

## Inhibition of Phosphatidylserine Recognition Heightens the Immunogenicity of Irradiated Lymphoma Cells In Vivo

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### Abstract

Strategies to enhance the immunogenicity of tumors are urgently needed. Although vaccination with irradiated dying lymphoma cells recruits a tumor-specific immune response, its efficiency as immunogen is poor. Annexin V (AxV) binds with high affinity to phosphatidylserine on the surface of apoptotic and necrotic cells and thereby impairs their uptake by macrophages. Here, we report that AxV preferentially targets irradiated lymphoma cells to CD8<sup>+</sup> dendritic cells for in vivo clearance, elicits the release of proinflammatory cytokines and dramatically enhances the protection elicited against the tumor. The response was endowed with both memory, because protected animals rejected living lymphoma cells after 72 d, and specificity, because vaccinated animals failed to reject unrelated neoplasms. Finally, AxV-coupled irradiated cells induced the regression of growing tumors. These data indicate that endogenous adjuvants that bind to dying tumor cells can be exploited to target tumors for immune rejection.

Key words: apoptosis • phagocytosis • cancer • adjuvants • dendritic cells

### Introduction

The swift recognition and the phagocytosis of apoptotic cells are the object of intense investigation (1, 2). However, the fate of the antigens they contain and the constraints limiting their immunogenicity in vivo are scarcely understood. Apoptotic cell antigens enter the MHC class I pathway of the phagocyte via the cytosol and become available for recognition by MHC-restricted cytotoxic T lymphocytes. After engulfment of apoptotic cells, monocytes/macrophages (M $\phi$ ) release antiinflammatory cytokines, such as IL-10 and TGF- $\beta$  (2). DCs that phagocytosed virus-infected apoptotic cells productively activate (cross-prime; reference 3) virus-specific cytotoxic T lymphocytes (4). DCs are unusually effi-

cient in processing internalized dying cells for presentation to MHC class I- and class II-restricted T lymphocytes. Accordingly, DCs that internalized dying tumor cells efficiently cross-activate tumor-specific T cells, both in vitro and in vivo (5). The presence of M $\phi$ , which release factors that prevent the maturation and the function of DCs, abrogates the cross-priming of T cells by phagocytosing DCs (4).

The in vivo immunogenicity of the highly tumorigenic Rauscher virus-induced H-2<sup>b</sup> RMA lymphoma dramatically abates after apoptosis induction (6). The efficacy of RMA lymphoma cells as vaccines was partially rescued in IL-10 knockout mice (6). Moreover, annexin V (AxV) hindering the recognition of exposed phosphatidylserine (PS) restored the ability of dying xenogeneic cells to evoke

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Abbreviations used in this paper: AxV, annexin V; ITC, irradiated tumor cell; M $\phi$ , macrophages; PS, phosphatidylserine.

antibody responses in vivo (7). Altogether, the results suggest that dying cells administered in vivo cause the secretion of factors that in turn restrict their immunogenicity (immunosuppressive clearance).

It is well established that PS serves as a recognition signal for the clearance of apoptotic cells. The redistribution of PS both on phagocyte and prey is involved (8). A well-characterized receptor mediates the PS-dependent uptake of apoptotic cells (PS receptor; reference 9), engaging a signaling pathway crucial for the rearrangement of phagocytes' actin-based cytoskeleton with eventual engulfment of the corpse. The activation of this receptor induces the release of immunosuppressive cytokines (9). Accordingly, animals lacking PS receptor accumulate uncleared apoptotic cells at the periphery and targeted mice develop tissue inflammation (1, 10, 11). AxV, which binds to exposed PS residues in vitro and in vivo, inhibits apoptotic cell uptake by M $\phi$  most likely through interference with the availability of PS for recognition (8, 12–14).

We hypothesized that the availability of AxV at the site of dying tumor cell clearance disrupts their PS-dependent recognition and, therefore, abrogates the immunosuppressive clearance. Indeed, addition of AxV strikingly heightened the immune response irradiated RMA cells elicit in vivo, including rejection of growing tumors.

## Materials and Methods

**Preparation of AxV.** We cloned and isolated chicken AxV used in our studies as described previously (15). In brief, AxV was synthesized in *Escherichia coli* upon induction with isopropyl thio- $\beta$ -galactoside and purified with a protocol based on the ability of AxV to interact reversibly with PS-containing liposomes in the presence of calcium. Human recombinant AxV was provided by Boehringer Ingelheim. The coding region of the mouse AxV cDNA (Genbank/EMBL/DDBJ; accession no. MMU29396) was amplified from corresponding cDNA clones (IMAGE; RZPD) by PCR and inserted in the vector pQE60 for recombinant expression in *E. coli* and purified as described before (15). Preparations were dialyzed against 50 mM Tris, 1 mM EGTA, pH 7.4, and purified on Poros HQ20 anion-exchange (Applied Biosystems) and on detoxi-gel affinity Pak (Pierce Chemical Co.) columns. The contamination with endotoxin was <5 EU/ml.

**Mice and Tumors.** C57BL/6 female mice, 6–8-wk-old (Charles River Laboratories), were housed in a pathogen-free animal facility and treated in accordance with the European Community guidelines. The Institutional Animal Care and Use Committee of the H. San Raffaele Scientific Institute gave approval for the animal experiments. The H-2<sup>b</sup> RMA lymphoma cell line was provided by V. Cerundolo (John Radcliff Hospital, Oxford, England, UK). The H-2<sup>b</sup> B16F1 melanoma cell line was purchased from American Type Culture Collection. Cell lines were routinely tested for mycoplasma infection.

