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Mechanism of dichotomy between CD8+ responses elicited by apoptotic and necrotic cells

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Apoptotic cells are significantly more immunogenic than necrotic cells, even though both forms are identical in antigenic content. When a combination of apoptotic and necrotic cells are used to immunize, the phenotype conferred by apoptotic cells, i.e., high immunogenicity, is dominant. However, necrotic cells are not immunosuppressive or tolerogenic. Apoptotic and necrotic cells are taken up by antigen-presenting cells in an equivalent manner. The priming of naïve T cell response is also equivalent. However, the CD8+ T cells elicited by apoptotic cells expand, accumulate, and express effector function, while those primed by the necrotic cells do not. This dichotomy does not extend to CD4+ cells. Apoptotic and necrotic cells elicit equivalent CD4+ T cell priming, accumulation, and function. The deficit in CD8+ T cell function elicited by necrotic cells can be overcome to varying degrees by anti-CD40 antibody and ligands for TLR4 and TLR9; conversely, the immunogenicity of apoptotic cells can be abrogated by blocking anti-CD154 antibody. Our results indicate that immunization with apoptotic cells leads to engagement of CD40 on antigen-presenting cells; this is essential for their ability to elicit mature functional CD8+ cells. The necrotic cells fail to engage CD40, and this failure is the basis of their lack of immunogenicity. These differences have consequences for the understanding of mechanisms of cross-presentation and for efforts toward immunotherapy of cancers and autoimmune pathologies.

Keywords: antigen-presenting cells, immunogenicity, apoptosis

Introduction

The immunological consequences of cell death have been the subject of speculation since Fuchs and Matzinger (1) suggested that apoptotic or programmed cell death is immunologically silent while necrotic cell death is not; apoptotic cell death is physiological and normal and, as such, poses no danger to the organism, while necrotic cell death may be a harbinger of danger and, hence, immunogenic. In experimental studies *in vivo*, Melcher *et al.* (2) demonstrated that apoptotic cells were poorly immunogenic, while cells killed by non-apoptotic means elicited highly potent and protective T cell response. The observations from studies *in vitro*, by Albert *et al.* (3), that apoptotic cells were readily taken up by dendritic cells (DCs) and their contents cross-presented by MHC I molecules of DCs to T cells, diverged from that view. In other studies, Gallucci *et al.* (4) and Sauter *et al.* (5) went on to show experimentally that necrotic lysates could mediate maturation of DCs; Basu *et al.* (6) demonstrated that hsp70 and hsp90 proteins were released into the extracellular space during necrosis but not apoptosis, and that these abundant intracellular molecules mediated maturation of DCs. These latter publications argued that necrotic death alone was immunostimulatory. This important difference in opinion—on whether apoptotic or necrotic death is immunostimulatory—has remained largely unresolved, even as the issue itself stands at the intersection of fundamental immunological phenomena, such as tolerance versus autoimmunity, as well as tumor and transplantation immunity. Mechanistically, it lies at the heart of the mechanism of cross-presentation in which exogenous antigens (in necrotic or apoptotic form) are taken up by antigen-presenting cells (APCs).

Understandably, attempts to resolve this issue, in studies *in vitro* or *in vivo*, have yielded a multiplicity of results and interpretations. In studies *in vitro*, bone marrow-derived dendritic cells (BM-DCs) could recognize, engulf, process, and cross-present antigen derived from apoptotic *or* necrotic cells, even as co-culturing BM-DCs only with necrotic cells (and not apoptotic cells nor their supernatants) induced DC maturation (4-6). Studies carried out *in vivo*, albeit preliminary, have shown inconsistent results. They are difficult to reconcile because of the differences in the manner in which cells were attenuated, and in the endpoints measured. Some studies use multiple freeze-thaw cycles to render cells necrotic (7, 8), others use a single cycle (9), while yet others initiate the killing response *in situ* by treating with drugs to render them necrotic (2, 10). Studies differ in the routes, concentrations, and schedules of immunization (7-9, 11). Some studies report that mice immunized with apoptotic cells mount a potent protective immune response, and those immunized with necrotic cells do not (7, 8). Other studies show equivalence between the two forms of immunization (11). In yet other studies, engulfment of apoptotic cells was reported to be tolerogenic and was suggested to be involved in suppressing immune responses to self (12, 13).

