermentation was performed in a semicontinuous manner using serum-free CHO-S-SFMII medium (Life Technologies, Inc.). Purification was done by affinity chromatography and size exclusion chromatography on Fractogel EMD SO3 650(S) and a cation exchange chromatography on Fractogel BioSic 650(S).

Monoclonal Antibodies. The following hybridomas were obtained from the American Type Culture Collection: OKT4 (anti-hCD4, T25G2b); OKT8 (anti-hCD8, T25G2a); 6D3 (anti-human monocytes, T25G1); HNK1 (anti-human NK cells, T25G); W6/32 (anti-HLA class I, monomorphic determinant, T25G2a); BB7.2 (anti-HLA-A2.1, T25G2b); and 9.3F10 (anti-human DR/DQ, monomorphic determinant, T25G2a). YBM61.10 (antimouse monocytes, rat IgG1) was obtained from the Swiss National Center for biologicals, Nutley, NJ, according to the TEPITOPE program (64). Mice received, in addition, twice per week either i.v. or i.t. injections of 5 × 105 BLM/BLM-gp100. The therapeutic treatment was started 5 days thereafter. Unstimulated PBLs or peptide-loaded DCs plus primed T cells (1–5 × 106) were injected i.v. once per week. Mice received, in addition, twice per week either i.v. or i.t. injections of 50 μg of anti-EGFR, 300 ng of IL-2, 500 ng of TNF, 50 μg of anti-EGFR plus 300 ng of IL-2, 50 μg of anti-EGFR plus 500 ng of TNF, 5 μg of IL-2-FuP, or 10 μg of TNF-FuP. The rationale for choosing the treatment dosage has been described in the accompanying manuscript, i.e., induction
of GvH disease by IL-2-FuP and TNF-FuP were the dose-limiting elements; IL-2 and TNF were applied at an amount corresponding in bioactivity to the FuPs. Spleen, draining lymph nodes, and the tumor were excised after 4–6 weeks. The organs were meshed and washed. The tumor cell suspension was layered on a Ficoll-Hypaque gradient and centrifuged to enrich for TILs.

**Proliferation Assay.** PBLs (2–0.25 × 10^7/well) were cultured with irradiated (30,000 rads) BLM or BLM-gp100 cells (1 × 10^4/well) for 3 days; 10 μCi/ml [3H]thymidine were added during the last 16 h of culture. Where indicated, cultures contained, in addition, 10 μg/ml anti-EGFR, 10 ng/ml IL-2, 20 ng/ml TNF, 10 μg/ml anti-EGFR plus 10 ng/ml IL-2, 10 μg/ml anti-EGFR plus 20 ng/ml TNF, 2 μg/ml IL-2-FuP, or 2 μg/ml TNF-FuP. Cells were harvested, and [3H]thymidine incorporation of triplicate cultures was determined in a beta counter.

**Cytotoxicity Assay.** NK activity was evaluated in freshly harvested SCs, TILs, and draining LNCs using YAC cells as target. LAK activity was evaluated after 48 h of culture in medium containing 2 ng/ml IL-2 and, in addition, the drugs, which the donor mouse had received. BLM cells were used as target. For the in vitro restimulation of CTLs, SC, draining LNCs, and TILs were cultured with irradiated (30,000 rads) BLM or BLM-gp100 cells for 8 days. The ratio of lymphocytes to irradiated tumor cells was in the range of 20–10:1. Cultures contained the drugs that the donor mouse had received at the same dose described above for the proliferation assay. After 8 days of culture, blasts were separated from dead cells by Ficoll-Hypaque gradient centrifugation and used for the evaluation of cytotoxic activity. Mφs were enriched by plating SCs, TILs, and draining LNCs in flat-bottomed 96-microtiter wells, where non-adherent cells were washed off after 2 h. Target cells were labeled with 51Cr and washed, and 10^4 cells were seeded in 96-well, round-bottomed plates at a ratio of 50–3:1 (E:T). All cytotoxicity assays contained the drugs at the doses indicated above. When determining CTL activity, cultures contained in addition cold target cells (A375, HLA-A2.1-, and EGFR+) for an experimental subtraction of LAK activity. The cold target: target ratio was 10:1. In some experiments, MHC restriction was controlled by adding the HLA-A2.1-specific mAB BB7.2 (10 μg/ml) and antigen specificity was controlled in experiments with primed T cells and BLM-gp100 target cells by addition of an excess of unlabeled BLM cells. Plates were incubated for 6 h (NK, LAK, and CTL) or 16 h (Mφ) at 37°C. After centrifugation, aliquots were removed, and released radioactivity was determined in a gamma counter. Cytotoxicity was calculated as % cytotoxicity = 100 × ([counts in test well − counts in control well]/(maximal releasable counts − counts in control well)). The spontaneous release was in the range of 7–12% (NK, LAK, and CTL) and of 12–20% (Mφ). SDs of triplicates were in the range of 1–5%.