**Cell Death.** We irradiated RMA cells using an UV source as described previously, treated them with mitomycin C (50  $\mu$ g/ml for 1 h at 37°C), and washed them extensively (16). We used FITC-labeled AxV (provided by V. Occhiena, Bender MedSystems, Torino, Italy) to assess the externalization of PS and propidium iodide to evaluate plasma membrane integrity. Cells were analyzed at 0, 3, 9, and 16 h after irradiation, in the presence or

absence of divalent cations. The specificity of the binding of AxV was also evaluated by treating irradiated cells with increasing amounts (range tested 1–500  $\mu$ g/ml) of unlabeled AxV or BSA and assessing the residual binding of FITC-AxV (5  $\mu$ g/ml) by flow cytometry. We analyzed samples with a FACScan apparatus (Becton Dickinson). When indicated, chicken albumin (Sigma-Aldrich) was coupled to the membrane of apoptotic tumor cells, using the three-step approach described previously (17, 18): apoptotic cells were biotinylated before incubation with 10  $\mu$ g/ml streptavidin (Biospa) in PBS containing 2% FCS (PBS/FCS) (30 min at 4°C), followed by 100  $\mu$ g/ml biotin–chicken albumin (45 min at 4°C).

**Dendritic Cells and M $\phi$ .** DCs were derived from bone marrow precursors propagated for 7 d in complete medium containing 1,000 U/ml recombinant murine GM-CSF and 5 ng/ml recombinant murine IL-4 (BD Biosciences). M $\phi$  were isolated from thioglycollate-elicited peritoneal lavages by plastic adherence (9).

**In Vitro Clearance of Irradiated Cells.** Biotinylated irradiated RMA cells were incubated with <sup>125</sup>I-labeled streptavidin (Amersham Biosciences) and washed extensively. We performed the phagocytosis either at 37°C or at 4°C using a 5:1 irradiated tumor cells (ITCs):phagocyte ratio. After 2 h, we added trypsin-EDTA at 37°C. We washed M $\phi$  monolayers till no residual noninternalized irradiated cells could be detected by phase contrast microscopy. In parallel, we separated DCs from noninternalized ITCs by density gradient centrifugation. We assessed incorporated radioactivity in a  $\gamma$ -counter after lysis with 1% Triton X-100. The assay was performed using ITCs incubated in medium containing either AxV or BSA (final concentration: 100  $\mu$ g/ml).

We also studied phagocytosis of ITCs by flow cytometry, according to the procedure described by Albert et al. (19). In brief, RMA cells were labeled before irradiation with the aliphatic red fluorescent dye PKH26-GL (Sigma-Aldrich) and cocultured with M $\phi$  or DCs that had been stained with the green fluorescent PKH67-GL dye (Sigma-Aldrich). Phagocytosis assays were performed at 37 or 4°C. If indicated, M $\phi$  and DCs were preincubated with 2 mM EDTA, 10  $\mu$ g/ml cytochalasin D, or 40  $\mu$ M lavendustin A, respectively. In selected experiments, we assessed the phagocytosis of AxV-coupled ITCs in the presence of EDTA or cytochalasin D. 2 h after addition of the apoptotic cells, we performed flow cytometry analysis and enumerated double positive cells (20). Phagocytosis percentage was calculated according to the following formula: (percentage of phagocytosing cells/percentage of phagocytosing cells when phagocytes were preincubated in medium)  $\times$  100.

We assayed culture supernatants for TNF- $\alpha$ , IL-1 $\beta$ , IL-10, and TGF- $\beta$  using commercial ELISA (DuoSet ELISA; R&D Systems).

**In Vivo Clearance of Irradiated Cells.** Sterile peritonitis was induced by intraperitoneal injection of 4% thioglycollate.  $50 \times 10^6$  CFSE-labeled apoptotic cells in 0.5 ml PBS, in the presence or in the absence of AxV, were injected 5 d after into peritoneal cavities. After 2 h, mice were killed and peritoneally lavaged with ice-cold HBSS. Elicited M $\phi$  were recovered and analyzed by flow cytometry after staining with PE-conjugated anti-CD11b mAb (BD Biosciences).

To evaluate clearance by DCs,  $100 \times 10^6$  CFSE-labeled ITCs in 0.3 ml PBS were injected intravenously. After 90 min, mice were killed, and spleens were removed. CD11c<sup>+</sup> DCs were retrieved by magnetic bead sorting with anti-CD11c mAb (Miltenyi Biotec). Cells were stained with anti-CD3 and anti-B220 Cy-Chrome-conjugated mAbs and with either PE-conjugated anti-CD8 or PE-conjugated anti-CD11b mAbs (BD Biosciences). Cells were analyzed by triple parameter flow cytometry.

**Immunization and Cure.** Mice were s.c. immunized in the footpad with ITCs ( $5 \times 10^6$ /mouse). ITCs were preincubated either with or without chicken AxV and mouse AxV, or physically coupled to chicken albumin (100  $\mu\text{g/ml}$ ) for 20 min at room temperature. On day 15, we boosted the mice in the right flank. On day 30, mice were s.c. challenged by injecting  $2.5 \times 10^4$  or  $5 \times 10^4$  viable RMA cells into the opposite flank. Animals were checked for the appearance of tumor twice weekly. We scored animals as tumor positive if the mean of the two perpendicular diameters of the tumor was  $>2$  mm and killed them when the tumor reached a diameter  $>10$  mm or ulcerated. We scored mice as tumor negative if no palpable tumor developed within 10 wk after the tumor challenge. Tumor-free mice were rechallenged by injecting  $2.5 \times 10^4$  RMA cells into the opposite flank to verify whether the protection was long lasting. We verified the specificity of the response in protected mice by s.c. administration of  $2.5 \times 10^4$  B16F1 melanoma cells.

