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*J Immunol* 1998; 160:5773-5780; ;
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IL-3 Enhances Both Presentation of Exogenous Particulate Antigen in Association with Class I Major Histocompatibility Antigen and Generation of Primary Tumor-Specific Cytolytic T Lymphocytes

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Recent studies have reported that APC can present particulate exogenous Ag in the context of class I MHC to CD8+ CTL, and our laboratory demonstrated that IL-3 could enhance CTL generation to exogenous Ag. In this paper, we wished to determine whether presentation of particulate Ag could be enhanced by IL-3. A T cell hybridoma, B3Z86/90.14 (B3Z) restricted to Ova/Kb, was used as an indicator for presentation of particulate Ag with class I MHC. When activated, this hybridoma expresses lacZ, allowing a simple colorimetric measurement of Ag-specific T cell stimulation. We demonstrated that bone marrow cells stimulated by IL-3 in vivo and in vitro exhibited significantly increased presentation of exogenous OVA linked to beads. Lysate from OVA-transfected line 1 murine lung adenocarcinoma cells (line 1/OVA) was also presented by IL-3-stimulated bone marrow cells, suggesting that these APC can process tumor fragments or debris. Studies using TAP1/2-deficient mice and Ag presentation inhibitors indicate that this exogenous Ag presentation is mediated via the conventional class I MHC pathway. Adoptive transfer of IL-3-stimulated bone marrow cells pulsed with lysate from line 1/OVA tumor cells into naive recipient mice led to the generation of a potent CTL response. These observations indicate that use of such cells may provide a new avenue for development of tumor vaccines. The Journal of Immunology, 1998, 160: 5773–5780.

The conventional Ag processing and presenting pathway for class I MHC uses antigenic peptides that are synthesized within the cell and transported into the endoplasmic reticulum (ER)5 via the transporters of Ag presentation (TAP), where they bind nascent class I MHC molecules. The antigenic peptides in association with class I MHC molecules are conveyed through the ER/Golgi transport and presented on the target cell surface (1). Expression of cytokines or costimulatory molecules by the APCs augments the response of the CTL to the peptide-MHC complex (2).

Recently, considerable attention has been focused on the finding that exogenous Ag prepared in particulate form, such as linked to beads, can be internalized by professional APC. The Ags are subsequently processed and the resulting peptides presented in the context of class I MHC (3–6), thus constituting an exception to the generalization that exogenous Ag is not presented by class I MHC molecules. This phenomenon provides a possible mechanism for stimulation of CD8+ CTL by professional APCs, even if the APC is not synthesizing the antigenic peptides endogenously. One physiologic situation in which this pathway may play a crucial role is in the generation of CTL reactive to tumors. In general, tumor cells fail to express potent costimulatory molecules or stimulatory cytokines, and are therefore inefficient at directly stimulating CTL. Thus, the effective induction of tumor-specific CTL responses probably requires the uptake of exogenous tumor Ag, processing and presentation by professional APCs. Factors that affect these processes, which are thought to be rather inefficient, might significantly enhance the generation of CTL. We have recently shown that IL-3 enhances the generation of exogenous Ag presenting APC and concomitantly enhances the generation of tumor-reactive CTL (7, 8).

These data suggested that IL-3 might be particularly effective in enhancing presentation of exogenous Ag if the Ag could be delivered in a form that was able to be internalized by professional APCs, as the increased number of APCs could then enhance the generation of CTL effector cells. Here we examined whether IL-3 could enhance the presentation of exogenous Ag by APC in vivo and in vitro, and whether cytokine-treated APC charged with Ag could induce a CTL response in naive recipients.

Materials and Methods

Animals

Hybrid (BALB/cByJ × C57BL/6)F1, mice (CBByB6F1/J) (H-2b), C57BL/6 mice (H-2b), and BALB/cByJ (H-2d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TAP1-deficient mice (9) were generously provided by Dr. Luc Van Kaer (Howard Hughes Medical Research
Institute, Vanderbilt University, Nashville, TN). All mice were used at 6 to 8 wk of age.

