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Research paper

IFNγ and TNFα account for a pro-clonogenic activity secreted by activated murine peritoneal macrophages

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Key words: F10-M3 cells, *Corynebacterium parvum*-, BCG- or *Listeria monocytogenes*-elicited macrophages, MHC class I antigens, lung colonization, macrophage pro-clonogenic acitivity, IFNγ, TNFα

Abstract

In the present study, we found that murine peritoneal macrophages elicited by BCG or *Listeria monocytogenes* release into the media an activity capable of stimulating the lung colonization as well as the expression of MHC class I antigens in B16 melanoma cells. A similar activity has previously been found in media conditioned by *Corynebacterium parvum*-elicited macrophages. Analysis by gel filtration chromatography of media conditioned by *Corynebacterium parvum*-, BCG- or *Listeria monocytogenes*-elicited macrophages revealed that the material responsible for the pro-clonogenic activity concentrated in chromatographic fractions corresponding to molecular weights (25 to 52 kDa) which are characteristic of certain cytokines. Thus, we challenged the various macrophage-conditioned media with polyclonal antibodies against IFNγ and TNFα, and found that the macrophage pro-clonogenic activity was completely abolished in the presence of anti-IFNγ antibodies, but only partially inhibited by anti-TNFα antibodies. This finding suggests a cooperative participation of the two cytokines to the pro-clonogenic activity of the media conditioned by *Corynebacterium parvum*-, BCG- or *Listeria monocytogenes*-elicited macrophages.

Introduction

In a previous study, we demonstrated that cultures of peritoneal macrophages elicited *in vivo* with *Corynebacterium parvum* contained a biological activity which stimulated the lung colonization in B16-F10 murine melanoma cells [1]. We also found that the pro-clonogenic activity stimulated the adhesiveness to endothelium, invasiveness through Matrigel and growth rate in melanoma cells. Moreover, melanoma cells exposed to the macrophage pro-clonogenic activity showed an increased expression of the major histocompatibility complex (MHC) class I antigens (K^b and D^b) [2].

In the present study, we tried to gain insight into the nature of the pro-clonogenic activity released by the elicited macrophages into their growth medium. Conditioned media of *C. parvum*- as well as of BCG- or *Listeria monocytogenes*-elicited macrophages were used as sources of a macrophage pro-clonogenic activity. These media were fractionated according to molecular weight by the use of gel filtration chromatography. The data obtained through this procedure suggested that the macrophage pro-clonogenic activity might derive from certain cytokines, a suggestion that was confirmed by the use of antibodies against IFNγ and TNFα.

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Materials and methods

Reagents

Corynebacterium parvum (Coparwax) was a gift from Wellcome Foundation (London, UK); BCG (OncoTICE) was supplied by Organon Teknika (Boxtel, The Netherlands), and *Listeria monocytogenes* was provided by Dr R. Dei (Department of Microbiology, University of Florence). AF6-88.5.3 mAb, specific for K^b determinant of MHC class I antigen (H-$2K^b$ antigen) was a gift from Dr S. Gattoni-Celli (Medical University of South Carolina, Charleston, South Carolina), rabbit polyclonal antibodies anti-murine TNFα and polyclonal antibodies anti-murine IFNγ were purchased from PeproTech EC (London, UK). Gel filtration calibration kit for molecular weight determination was purchased from Pharmacia (Uppsala, Sweden).

Table 1. Change of lung-colonizing potential and expression of H-2K^b antigen in F10-M3 melanoma cells grown in media conditioned by *C. parvum*-, BCG-, or *L. monocytogenes*-elicited macrophages.[1]

Growth conditions	No. of animals in the experiment	Lung colonies	H-2K^b antigen expression
Standard medium	5	4 ± 1[2]	6–15[3]
Macrophage-conditioned medium:			
BCG	3	> 300[4]	72–96
L. monocytogenes	5	158 ± 35[4]	54–88
C. parvum	3	220 ± 15[4]	76–98

[1] Data reported in the Table are derived from a typical experiment.
[2] Values represent the mean ± SEM of lung colonies found in the experimental animals.
[3] % of cells positive for H-2K^b antigen as assayed by flow cytometry using a specific mAb.
[4] Significantly different at $P < 0.03$ from cells grown in a standard medium.