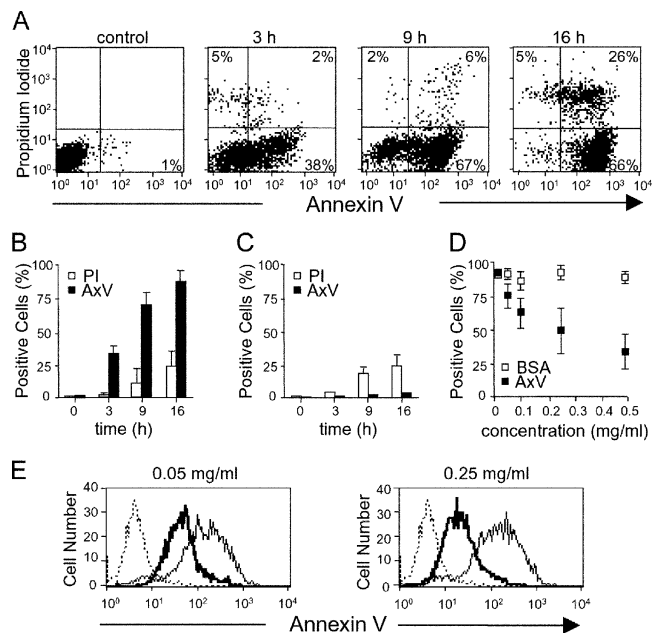
To evaluate the effect of different treatments on growing RMA lymphomas, we s.c. injected  $2.5 \times 10^4$  living RMA cells in the left flank. We allowed tumors to grow for 3 d before immunization in the opposite flank with  $5 \times 10^6$  ITCs treated or not with AxV (1 immunization/wk for 3 wk). We evaluated tumor growth as described before.

**Statistical Analysis.** Statistical analyses were performed using either log-rank, two-tailed Student's *t* test and  $\chi^2$  (SPSS 10.0.7, SPSS Inc.; InStat 2.01; GraphPad Software). Results were considered statistically significant for  $P < 0.05$ .

## Results

**AxV Specifically Binds to Irradiated RMA Cells.** After irradiation, RMA lymphoma cells acquired the ability to bind FITC-labeled AxV (Fig. 1, A and B). After 16 h,  $>60\%$  of the cells were AxV positive/propidium iodide negative and  $<30\%$  double positive. The binding of FITC-labeled AxV was dependent on divalent cations (Fig. 1 C) and abated when cells were preincubated with unlabeled AxV (Fig. 1, D and E).

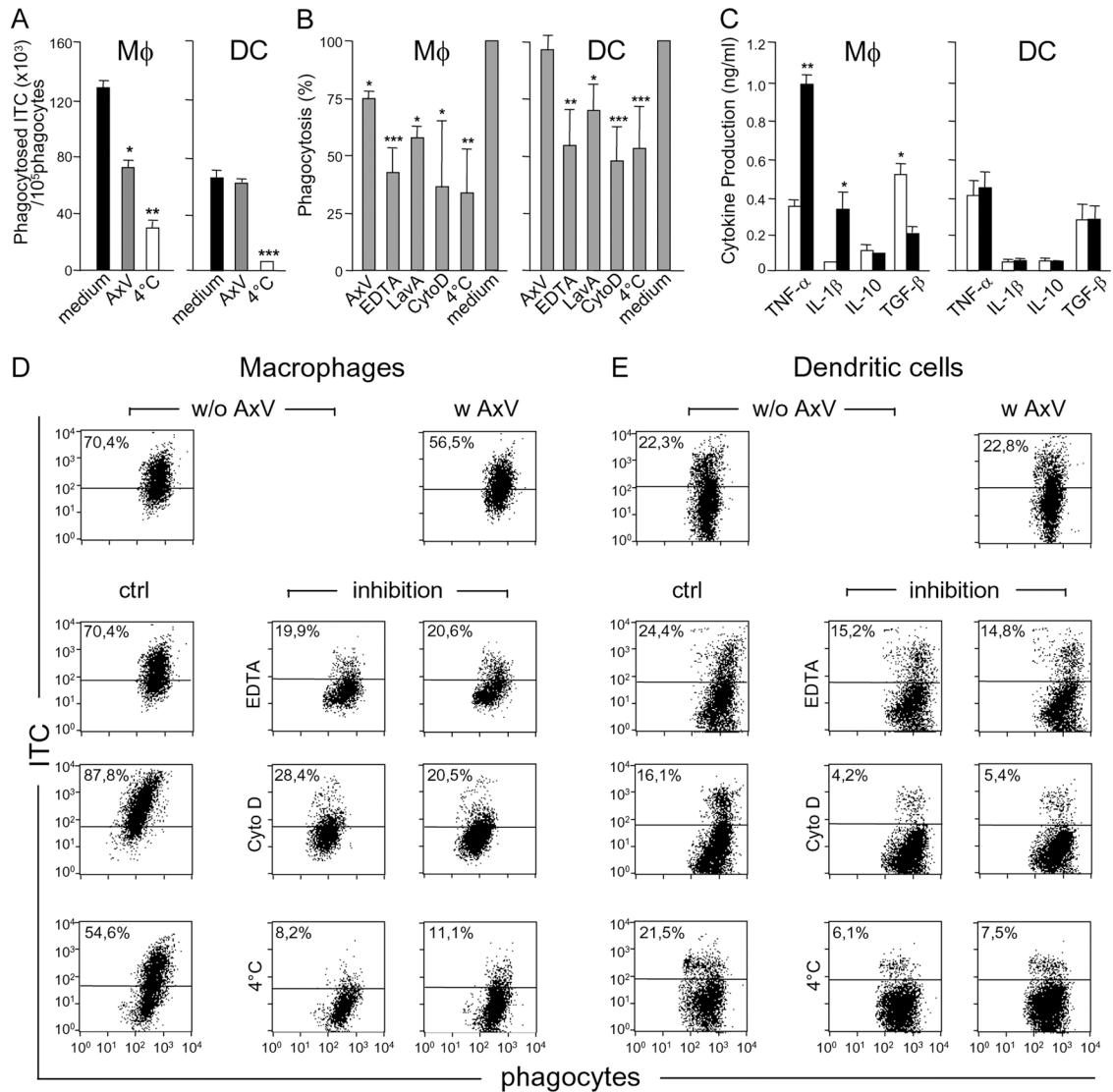
**AxV Skews the Phagocytosis of Irradiated Cells *In Vitro* and *In Vivo*.** We assessed whether interference with PS recognition influenced the phagocytic clearance of ITCs. To this aim, we derived thioglycollate-elicited M $\phi$  from peritoneal lavages or immature DCs from bone marrow precursors and assessed their ability to phagocytose  $^{125}\text{I}$ -conjugated or fluorescent ITCs. M $\phi$  internalized ITCs at  $37^\circ\text{C}$  more efficiently than DCs ( $127,475 \pm 1,246$   $^{125}\text{I}$ -conjugated ITCs/ $10^5$  M $\phi$  vs.  $64,939 \pm 230/10^5$  DCs). Furthermore, a larger fraction of M $\phi$  were actually involved in phagocytosis of fluorescent ITCs, as assessed by flow cytometry ( $71.9 \pm 3.3\%$  M $\phi$  vs.  $20.9 \pm 2.5\%$  DCs). The process was greatly inhibited when experiments were performed at  $4^\circ\text{C}$  (i.e. at a temperature that does not permit actual phagocytosis) in the presence of cytochalasin D, an inhibitor of cytoskeletal function or of EDTA, demonstrating that divalent cations are required (Fig. 2, A and B). Protein tyrosine kinase integrity is also necessary for internalization of apoptotic cells by DCs (19); in agreement the protein tyrosine kinase inhibitor, lavendustin A substantially inhibited phagocytosis of ITCs. AxV inhibited the uptake of ITCs by M $\phi$  (Fig. 2 B). No further significant