**Statistics.** Significance of differences was evaluated by the two-tailed Student’s t test.

**RESULTS**

We have shown in a previous report (58) that the systemic and, more so, the local application, of IL-2-FuP and TNF-FuP significantly prolong the survival time of tumor-bearing SCID mice, which had been “reconstituted” i.v. with HLA-A2.1-matched PBLs. The therapeutic efficacy of the drugs largely depended on the presence of human leukocytes. This indicated that the drugs may have facilitated the in vivo activation of leukocytes. To explore the underlying mechanisms, leukocytes were recovered from the tumor-bearing mice and analyzed for composition and functional activity.

**Influence of IL-2-FuP and TNF-FuP on the in Vivo Expansion of Human PBLs in the Tumor-bearing SCID Mouse.** It should be stated, in advance, that in most experiments animals not receiving a tumor had been included as a control. In these mice, a high percentage of the anti-EGFR antibody as well as of the FuPs were trapped in the spleen, which was likely attributable to Fe receptor binding. In addition, human lymphocytes were only recovered from the spleen. As a consequence of the retention of human PBLs as well as the drugs in the spleen, lymphocytes became activated, and most of the mice developed huge spleens, the number of spleen cells exceeding those of untreated SCID mice by up to a factor of 30. Furthermore, these mice rapidly succumbed with GvH disease. Thus, this control group provided clear evidence that in the tumor-bearing SCID mouse, the response of the human PBLs was redirected toward the tumor. Nonetheless, for clarity of presentation these data will not be explicitly shown.

When tumor-bearing mice received only human PBLs, the recovery of SCs increased with time (data not shown) but remained rather poor (Fig. 1A). Recovery of cells was slightly improved by systemic or local (data not shown) application of anti-EGFR. The same findings were true for application of IL-2 or IL-2 plus anti-EGFR or TNF plus anti-EGFR, whereas the application of TNF alone had no effect. Application of IL-2-FuP or TNF-FuP led to a further increase in the number of SCs as compared with treatment with the mixture of antibody and cytokine. The recruitment of leukocytes to the tumor differed significantly in dependence on the local versus the systemic route of application (Fig. 1B). After systemic application, an increased number of TILs was only seen with IL-2-FuP, whereas after local application a high number of TILs were recovered in mice receiving either the mixtures or IL-2-FuP or TNF-FuP. Draining lymph nodes (Fig. 1C) remained empty or were poorly populated when the drugs were applied systemically. However, draining lymph nodes of the SCID mouse became populated after an i.t. application of IL-2-FuP and, though less pronounced, of TNF-FuP.

Thus, IL-2-FuP as well as TNF-FuP supported survival and expansion of human PBLs in the SCID mouse, particularly after i.t. application. Irrespective of the route of administration, IL-2-FuP triggered the expansion of human PBLs more efficiently than TNF-FuP.

**Different Leukocyte Subpopulations Expand in Vivo after Administration of IL-2-FuP and TNF-FuP.** In the spleen, the systemic application of anti-EGFR had no impact on the distribution of leukocyte subsets. The systemic application of IL-2 or of IL-2 plus anti-EGFR or of IL-2-FuP led to an increase in the percentage of CD4+ cells. After the systemic application of TNF by itself or together with the antibody, an increased number of human Mφs were recovered. In addition, a slight increase in CD4+ and CD8+ cells was observed after application of TNF-FuP. Accordingly, the percentage of murine
Mφ decreased in mice receiving TNF-FuP and, most strongly, in mice treated with IL-2-FuP (data not shown).

The infiltration of lymphoid cells into the tumor (Fig. 2A) upon treatment with FuPs was of central importance for judging their therapeutic functions. The local application of either IL-2-FuP or the mixture of the cytokine and the antibody had a major impact, i.e., a higher percentage of CD4⁺, CD8⁺, and HNK1.1⁺ cells was recovered after i.t. than after i.v. application. Similarly, human Mφ were only recruited by i.t. but not by i.v. application of IL-2-FuP. In mice treated with TNF-FuP, Mφ of human origin were markedly enriched in TIL preparations, irrespective of the route of FuP application. Furthermore, TNF-FuP, when applied locally, had some impact on the level of CD4⁺ and CD8⁺ T cells. Finally, it should be mentioned that the local application of TNF (data not shown), TNF plus anti-EGFR, and TNF-FuP as well as IL-2-FuP resulted in a moderate increase of murine Mφ in the tumor.