Cell lines and reagents

Line 1 (L1), a BALB/c (H-2d) alveolar cell lung adenocarcinoma, was originally obtained from Dr. John Yuhas (Oak Ridge National Laboratory, Oak Ridge, TN) (10). IL-3-transfected line 1 (L1/IL-3), which produces 2500 pg/ml IL-3, and IL-4-transfected line 1 (L1/IL-4), which produces 250 ng/ml IL-4, were described previously (7, 11). Line L1/OVA is a stable OVA-expressing line 1 transfectant (8). EL-4 is a thymoma from C57BL/6 (H-2b) mice (12), and EL-4/OVA is a stable OVA-expressing EL-4 line derived by transfection (4). YAC-1 is an A/Sn leukemia (13). B3Z, a T cell hybridoma that recognizes K\(^\text{b}\) with peptide 257-264 (SIINFEKL) of OVA, was previously described (14). B3Z contains a DNA construct coding for the lacZ gene, under the control of the IL-2 regulatory elements. Upon activation of B3Z through the TCR, the lacZ gene is expressed, allowing determination of the activation of the T cell hybrid through colorimetric assays (14). All cell lines were maintained in vitro in EXCELL-300 (JRH Biosciences, Lenexa, KS) supplemented with 2 or 5% FBS (HyClone, Logan, UT).

Sodium azide, 2-deoxyglucose, cytochalasin B, brefeldin A (BFA), and OVA were obtained from Sigma (St. Louis, MO). Tosyl-activated beads (M-280) were purchased from Dynal (Great Neck, NY); OVA was covalently bound to the beads according to the manufacturer’s instructions and the conjugate was designated as particulate OVA. The BCA protein assay (Pierce, Rockford, IL) was utilized to determine the amount of OVA linked to the beads by the difference in OD between the solution of OVA before and after the conjugation. The concentration of OVA was 80 to 100 μg/ml of beads. IL-3 supernatant and L1 supernatant were prepared as previously described (15). Recombinant mouse IL-3 (rIL-3) was purchased from Collaborative Research Products (Bedford, MA).

In vivo bone marrow and spleen isolation

Tumor cells (10\(^5\) parental line 1 or L1/IL-3) in a volume of 50 μl were injected i.m. into the left hind thighs of CByB6F1/J mice. Bone marrow and spleen were isolated and dissociated into single-cell suspensions 10 or 20 days later.

In vitro bone marrow culture

Bone marrow cells were isolated from the femurs of naive CByB6F1/J mice and plated at 2 × 10\(^5\) cells/well in 12-well plates or 2 × 10\(^5\) in 100-mm plates. The cells were cultured for 4, 7, and 10 days with recombinant IL-3, L1/IL-3 supernatant, or L1 supernatant as indicated in the appropriate figure legends. Cells were harvested, and tested using the B3Z T hybridoma activation assay.

Generation of L1/OVA tumor cell lysate

L1/OVA (H-2d) tumor cells were treated with DMSO for 7 days to induce class I MHC expression (16). To make allogeneic CTL, each well received 5 × 10\(^5\) C57BL/6 spleen cells stimulated with 2.5 × 10\(^5\) irradiated BALB/cByJ spleen cells (2000 rad) in 24-well plates. The final volume for each well was 2 ml. Four days later, cells were harvested and the cytotoxicity was tested against DMSO-induced line 1 (high class I expression). These allogeneic CTL exhibited 65% specific lysis of DMSO-induced line 1 at an E:T ratio of 100:1. To make tumor cell lysate, 1 × 10\(^6\) DMSO-induced L1/OVA cells were incubated with 1 × 10\(^6\) allogeneic CTL in 12-well plates for 24 h. Lysates were collected and centrifuged at 1200 × g for 30 min. The supernatant was removed and the pellet was resuspended with 5% FBS-supplemented EXCELL. The Ag concentration of tumor cell lysate as indicated in the figure legends was determined by the method of L1/OVA cells or the concentration of OVA by ELISA as previously described (4). B3Z T hybridoma activation assays

Activation of B3Z cells was measured by lacZ activity, as previously described (14). Briefly, 5 × 10\(^5\) putative APCs (as indicated in the appropriate figure legends) and 5 × 10\(^5\) B3Z cells were mixed in 24-well culture plates, in the presence of varying concentrations of particulate OVA. A total of 16 to 20 h later, the supernatant was removed, the cultures were washed with PBS, and the cells were fixed with cold 2% formaldehyde/0.2% glutaraldehyde for 10 min at 4°C. They were washed again with PBS and overlaid with 250 μl of 0.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Fisher Biotech, Pittsburgh, PA). The total number of blue cells in the cultures was examined using a microscope after an 8- to 12-h incubation at 37°C.