**Figure 1.** AxV specifically binds to irradiated RMA lymphoma cells. (A) We monitored by flow cytometry the staining with FITC-labeled annexin V (x axis) and propidium iodide (y axis) of RMA lymphoma cells at different time points after UV irradiation (0, 3, 9, and 16 h). Percentages are indicated. The experiment shown is representative of four independent experiments. (B) We monitored the staining with FITC-labeled AxV (shaded columns) and propidium iodide (PI, unshaded columns) of RMA lymphoma cells at different time points after UV irradiation (x axis, hours). Incubations were performed in the presence of calcium and magnesium. Results are expressed as percentage of positive cells  $\pm$  SD from four independent experiments (y axis). (C) We monitored the staining with FITC-labeled AxV (shaded columns) and propidium iodide (PI, unshaded columns) of RMA lymphoma cells at different time points after UV irradiation (x axis, hours) in the absence of divalent cations. Results are expressed as percentage of positive cells  $\pm$  SD from four independent experiments (y axis). (D) We treated with increasing amounts of unlabeled AxV (closed squares) or of BSA (open squares) (x axis concentration, mg/ml) irradiated lymphoma cells, before assessing the binding of FITC-labeled AxV. Results are expressed as percentage of positive cells  $\pm$  SD from three independent experiments (y axis). (E) We treated with unlabeled AxV (bold lines, 0.05 or 0.25 mg/ml) ITC, before assessing the binding of FITC-labeled AxV. Thin lines represent the binding of FITC-labeled AxV to irradiated RMA lymphoma cells in the absence of competitors. Dotted lines indicate the fluorescence background. The experiment shown is representative of three independent experiments.

inhibition in the uptake of AxV-coupled ITCs occurred in the presence of EDTA, cytochalasin D, or at  $4^\circ\text{C}$ . AxV did not significantly influence the phagocytosis of ITCs by immature DCs (Fig. 2, D and E).

Thioglycollate-elicited M $\phi$  challenged with AxV-coupled ITCs secreted significantly higher amounts of TNF- $\alpha$  ( $P = 0.008$ ) and IL-1 $\beta$  ( $P = 0.036$ ). In contrast, the production of TGF- $\beta$  abated, and the difference was statistically significant ( $P = 0.040$ ). IL-10 was unaffected. DCs challenged with ITCs released a similar array of cytokines in the presence or in the absence of AxV (Fig. 2 C). The effects of chicken AxV on cytokine production were confirmed by experiments using recombinant human AxV. Of notice, AxV alone did not induce the production of cytokine by both phagocytes (Table I).