Draining lymph nodes (Fig. 2B) were only populated after i.t. treatment with FuPs. IL-2-FuP led to an increased recovery of CD4⁺ and CD8⁺ cells, whereas TNF-FuP led to an increased in CD8⁺ cells only.

These findings accounted for SCID mice that had been "reconstituted" with unstimulated human PBLs. Because IL-2-FuP, in particular, led to a very efficient recruitment of human leukocytes into the tumor, we asked whether the application of primed T cells may be of further advantage. PBLs of an HLA-A2.1⁺ donor were primed on DCs loaded with an MHC class I- and an MHC class II-restricted peptide of the gp100 tumor antigen as described in "Materials and Methods." The mixture of peptide-pulsed DCs plus primed T cells was transferred into SCID mice bearing a gp100 cDNA transfected BLM clone (BLM-gp100). In these mice (Fig. 2C), a higher percentage of CD4⁺ cells, CD8⁺ cells, and NK cells was recovered from the tumor. The i.t. application of IL-2-FuP further enhanced the expansion of these leukocyte populations in the tumor and, particularly of CD4⁺ and CD8⁺ cells, in the draining lymph nodes.

Taken together, human leukocytes were more efficiently recruited toward the tumor by the i.t. application of both FuPs than by the respective mixtures. Because IL-2-FuP and TNF-FuP recruited different populations of leukocytes, it became tempting to speculate that the effect on tumor growth retardation may have been based on distinct immune mechanisms. To examine this, we analyzed cytokine expression and proliferative activity as well as cytotoxic activity of Mφ, NK cells, and CTLs.

**Induction of Cytokine Expression by IL-2-FuP and TNF-FuP.** We first evaluated endogenous IL-2 expression as a general marker of T-cell activity. After systemic application of IL-2, IL-2 plus anti-EGFR, or IL-2-FuP, the percentage of IL-2-producing SCs was increased as compared with mice reconstituted with PBLs only. TNF exerted no effect, and TNF plus anti-EGFR as well as TNF-FuP was far less efficient than

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**Fig. 1** Recovery of leukocytes from tumor-bearing mice treated with IL-2-FuP or TNF-FuP. SCID mice received a s.c. injection of 5 × 10⁴ BLM cells, and starting 5 days after the tumor cell application, mice received twice per week an i.v. or peritumoral/i.t. injection of 50 mg of anti-EGFR, 300 ng of IL-2, 500 ng of TNF, 50 μg of anti-EGFR plus 300 ng of IL-2, 50 μg of anti-EGFR plus 500 ng of TNF, 5 mg of IL-2-FuP, or 10 mg of TNF-FuP. Mice received, in addition, weekly i.v. injections of 1–5 × 10⁶ human PBLs (HLA-A2.1⁺). Mice (three/group) were killed after 5 weeks, and the number of SCs (A), TILs (B), and draining LNCs (C) was evaluated. Numbers represent the mean of three mice/group. The experiment was repeated three times and revealed similar results. *, significance of differences as compared with mice receiving only PBLs.
IL-2 and IL-2-FuP. This was different for expression of IFN-γ, which was equally well supported by IL-2 plus anti-EGFR, IL-2-FuP, and TNF-FuP. The systemic application of none of the drugs supported human or murine TNF expression (data not shown).

A similar pattern of cytokine expression was seen in TILs after systemic application of the drugs, with the additional feature of TNF-FuP having a positive effect on the expression of human TNF. Regarding the expression of murine TNF, an increase was seen in mice receiving TNF, TNF plus anti-EGFR, and TNF-FuP, as well as IL-2-FuP. The local application of IL-2-FuP very efficiently supported IL-2 and IFN-γ expression, whereas the local application of TNF-FuP increased the percentage of IFN-γ and of human and murine TNF-expressing cells (Fig. 3A). The pattern of cytokine expression in the draining lymph nodes was similar to the one detected in TILs (Fig. 3B). When mice had received IL-2-FuP and primed T cells (Fig. 3C), a general increase in the frequency of cytokine expression by TILs and draining LNCs was seen. These effects were particularly pronounced in mice receiving i.t. application of IL-2-FuP.