Cell line and culture conditions

F10-M3 cells, a clone isolated from B16-F10 cell line [3], were kindly provided by Dr S. Gattoni-Celli (Medical University of South Carolina, Charleston, South Carolina). Cells were grown in Dulbecco's modified Eagle medium containing 4,500 mg/l glucose (DMEM 4500) (GIBCO, Life Technologies, Italy) supplemented with 10% fetal calf serum (FCS) (Boehringer Mannheim, Germany), at 37 °C in a 10% CO_2-humidified atmosphere. 5.0×10^5 cells were seeded in 100 mm Falcon dishes and propagated every three days by incubation with a trypsin solution (GIBCO).

Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test [4].

Preparation of macrophage-conditioned media

As previously reported, monolayers were established from macrophages isolated from a lavage of the peritoneal cavities of female C57Bl/6 or CBA mice which had received an intraperitoneal injection of 1 ml of a suspension containing 0.7 mg/ml of killed *C. parvum*, or 0.4 ml of a suspension of BCG containing 5×10^6 CFU/ml or 1 ml of a suspension of *L. monocytogenes* containing 15×10^4 CFU/ml. Macrophage-conditioned media were prepared from monolayers of *C. parvum*-, BCG- or *L. monocytogenes*-elicited macrophages (3-5 $\times$ 10^4 macrophages/cm^2) incubated for 24 h in DMEM 4500 supplemented with 250 μg/ml of bovine serum albumin (BSA) [1, 2].

Media conditioned by BCG- or *L. monocytogenes*-elicited macrophages were found to contain amounts of NO and TNFα comparable to those found in media conditioned by *C. parvum*-elicited macrophages.

Determination of the pro-clonogenic activity in media conditioned by C. parvum-*, BCG-, or* L. monocytogenes-*elicited macrophages*

Melanoma cells were grown in a standard medium for 24 h and then for a further 24 h period in macrophage-conditioned media that were supplemented with FCS to a final concentration of 10%. Melanoma cells grown for 48 h in a standard medium were used as a control. Cultures were harvested by trypsinization, washed by centrifugation in PBS, and then resuspended at 5×10^5 cells/ml in serum free DMEM 4500. 0.2 ml of this suspension were injected intravenously into female C57Bl/6 mice, which were sacrificed 21 days later. Metastatic nodules on the lung surfaces were counted using a dissecting microscope.

Expression of H-2K^b antigen in melanoma cells grown in a standard medium or in a medium conditioned by C. parvum-*, BCG- or* L. monocytogenes-*elicited macrophages*

The expression of H-2K^b antigen on melanoma cells grown in a standard medium or in a macrophage conditioned medium was measured by FACS analysis (FACScan, Becton Dickinson, Mountain View, California) using a AF6-88.5.3 mAb.

Analysis of macrophage-conditioned media by size-exclusion chromatography

The conditioned media were concentrated by lyophilization, dissolved in water and loaded on a Sephacryl S-300 column (Pharmacia) (1.5 $\times$ 40 cm) equilibrated with DMEM 4500. Chromatography was performed at RT with a flow-rate of 0.4 ml/min, and the eluted material was monitored at 280 nm. The chromatographic fractions were collected, supplemented with FCS to a final concentration of 10%, and then transferred into 24 multiwell dishes, where melanoma cells had been previously layered. After a 24-h period of incubation at 37 °C, melanoma cells were removed and tested for the expression of H-2K^b antigen.

The molecular weights of the bioactive material eluted from the Sephacryl column was determined by the use of a calibration kit containing ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), and BSA (67 kDa).