**Figure 2.** AxV skews the phagocytosis of irradiated cells in vitro. (A) We incubated thioglycollate-elicited M $\phi$  or bone marrow-derived immature DCs with radioactive irradiated tumor cells (ITC, x axis), at 37°C in the presence (gray columns) or absence (black columns) of AxV. As a control, both phagocytes were incubated with ITC at 4°C in the presence (white columns). Results are representative of seven experiments and expressed as number of engulfed <sup>125</sup>I-conjugated ITC per 10<sup>5</sup> M $\phi$  or DC  $\pm$  SD (y axis). Actual cpm per <sup>125</sup>I-conjugated ITC was 0.06, and maximum incorporated radioactivity in phagocytes (at 37°C and in the absence of AxV) was 7654  $\pm$  97 cpm. For other experiments, these values were respectively: 0.01 cpm/cell and 1,021  $\pm$  97 cpm; 0.02 cpm/cell and 9,890  $\pm$  343 cpm; 0.01 cpm/cell and 1,629  $\pm$  6 cpm; 0.02 cpm/cell and 3,489  $\pm$  354 cpm; 0.01 cpm/cell and 1,696  $\pm$  180 cpm; and 0.09 cpm/cell and 539  $\pm$  66 cpm. (B) Peritoneal thioglycollate-elicited M $\phi$  or bone marrow-derived DCs were stained with the aliphatic PKH26-GL green fluorochrome and ITC with the aliphatic PKH26-GL red fluorochrome. Phagocytosis was assessed by flow cytometry after 120 min as described in Materials and Methods. 100% represents the phagocytosis observed when phagocytes were preincubated in culture medium at 37°C (medium). Percentage of phagocytosing cells ranged in different experiments from 54.6 to 87.8% for M $\phi$  and from 16.1 to 24.4% for DCs. Bars refer to the phagocytosis percentage (calculated as described in Materials and Methods) observed in the presence of AxV, EDTA, lavendustin A (LavA), cytochalasin D (CytoD), or at 4°C. All results represent mean  $\pm$  SD from at least four experiments. Results obtained were compared with results obtained in medium alone using two-tailed Student's *t* test: \*, *P* < 0.01; \*\*, *P* < 0.005; \*\*\*, *P* < 0.001. (C) We retrieved the supernatants of peritoneal thioglycollate-elicited M $\phi$  or bone marrow-derived DCs challenged for 24 h with ITC coupled (shaded bars) or not (unshaded bars) with AxV (ITC:phagocyte ratio of 5:1). The release of TNF- $\alpha$ , IL-1 $\beta$ , IL-10, and TGF- $\beta$  was assessed by ELISA as described in Materials and Methods. Results are expressed as ng/ml. Results obtained with AxV-coupled ITC were compared with results obtained with ITC using two-tailed Student's *t* test: \*, *P* < 0.01; \*\*, *P* < 0.005. Phagocytosis of ITC by M $\phi$  (D) or DC (E) was evaluated by flow cytometry with or without the indicated inhibitors, alone or in combination. Percentages of phagocytosing cells are indicated. Results shown are representative of at least four independent experiments.

To determine whether AxV was equally effective in vivo, we challenged animals with CFSE-labeled ITCs in the presence or in the absence of AxV, and evaluated the clearance of dying cells by two alternative approaches. First, we injected fluorescent ITCs into the peritoneal cavity 5 d

after induction of sterile peritonitis with thioglycollate. Phagocytosis of ITCs by elicited peritoneal M $\phi$  was impaired, and a statistically significant difference in the fraction of engulfing M $\phi$  was evident in AxV-treated mice after 2 h (27.9  $\pm$  3.6% vs. 12.6  $\pm$  1.1; *P* = 0.028; Fig. 3, A



**Table I.** *AxV Biases towards Inflammation the Phagocytic Clearance of ITCs*

Cytokines	Macrophages				Dendritic cells			
	Medium		AxV		Medium		AxV	
	Alone	w/ ITC	Alone	w/ ITC	Alone	w/ ITC	Alone	w/ ITC
	<i>pg/ml</i>	<i>pg/ml</i>	<i>pg/ml</i>	<i>pg/ml</i>	<i>pg/ml</i>	<i>pg/ml</i>	<i>pg/ml</i>	<i>pg/ml</i>
TNF- $\alpha$	125 $\pm$ 15	335 $\pm$ 30	117 $\pm$ 24	978 $\pm$ 48 <sup>a</sup>	267 $\pm$ 137	392 $\pm$ 79	245 $\pm$ 121	426 $\pm$ 78
IL-1 $\beta$	21 $\pm$ 5	44 $\pm$ 7	14 $\pm$ 1	322 $\pm$ 85 <sup>b</sup>	10 $\pm$ 4	46 $\pm$ 4	8 $\pm$ 3	47 $\pm$ 3
IL-10	88 $\pm$ 18	105 $\pm$ 30	129 $\pm$ 11	90 $\pm$ 8	42 $\pm$ 1	51 $\pm$ 8	42 $\pm$ 2	43 $\pm$ 4
TGF- $\beta$	82 $\pm$ 10	505 $\pm$ 54	105 $\pm$ 11	193 $\pm$ 26 <sup>b</sup>	230 $\pm$ 72	266 $\pm$ 76	238 $\pm$ 93	261 $\pm$ 65

We retrieved the supernatants of thioglycollate-elicited peritoneal M $\phi$  or bone marrow-derived DCs challenged for 24 h with ITC coupled or not (medium) with chicken AxV (ITC:phagocyte ratio of 5:1). The supernatants were also retrieved from cells incubated for 24 h in medium (alone) with or without chicken AxV. The release of TNF- $\alpha$ , IL-1 $\beta$ , IL-10, and TGF- $\beta$  was assessed by ELISA as described in the Materials and Methods. Results are expressed as pg/ml (mean  $\pm$  SD). Results obtained with AxV-coupled ITC were compared with results obtained with ITC using two-tailed Student's *t* test.

<sup>a</sup>P < 0.005.

<sup>b</sup>P < 0.01.

and B). This resulted in an actual delay in the clearance of the apoptotic ITCs from the peritoneum.

Second, we injected fluorescent syngeneic ITCs intravenously and assessed the clearance within the spleen by magnetic bead sorting of CD11c<sup>+</sup> DCs and triple parametric analysis by flow cytometry. A fraction of CD8<sup>+</sup>, CD11b<sup>-</sup> DCs was consistently engaged in phagocytosis of fluorescent ITCs and the presence of AxV significantly increased this value (3.1  $\pm$  0.1% vs. 7.34  $\pm$  2%; P = 0.025). We found that among CD8<sup>-</sup>, CD11b<sup>+</sup> splenic DCs only a small percentage of cells was actively engaged in phagocytosis, in both normal and AxV-injected mice (Fig. 3, C and D).

