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*References*

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The Role of Adoptively Transferred CD8 T Cells and Host Cells in the Control of the Growth of the EG7 Thymoma: Factors That Determine the Relative Effectiveness and Homing Properties of Tc1 and Tc2 Effectors

Brian K. Helmich and Richard W. Dutton

We had previously examined the factors that regulate the response of OVA-specific TCR-transgenic CD8 T cells to the B16 OVA melanoma, growing as lung metastases. We examine here whether the same parameters operate for EG7, growing intradermally. Tc1 or Tc2 CD8 effector cells from OT-1 mice were injected either mixed with the tumor or i.v., at day 0 or 7. Tc2 were one-fifth to one-tenth as effective as Tc1 when injected with the tumor, in controlling tumor growth, but were only 1/20 to 1/100 injected i.v. Tc1 injected i.v. entered the draining lymph nodes faster than Tc2 and caused a faster accumulation of host cells. Both caused an abrupt termination of host cell entry into lymph nodes and spleen after tumor elimination, but this occurred earlier for Tc1 than for Tc2. Host responses were ineffective in the absence of adoptive transfer but were essential after transfer. Perforin expression in the donor cells plays no role in adoptively transferred Tc1 or Tc2 control of the tumor, and neither IL-4 nor IL-5 is needed for Tc1 or Tc2 function. Tc1 cells from mice lacking IFN-γ, however, control tumor growth less well, whereas Tc2 effectors lacking IFN-γ are unaffected. Tc1 from IFN-γ-deficient mice attract fewer host cells to the draining lymph node, whereas Tc1 cells from perforin-deficient donors are unimpaired. We conclude that host cell recruitment is a crucial element in adoptive immunotherapy. The differences between the EG7 and the previous B16 melanoma model are discussed. The Journal of Immunology, 2001, 166: 6500–6508.

Much attention has been focused in recent years on the factors that control the induction of immune responses to tumor cells (1). We focus here on the effector mechanisms that lead to the control of tumor growth once the immune response has been induced by starting with already activated effector cells specific for a tumor Ag. The effector phase itself is still very complex. Effector cells may have a direct effect on the tumor cells. These effects may be mediated by direct cell contact and lysis, involving perforin, Fas ligand, or membrane-bound TNF or by the secretion of cytokines (2). These effects require that the effector cells are able to migrate to the site of tumor in effective numbers. The effector cells may influence their own migration patterns by the secretion of cytokines that affect the expression of adhesion molecules, the secretion or induction of chemokines, and the induction of chemokine receptor expression (3). They may also influence tumor growth indirectly by altering the behavior of other cells of the innate or acquired immune system, and again cytokines, chemokines, adhesion molecules, and chemokine receptor expression are involved in these effects. The tumor may also react in such a way as to deflect or suppress the effector cells or may become invisible by the down-regulation of the cell surface molecules that are recognized by the effector cells (4).

A number of investigators have used the adoptive transfer model to investigate the role of the immune system in the response to tumor Ags (5–14), and CD8 T cells have been shown to have a major therapeutic role in antitumor responses in a number of experimental tumor models (5). In these studies, investigators have used in vitro activated tumor-specific CD8 T cells from wild-type mice (6) or CD8 T cells from mice possessing the transgenic TCR specific for an Ag introduced into the tumor (7–10) or a naturally occurring tumor Ag (12). In some studies naive or memory T cells were transferred (12); in others, CD8 T cells from recently immunized mice were used (13, 14); whereas in yet others, cytotoxic CD8 T cell clones were transferred (5). Variable results were obtained, in part due to the different tumor models used but in part due to the activation state of the transferred cells.