The analysis of cytokine expression revealed two important facts:

(a) The preferential induction of IL-2 and IFN-γ expression by IL-2-FuP as well as the preferential induction of IFN-γ and TNF by TNF-FuP corresponds to the preferential recruitment/expansion of CD4+ and CD8+ cells versus Mφ and CD8+ cells, respectively.

(b) The systemic and, more so, the local application of both FuPs supported cytokine expression in TILs and draining LNCs much stronger than the individual components or their mixtures. This was a clear indication that the FuPs are more efficient in the recruitment to the tumor and the draining lymph nodes as well as in the activation of human leukocytes in these compartments.

Augmentation of Lymphocyte Proliferation in Response to BLM Tumor Cells by IL-2-FuP and TNF-FuP. As could have been expected, SCs from mice treated with IL-2 drugs, i.e., cytokine or mixture or FuP, showed a marked enhancement of proliferative propensity after 5 weeks of systemic treatment. When mice had been treated with the corresponding TNF drugs, a slight increase in the proliferative activity of SCs was only noted with TNF-FuP and the mixture (data not shown). TILs responded to the systemic application of all three IL-2 drugs, whereas a response to TNF was only seen when applied as FuP or, weakly toward application of TNF plus the antibody (Table 1). Although the local versus the systemic application of
the cytokines plus the antibody had no additional impact on the proliferative responses, a significant increase was seen after the local application of IL-2-FuP and TNF-FuP. Draining LNCs showed a strong proliferative response when mice had received IL-2-FuP locally or systemically. TNF-FuP was rather ineffective, irrespective of the route of application. When mice had received primed T cells, the proliferative response of draining LNCs and TILs was significantly augmented by the i.t. as compared with the systemic application of IL-2-FuP.

Activation of Cytotoxic Activity by Elements of the Nonadaptive and Adaptive Immune Defense via IL-2-FuP and TNF-FuP. We next asked whether the drugs in mice “reconstituted” with human PBLs would support NK and LAK activity (Fig. 4). NK activity was evaluated using YAC as target in freshly harvested lymphoid cells from tumor-bearing mice and in SCs of mice that had not received the tumor. LAK activity was tested after 48 h of culture in the presence of IL-2.

As already mentioned, mice receiving human PBLs and the drugs, but not the tumor, succumbed with GvH disease earlier than tumor-bearing mice. This may have been partly because of very high levels of NK activity in mice receiving only PBLs. In the tumor-bearing mouse, NK activity of SCs was significantly lower (Fig. 4A). In TILs and draining LNCs, NK activity did not reach the levels observed in SCs from tumor-free animals (Fig. 4B). As could have been expected, NK and LAK activity correlated in all three organs. Thus, the systemic as well as the local application of the anti-EGFR mAb increased LAK activity mainly in the tumor. NK and LAK activity was further increased when mice received the antibody plus IL-2 or IL-2-FuP. Yet, IL-2-FuP was hardly more efficient than the mixture. Furthermore, when mice had been reconstituted with native PBLs, LAK activity did not differ significantly, depending on the route of drug administration. Instead, when mice had been “reconstituted” with DCs together with primed T cells, stronger LAK activity of TILs and draining LNCs was seen after the local as compared with the systemic application of IL-2-FuP (Fig. 4C). Neither TNF nor TNF-FuP influenced LAK activity in the tumor-bearing mouse. This was independent of the route of application and accounted for TILs, draining LNCs, and SCs (data not shown).

Because IL-2-FuP as well as TNF-FuP supported the expansion of CD8+ cells as well as expression of IFN-γ, it became likely that both drugs would efficiently induce CTL activity. The effect of the different cytokine drugs on CTL activity was explored after in vitro restimulation. To minimize a contribution of LAK activity, a 10-fold excess of unlabeled A375 cells, which are EGFR+ and HLA-A2.1+, was included during the cytolysis assay. With this correction, it was found that the

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**Fig. 3** Induction of cytokine expression by IL-2-FuP and TNF-FuP in tumor-bearing SCID mice. Mice were treated as described in Fig. 2. Organs were excised 5 weeks after tumor cell application, and the percentage of cells expressing human IL-2, IFN-γ, and human or murine TNF was evaluated by flow cytometry after fixation and permeabilization of TILs (A and C) and draining LNCs (B and C). Values represent the mean percentage of stained cells of three mice/group. *, significance of differences. First row, significance of differences was evaluated in comparison to mice receiving only PBLs; second row, significance of differences was evaluated between systemic versus local application of the drugs. Repeated experiments revealed similar results.