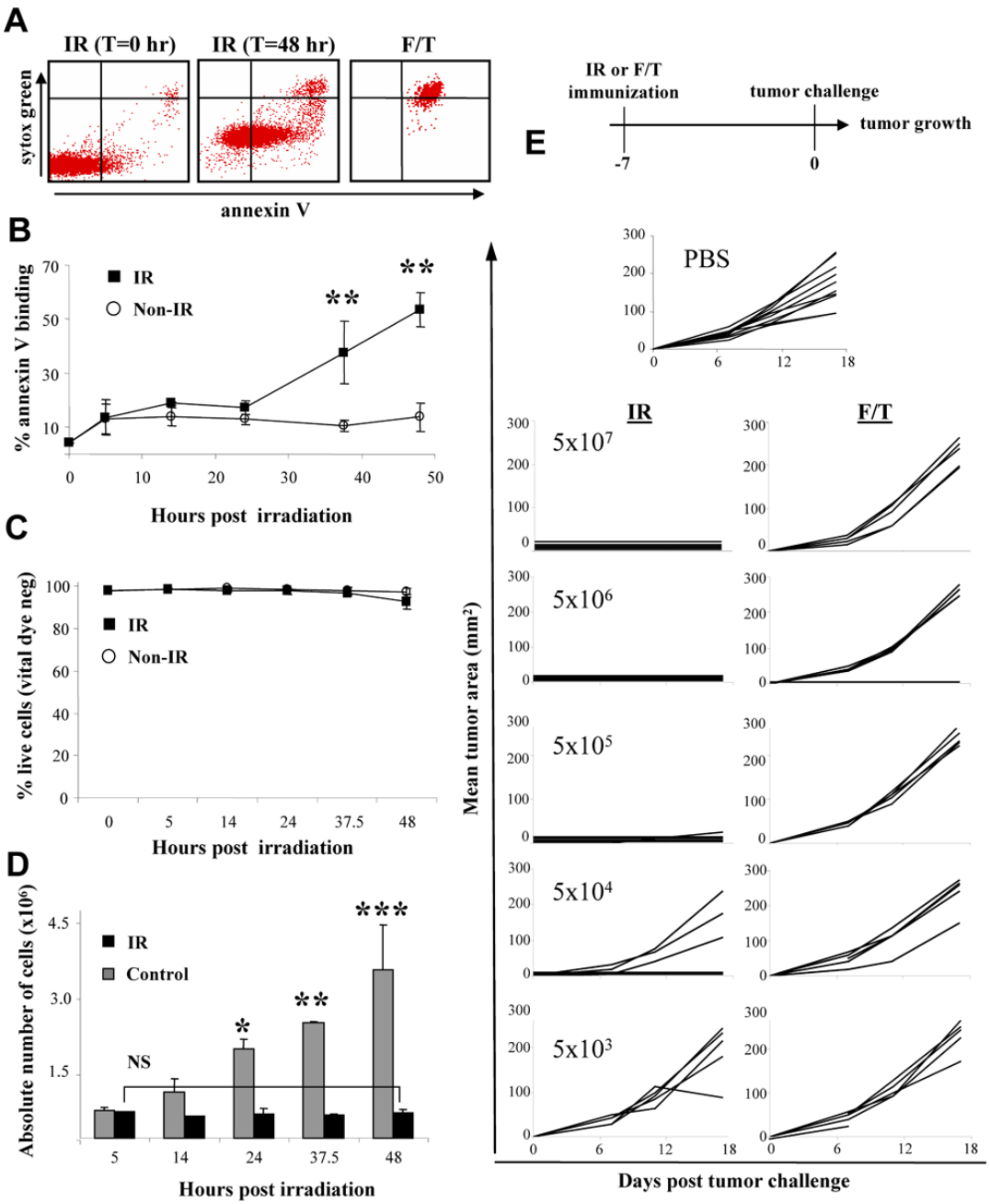
Here, we attempt to address the issue of immunogenicity (or lack of it) of apoptotic or necrotic cells comprehensively and mechanistically. We have chosen two distinct systems that permit distinct endpoints—a tumor immunization system that allows monitoring of results through a demanding and physiologically relevant endpoint of tumor rejection, and an ovalbumin (OVA)-containing system, which allows, in addition, a detailed mechanistic enquiry into the phenomena observed.