Challenge of macrophage-conditioned media with anti-IFNγ or -TNFα antibodies

The macrophage-conditioned media, intact or diluted with fresh medium (DMEM 4500 plus BSA), were challenged with various concentrations of rabbit polyclonal antibodies against murine TNFα (1:64.000-fold dilution of a 1 mg/ml antibody reacts with 1 μg/ml TNFα in a ELISA test) or with polyclonal antibodies against murine IFNγ (ND_{50} of

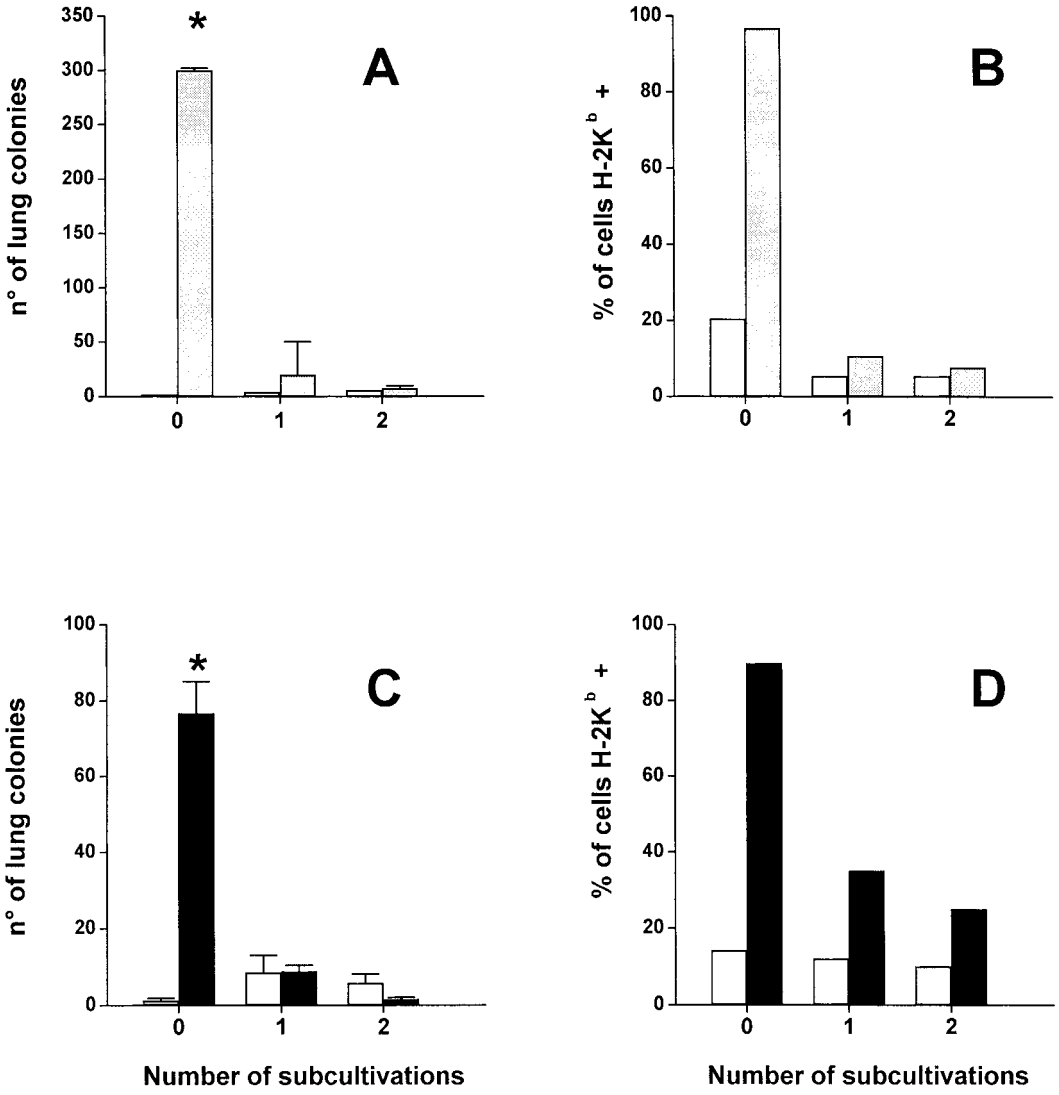

Figure 1. Lung-colonizing potential (A, C) and expression of H-2K^b antigen (B, D) in F10-M3 melanoma cells grown in media conditioned by BCG- (A, B) or *L. monocytogenes*- (C, D) elicited macrophages, and then subcultivated in a standard medium for two passages. Tumor cells grown in a standard medium for the entire experimental protocol (empty columns) served as a control. Values represent the mean ± SEM of lung colonies found in the experimental animals (groups of 3–4 mice). *Significantly different at $P < 0.02$ from cells grown in a standard medium.