Inhibition studies of Ag presentation

Naive CByB6F1/J bone marrow–cultured cells treated with or without 250 pg/ml of IL-3 supernatant for 7 days were harvested. In all, 5 × 10\(^5\) cells/well were plated in 24-well plates in fresh medium overnight before the inhibition assay. In some experiments, cells were incubated with sodium azide (10 mM) and 2-deoxyglucose (60 mM), or cytchalasin B (20 μM) for 1 h at 37°C, and then Ag (tumor cell lysate) was added for another 18 h in the presence of inhibitors. For BFA, bone marrow–cultured cells were first incubated with Ag for 2 h, and then BFA (20 μM) was added for another 17 h in the presence of Ag. Subsequently, cells were fixed with 1% paraformaldehyde, washed twice with medium, and then incubated with 5 × 10\(^5\) B3Z cells to evaluate Ag presentation.

Priming with particulate Ag-pulsed cells from bone marrow cultures

Bone marrow cells were harvested from femurs of CByB6F1/J mice and plated at 2 × 10\(^6\) in 100-mm plates for 7 days with 250 pg/ml IL-3 supernatant or control line 1 supernatant. Using L1/OVA tumor cell lysate as exogenous Ag, cells were incubated with lysate prepared from 8 × 10\(^6\) L1/OVA tumor cells at 37°C for 24 h. After incubation, the cells were collected, washed twice with medium, counted, and injected i.p. into naive CByB6F1/J mice. Additional control animals were injected with L1/OVA tumor cell lysate alone, or were left untreated. Two weeks later, spleens were harvested and 2 × 10\(^6\) splenocytes were restimulated in vitro with 2 × 10\(^3\) irradiated EL-4/OVA for 5 days in EXCELL-300 with 5% FBS. Cytotoxicity was measured in a 5-h ³¹Cr-release assay as previously described by Maryanski et al. (17). Target cells tested were EL-4 and EL-4/OVA.

Results

IL-3 enhances presentation of exogenous particulate OVA in association with class I MHC

Our previous work suggested that IL-3 enhances the presentation of exogenous tumor Ags in vivo (8). To further examine this process, we devised a method that delivered the cytokine IL-3 in vivo but enabled us to examine Ag presentation in vitro. IL-3-transfected line 1 (L1/IL-3), the parental line 1 (L1), or control line 1 transfectant with IL-4 (L1/IL-4) tumor cells were injected into CByB6F1/J mice (H-2\(^\text{b}\text{b}\)). The tumors were allowed to grow and produce cytokine for 10 or 20 days and then bone marrow and spleen cells were harvested. The efficiency of exogenous Ag presentation with class I MHC was evaluated using a K\(^\text{b}\)/OVA peptide–specific T cell hybridoma reporter system. B3Z is a T cell hybrid that expresses the lacZ gene upon recognition of an OVA peptide with the class I MHC molecule K\(^\text{b}\) (14). As the host cells express H-2\(^\text{b}\), but the line 1 cells, which are H-2\(^\text{b}\), do not, only host cells can activate the B3Z cells (8). Activated B3Z cells turn blue in the presence of X-gal substrate, allowing simple determination of the number of activated B3Z. For exogenous particulate Ag, we first used OVA-conjugated beads, because this system has been well characterized (3).