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**Fig. 4** Induction of cytokine expression by IL-2-FuP and TNF-FuP in tumor-bearing SCID mice. Mice were treated as described in Fig. 2. Organs were excised 5 weeks after tumor cell application, and the percentage of cells expressing human IL-2, IFN-γ, and human or murine TNF was evaluated by flow cytometry after fixation and permeabilization of TILs (A and C) and draining LNCs (B and C). Values represent the mean percentage of stained cells of three mice/group. *, significance of differences. First row, significance of differences was evaluated in comparison to mice receiving only PBLs; second row, significance of differences was evaluated between systemic versus local application of the drugs. Repeated experiments revealed similar results.
cytotoxic activity of draining LNCs and TILs was low when mice were treated systemically with IL-2 plus anti-EGFR or IL-2-FuP (Fig. 5A). When these drugs were administered locally, CTL activity of TILs and draining LNCs was significantly increased, a preferential stimulation by IL-2-FuP over the mixture being particularly observed with draining LNCs.

The same observation also accounted for TILs and draining LNCs from mice receiving DCs together with primed T cells (Fig. 5B). In this setting, BLM-gp100 cells were used as target and A375 as cold target. As an additional control for specificity and HLA restriction of the CTLs, either BLM cells, which differ only by the absence of gp100, were used as cold target or the cytotoxicity assay was performed in the additional presence of an anti-HLA-A2.1 mAB (BB7.2). Cytotoxic activity in the presence of cold target BLM cells was only slightly reduced, indicating that the majority of CTL was gp100 specific. In the presence of BB7.2, hardly any cytotoxic activity was recovered, i.e., the cytotoxic effector cells were HLA restricted.

Finally, it should be noted that the effect of TNF plus anti-EGFR was rather weak. In contrast, the systemic and even more efficaciously the local application of TNF-FuP induced the activation of CTLs in TILs and draining lymph nodes. Notably, activation of CTLs was achieved more efficiently by TNF-FuP than by IL-2-FuP (Fig. 5A).

Comparison of the Potency of i.t. and Systemic Application of IL-2-FuP and TNF-FuP. Because treatment with FuP influenced both the repopulation of relevant compartments with human lymphoid cells as well as their functional activity, it was mandatory to compare the potency of both FuPs by the use of compounded parameters. To this end, we arbitrarily defined 1000 cpm/10⁵ cells as 1 proliferative unit and 10⁵ cpm/10⁵ cells as 1 lytic unit. When judged with these parameters, as summarized in Table 2, it was confirmed that the i.t. as compared with the systemic administration of both FuPs resulted in an overall increased activation of TIL. This accounted for proliferative activity and lytic units of CTLs. LAK activity of TILs after IL-2-FuP administration was the only exception, inasmuch as in this respect the systemic route was equally effective to the local one. A different picture was obtained in the draining lymph nodes. For TNF-FuP, the relative increase in proliferative and lytic units by the i.t. application was comparable with the one observed with TILs. On the other hand, proliferative and lytic CTL units increased in the draining lymph node 10-fold by the local versus the systemic application of IL-2-FuP. The finding is well in line with our hypothesis that a generalized TH response is only induced by the local application of IL-2-FuP.

The comparison of IL-2-FuP with TNF-FuP at the level of compounded response parameters further corroborated that proliferation and LAK activity of TILs were predominantly induced by IL-2-FuP, whereas CTLs were almost equally stimulated by both FuPs. Finally, we could confirm that TNF-FuP hardly induced a systemic response, i.e., for draining LNCs the unit ratios of TNF-FuP to IL-2-FuP were very low.

**DISCUSSION**

Tumor cells, although they may express immunogenic entities, do not induce an effective immune response (4–8). Several strategies for circumventing the problem have been proposed, cytokine-FuPs being among the more recent concepts (35). They are composed of an antitumor antibody and a cyto-

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Note: * SCID mice received 5 × 10⁶ BLM cells or BLM-gp100 cells s.c. Mice received, in addition, weekly i.v. injections of 1–5 × 10⁶ freshly harvested human HLA-A2.1 PBLs or (BLM-gp100 tumor-bearing mice) gp100-primed PBLs plus peptide-loaded DCs as described in “Materials and Methods.”