In our own studies (8–10), we examined the role of adoptively transferred Tc1 and Tc2 cytokine polarized subsets of CD8 effector T cells in the control of the growth of an already established tumor, the B16 melanoma, which grows in the lung. We used in vitro-generated cytokine Tc1 and Tc2 polarized effector cells from naive CD8 populations from OT-I mice that bear the α- and β-chains of the TCR specific for the OVA-derived peptide presented on K b and OVA-transfected B16 melanoma cells. The tumor cells were injected i.v. 7 days before adoptive transfer. Graded numbers of effector Tc1 or Tc2 cells were injected i.v., and the survival times and numbers of lung metastases were determined in each case. We found that Tc1 cells were more effective than Tc2 in controlling tumor growth and in prolonging the survival times of tumor-bearing mice. Approximately 25 times more Tc2 cells were required to bring about the same degree of control as for Tc1 cells (8). Under these conditions, the tumors grew out at later times; but when fewer tumor cells were used, the adoptively transferred cells were able to prolong survival indefinitely. Tumor cells were...
still present, however, in the lungs of healthy mice, apparently con-
trolled by the presence of CD8 T cells and other cells (9). In further
studies (Ref. 10 and M. J. Dobrzanski and R. W. Dutton, unpublished observations), we determined the role of various donor effector mol-
ecules by generating effectors from OT-1 mice crossed to various
deficient mice. In these studies, we found that donor production of
IFN-γ was essential for full Tc1 activity but not for Tc2 activity (10).
Effector cell-derived IL-4 or IL-5 was equally important for Tc2, but
not Tc1, activity, whereas perforin deficiencies had no effect on either
Tc1 or Tc2 activity (M. J. Dobrzanski and R. W. Dutton, unpublished observations). IFN-γ was shown to inhibit tumor growth in vitro and
to up-regulate class I and class II MHC, CD95, and TNF receptor gene
expression and to induce the secretion of IFN-γ-inducible protein 10 and
other chemokines (10). We also showed that although the adoptively
transferred cells destroyed most of the tumor cells shortly after trans-
fer, host cells were required to control further growth of surviving
tumor cells (10). We found that Tc2 cells, that were equally effective
whether they came from IFN-γ-deficient or wild-type mice, were not
effective if the host were IFN-γ deficient (M. J. Dobrzanski and R. W.
Dutton, unpublished observations).

In the current studies, we sought to determine whether the same
parameters were important in the control of a different
tumor, growing in a different anatomical location. We used the
ova-transfected EL4 thymoma, EG7, and injected the tumor
intradermally into the flank of the recipient mice. In the absence
of adoptive transfer, the tumor grows progressively, and the
mice die if left untreated. The adoptively transferred cells either
were injected mixed with the tumor, as in the classic Winn assay (15),
or were injected i.v. at the same time as the tumor or
7 days after tumor injection. We found substantial differ-
ences in the relative effectiveness of the Tc1 and Tc2 effectors
under the different conditions. Tc2 were one-fifth to one-tenth
as effective as Tc1 when injected together with the tumor but
were only one-twentieth to one-one hundredth as effective when
injected i.v. We used Thy-1 congenic donor and recipient combina-
tions to monitor the entry of donor cells into the draining and
contralateral lymph node and spleen and also determined
the accumulation of host cells in these locations. We found that
Tc1 cells entered the draining lymph nodes more rapidly than
Tc2 cells and caused a more rapid accumulation of host cells at
the same site. Both populations brought about an abrupt termination of
host cells migration into and exit from the nodes and spleen after
elimination of the tumor, but this took place earlier after transfer of
Tc1 than Tc2. It appeared that the host cell response was ineffective in
the absence of adoptive transfer but was an essential component of
the response after transfer.

We examined next which functions of the Tc1 and Tc2 effectors
are involved in the mechanism of tumor rejection by generating
effectors from perforin- or cytokine-deficient mice and comparing
their activity with comparable effectors from wild-type mice. Per-
forin expression in the donor cells appears to play no role in either
the Tc1 or Tc2 control of the tumor, and neither IL-4 nor IL-5 seem to
be needed for Tc1 or Tc2 function. Tc1 cells lacking IFN-γ gene
expression, however, are severely handicapped in the function,
whereas Tc2 lacking IFN-γ gene expression are unaffected. In
vitro exposure of the EG7 to IFN-γ elevates the already high ex-
pression of class I MHC but does not appear to induce any other
changes that might render the tumor less able to grow in vivo. We
show that lack of perforin has no effect on the accumulation of
donor or host cells in the ipsilateral lymph nodes. Cells from IFN-
γ-deficient donors, however, are markedly less able to bring about
host cell accumulation.

Materials and Methods

Animals

TcR-transgenic OT-1 mice (16) specific for the OVA peptide, SIINFEKL,
were used as the source of donor cells. In some experiments, donor cells
were from OT-1 mice, crossed to C57BL/6 perforin−/−, IFN-γ−/−, IL-
4−/−, and IL-5−/− mice to produce OT-1 mice that were deficient for each
of the single genes. Syngeneic C57BL/6, PL.Thy-1.1, or C57BL/6 SCID
mice were used as recipients.

Cell lines

The H-2b cell lines EG7 and EL4 were obtained from American Type
Culture Collection (Manassas, VA).

Monoclonal Abs

The following mAbs were used for immunofluorescent staining: Cy-
Chrome anti-CD8 (BD PharMingen, San Diego, CA); FITC anti-CD4 (BD
PharMingen; GK1.5); FITC anti-CD62L (BD PharMingen; clone MEL-
14); FITC anti-CD44 (BD PharMingen; clone IM7); FITC anti-CD25 (BD
PharMingen; clone 2a3); FITC anti-Ly6C (BD PharMingen;
gen; clone AL-21). For details of additional specific Abs used in these
studies, see figure legends. Isotype-matched Abs were used as negative
controls. Cell populations were analyzed by multiparameter flow cytometry
using a Becton Dickinson FACSCalibur or FACSscan instrument (Becton
Dickinson Immunocytometry Systems, Mountain View, CA). Surface
marker analysis was performed using CellQuest software (Becton Dickin-
son Immunocytometry Systems).