Treatment of both bone marrow and spleen cultures with IL-3 resulted in a population of cells with an enhanced ability to present particulate OVA (Fig. 1). The effect on the bone marrow cells, the more potent of the populations tested, was greatest at day 10, but also present at day 20. Line 1 cells transfected with IL-3 also enhanced stimulation of B3Z by spleen cells. Neither the parental line 1 cells alone nor line 1 cells secreting IL-4 altered the ability of these cells to present OVA with class I MHC. To eliminate the possibility that the enhanced Ag presentation of cells by IL-3 resulted from an increased expression of class I MHC, we examined the class I MHC level of cells by flow cytometric analysis. Both bone marrow and spleen cells isolated from animals bearing L1/IL-3 tumors expressed class I MHC molecules at a comparable level to cells from animals bearing control tumors (data not shown).
IL-3 enhances presentation of particulate OVA by bone marrow and spleen cells. CB6F1/J mice were injected with 10^5 tumor cells of the parental line 1 (L1), IL-3 transfected line 1 (L1/IL-3), or IL-4-transfected line 1 (L1/IL-4). Bone marrow and spleen cells were harvested 10 and 20 days later and tested for their ability to present particulate OVA. In all, 5 × 10^5 cells of the indicated APC sources and 5 × 10^5 B3Z cells were cultured with 5 μg/ml of particulate OVA (OVA-conjugated beads) for 16 to 20 h in 24-well plates. Fixation and X-gal processing were performed as described in Materials and Methods in this and all subsequent relevant figures. Data are presented as frequency (stimulating cells/10^6 cells). This experiment has been repeated three times with similar results.

**FIGURE 1.** IL-3 enhances presentation of particulate OVA by bone marrow and spleen cells. CB6F1/J mice were injected with 10^5 tumor cells of the parental line 1 (L1), IL-3 transfected line 1 (L1/IL-3), or IL-4-transfected line 1 (L1/IL-4). Bone marrow and spleen cells were harvested 10 and 20 days later and tested for their ability to present particulate OVA. In all, 5 × 10^5 cells of the indicated APC sources and 5 × 10^5 B3Z cells were cultured with 5 μg/ml of particulate OVA (OVA-conjugated beads) for 16 to 20 h in 24-well plates. Fixation and X-gal processing were performed as described in Materials and Methods in this and all subsequent relevant figures. Data are presented as frequency (stimulating cells/10^6 cells). This experiment has been repeated three times with similar results.

**FIGURE 2.** IL-3 treatment of bone marrow cells in vitro enhances exogenous Ag presentation of particulate OVA. Bone marrow cells were harvested from naive (CB6F1/J) mice, cultured for 4, 7, or 10 days, and then harvested and trypan blue-excluding cells counted. In all, 5 × 10^5 cells were incubated with 5 × 10^5 B3Z in the presence of 2.5 μg/ml particulate OVA for 18 h in 24-well plates. For A and B, the bone marrow cells were cultured with dilutions of L1/IL-3 supernatant (closed symbols) containing 62.5 (circles), 125 (squares), or 250 pg/ml (triangles) of IL-3 or equivalent concentrations of the control supernatant from L1 (open symbols). For C and D, the bone marrow cells were cultured with L1/IL-3 supernatant (closed triangles) or rIL-3 (open triangles) at 250 pg/ml. Data are presented as (A, C) frequency (stimulating cells/10^6 cells) or as (B, D) the total number of stimulating cells (frequency × number of cells recovered).