the biological activity of 0.3 ng/ml of mIFNγ requires a concentration of 0.03–0.05 μg/ml of antibody). The conditioned media-antibody mixtures were incubated at 37 °C, for 1.5 h.

Statistical analysis

The statistical significance of the differences between the lung colonization of melanoma cells grown in a standard medium and in a medium conditioned by *C. parvum*-, BCG-, or *L. monocytogenes*-elicited macrophages was determined by the use of the Mann–Whitney test.

Results

As shown in Table 1, growth in media conditioned by BCG- or *L. monocytogenes*-elicited macrophages enhanced the lung-colonizing potential and stimulated the expression of H-2K^b antigen in F10-M3 melanoma cells to levels comparable to that of melanoma cells grown in media conditioned by *C. parvum*-elicited macrophages. As previously found with B16 melanoma cells grown in media conditioned by *C. parvum*-elicited macrophages [2], the lung-colonizing potential and expression of H-2K^b antigen in melanoma cells stimulated by the pro-clonogenic activity released by BCG- or *L. monocytogenes*-elicited macrophages declined to the same level as that found in unstimulated cells after two subcultivations in a standard medium (Figure 1).

Figure 2 gives the profiles of the pro-clonogenic activity detected in the various fractions obtained by gel filtration chromatographic analysis of media conditioned by *C. parvum*-, BCG- or *L. monocytogenes*-elicited macrophages. Regardless of the eliciting agent, the highest levels of the macrophage pro-clonogenic activity were found in chromatographic fractions corresponding to molecular weights ranging between 25 and 52 kDa (maximum peak of activity at 35–38 kDa.). These values are compatible with various cytokines, in particular IFNγ (34 kDa) [5] and TNFα (51 kDa) [6]. At this point, we investigated