Cell preparations

CDS T cells were isolated from the spleen and lymph nodes of the OT-1
TCR-transgenic mice and enriched by passage through nylon wool and
treatment with anti-CD4 (RL172.4), anti-heat-stable Ag (J11D), anti-class
II MHC (D3.137, M5114, CA4) mAbs and complement. Small resting
CDS T cells were harvested from the bottom interphase of a Becton
Dickinson Percoll gradient (Sigma, St. Louis, MO). The freshly isolated T cell pop-
ulations were 90–95% CD8+ T cells. Splenic APCs were enriched from
C57BL/6 mice by T cell depletion using anti-Thy-1.2 (HO13.14 and
F7D5), anti-CD4 (RL172.4), and anti-CD8 (3.155) mAbs.

Tc1 and Tc2 effector generation

CDS cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA)
supplemented with penicillin, streptomycin, glutamine, 2-ME, and 10% FCS
(HyClone Laboratories, Logan, UT). C57BL/6 3-day LPS-stimulated
B cell blasts were used as APCs and were loaded with the OVA peptide (11
mM) at 37°C for 30 min, treated with mitomycin C (100 μg/ml; Sigma) at
37°C for 40 min, and washed three times before use. CDS effector T cells
were prepared from the OT-1-transgenic mice by 4-day culture under po-
larizing conditions as previously described (8). On day 4 of culture, effec-
tors were 95–99% CD8+ T cells.

Analysis of cytokine production

Tc1 and Tc2 effector cells were restimulated in vitro and supernatants were
collected at 24 and 36 h. IFN-γ, IL-4, and IL-5 cytokine levels were de-
termined by ELISA as previously described (8).

CTL assays

Tc1 and Tc2 effector cells cytolytic activity was determined in a 51Cr
release assay (8).

RNase protection assays

Pelleted cells were resuspended in Trizol reagent (Life Technologies,
Gaithersburg, MD). Total RNA was isolated by chloroform extraction and
ethanol precipitation and analyzed using the RiboQuant Multiprobe RNase
Protection Assay system (BD PharMingen). mCK-1, mCK-3, mCK-4 cy-
tokine; mCR-1 cytokine receptor; mCK-5 chemokine; mCR-5 and mCR-6
chemokine receptor; and mAPO-3 “death gene” mRNA detection multi-
probe system (BD PharMingen). mCK-1, mCK-3, mCK-4 cy-
tokine; mCR-1 cytokine receptor; mCR-5, mCR-6
chemokine receptors, and mAPO-3 “death gene” mRNA detection multi-
probe template sets were then used. Bands were detected and quantified
using the Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA),
and the Quantity One software analysis package (Bio-Rad). All values reported
were normalized as a percent of the band intensity measured for the L32
housekeeping gene.
**Intradermal tumor establishment**

Syngeneic C57BL/6, B6.PL-Thy-1, or C57BL/6 SCID mice were injected intradermally with EG7 tumor cells (typically 3 × 10^6 cells) in 100-μl volumes of sterile PBS. Tumor cells for injection were recovered from log phase in vitro growth and were injected into the right flank skin of recipient mice. Tumors were clearly visible after 1 wk and grew progressively, in an encapsulated fashion.

**Tumor measurement and volume approximation**

Tumors were measured on two perpendicular axes using a vernier caliper. Tumor volumes were approximated by multiplying the measured length by the measured width by the calculated mean of these measured values and were presented as the mean of (typically) five identically treated mice ± SEM. Tumor size was determined weekly.

**Adaptive immunotherapy of tumors**

Varying numbers of Tc1 and Tc2 effector cells were injected in 500-μl volumes of PBS. Injections were delivered i.v. unless otherwise stated.

**In vivo trafficking of transferred effector cells**

B6.PL-Thy-1 mice receiving 10^7 polarized Thy-1.2 effector cells on day 0 were sacrificed (in duplicate) on days 1, 3, 5, 7, 10, and 13 (or 14) after adoptive transfer. Inguinal and cervical lymph nodes were collected and pooled from the tumor-ipsilateral or tumor-contralateral sides of sacrificed mice. Splenectomies were also collected at this time. Recovered populations were stained with mAbs specific to mouse CD8 (2.43), CD4 (GK1.5), Thy-1.2 (53-2.1), CD19 (ID3), NK1.1 (4D11), Mac-1 (M1/70), and Ly-6G (RB6) and analyzed by three-color multiparameter flow cytometry. Numbers of individually identified cell populations in each organ preparation were calculated by multiplying the total organ cell count by the frequency of cells that stained with each appropriate cell surface phenotype.