To determine whether our stimulation of APC in vivo by IL-3 could be replicated in a simpler in vitro environment, bone marrow cells from unmanipulated mice were cultured in vitro with IL-3 (from L1/IL-3 supernatant), or control line 1 supernatant. After 4, 7, or 10 days, the cells were harvested, counted, replated at equal cell numbers, pulsed with particulate OVA, and tested for stimulation of B3Z cells. Similar to the in vivo data obtained above, cultures of bone marrow cells treated with IL-3 supernatant in vitro exhibited significantly higher presentation of particulate OVA than did the control cultures (Fig. 2A). The increase in the ability of the cells to present Ag, which was greatest after 7 days in culture, did not appear to be influenced by the dose of IL-3 in that 62.5 pg/ml was as effective as 250 pg/ml if a constant number of cells were used (Fig. 2A). However, there was a dose dependence on the total number of cells recovered from the cultures. The total number of cells in the IL-3-treated cultures increased four- to fivefold in number, whereas the control cultures decreased in cell number by 10-fold (data not shown). The total number of stimulating cells in the cultures, which takes into account both the increase in cell number and the enhanced Ag-presenting activity, is shown in Figure 2B and illustrates that the proliferation of the APC was dependent on the amount of IL-3 used for stimulation. Thus, the higher doses of IL-3 appear to stimulate the proliferation of both the APC as well as other cells in the bone marrow cultures. Whereas the ability of the cells to present particulate OVA was similar at all of the IL-3 doses tested (Fig. 2A), the total yield of APC was highest at the greatest concentration of IL-3 (Fig. 2B). To ensure that the effects we observed with the L1/IL-3 were indeed due to IL-3 and not to a combination of IL-3 and other substances that might be present in the supernatants from the L1/IL-3 cells, we repeated these experiments using recombinant IL-3 at the concentration that had given the best response in the initial experiments (250 pg/ml). Recombinant IL-3 gave very similar results to L1/IL-3 in terms of the ability of the cells to activate B3Z (Fig. 2C) as well as the total yield of stimulating cells (Fig. 2D). There was a greater drop in total yield at day 10 with L1/IL-3 supernatant, probably due to a
greater depletion of media nutrients. Due to the similar effects and the lower cost of L1/IL-3 supernatant, this reagent was used in all subsequent experiments. In summary, IL-3 increases both the ability of the cells to present exogenous particulate Ag in the context of class I and the total number of cells present. Further, the enhancement by IL-3 of Ag presentation with class I occurs in vitro as well as following in vivo exposure.

Phenotype of APC in bone marrow cultures stimulated in vitro with IL-3

To characterize the APC in the bone marrow cultures stimulated in vitro with IL-3, we determined the cell surface phenotype of the presenting cells. Because of the heterogeneous nature of the cultures, this was done by treating the cells with a variety of Abs, followed by complement treatment and the Ag-presenting activity of the remaining cells analyzed in the B3Z assay. These results are illustrated in Figure 3. Abs to CD8, CD4, and B220 all had little effect, indicating that the APC are neither T nor B cells. The greatest depletion of activity was seen following treatment with Abs to the leukocyte common Ag (CD45) and to the macrophage markers MP20 and Mac-1. The APC activity was also depleted by treatment with Abs to MHC class II, B7-1, and B7-2. Thus, the cells have the attributes of professional APC such as macrophages or dendritic cells (DC). Abs to the macrophage markers F4/80 and FcR also depleted the activity. Abs to two different DC markers, 33D1 and NLDC, gave somewhat different results in that anti-NLDC depleted ~45% while the anti-33D1 depleted only about 30%. These results are perhaps not surprising given the heterogeneity of the cells within the cultures. The APC may be macrophages or transitional cells with characteristics of both DC and macrophages. Alternatively, more than one cell type may be responsible for the Ag-presenting activity we observe.

Presentation of exogenous tumor cell lysate by bone marrow cells is enhanced by IL-3

To determine the potential relevance of our observation that IL-3 can enhance exogenous particulate Ag presentation associated with class I MHC, we evaluated the efficacy of these cells in presenting tumor fragments/debris. Bone marrow cells from unmanipulated mice were cultured in vitro with L1/IL-3 supernatant or control line 1 supernatant for 7 days. Cells were harvested and pulsed with titrated concentrations of L1/OVA tumor cell lysate and tested for stimulation of B3Z cells. The tumor cell lysate contained no viable cells as detected by trypan blue exclusion. In all, 10^8 L1/OVA lysed cells contained 100 ng of OVA protein as measured by Ab techniques (ELISA) (4). Figure 4 illustrates the appearance of the APC pulsed with tumor lysate and mixed with the B3Z cells following development of the assay with the X-gal substrate. Many more blue, activated B3Z cells were observed in the wells containing the IL-3-stimulated APC, and the activated T cells were often in clusters. In contrast, wells with cells from the control line 1-treated cultures had only an occasional cluster. Our previous results with this assay (8) have indicated that each of these clusters usually contains a single APC-like cell. Thus, the frequency of APC capable of presenting exogenous tumor Ag is enhanced by IL-3. The number of activated B3Z cells was quantified as illustrated in Figure 5. The cultures of bone marrow cells treated in vitro with IL-3 were significantly better in presenting
The experiment was repeated twice with similar results. This observation suggests that the presentation of tumor cell lysate by the cultured bone marrow cells uses the classical class I pathway requiring the TAP1-TAP2 transporter system to transfer the internalized tumor cell lysate from the cytosol into the ER.