a Starting 5 days after tumor cell application, mice received twice per week an i.v. or peritumoral/i.t. injection of either 50 μg of anti-EGFR, 300 ng of IL-2, 50 μg of anti-EGFR plus 300 ng of IL-2, 50 μg of anti-EGFR plus 500 ng of TNF, 5 μg of IL-2-FuP, or 10 μg of TNF-FuP. Applications of the stimuli were repeated twice per week.

b Animals (three mice/group) were killed 5 weeks after tumor cell application. Draining LNCs and the tumor were excised. To enrich for TILs, tumor cell suspensions were passed over a Ficoll-Hypaque gradient, collecting cells from the interphase. Draining LNCs and TILs were titrated from 2-0.25 × 10⁶ cells/well and were cultured with 1 × 10⁶ irradiated tumor cells (BLM and BLM-gp100, respectively) for 3 days, adding [³H]thymidine during the last 16 h of culture. Cells were harvested, and incorporation of [³H]thymidine was determined in a beta counter. The mean cpm ± SD of triplicate cultures containing 1 × 10⁶ lymphocytes are shown.
d,e The significance of differences is indicated only for: d the comparison of cytokine plus anti-EGFR versus FuP; and e the systemic versus the i.t. application.

**Table 1 In vivo induction of a proliferative response to the BLM tumor by IL-2-FuP and TNF-FuP**
kine, thus providing a targeted stimulus for lymphocyte activation. The analysis of the immunostimulatory potential of antibody-cytokine FuPs was mostly performed with IL-2 FuPs (37, 38, 40, 41, 43, 66–70). We have described recently the use of IL-2 FuP and TNF FuP (58), both of which were capable of a significant growth retardation of a human tumor transplant in SCID mice that were reconstituted with HLA-A2.1-matched human leukocytes. A targeting effect and its therapeutic consequences were observed after systemic application, but the overall effect was clearly stronger after local administration. Furthermore, results of an in vitro analysis of leukocyte activation in the presence of IL-2 FuP and TNF FuP suggested that the two FuPs may function via activation of different leukocyte subsets. We provide evidence that this is, indeed, the case in the tumor-bearing SCID mouse, i.e., IL-2 FuP acts mainly by the activation of helper T cells, which recruit NK cells, but also Mφ, whereas TNF FuP is a potent stimulus for Mφ and CD8+ CTLs. In view of the clinical relevance of the data, we will discuss: (a) the possible reasons of the superiority of FuPs as compared with the mixtures; (b) the efficacy of the i.t. versus the systemic route of application; and (c) the distinct activity profiles of IL-2 FuP as compared with TNF-FuP.

**Efficacy of FuP versus Individual Components.** Although both FuPs were applied at a lower dose than the antibody, cytokines were given at a dose that matched the one of the cytokines contained in the FuPs. The higher dose of antibody, which corresponded to doses usually applied to patients, was maintained because doses corresponding to FuP displayed no effect at all (data not shown). The dose of FuP was dictated by the very efficient induction of GvH reactions in animals receiving systemic application of the FuPs plus human PBLs. Because the application of higher doses of cytokines (plus anti-EGFR) and human PBLs did not induce GvH reactions (data not shown), it could be concluded that activation of leukocytes was more efficiently achieved by the FuPs than by the mixture of their components. We assume that the critical parameter for GvH stimulation is the longevity of the cytokines in their FuP form.

IL-2 supported the survival and expansion of human leukocytes in the spleen of the SCID mouse. The simultaneous
application of the anti-EGFR, as could have been expected, supported LAK activity and, to a low degree, IL-2 expression. Yet, systemic application of neither IL-2 by itself nor of IL-2 together with the antibody supported infiltration in the tumor. TNF exerted some effect on Mφ activation but had no impact on T-cell survival or activation. With respect to the combination of TNF plus the antibody, there was no evidence for a direct cooperativity. Thus, a genuine superiority of FuPs over the mixtures of antibody and cytokines was observed in three major aspects: proliferative propensity of TILs (both FuPs) and draining LNCs (mainly IL-2-FuP), which lead to an increased cellularity in lymphatic compartments; production of endogenous cytokines; and induction of CTL activity. LAK activity was assigned under defined circumstances, i.e., in the draining lymph nodes of mice that received primed T cells, and local application of the drug, more strongly supported by IL-2-FuP than by the mixture. Importantly, all of these effects predominated in TILs and draining LNCs, i.e., in lymphoid compartments in (close) proximity to tumor cells.