**Results**

**The EG7 thymoma model**

EG7 is a derivative of the H-2^b^ thymoma EL4, which was transfected with cDNA encoding full-length chicken OVA (17) and displays the immunodominant epitope of chicken OVA on its surface, identified as the eight-residue peptide OVA257–264, with the amino acid sequence SIINFEKL (18, 19). On intradermal inoculation of C57BL/6 mice with EG7 cells, tumors grew progressively and in a dose-dependent manner in the skin of the injected animals (Fig. 1). Although detectable tumors could be produced with as few as 10^5 EG7 cells per mouse, reliable tumor establishment required injection of at least 3 × 10^6 tumor cells. Accordingly, 3 × 10^5 cells was adopted as the minimum dose for reliable intradermal establishment of tumors. Tumors were measured on two perpendicular axes using a vernier caliper. The two measurements were multiplied together with their average to approximate the total volume of the intradermal tumor mass. Mean tumor volume represents the mean of the approximated values for each cohort of replicate mice. Tumor frequencies indicate the number of mice bearing palpable tumors 29 days after injection of EG7 cells.

**Effect of adoptively transferred CD8 effector cells on tumor growth**

In the first experiments, the effects of the Tc1 and Tc2 effectors on EG7 were compared in a Winn assay (15). Polarized effector cell populations were prepared as described, and graded numbers of effector cells were mixed with a constant number (3 × 10^5) of EG7 cells before coinjection into the skin of recipient C57BL/6 mice. After injection, recipient mice were monitored for the presence of tumors and the tumor sizes were measured as described in Materials and Methods. It can be seen (Fig. 2A) that both Tc1 and Tc2 were effective at controlling tumor growth but that Tc1 were more effective than Tc2. Approximately 5–10 times as many Tc2 were required to achieve the same measure of control as with Tc1. Approximately 3 × 10^5 Tc1 cells were required to give complete suppression of tumor growth.

We next examined the effect of adoptively transferred cells on the growth of tumor when the CD8 T cell populations were injected i.v. immediately before tumor inoculation. Both populations were effective, as can be seen in Fig. 2B, but under these conditions Tc2 cells were considerably less effective than Tc1, requiring 20–100 times more Tc2 cells than Tc1 cells to achieve the same measure of control. However, Tc1 cells were almost as effective when injected i.v. as when co-injected with the tumor.

In a third set of experiments (Fig. 2C), we determined the effect of transferred cells on established tumors, injected 7 days before adoptive transfer. Again, Tc2 were less effective than Tc1, with ~20–25 times as many cells being required to achieve the same effect. More than 10 times as many Tc1 cells were now required when the adoptive transfer was delayed to day 7 rather than day 0. The transfer of even 10^7 naive CD8 T cells had no effect on tumor growth (data not shown).

**Trafficking of donor and host T cells**

In a further series of experiments, we investigated the entry and the accumulation of donor and host cells into the draining and contralateral lymph nodes and the spleen. Donor cells came from the OT-1 Thy-1.2-positive donors which were transferred into C57BL/6 Thy-1.1 recipients (PL.THy-1.1). The lymph nodes and
FIGURE 2. Growth of EG7 tumors in untreated mice and mice injected with CD8 effectors. Polarized effector cell populations were prepared as previously described, and varying numbers were injected into mice also injected with a constant number of EG7 cells (3 × 10^6). Recipient mice were monitored for the presence of tumors, and the tumor size was measured as described in Materials and Methods. A, OT-I Tc1 or Tc2 effector cells and EG7 co-injected intradermally. Polarized effector were mixed in vitro with EG7 cells before injection into the skin. Error bars represent the SEM of four identically treated animals in each case. Data are representative of three independent replicate experiments. B, OT-I Tc1 or Tc2 effector cells were injected i.v. at the same time that the EG7 was injected intradermally. Each data point represents the mean size of tumors seen in five identically treated animals, and error bars depict the calculated SE of each mean. These results are typical of two independent experiments. C, OT-I Tc1 or Tc2 effector cells were injected i.v. 7 days after the EG7 was injected intradermally. Data are representative of 18 independent experiments.
spleen were harvested from recipient mice at various times after adoptive transfer of donor CD8 effectors and analyzed by flow cytometry for donor (Thy-1.2\(^{+}\)) and host (Thy-1.2\(^{-}\)) cell content, and the total number of cells was determined. The host (Thy-1.2\(^{-}\)) cells were further characterized as CD4, CD8, B cells, NK cells, or neutrophils, using the Abs indicated in the figure legends.