*AxV Enhances the Immunogenicity of Irradiated Cells: Vaccination.* Mice injected with 2.5  $\times$  10<sup>4</sup> living RMA cells developed the tumor with a mean latency of 13.7  $\pm$  1.9 d (Fig. 4 A). The s.c. vaccination with ITCs protected the 25% of the animals from tumor growth after the challenge with living RMA cells (Fig. 4 B). AxV-coupled ITCs were consistently more efficient vaccines than ITCs alone (Fig. 4 B). 90% of the animals vaccinated with AxV-coupled ITCs rejected the tumor after the challenge, compared with only 25% of the animals vaccinated with ITCs without AxV (P = 0.0015; Fig. 4 B). Even in the few animals that were not protected, the latency of tumor appearance and survival time after tumor development increased. The mean latency increased from 16.2  $\pm$  3.3 to 23  $\pm$  9.5 for animals treated with irradiated cells in the absence or in the presence of AxV (P = 0.0064; Fig. 4 B). The mean survival increased from 24.1  $\pm$  4.9 to 33 d (P = 0.0024; Fig. 4 C). Significantly enhanced latency and survival were observed when vaccinated mice were challenged with higher numbers of living lymphoma cells (Table II); we compared the ability of AxV of different origins (xenogeneic vs. syngeneic) to enhance the immunogenicity of ITCs. Both mouse and

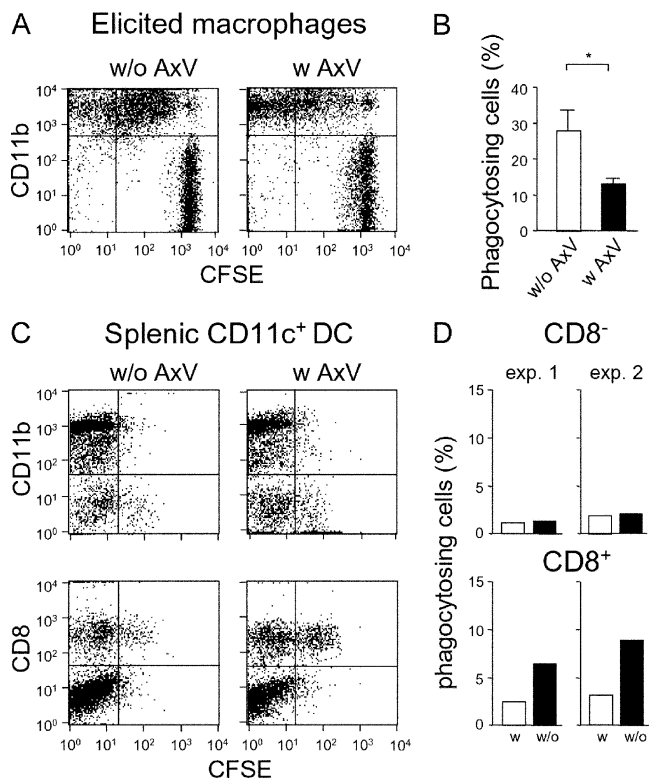
chicken AxV associated with ITCs significantly delayed (from 15  $\pm$  8.3 to 24.1  $\pm$  8 and 27.1  $\pm$  6.2, respectively; P < 0.005) the appearance of the tumor in mice vaccinated as described before and challenged with 5  $\times$  10<sup>4</sup> living RMA cells. The survival and the protection were significantly enhanced as well (P < 0.005; Table II). In contrast, no significant difference in tumor take, animal survival, or elicited protection was observed between animals treated with ITCs in the presence of mouse or chicken AxV. Moreover, we failed to increase the immunogenicity of ITCs by physical coupling with another xenogeneic protein, chicken ovalbumin (Table II). Altogether, these findings rule out a bias due to the xenogeneic origin of chicken AxV.

We rechallenged protected mice in the opposite flank (2.5  $\times$  10<sup>4</sup> living RMA cells/animal). In all cases but one, mice protected by the vaccination with ITCs rejected the tumor (Fig. 4 B). We challenged protected animals with an unrelated tumor, the B16F1 melanoma. All mice developed the melanoma, with a latency and a survival identical to nonimmunized mice (unpublished data). Therefore, the response we elicited was long lasting, endowed with memory and specific.

*AxV Enhances the Immunogenicity of Irradiated Cells: Cure of Growing Tumors.* We injected naive mice with 2.5  $\times$  10<sup>4</sup> viable RMA cells. We allowed a 3-d period for the tumor to develop before injection of ITCs, coupled or not with AxV. The tumor did not grow in the 10% of the animals treated with ITCs alone (Fig. 4 D). In contrast, 60% of the mice treated with AxV-coupled ITCs were cured (Fig. 4 H). The result was statistically significant (P = 0.019).