Previous reports have shown that for the presentation of exogenous particulate Ag by bone marrow macrophages, Ags need to be internalized, requiring an energy-dependent process (3). To elucidate whether internalization and energy were indispensable for the optimal presentation of the tumor-lysate form of Ag in our bone marrow culture system, cells were incubated with 2-deoxyglucose and sodium azide (ATP blockers), or cytochalasin B (which interrupts microtubules that inhibits phagocytosis) for 1 h before the addition of the tumor cell lysate. Treatment with the inhibitors reduced the efficiency for activation of B3Z of both the IL-3-stimulated and control bone marrow cells by over 60% (Fig. 7). The experiment was repeated twice with similar results. This observation clearly indicates that energy and phagocytosis are needed for the presentation of exogenous tumor cell lysate.

FIGURE 6. Enhanced presentation of L1/OVA tumor cell lysate by IL-3-treated bone marrow-cultured cells is Ag dose and TAP1 dependent. Bone marrow cells were harvested from naive (CBByB6F1/J) mice (squares) or TAP1-deficient (H-2b) mice (circles), and cultured with L1/IL-3 supernatant (closed symbols) or the control supernatant from L1 (open symbols). After 7 days, cells were harvested and trypan blue-excluding cells counted. A total of $5 \times 10^5$ cells were incubated with a serial dilution of L1/OVA tumor cell lysate for 24 h in 24-well plates. These cells were washed with medium twice to remove the residual Ag, and $5 \times 10^5$ B3Z hybridoma cells were added and incubated for another 16 to 18 h. Fixation and X-gal processing were performed. The $x$-axis is the concentration of tumor cell lysate presented as the number of lysed cells. Data are presented as frequency (stimulating cells/$10^6$ cells).

FIGURE 7. Sodium azide, 2-deoxyglucose, cytochalasin B, and BFA inhibit the presentation of L1/OVA tumor cell lysate with class I MHC molecules. Bone marrow cells were harvested from naive (CBByB6F1/J) mice and cultured with L1/IL-3 supernatant (closed bars) or the control supernatant from L1 (open bars). After 7 days, cells were harvested and trypan blue-excluding cells counted. A total of $5 \times 10^5$ cells were incubated with sodium azide (10 mM) and 2-deoxyglucose (60 mM), or cytochalasin B (20 mM) for 1 h at 37°C, and then lysate from $5 \times 10^5$ tumor cells was added for another 18 h in the presence of inhibitors. For BFA, $5 \times 10^5$ cells were first incubated with Ag for 2 h and then BFA (20 mM) was added for another 17 h in the presence of Ag. Subsequently, cells were fixed with 1% paraformaldehyde, and Ag presentation was evaluated by the B3Z activation assay ($5 \times 10^5$ cells per well). Data are presented as frequency (stimulating cells/$10^6$ cells). The experiment was repeated twice with similar results.
against OVA (Fig. 8). In contrast, mice immunized with the con-
was tested by assaying the cytotoxicity of restimulated splenocytes
these Ag-charged cells i.p. into naive mice. Generation of CTL
were pulsed with L1/OVA tumor cell lysate for 24 h. We injected
for 7 days in the presence of IL-3 or control line 1 supernatant
3-treated cells could stimulate CTL. Bone marrow cells cultured
stimulated in vitro with IL-3 led us to examine whether these IL-
OVA with MHC class I molecules by naive bone marrow cells
The high efficiency of processing tumor cell lysate and presenting
potent CTL response in naive recipients
appears to be the same for both forms of Ag.

Discussion
This report demonstrates that the cytokine IL-3 can enhance pre-
sentation of exogenous particulate Ag in the context of class I
MHC by bone marrow-derived APC. An earlier study reported that
LPS, IFN-γ, granulocyte macrophage (GM)-CSF and, as in this
study, IL-4, did not enhance the presentation of exogenous particu-
ate Ag by bone marrow macrophages (19). In contrast to the
cytokines tested in this earlier study, we have found that IL-3
greatly enhances presentation of particulate OVA, in both the bead
and tumor cell lysate forms. Although IL-3 has been well docu-
umented to stimulate myeloid and other hematopoietic cell develop-
ment (20), this paper has shown that IL-3 can also enhance both
the absolute number and frequency of cells capable of presenting
exogenous Ag in a class I-restricted fashion.