Results

Immunogenicity of apoptotic and necrotic cells

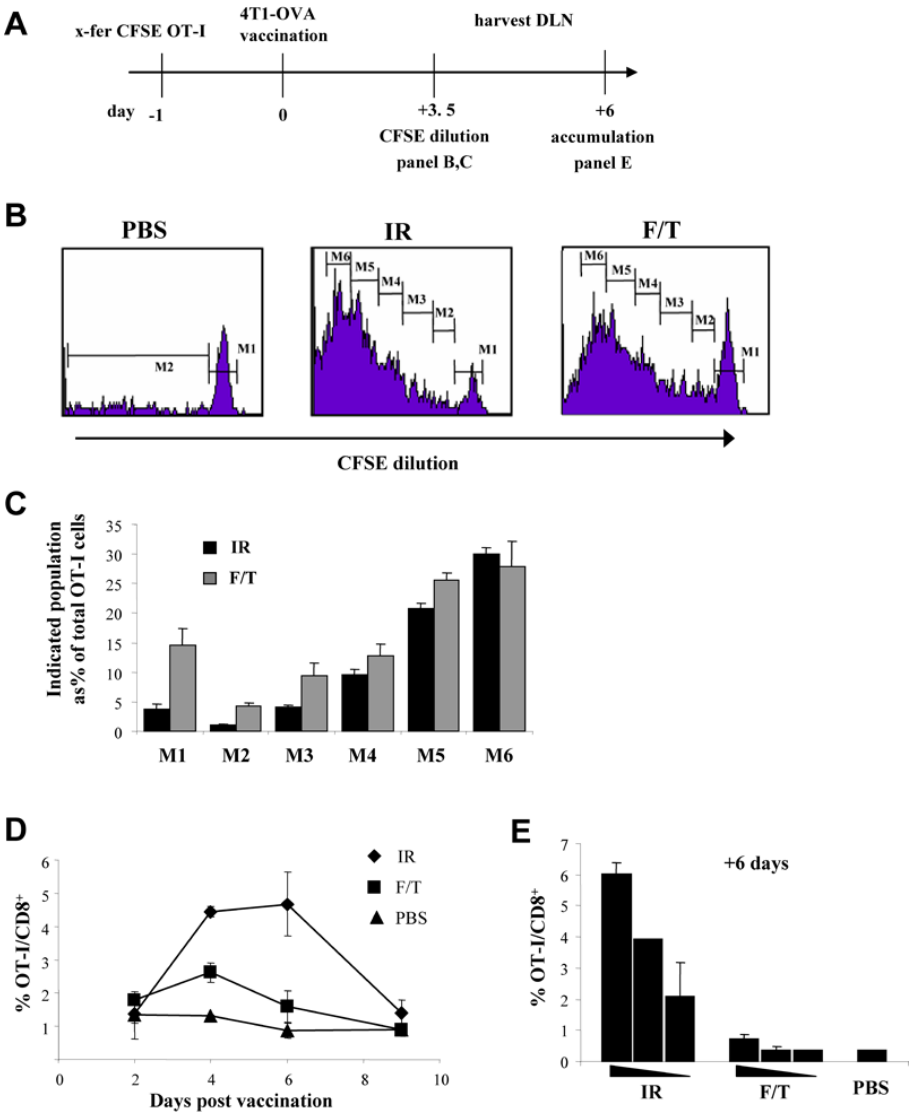
Meth A fibrosarcoma cells, grown in ascites form, were irradiated (IR) or treated to three cycles of freezing and thawing (F/T), as described in Materials and Methods. Untreated, IR, and F/T cells were analyzed for surface expression of phosphatidylserine (by Annexin V binding) and structural integrity (by exclusion of SYTOX Green) (Figure 1A). An over-

Figure 1



Immunization with IR or F/T cells protects from tumor challenge. (A) Induction of apoptosis and necrosis following IR and F/T treatments. Meth A cells were untreated, induced to undergo apoptosis by subjecting them to 7,500 rads of γ-radiation, or induced to undergo necrosis by 3 rounds of freezing and thawing, as indicated. Cells were analyzed for Annexin V binding and vital dye (SYTOX Green) exclusion by FACS analysis. (B) Kinetics of apoptosis following irradiation. IR or non-IR Meth A cells were cultured under standard conditions post-irradiation. At the indicated time points, 10^6 cells were assayed for Annexin V binding. (C) Time course analysis of viability of Meth A cells following irradiation. Meth A cells were analyzed for membrane integrity at various time points following irradiation. Data are shown as the percentage of cells able to exclude vital dye. (D) IR inhibits proliferation of Meth A cells, without causing acute cell death. Meth A cells were irradiated or mock irradiated and cultured under standard conditions. At the indicated time points, an aliquot was counted and total (trypan blue negative) cell numbers were determined. (E) Tumor protective capacity elicited by immunizing with apoptotic or necrotic Meth A cells. Groups of BALB/c mice were immunized on the left dorsal flank with titrated amounts ($5x10^7$ - $5x10^3$) of IR or F/T Meth A cells. Seven days post-immunization, all groups were challenged with 10^5 live Meth A cells on the right dorsal flank. Mice were monitored for tumor growth over time and data are presented as mean tumor area for individual mice. For panels B, C, and D, results of 3 independent experiments are plotted, with error bars representing standard deviation (SD) between replicate samples. *p* values were determined by Student's *t*-test: * = *p* value between 0.05 and 0.01; ** = *p* value between 0.01 and 0.001; and *** = *p* value < 0.001.