## Discussion

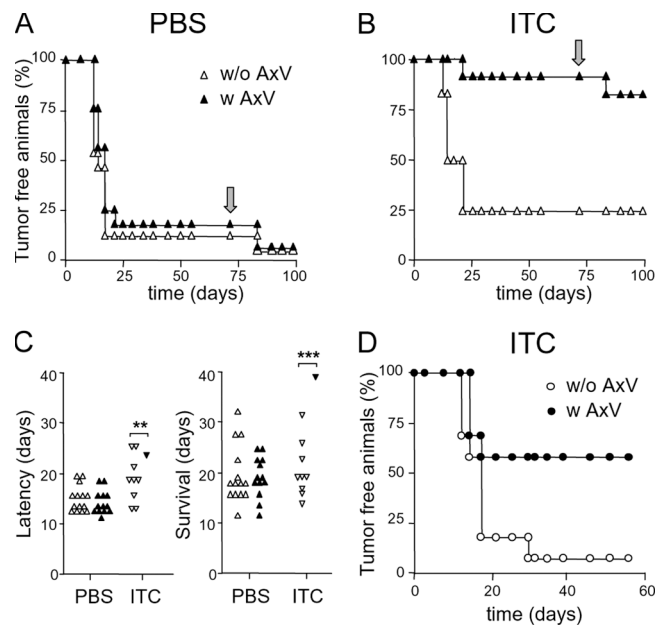
Scavenger cells recognize exposed moieties on dying cells. The outcome is the swift phagocytosis of the corpses,



**Figure 3.** AxV skews the phagocytosis of irradiated cells in vivo. (A) The in vivo clearance of CFSE-labeled fluorescent ITC by elicited CD11b<sup>+</sup> M $\phi$  in the absence (w/o AxV) or in the presence (w AxV) of AxV was evaluated by flow cytometry. (B) The percentage of phagocytosing M $\phi$  (mean of three independent experiments  $\pm$  SD), in the absence (w/o, unshaded bar) or in the presence (w, shaded bar) of AxV is shown. The difference was statistically significant. \*,  $P < 0.05$ . (C) The clearance of i.v. injected CFSE-labeled ITC by splenic DCs was evaluated by flow cytometry. CD11c<sup>+</sup> DCs were retrieved by magnetic bead sorting (purity >95%). CD11b<sup>+</sup>, CD8<sup>-</sup> DCs (top) and CD11b<sup>-</sup>, CD8<sup>+</sup> DCs (bottom) phagocytosing ITC in the absence (w/o AxV) or in the presence of AxV (w AxV) were identified by triple parameter flow cytometry. (D) The percentage of phagocytosing CD11b<sup>+</sup>, CD8<sup>-</sup> (CD8<sup>-</sup>, top histograms) and CD11b<sup>-</sup>, CD8<sup>+</sup> (CD8<sup>+</sup>, bottom histograms) DCs in the absence (w/o, unshaded bars) or in the presence (w, shaded bars) of AxV in two independent experiments is shown (exps. 1 and 2).

which prevents secondary necrosis and the ensuing tissue damage. The recognition of exposed PS via its receptor (9) triggers the release of immunosuppressive cytokines, in particular TGF- $\beta$ , which quench inflammation and prevent the maturation of antigen-presenting DCs. Tolerance induction is the likely default outcome (21, 22). The events recruited by the recognition of exposed PS residues, including the release of immunosuppressive cytokines, possibly contribute to the tolerogenic outcome (23). However, environmental signals, including signals recruiting the CD40 receptor (24), delivered by local cytokines, or possibly released by the dying cells themselves (25, 26), switch the response toward active cross-priming (3).

Dying tumor cells represent a source of antigens for active antineoplastic immunotherapy. Antigen-presenting DCs efficiently phagocytose dying tumor cells and prime immune responses against tumor antigens both in vitro and, in suit-



**Figure 4.** AxV-coupled ITC protect and cure mice from lethal tumor challenge and determine long-lasting antitumor immunity. C57Bl/6 mice were immunized twice with PBS (A) or ITC (B) in the presence (closed triangles) or not (open triangles) of AxV (see Materials and Methods). On day 0, we challenged immunized mice s.c. in the opposite flank with  $2.5 \times 10^4$  viable RMA lymphoma cells and we monitored at different times (x axis, days) the percentage of tumor-free animals (y axis). To evaluate whether the elicited response was long lasting, we rechallenged protected animals on day 72 with  $2.5 \times 10^4$  viable RMA lymphoma cells (arrow) and the survival time (y axis, survival) in vaccinated animals that were not protected (see Materials and Methods). Each symbol refers to a single mouse; only mice developing the tumor are depicted. Statistical analysis was performed with the  $\chi^2$  test, choosing as cut-off 18 d for the latency and 28 d for the survival, comparing the results obtained in all treated animals in four independent experiments. 25 mice were vaccinated with PBS, 18 mice were vaccinated with AxV, 17 mice were vaccinated with ITC alone, and 22 mice were vaccinated with ITC + AxV. \*,  $P < 0.01$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.001$ . (D) We injected  $2.5 \times 10^4$  living RMA cells s.c. in the left flank of C57Bl/6 mice. On day 4, ITC were injected into the opposite flank alone (open symbols) or in the presence of AxV (closed symbols). The percentage of tumor free animals (y axis) was evaluated at different time points after treatment (x axis, days). Differences were statistically significant, as ascertained by log-rank test (for the vaccination experiments,  $P = 0.0015$ ; for the cure experiments,  $P = 0.019$ ).

able conditions, in vivo. However, this is not the rule in vivo. We reasoned that disrupting the local PS-dependent clearance of dying cells by M $\phi$  would result in “unscavenged” dead tumor cells, available for uptake and processing by DCs. Indeed, we confirmed that the PS-binding protein AxV is a suitable tool to obtain this proof of concept because it interferes with the in vitro clearance of dying ITCs by M $\phi$ , consistent with previous observations (8, 12–14).