From a mechanistic point of view, FuPs may be superior to the mixture because they retain the activational cytokines in proximity to the tumor cell such that lymphocytes can be synchronously targeted to and activated against antigenic entities of the tumor. It is important to note that an efficient activation of TH cells and CTLs was only observed with the FuPs but not the mixtures. Instead, response elements, which are antigen-independent, i.e., LAK and Mφ, were found to benefit from the mixtures as well as from the FuPs. Additional parameters that may have facilitated induction of a T-cell response are the repeated application of PBLs and the drugs (71) and, possibly, the fact that the BLM tumor in the treated animals expressed MHC class II molecules (data not shown).

**Efficacy of the i.t. versus the Systemic Application.**

The i.t. application of cytokines, particularly of TNF, may be a means of circumventing severe side effects, but its putative advantages have not been explored systematically. We consider the following aspects as being of relevance for the higher therapeutic efficacy of an i.t. versus a systemic application. By the local application of FuPs, the retention in the tumor was prolonged, and the therapeutic efficacy was improved (see previous report, Ref. 58). However, with some delay a sizable spillover into the circulation was observed such...
that lymphoid cells outside the tumor were also exposed to the FuP. These kinetics may have favorable consequences inasmuch as an exhaustion of the immune system attributable to the initial bolus of an i.v. injection can be avoided. In fact, we observed a steady increase in cell numbers as well as in their state of activation (data not shown). In the reconstituted SCID mouse, i.v. injection of FuP (and mAB) had the additional “side effect” of a transient but pronounced trapping in the spleen, which most likely was mediated by Fc receptor-positive cells. Furthermore, the recruitment and activation of CD8\(^+\) cells. The observation that the efficacy of IL-2-FuP was strongly increased when applied concomitantly with peptide-pulsed DCs (53), which, in turn, facilitates the recruitment of lymphocytes and supports the activation of cytotoxic T cells (73). By secretion of IFN-\(\gamma\), which we have shown to be significantly increased in TILs of mice receiving TNF-FuP i.t., T cells may have further facilitated the recruitment and activation of Mφ. Thus, TNF-FuP apparently induces a circuit of nonadaptive-adaptive responses, which are mutually supportive.

As already mentioned, the efficacy of IL-2-FuP has been explored in a variety of human tumors in the SCID mouse as well as in syngeneic mouse tumor models. By the systemic application of FuP, activation of CD8\(^+\) CTLs as well as of antibody-lymphotoxin-FuP, asialo GM1\(^-\)positive cells, including NK/K cells mediating antibody-dependent cellular cytotoxicity has been described (40, 43, 67, 68, 70, 74, 75). Using an antibody-lymphotoxin-FuP, asialo G\(_{M1}\)-positive cells, including NK cells and B cells, appeared to be important (46). The involvement of CD4\(^+\) cells, although essential for the induction of a sustainable immune response including memory (19), has not been explicitly evaluated. In our system, the therapeutic effect of IL-2-FuP was clearly based on the activation of TH cells. The dominating feature was an increase in the percentage of CD4\(^+\) cells and, accordingly, of IFN-\(\gamma\)- and IL-2-expressing cells. The observation that the efficacy of IL-2-FuP was strongly increased when applied concomitantly with peptide-pulsed DCs plus primed T cells supports our interpretation of the importance of a TH cell activation. Furthermore, only in this setting, TILs and draining LNCs exerted increased LAK activity as compared

### Table 2
Comparative evaluation of leukocyte activation by local versus systemic application of IL-2-FuP and TNF-FuP

<table>
<thead>
<tr>
<th>PBL + Stimulus*</th>
<th>Proliferative units</th>
<th>i.t.: i.v. ratio</th>
<th>TNF-IL-2 ratio</th>
<th>LAK lytic units</th>
<th>i.t.:i.v. ratio</th>
<th>TNF-IL-2 ratio</th>
<th>CTL lytic units</th>
<th>i.t.:i.v. ratio</th>
<th>TNF-IL-2 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL2-FuP, i.v.</td>
<td>662.2</td>
<td>4.06</td>
<td>76.5</td>
<td>1.22</td>
<td>64.4</td>
<td>2.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL2-FuP, i.t.</td>
<td>2690.4</td>
<td>0.40</td>
<td>93.0</td>
<td>0.24</td>
<td>161.2</td>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-FuP, i.v.</td>
<td>1060.8</td>
<td>3.98</td>
<td>18.1</td>
<td>3.98</td>
<td>120.6</td>
<td>2.97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-FuP, i.t.</td>
<td>8.8</td>
<td>0.23</td>
<td>1.3</td>
<td>0.20</td>
<td>19.8</td>
<td>3.25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SCID mice received 5 \(\times\) 10^6 BLM cells s.c., and starting 5 days thereafter, mice received twice per week systemic or local applications of 5 \(\mu\)g of IL-2-FuP or 10 \(\mu\)g of TNF-FuP. All mice received i.v. injections of PBLs, once per week.