It can be seen that, in the absence of adoptive transfer, there is a substantial accumulation of host cells into the draining lymph node with time but not the contralateral node (Fig. 3, \(\text{f}\)). There is also an increase of host cells in the spleen. The analyses of cell phenotype showed that CD4, CD8, B cells, and macrophages were recruited in substantial numbers to the ipsilateral node. Fewer NK cells and neutrophils were also seen to accumulate. All of the categories of cells measured were seen to increase in number in the spleen.

In mice injected with Tc1 effectors (Fig. 3, \(\text{G}\)), Tc1 donor cells entered the ipsilateral or draining lymph nodes and spleen very rapidly but did not enter the contralateral nodes. In mice injected with Tc2 effectors (\(\text{C}\)), Tc2 cells also entered the draining nodes and spleen but in smaller numbers, and the entry into the draining LN (but not the spleen) was delayed compared with Tc1 cells.

Donor Tc1 T cells appeared in the draining lymph nodes within 3 days of transfer and were tetramer positive when stained with K\(^{+}\) SIINFEKL tetramers (data not shown). The arrival of Tc2 cells was delayed for several days and peaked at day 10. The rate of accumulation host B cells, NK cells, and neutrophils into the draining LN was accelerated after adoptive transfer of Tc1 cells but not Tc2 cells. The accumulation of cells in the draining lymph node and the spleen is severely truncated after elimination of the tumor.

**Role of host response.** There is clearly a host response in the absence of adoptive transfer as seen in Fig. 3, but the growth of the tumor does not appear to be limited. This was further investigated by comparing wild-type and SCID mice as recipients (Fig. 4). The tumors grew at the same initial rate in the SCID mice as in wild-type controls (data not shown). It can be seen that the initial control of the tumor by adoptively transferred cells is the same as in the wild-type recipients but that the tumor grows out in SCID recipients at later times (day 22), in contrast to the situation in wild-type recipients, even after the transfer of \(10^7\) Tc1 effectors.

**Mechanisms of donor cell function.** We prepared CD8 Tc1 and Tc2 effector populations from OT-1 mice crossed to perforin or cytokine-deficient mice (IFN-\(\gamma\), IL-4, or IL-5). The effector populations were examined for cytolytic activity and for the ability to secrete cytokines on restimulation with Ag in vitro. Each of the populations was defective only in the function controlled by the deleted gene (data not shown). Effectors from all but the perforin-deficient mice had comparable cytolytic activity, whereas effectors from only IFN-\(\gamma\), IL-4, or IL-5-deficient mice lacked the ability to produce the cytokine controlled by the deleted gene. We next determined how each deletion affected the ability of the adoptively transferred cells to control tumor growth. It can be seen (Fig. 5A) that the performance of both Tc1 and Tc2 cells was independent of perforin

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**FIGURE 3.** Cell recovery and calculated cell distributions in the ipsilateral lymph node (ILN), contralateral lymph node (CLN), and spleens of tumor-bearing untreated control mice and mice injected with CD8 effectors. At day −7, 12 B6.PL-Thy-1 mice were injected intradermally with \(3 \times 10^6\) EG7 thymoma cells, which subsequently grew into measurable intradermal tumor masses by day 0. On the indicated days, two mice were randomly selected from this cohort and sacrificed to allow pooled recovery of the inguinal and cervical lymph nodes ipsilateral to the tumor, as well as those contralateral, and also the spleen. These organs were dissociated into single-cell suspensions and counted. Aliquots of these suspensions were stained with mAbs specific for CD8, Thy-1.2, CD4, CD19, NK1.1, Mac-1, and Ly-6G and analyzed by FACS to obtain percentages of the overall populations that corresponded to CD8 T cells, donor CD8 T cells, CD4 T cells, B cells, NK cells, macrophages, and neutrophils. The total number of each cell population present in each sample was then calculated. Mice were injected with tumor on day −7 and left untreated. Results from four independent experiments were then averaged, with mean values presented here, \(\pm\) SEM. Mice were injected with tumor on day −7 and treated with i.v. Tc1 effectors on day 0. Results are from four independent experiments, mean \(\pm\) SEM. Mice were injected with tumor on day −7 and treated with i.v. Tc2 effectors on day 0. Results are the mean \(\pm\) SEM from four independent experiments.
expression, there being no difference in the number of defective and wild-type cells required to achieve a given level of control of tumor growth. The same finding was true for effectors prepared from IL-4 or IL-5-deficient mice as can be seen in Fig. 5, B and C. Tc1 and Tc2 from IL-4- or IL-5-deficient mice were as effective as the corresponding populations from wild-type mice. When we examined the performance of CD8 effector T cells prepared from IFN-γ-deficient mice, we found that the effect of Tc1 cells from deficient mice was markedly impaired (Fig. 5D). Approximately 20–50 times as many Tc1 effectors from IFN-γ-deficient mice were required to achieve the same level of control as with cells from wild-type mice. The performance of the Tc2 cells was unaffected, and the Tc1 populations from IFN-γ-deficient mice were indistinguishable from that of Tc2 populations from either wild-type or IFN-γ-deficient mice in their ability to control tumor growth.