The engulfment of apoptotic cells is a two-step process. The first involves binding without actual internalization. Ingestion of cell corpses is the second step (27). Several receptors, including CD14, CD36, and  $\alpha_v$  integrins, are involved in the recognition of apoptotic cells (“tethering” receptors). However, the ingestion by macropinocytosis requires recognition of PS on the membranes of the apop-

**Table II.** *AxV Increases the Immunogenicity of ITCs*

	Take	Survival	Protection
PBS	9.3 ± 1.9	14.2 ± 1.9	0/10
mAxV	8.2 ± 1.6	13.8 ± 2	0/4
cAxV	7.6 ± 1.3	12.4 ± 1.7	0/4
cOVA	11 ± 2.6	15.8 ± 2.8	0/4
ITC	15 ± 8.3	16.8 ± 7	2/10
ITC + mAxV	24.1 <sup>a</sup> ± 8	26.1 <sup>a</sup> ± 5.3	6/10 <sup>a</sup>
ITC + cAxV	27.1 <sup>a</sup> ± 6.2	28 <sup>a</sup> ± 4.2	6/10 <sup>a</sup>
ITC <sup>biot</sup>	19.8 ± 6.1	24 ± 3.7	1/4
ITC <sup>biot</sup> + cOVA	18.6 ± 8.2	22.3 ± 5.9	3/10

Results are expressed as the tumor take (days elapsing between the challenge with living tumor cells and the appearance of the lymphoma *in vivo*; mean ± SD), the survival (days elapsing between the challenge with living tumor cells and the death; mean ± SD), and the elicited protection (number of protected animals/number of vaccinated animals) of mice vaccinated with saline, living, and apoptotic melanoma cells, alone or coupled with AxV of mouse (mAxV) or chicken (cAxV) origin. As a further negative control, ITC were biotinylated and used as an immunogen, coupled or not with another irrelevant xenogeneic protein (chicken ovalbumin [cOVA]).

<sup>a</sup>P < 0.005, significantly different from ITC alone and ITC<sup>biot</sup> + cOVA.

totic cells (28). Our results fit well with this model. AxV efficiently binds to exposed PS. Without the PS “tickle” signal, which is necessary for the reorganization of the actin-based cytoskeleton upon activation of Rho GTPases (27), Mφ internalization fails. PS recognition also elicits the release of immunosuppressive cytokines, namely TGF-β (29), and we found that thioglycollate-elicited Mφ clearing ITCs in the presence of AxV secreted a significantly lower amount of TGF-β and higher amounts of IL-1β and TNF-α (Table I).

Somersan and Bhardwaj predicted that immature DCs, which are highly macropinocytic and express the constitutively activated Rho GTPase Cdc42, would be less dependent than Mφ on tickling signals to internalize tethered apoptotic cells (30). Our results are in agreement with this prediction: AxV, which hinders PS-elicited internalization of apoptotic cells, but not their “tethering” via CD36 and the α<sub>v</sub> integrin (1, 2), did not influence phagocytosis by DCs (Fig. 2). Actually, possibly due to the decreased *in vivo* clearance by elicited Mφ (Fig. 3, A and B), splenic CD11c<sup>+</sup> DCs phagocytosed more efficiently ITCs injected in the presence of AxV (Fig. 3, C and D). Of importance, the clearance by DCs both in the presence or in the absence of AxV was restricted *in vivo* to CD8<sup>+</sup> DCs (Fig. 3, C and D). This is an agreement with papers that implicate CD8<sup>+</sup> DCs in the cross-presentation of cell-associated antigens (31–36). Accordingly, AxV significantly increased the fraction of CD8<sup>+</sup> DCs that phagocytose ITCs, but did not influence the phagocytosis by CD8<sup>-</sup> DCs (Fig. 3). The molecular basis of the different ability of Mφ, CD8<sup>+</sup>, and

CD8<sup>-</sup> DCs to phagocytose dying cells is the object of intense investigation. However, it has been suggested that CD8<sup>+</sup> DCs selectively express a receptor for apoptotic cells (33, 37). Our results suggest that recognition via this yet unidentified moiety is not affected by the two-dimensional crystalline lattice AxV forms on membrane phospholipids.

CD8<sup>+</sup> DCs participate in both cell priming and cell tolerance to cell-associated antigens (38–42). The factors dictating the outcome of cross-presentation include microbial products and endogenous proinflammatory signals, which convert a tolerogenic response by CD8<sup>+</sup> DCs into productive immunity (40). In our case, endogenous factors possibly released as a consequence of delayed clearance by scavenger phagocytes are likely to be involved (43).

In agreement, the administration of AxV-coupled ITCs into naive animals protected them from the challenge with living lymphoma cells. The protection we achieved was long lasting and specific. AxV-coupled cells were significantly more immunogenic than their untreated counterparts (Fig. 4). This number of cells proved highly efficient in causing the rejection of tumors already growing *in vivo*. The result required the presence of AxV, with statistically significant difference in the latency with which the tumor appeared, in the survival of vaccinated mice and in the number of protected mice (Fig. 4).

Immunity does not spontaneously initiate when tissue cells die by necrosis and release autoantigens and intracellular adjuvants (25). This highlights a role for immunoregulatory activities recruited at the site of dying cell clearance. Scavenger Mφ releasing immunosuppressive cytokines are possibly involved. Intriguingly, the failure in the function of Mφ is involved in the pathogenesis of the prototypic autoimmune disease systemic lupus erythematosus, and the defective clearance of dead cells associates to persistent autoimmunity *in vivo* (7, 44, 45).

Tumor-associated antigens, contained in dying cells, can elicit immune responses. However, they normally fail to do so. Identical constraints are likely to restrict the onset of noxious autoimmune responses and protective tumor-specific immunity (46). We increased the immunogenicity of dying tumor cells by interfering with the PS-dependent recognition (47) and immunosuppressive clearance of dying cells, a mechanism involved in the physiological maintenance of peripheral tolerance.

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J.R. Kalden holds a patent on the use of AxV as adjuvant for apoptotic cells. A.A. Manfredi, M. Herrmann, and R. Voll are listed as inventors. M. Herrmann is a partner of a company that owns a license of this patent.

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