* Mice were sacrificed 5 weeks after tumor cell inoculation, draining LNCs, and tumors were excised, and TILs were enriched as described in “Materials and Methods.” Cells were counted and assayed for proliferative and lytic activity toward BLM cells as described. One proliferative unit was arbitrarily defined as 1000 cpm/10^5 cells; 1 lytic unit was defined as 10% lysis/10^6 cells.
with the individual components. Thus, we assume that under these conditions LAK became activated in a physiological way, i.e., via endogenous IL-2, which has been provided by activated TH cells. Obviously, activated TH also supported the expansion and activation of CTLs. Such a physiological cascade of immune activation events, which is initiated by antigen-specific TH and extends into the recruitment and activation of nonadaptive effector mechanisms is, in our view, the ultimate goal in immunotherapy of cancer.

Our findings are at variance with other reports on the efficacy of IL-2-FuP in the humanized SCID mouse (67, 70, 74), which describe that the systemic application of IL-2-FuP induces an efficient activation of LAK activity (although no T-cell specific response) in the absence of GvH reactions. The differences between these observations and our own findings likely are attributable to the experimental settings: (a) we used naive or antigen-specific T cells, whereas otherwise mainly LAK cells were used (67, 70, 74), i.e., there was an a priori bias toward LAK; (b) in many instances, LAKs were not HLA matched (67, 70, 74, 76). Taking into account that every second lymphocyte proliferates in response to a fully allologenic stimulus, it is reasonable to assume that the response toward the allogeneic tumor retracted lymphocytes from the xenogeneic response. Instead, in our system PBLs were at least matched for the HLA-A haplotype of the tumor; (c) to avoid in our setting a rapid onset of GvH reactions, we reduced the doses of FuP to a level that allowed for an onset of GvH disease beyond the survival time of tumor-bearing mice receiving only PBLs. Under these circumstances, we did not see, at least in the tumor-bearing mouse, an excessive activation of NK/LAK in the spleen by either IL-2 or IL-2-FuP. Nonetheless, we observed increased levels of “targeted” LAK activity in the tumor and in draining lymph nodes after reconstitution with activated T cells and i.t. application of IL-2-FuP. Such a physiological (see above) and persistent activation of LAK may well be advantageous in clinical settings.

A comparison of IL-2-FuP with TNF-FuP by the use of proliferative and lytic unit parameters confirmed the outlined view. Proliferative activity and “targeted” LAK activity were predominantly induced by IL-2-FuP, whereas TNF-FuP predominantly activated M6 (which is not directly reflected by the units). The cytotoxic potential of CTLs was increased by both FuPs in a similar manner.

Some major findings can be extracted from the results obtained in our SCID model system:

(a) with respect to both FuPs our data led to the conclusion that analyses of TILs and LNCs closely reflected the relevant activational events and the therapeutic outcome, whereas response analyses of SCs were not very telling.

(b) TNF-FuP showed a global stimulatory effect that was different to but not dramatically weaker than the effect of IL-2-FuP. IL-2-FuP led to expansion and activation of TH-1 lymphocytes within the tumor and the draining lymph node, which recruit LAKs and monocytes and support the activation of CTLs. The effect of TNF-FuP was attributable to the recruitment and activation of monocytes and CTLs within the tumor. (c) It is important to stress that the action spectrum of both FuPs was greatly enhanced upon i.t. injection.

(d) Finally, it has to be considered that the SCID situation with a human tumor transplant and human PBL “reconstitution” is artificial in the sense that a plethora of HLA antigens, in addition to the matching HLA-A2.1, are most likely divergent and may work as operational tumor antigens, which confer an extra degree of “immunosensitivity” to the system. Although these “operational” tumor antigens will be missed in the patient, the actual tumor-directed activities will not be diminished by a “deviation” toward GvH reactions in the patient.

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Interleukin 2-Antibody and Tumor Necrosis Factor-Antibody Fusion Proteins Induce Different Antitumor Immune Responses in Vivo

Oliver Christ, Siegfried Matzku, Christa Burger, et al.


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