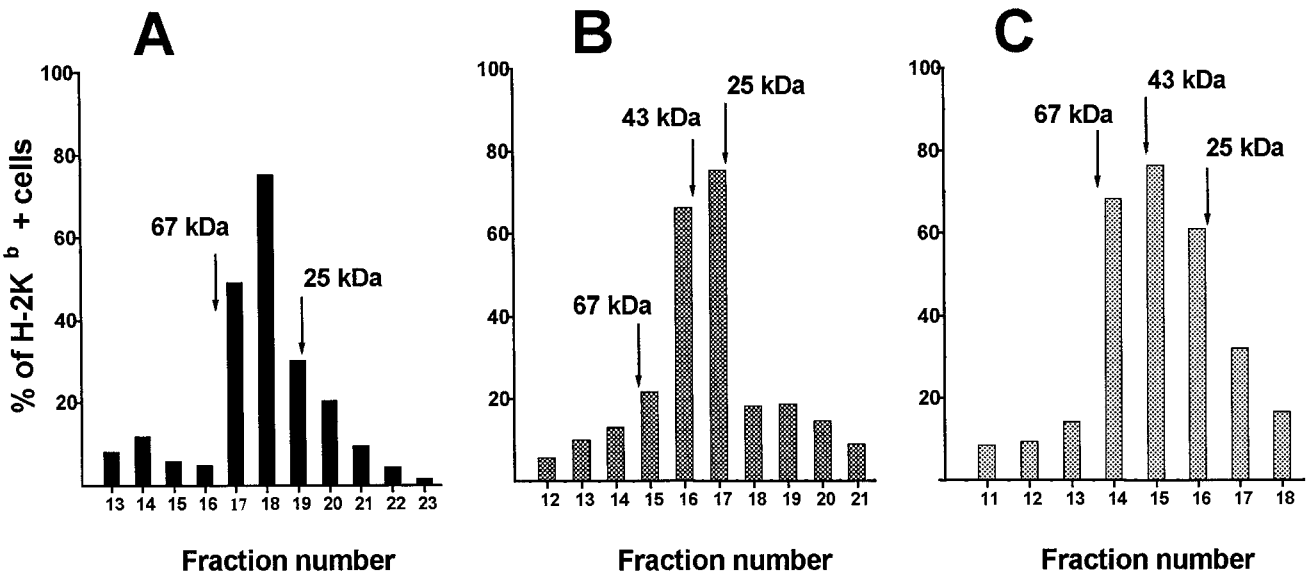

Figure 2. Gel filtration chromatography of media conditioned by macrophages elicited with *C. parvum* (A), BCG (B), or *L. monocytogenes* (C). The histograms correspond to the % of F10-M3 melanoma cells which expressed the H-2K^b antigen after exposure to the various chromatographic fractions. The chromatographic elution positions of chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and BSA (67 kDa) are reported as size standards.