**Direct effects of IFN-γ on EG7 tumor cells**

We had shown in previous studies in a related tumor model using the OVA-transfected B16 melanoma that IFN-γ had a number of effects on the tumor cultured in vitro that could be expected to hamper tumor growth or survival. Thus tumor growth was inhibited and the expression of class I and class II MHC, CD95, and TNFR55 were up-regulated when B16 melanoma cells were cultured with 1000 U/ml IFN-γ for 24–36 h. The tumor was also induced to express message for IFN-γ-inducible protein 10 and other chemokines. Experiments were therefore undertaken to identify what direct effects Tc1-derived IFN-γ might have on EG7 cell expansion and protein expression. In these experiments, EG7 cells were harvested from log phase in vitro growth and resuspended at 10^5 cells/ml in complete RPMI medium ± 1000 U/ml IFN-γ. After additional culture for 24, 48, or 72 h, the tumor cells were recovered from culture, counted, and stained for cell surface H-2Kb class I MHC, I-A^b class II MHC, and Fas protein expression (Fig. 6B). Whereas exposure to 1000 U/ml IFN-γ has been shown to profoundly suppress cell recovery of an OVA-transfected melanoma tumor line used in similar studies in the laboratory (10), no deleterious effect was seen in the growth kinetics of EG7 thymoma cells (Fig. 6A). However, the presence of exogenous IFN-γ was seen to induce a roughly 10-fold enhancement of class I MHC expression in EG7, and a modest up-regulation of Fas. Neither of these effects was believed to significantly impact the overall immunogenicity of these tumor cells, however, because EG7 cells already demonstrated ample expression of both class I MHC and Fas in the absence of added IFN-γ (Fig. 6B). In this same experiment, 10^7 cells from each treatment group were collected at 24, 48, and 72 h of culture for use in the preparation of total RNA. Using these samples, RNase protection assays were then conducted to determine the expression of the genes of these cells encoding a fairly comprehensive list of cytokines and cytokine receptors and chemokines, chemokine receptors, and so-called death genes. Generally, very little expression of these genes was observed among EG7 cells, regardless of their exposure to IFN-γ in vitro. Still, bands were identified that corresponded to hybridized IL-2, TNF-α, TGF-β1, IL-11, monocyte-CSF, and leukocyte-inhibitory factor cytokine mRNA. Also, some expression was seen for the IL-7, IL-9, IL-13, IL-15, IL-4, and IL-2R α-chains, common γ-chain, and IL-2R β-chain. Although EG7 cells did not appear to express any of the chemokine gene products assayed, these cells were observed to express significant levels of the chemokine receptor CXCR4, as well as more limited amounts of CCR4.
mRNA. The expression of death genes did not appear to be affected by culture in IFN-γ.

Migration patterns of donor and host cells on transfer of perforin or IFN-γ-deficient effectors

We showed above that the adoptive transfer of Tc1 effector cells led to an increased rate of host cell entry into the draining lymph nodes and that this correlated with their superior efficiency in the control of tumor growth. We next determined the effect of gene deficiencies in the Tc1 effector cells on the recruitment of cells into the draining lymph nodes as described in Materials and Methods. Tc1 4-day effectors were prepared from perforin-deficient OT-1 mice and were injected into mice that had been injected with tumor on day −7. The accumulation of host cells in the ipsi- and contralateral nodes and the spleen was determined as before. It can be seen in Fig. 7 that the accumulation of host cells was the same in mice that received wild-type (●) or perforin-deficient effector cells (○), with a possible slight diminution of the accumulation of B cells, neutrophils, and macrophages. In contrast, mice receiving Tc1 effectors from IFN-γ-deficient OT-1 showed a reduced rate of host cell entry (■). The entry of CD4 cells, CD8 cells, and macrophages was markedly reduced. The entry of B cells was slightly reduced, and the entry of neutrophils was delayed.