Figure 2



Immunization with IR or F/T 4T1-OVA cells elicits cross-presentation of SIINFEKL to CD8+ T cells. (A) Experimental design for cross-presentation studies. OT-I T cells were adoptively transferred to WT C57BL/6 mice (where indicated, OT-I cells were labeled with CFSE). One day following transfer, mice were immunized with IR or F/T 4T1-OVA cells. DLNs were harvested on d 3.5 or d 6 post-immunization and analyzed for CFSE dilution or OT-I accumulation as indicated. (B) Cross-presentation of IR or F/T 4T1-OVA cells to OT-I T cells. CFSE-labeled OT-I cells (10^6) were adoptively transferred. DLNs were harvested from mice immunized with PBS, IR, or F/T 4T1-OVA cells, as indicated, 3.5 d post-immunization and analyzed for CFSE dilution. Representative histograms are shown for 3 independent experiments. (C) Immunization with IR or F/T 4T1-OVA cells elicits similar levels of OT-I proliferation. Numbers of OT-I cells that had diluted CFSE were compared by calculating the percentage of cells, which fell within indicated gates, to the total number of OT-I cells. Results are shown as the mean of triplicate samples (±SD), and representative data are shown for 1 of 3 independent experiments. (D) Kinetics of T cell accumulation in the DLNs following immunization with IR or F/T 4T1-OVA cells. Groups of mice were transferred with OT-I cells and immunized. At the indicated times post-immunization, the percentage of OT-I cells in the total CD8+ population was determined by FACS analysis. Results of at least 3 independent experiments were averaged and error bars represent SD between the averaged samples for each time point. (E) Accumulation of OT-I cells in the DLNs 6 d post-immunization. Mice received OT-I cells and were immunized with titrated amounts (10^6, $2x10^5$, $5x10^4$) of IR or F/T 4T1-OVA cells. Six days post-immunization, DLNs were harvested and the percentage of OT-I cells/CD8+ population was determined for each group. Results are presented as the mean (±SD) and are representative of 3 independent experiments.

whelming majority of untreated cells showed no surface Annexin V binding and were structurally intact. In contrast, IR cells retained their structural integrity and showed abundant Annexin V binding, while F/T cells completely lost membrane integrity. The IR cells were specifically analyzed for these same characteristics at various time points post-irradiation (0-48 h), as indicated (Figure 1, panels B and C); they showed a time-dependent increment in the proportion of Annexin V+ cells, while remaining structurally intact all the way through. In addition, untreated and IR cells were placed in culture for 48 h and absolute numbers of cells were counted at various time points (Figure 1D); the untreated cells proliferated, while the IR

cells neither proliferated nor decayed and remained constant in number during this period. F/T cells were observed microscopically and no intact cells were detected. These F/T preparations were also placed in culture after each of the three F/T cycles. While some viable colonies grew after the first two F/T cycles, none was detected after the third F/T cycle (data not shown). In addition, the three forms of cells were examined by mitochondrial depolarization (by JC-1 incorporation) and scanning electron microscopy. The IR cells showed significantly enhanced JC-1 accumulation, cell shrinkage, and surface blebbing, as compared to live cells (data not shown). The IR and F/T cells thus fulfill the criteria for being apoptotic and necrotic, respectively. While these data are shown only for the Meth A cell line, grossly similar results were obtained with other cells including the CMS5 fibrosarcoma and 4T1 mammary carcinoma lines.