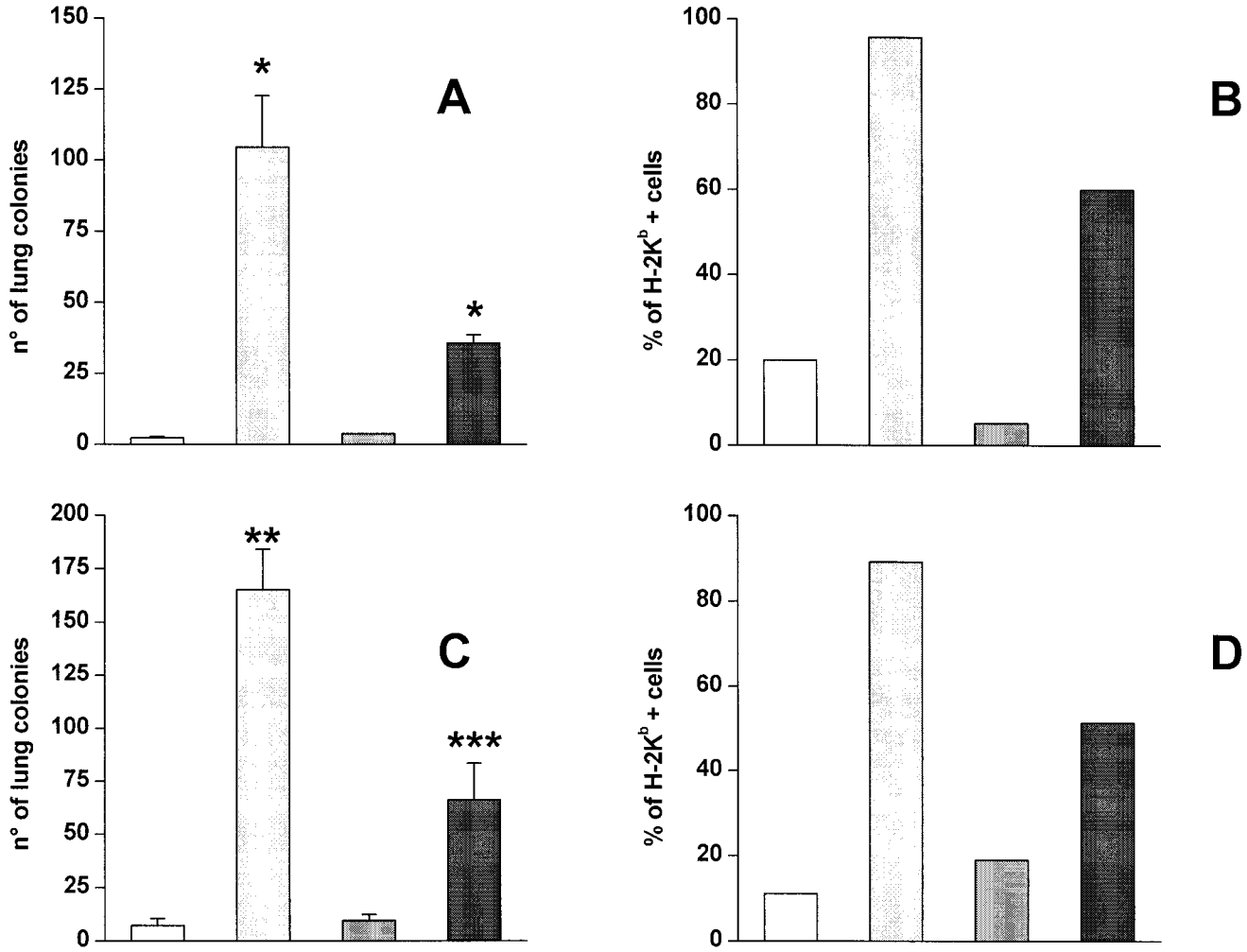

Figure 3. Change of the lung-colonizing potential (A, C) and expression of H-2K^b antigen (B, D) in F10-M3 cells grown in media conditioned by BCG- (A, B) or *L. monocytogenes*- (C, D) elicited macrophages (light grey columns), and in the same media challenged with anti-IFNγ (dark grey columns) or anti-TNFα antibodies (black columns). Tumor cells grown in a standard medium for the entire experimental protocol (empty columns) served as a control. Data reported in the Figure are derived from a typical experiment. Values represent the mean $\pm$ SEM of lung colonies found in the experimental animals (groups of 4–5 mice). Significantly different at $^*P = 0.02$, $^{**}P = 0.008$, $^{***}P = 0.01$ from cells grown in a standard medium.