Discussion

It is clear that both Tc1 and Tc2 effectors can kill tumor cells in vitro and are about equally effective, data not shown. They can also mediate the complete rejection of coinjected tumors in vivo, and the Tc2 are one-fifth to one-tenth as active as Tc1 (Fig. 2A). When the CD8 effectors are injected i.v., however, whether simultaneously (Fig. 2B) with the tumor or 7 days later (Fig. 2C), the Tc2 are 20–100 times less effective than Tc1. More cells were required, however, if the adoptive transfer was delayed to day 7, presumably because there were more tumor cells to control. We presume that the difference between i.v.-injected Tc1 and Tc2 may be due, in part, to the relative effectiveness of the two populations in gaining entry to the draining lymph node and in the ability to recruit host cells to the site. Indeed, we have shown (Fig. 3) that although both populations enter the spleen early, the entry of Tc2 cells into the draining lymph node is less rapid for Tc2 and that they are less effective at recruiting host cells to the nodes. We have shown, however, that the in vitro killing in a 4-h chromium release assay is perforin mediated (20) whereas the effectiveness of CD8 T cells injected i.v. appears to be independent of perforin secretion (Fig. 5A), and the actual method of tumor eradication in vivo is unclear. It is therefore possible that factors other than lymph node homing may also be important causing the reduced effectiveness of Tc2 cells.

The measurement of host cell accumulation in the absence of adoptive therapy shows that there is a considerable immune response to the tumor Ag, which is, however, unable to limit tumor growth (Fig. 3). This has been seen in a number of tumor models and it has been suggested that tumor Ags elicit low affinity responses because high affinity responses, to what is essentially a self Ag, have been deleted. The Ag here, however, is OVA, and there is no a priori reason why this should elicit T cells with low affinity receptors. It is possible that the T cells are in some other way qualitatively different in the response of untreated mice, and this is currently the focus of further study. The accumulation of host cells in the untreated mice occurs both in the draining lymph nodes and in the spleen but is not seen in the contralateral node. This accumulation includes CD4 and CD8 T cells, B cells, NK cells, neutrophils, and macrophages. Adoptive transfer of Tc1 effectors accelerates the accumulation of host cells, especially host B cells, NK cells, and neutrophils in the draining lymph nodes, and it is tempting to believe that these cells may have a role in controlling tumor growth. There are more CD4 and CD8 host cells at the height of the response in the untreated mice than there are when the Tc1-treated mice undergo tumor rejection (Fig. 3). NK cell and eosinophils, however, reach higher levels in the treated mice than the untreated and are perhaps the key to successful control of the tumor growth. The adoptive transfer of Tc2 cells (Fig. 3) has little effect on the accumulation of host cells (probably because they themselves were delayed in their entry into the draining nodes), and this may correlate with their lesser effectiveness in controlling tumor growth.

Histological analyses of frozen sections showed that Tc1 and Tc2 cells also infiltrated the tumor (data not shown), but no definitive conclusion could be drawn about the kinetics or relative amounts of infiltration of the two populations. The positive correlation between the degree of host cell migration into the draining lymph node and the control of tumor growth in both wild-type Tc1 vs Tc2 and Tc1 wild-type vs Tc1 IFN-γ−− provides circumstantial evidence that factors that control both donor and host cell migration play a very important role in tumor rejection.

Although the host response in the absence of adoptive transfer was not able to control the growth of the tumor, it clearly played a role after adoptive transfer in that cells accumulated more extensively in the draining lymph nodes and the host cells departed abruptly after tumor rejection. The role of the host response was also seen when C57BL/6 SCID mice were used as recipients (Fig. 4). The initial control of tumor growth was indistinguishable from that in wild-type host, but at later times the tumor growth was not controlled in SCID recipients and tumors grew out after a delay of only 7–10 days.
The departure of both host and donor cells after the initial control of the tumor is reminiscent of the results reported by Shrikant and Mescher (7) in which adoptively transferred cells first migrated into the peritoneal cavity where the tumor was growing but left subsequently, allowing the tumor to grow out at later times. One can also speculate that the failure of adoptively transferred CD8 T cells to control more established tumors while rejecting newly injected tumors, seen by Hanson et al. (11) may be dependent on differences in cell migration into the tumor and may be a consequence of the fact that the older tumor no longer secretes chemotactic factors. Alternatively, the secretion of excessive concentrations of chemokine might lead to the down-regulation of chemokine receptors and the failure of further migration before the donor or host cells reached the tumor.

Finally, the effector cells used in this study were very efficient in controlling tumor growth, and this may be because they were effector cells requiring no additional activation. We have shown in a different model that effector CD8 T cells, expressing chemokine receptors CCR5 (Tc1) or CCR4 (Tc2), migrate rapidly into sites of inflammation (21) whereas naive cells and memory cells require activation before they can migrate (22). In the current studies, naive OT-1 CD8 T cells were totally unable to control tumor growth on adoptive transfer even at the highest cell number, whereas memory cells were less effective than memory cells on a per cell basis (data not shown).