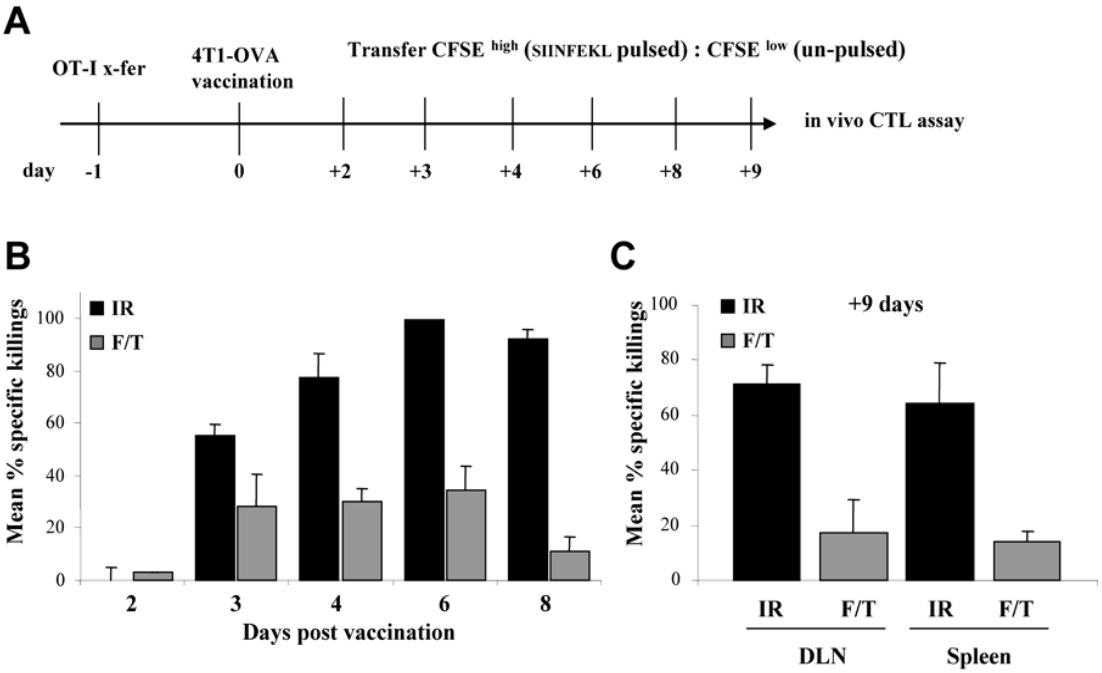
The immunogenicity of IR and F/T Meth A cells was determined. BALB/c mice were immunized once with intradermal injections of titrated amounts ($5x10^7$ - $5x10^3$) of IR or F/T cells and challenged with live 10^5 Meth A cells on the contralateral flank. Individual mice were monitored for kinetics of tumor growth (Figure 1E). The IR cells were observed to elicit potent protective immunity, while the F/T cells did not. The protective effect of IR cells was titratable and F/T cells failed to elicit protective immunity at any cell equivalent tested.

If IR cells were subjected to freeze-thaw cycles, and then used to immunize, they behaved like F/T cells, thus eliminating the possibility that the differences in the immunogenicity of IR and F/T cells could be attributable to new antigens induced by irradiation (data not shown). The potent immunogenicity of apoptotic cells and a poor immunogenicity of necrotic cells were also observed in the CMS5 fibrosarcoma and 4T1 mammary carcinoma tumor models tested (data not shown).

Priming, accumulation, and effector function of CD8+ T cells elicited by apoptotic and necrotic cells