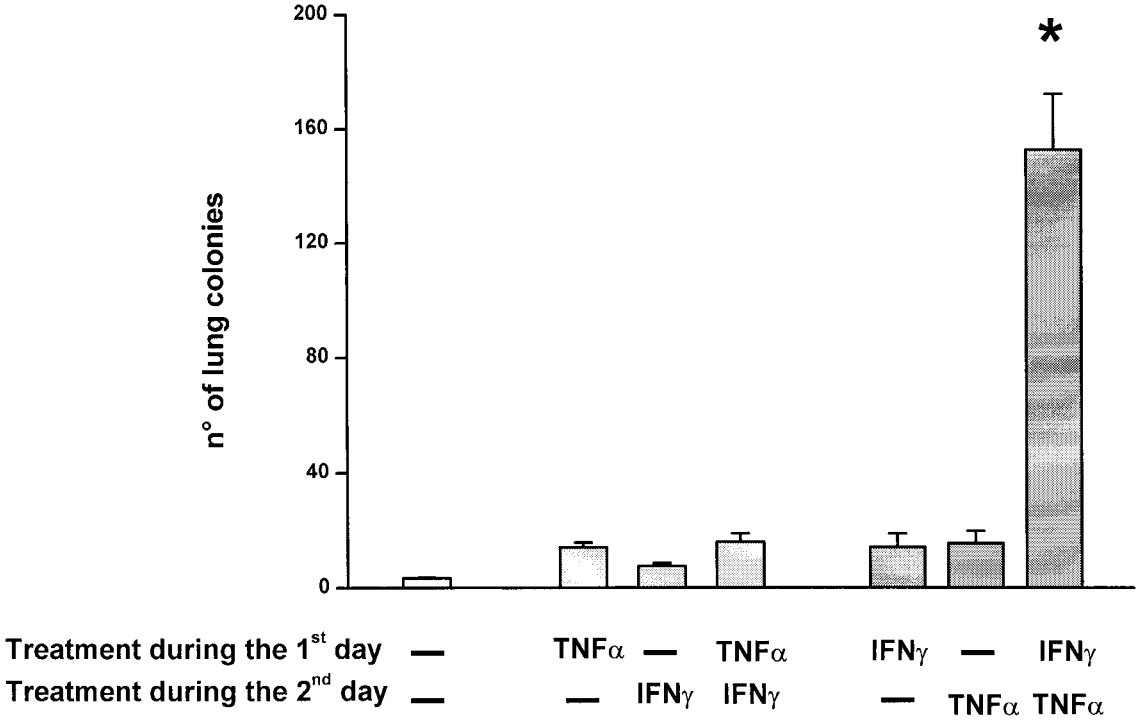

Figure 4. Change of the lung-colonizing potential in F10-M3 melanoma cells treated with exogenous IFNγ and/or TNFα. Melanoma cells were grown in media supplemented with IFNγ (25 U/ml) or TNFα (25 μg/ml) following the indicated sequence. Tumor cells grown in a standard medium for the entire experimental protocol (empty columns) served as a control. Values represent the mean $\pm$ SEM of lung colonies found in the experimental animals (groups of 5–6 mice). *Significantly different at $P = 0.008$ from cells grown in a standard medium.

whether the treatment of macrophage-conditioned media with anti-IFNγ and anti-TNFα antibodies affected the pro-clonogenic activity present in these media. As shown in Figure 3, the capacity of the media conditioned by BCG- or *L. monocytogenes*-elicited macrophages of up-regulating H-2K^b antigen as well as enhancing the lung-colonizing potential in melanoma cells was lost after treatment with anti-IFNγ antibodies. Treatment of macrophage-conditioned media with anti-TNFα antibodies only partially affected their pro-clonogenic activity.

Discussion

The present study revealed that a pro-clonogenic activity, previously demonstrated in media conditioned by *C. parvum*-elicited macrophages [1, 2], is also present in media conditioned by BCG- or *L. monocytogenes*-elicited macrophages. Moreover, as in the case of *C. parvum*-elicited macrophages [2], the pro-clonogenic activity generated by BCG- or *L. monocytogenes*-elicited macrophages enhanced the expression of MHC class I antigens in F10-M3 melanoma cells.

The gel filtration chromatographic analysis of macrophage-conditioned media revealed that the molecular weight of the pro-clonogenic activity present in these media ranged between values compatible with certain cytokines, such as IFNγ and TNFα. Indeed, the challenge of the macrophage-conditioned media with anti-IFNγ antibodies abolished their capacity of increasing the lung-colonizing potential as well as up-regulating the expression of H-2K^b antigen in melanoma cells. The recent finding that macrophages, besides lymphocytes, can secrete IFNγ [7–9] sustains the participation of IFNγ in the pro-clonogenic activity released by the elicited macrophages. Our observation that IFNγ is involved in the macrophage pro-clonogenic activity is analogous to the finding that treatment of tumor cells with IFNγ enhances their colonization in secondary organs [10–15]. The pro-clonogenic effect of IFNγ has been attributed to an inhibition of the anti-metastatic NK cell activity caused by the IFNγ-mediated enhancement of MHC class I antigens [16–18].

The limited inhibitory effect of anti-TNFα antibodies on the macrophage pro-clonogenic activity suggests a different contribution of TNFα as compared to IFNγ in promoting organ colonization of melanoma cells. It is possible that a prior stimulation by IFNγ is required for TNFα to produce the maximal pro-clonogenic activity. This hypothesis is sustained by our observation, reported in Figure 4, that the lung-colonizing potential is enhanced in melanoma cells treated with exogenous TNFα provided the cells were previously exposed to IFNγ, while the reversal of this sequence was not effective. The possibility that TNFα displays a pro-clonogenic activity implies the presence of specific receptors whose expression, as recently reported [19], is promoted by IFNγ.

Our observation that TNFα and IFNγ contribute to the pro-clonogenic activity generated by elicited macrophages is in contrast with the *in vivo* therapeutic use of IFNγ and TNFα as anti-metastatic agents [20–23]. This discrepancy might be explained on the basis of the differences between the *in vivo* and *in vitro* conditions. Indeed, *in vivo* treatment

with INFγ and TNFα may evoke a complex array of effects in different homeostatic systems of the host, due to the pleiotropic properties of these cytokines [7–24]. Nevertheless, use of *in vitro* experimental protocols offers the advantage of exploring specific tumor cell/host cell interactions under well controlled conditions.

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