We have begun here the first phase of an analysis of the factors that control these events and lead to more effective control of tumor growth by using effector cells generated from gene knockout mice. OT-1 mice were crossed to perforin, IFN-γ, IL-4, or IL-5 knockout mice and bred to produce OT-1-positive mice that were homozygous for each of the gene defects. We showed that for each knockout combination, the mice were defective only for the deleted gene and that the ability to kill target cells (except for the perforin knockouts) or secrete other cytokines was not affected. The first factor that we examined was perforin; it was somewhat surprising to find that effectors from Tc1 or Tc2 from perforin knockout mice were as effective as those from wild-type donors, and it is clear that control of tumor growth is not dependent on direct perforin-mediated lysis (Fig. 5A). This was surprising because the effectors clearly killed EG7 cells in an in vitro assay by perforin-mediated lysis. Kagi et al. (23) have shown a key role for perforin-mediated lysis in CD8 and NK lysis of a variety of target cells, whereas others have shown that Fas ligand (24)- or TNF-α (25)-mediated killing may be important in responses to tumors. We are currently breeding the OT-1 transgene onto the Fas ligand-deficient (GLD) and the TNF-α-deficient backgrounds to test for the role of these alternate cytolytic mechanisms in this model.

Similar experiments were conducted with Tc1 and Tc2 effectors prepared from OT-1 IL-4- and IL-5-deficient mice. No effect was seen with either Tc1 or Tc2 for either cytokine, and effectors from wild-type and knockout mice were equally effective as shown in Fig. 5, B and C. This finding is contrast to our previous finding with the B16 melanoma tumor model (2) in which we had shown that the effectiveness of the Tc2 (but not Tc1) effectors was severely compromised.
in IL-4 or IL-5 knockout mice. Whether the difference in findings is dependent on a difference between the two tumors (melanoma vs thymoma) or is a consequence of the different locations of the two tumors (lung vs intradermal) is not known.

In contrast, Tc1 effectors from OT-1 mice deficient in IFN-γ were significantly less able to control tumor growth (Fig. 5D) than those from wild-type mice, as had been seen in the B16 melanoma model (10). The Tc2 cells from the IFN-γ knockout mice, however, were as effective as Tc2 effectors from wild-type mice, indicating that IFN-γ played no role in the function of the Tc2 cells. The effectiveness of Tc1 effector cells from Tc1 IFN-γ-deficient mice was equal to that of some Tc2 effectors from wild-type mice, suggesting that there might be some basic mechanism common to both Tc1 and Tc2 effectors and that the Tc1 had an additional IFN-mediated effect.

It is clear that Tc1 secretion of IFN-γ is a significant factor in the ability of the effector cells to control tumor growth. In addition to its role as a powerful immunoregulatory cytokine, IFN-γ has also been shown to exert a variety of direct inhibitory effects on a number of different tumor cell types. For example, in tumors that express only meager amounts of cell surface class I MHC, exposure to IFN-γ has been shown to markedly up-regulate class I expression, enhancing the functional antigenicity of the tumor cells (26). Also, IFN-γ can induce cell cycle arrest of tumor cells (27) and promote an overall state of tumor dormancy in vivo (28). Furthermore, exposure to IFN-γ has been shown to also directly enhance the expression of Fas on the surface of renal carcinoma cells, heightening their susceptibility to Fas/Fas ligand-mediated mechanisms of killing (29). In the B16 model we showed that high concentrations of IFN-γ prevented in vitro growth of the tumor and led to an up-regulation of class I and class II MHC, Fas, and TNF receptors, all potentially increasing the vulnerability of the tumor cells (30). Furthermore, exposure to IFN-γ has been shown to also directly enhance the expression of Fas on the surface of renal carcinoma cells, heightening their susceptibility to Fas/Fas ligand-mediated mechanisms of killing (29). In the B16 model we showed that high concentrations of IFN-γ prevented in vitro growth of the tumor and led to an up-regulation of class I and class II MHC, Fas, and TNF receptors, all potentially increasing the vulnerability of the tumor cells (30).

The expression of death genes was not affected. It seems likely that the Tc1 had an additional IFN-mediated effect.

We found, however, that effectors from IFN-γ-deficient mice were significantly impaired in their ability to recruit host cells into the draining lymph nodes, whereas effectors from perforin-deficient mice were unimpaired. It seems likely that the IFN-γ plays a significant role in the recruitment of host cells and that these are essential for complete control of tumor growth. This provides yet another example of the correlation between the ability of donor cells to cause a host cell accumulation in the draining node and the ability to limit tumor growth observed in the accompanying study, and it suggests that the control of migration is a key factor in the ability of the immune response to bring about tumor rejection. The role of chemokines and chemokine receptor expression in this process is currently under investigation.