The experiments presented here suggest that the percentage of
APC capable of presenting either bead or tumor cell lysate is ex-
tremely low in untreated bone marrow. Interestingly, Reis e Sousa
and Germain also found that only a small portion of Ag-presenting
thioglycollate-induced macrophages were susceptible to lysis by
CTL, implying that only a subset of cells was presenting antigenic
peptide with class I MHC (21). In a similar vein, Norbury et al.
obtained that a small percentage of their bone marrow macro-
phages could present exogenous Ag to the B3Z hybridoma (22).
After treatment with IL-3, we found that the presenting ability of
these bone marrow APC increased by over 50-fold, and they dis-
played CTL-priming ability whereas bone marrow cells that were
not treated with IL-3 were not able to prime T cells effectively.
Although it remains formally possible that some of the increase is
due to better Ag presentation by the same cell, given the prolifer-
aton observed in the IL-3-treated cultures and the direct evidence
of increased numbers of clusters, we think the simplest and most
conservative interpretation is that the cells with this activity are
very rare. Consequently, the number of these APC may be a limiting
step in determining the magnitude of antitumor CTL responses.
Thus, cytokines that enhance the generation of APC capable of
representing exogenous Ag could have a pivotal role in determin-
ing the antitumor CTL response to tumors (23).

To characterize the phenotype of the IL-3-treated bone marrow
APC responsible for exogenous Ag presentation, we used Ab and
complement depletion studies, combined with functional studies of
Ag presentation. The presenting bone marrow cells have many of
the expected properties of professional APC, including expression
of leukocyte common antigen (LCA), class II Ags, and costimula-
tory molecules such as B7-1 and B7-2. Interestingly, however,
the results with macrophage and DC markers did not clearly de-
lineate these lineages. One possibility is that the IL-3-treated bone
marrow cultures contain a heterogeneous population of APC, pos-
sibly containing both macrophages and DC, each with exogenous
APC ability. Alternatively, these results may reflect a single cell
with properties of both cell types. In this regard, some recent re-
ports have indicated that the traditional criteria used to distinguish
macrophages and DC, including stimulation of MLR, morphology,
surface markers, adherence ability, and phagocytic function, may not hold, especially during the immature or precursor stages (24, 25). For instance, DC progenitors have been shown to phagocytose particulate Ag containing Bacillus Calmette-Guérin organisms (26); likewise, monocyte-derived macrophages have been reported to stimulate an allogeneic MLR (27). In addition, Szabolcs et al. have shown that both macrophages and DCs can be stimulated from a common progenitor cell in human bone marrow (28). Therefore, according to the developmental plasticity of the myeloid lineage, our IL-3 treatment of bone marrow APC revealed cells with both macrophage and DC traits is not without precedent (24, 25).

Presentation of exogenous Ags with class I MHC was first demonstrated by Bevan (29) for minor histocompatibility Ags, Gordon et al. (30) for H-Y Ags, and Gooding and Edwards (31) for SV40 Ags in vivo. More recent studies have extended this concept and utilized particulate Ags such as latex beads to demonstrate this alternative class I MHC presentation (32). In agreement with other reports (3, 33), the bone marrow-derived APC described here were able to efficiently process and present exogenous Ags in the context of class I MHC, when the Ag was conjugated to beads. Importantly, when pulsed with lysate from killed Ag-expressing tumor cells, a form of Ag that might be encountered in vivo, the IL-3-cultured bone marrow cells presented the Ag very effectively. Although it is difficult to precisely determine the amount of Ag in these preparations, based on an ELISA determination, the concentration of OVA present in the tumor lysate is low (~1 ng/10⁶ cells). However, it should be noted that this may be an underestimation of the total amount of OVA since the antigenic determinants recognized by the Abs used in the ELISA assay are probably different from the antigenic peptides recognized by the T cells, and some of the Ab epitopes likely depend on conformation of the molecule. Nevertheless, these estimates suggest that the process of representation in these cells is remarkably efficient. Interestingly, Ags such as those complexed with tumor-derived heat shock proteins have been reported to be highly antigenic in vivo (34). Further, the use of cell debris or irradiated whole mammalian cells as exogenous Ags to prime CTL responses in vivo has been reported (35, 36), and has been used for a number of years in tumor immunology. Debrick, Campbell, and Staerz found that mice immunized i.v. with irradiated, OVA-expressing EL-4 tumor cells or sonicated equivalents could induce OVA-specific CTL (26). These experiments also suggested that macrophages may act as accessory cells for this class I MHC-restricted immune response (26). Carbone and Bevan also reported that the transfer of irradiated OVA-expressing, syngeneic splenocytes into naive recipients can result in the development of CTL immunity (35). The cells obtained from the IL-3-treated bone marrow cultures may be very useful in further characterizing how this process occurs.

Using the bone marrow from TAP1/2 knockout mice and inhibitors, the presentation of Ag-expressing tumor cell lysate by IL-3-treated, bone marrow-derived APC was found to require energy, phagocytosis, and access to the class I MHC pathway to stimulate CD8⁺ T cells (Fig. 7). This linking of the phagosome-to-cytosol pathway with the conventional class I MHC pathway has been reported by Kovacsics-Bankowski and Rock using OVA-conjugated latex beads as Ag and peritoneal and bone marrow macrophages as APC (36). Supporting these previous results, we performed similar experiments, using the Ag-coupled beads as an exogenous Ag. An identical inhibition pattern was found (K. Yeh, J. Frelinger, and E. Lord, unpublished observations). Furthermore, these results suggest that the same mechanisms for Ag internalization and processing are used for both the Ag-expressing tumor cell lysate and the Ag-coupled beads.

An interesting issue is what might initiate this process of tumor fragmentation in vivo. Such fragments might originate from a direct attack of the tumor by nonspecific cells of the immune system such as macrophages, granulocytes, or NK cells. This would be consistent with reports demonstrating a link between the innate and the adaptive immune responses to tumors (37). Alternatively, physical stresses, such as insufficient vascularization resulting in nutrient or oxygen deprivation, could also result in apoptosis and subsequent release of tumor fragments. In either scenario, host APC could then take up these tumor fragments and re-present tumor Ag to CTL precursors. In this regard, we have shown that IL-3-treated bone marrow APC internalize Ag-expressing tumor cell lysate, and engender a potent CTL response when transferred to naive recipients (Fig. 8). Thus, presentation of exogenous Ag by bone marrow APC may be very relevant to the in vivo situation. Interestingly, GM-CSF, a cytokine that is functionally related to IL-3 though generally thought to act on a more differentiated population in hemopoiesis, has been used as an adjuvant to elicit CTL responses in some tumor models (38). In mouse chimera studies, GM-CSF was found to promote the differentiation of host APC derived from the bone marrow, leading to tumor-reactive CTL generation that could only arise from re-presentation (39). Our previous data that IL-3 enhances CTL development, via the increased presentation of tumor Ag by rare host APC (8), also suggest that host APC can play a significant role in the generation of tumor-reactive CTL. Understanding the precise mechanisms of how cytokines such as GM-CSF and IL-3 function will be important in effectively using them as adjuvants in cancer immunotherapy.

In summary, this paper has clearly demonstrated that IL-3-treated bone marrow cells can act as potent APC for presenting tumor Ags to CTL. This has profound implications for the development of new methods to enhance the generation of tumor-reactive immune cells. IL-3 may be just one of a cascade of cytokines needed to generate both specific CTL and helper cells as well as nonspecific effector cells. It is possible that tumor cell lysates could be used in conjunction with IL-3 and GM-CSF to provide maximal stimulation of APC capable of optimally presenting Ag to a variety of T cell types. Such APC might also produce additional cytokines such as IL-12 to further enhance antitumor immunity. If, as our data suggest, APC are the limiting cells in this process, methods such as those suggested in this study have considerable potential for enhancing immunotherapy, even in tumors in which the tumor Ag has not yet been identified.

References