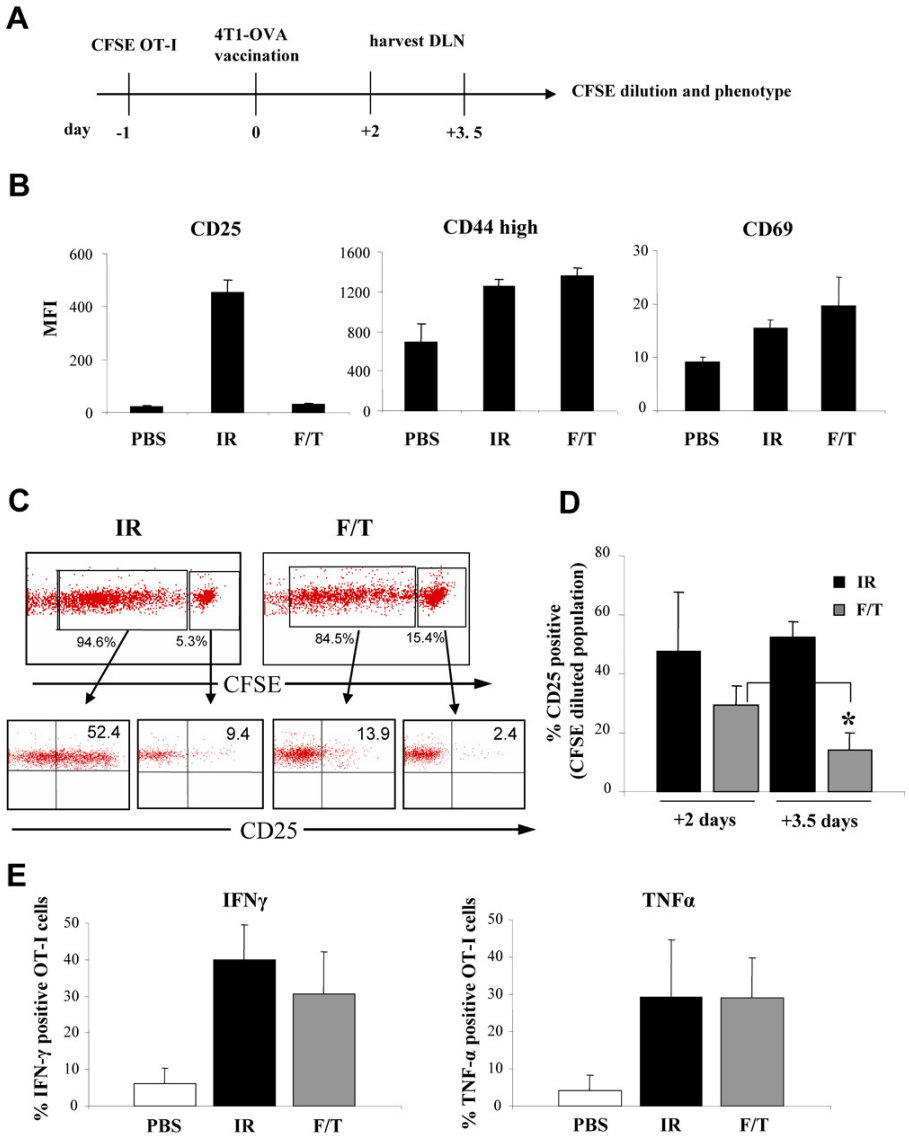
The immunization and tumor challenge model used in Figure 1 demonstrates rather dramatically the differences in immunogenicity of apoptotic and necrotic cells; however, in lack of suitable reagents, it does not permit a dissection of its mechanistic basis. An OVA-based system was used for this purpose. BALB/c mammary carcinoma 4T1 cells were electroporated to contain OVA as described in Materials and Methods, and the 4T1-OVA cells were irradiated or freeze-thawed, as in Figure 1. In order to determine the immunogenicity of IR and F/T 4T1-OVA cells, CFSE-labeled OT-I T cells were introduced into groups of C57BL/6 mice, which were immunized the following day with IR and F/T 4T1-OVA cells. Draining lymph nodes (DLNs) were harvested on d 3.5 post-immunization and analyzed for CFSE dilution of OT-I cells as a marker for priming of T cell response (Figure 2A). Immunization with PBS demonstrated minimal OT-I proliferation as evidenced by CFSE dilution. In contrast, immunization with IR or F/T 4T1-OVA cells demonstrated substantial and similar CFSE dilution profiles (Figure 2B). In order to quantitate these results, incremental gates were esta-

Figure 3



Immunization with F/T cells elicits CTLs with moderate and transient effector function. (A) Experimental design for determining the development of CTL effector function following immunization with IR or F/T 4T1-OVA cells. Groups of C57BL/6 mice were adoptively transferred with OT-I T cells ($2.5x10^5$/mouse) 1 d prior to immunization with IR or F/T 4T1-OVA cells. At the indicated time points, all mice received a 1:1 mixture of target cells (SIINFEKL-pulsed $CFSE^{high}$:unpulsed $CFSE^{low}$ splenocytes) as described in Materials and Methods. Mice were harvested 20 h post-transfer and the DLNs were analyzed for specific killing of target cells. (B) Kinetics of effector function acquisition by CTLs following immunization with IR or F/T 4T1-OVA cells. Data are shown as the mean percent specific killing compared to mice immunized with PBS. Results from 3 to 4 independent experiments were averaged per time point and error bars represent SD between samples. (C) CTL activity *in vivo* in DLNs and spleen, 9 d following immunization with IR or F/T 4T1-OVA cells.

Figure 4



Immunization with IR or F/T elicits activated OT-I cells of distinct phenotypes. (A) Experimental approach used to analyze phenotype and cytokine production of OT-I cells following immunization with IR or F/T 4T1-OVA cells. Groups of C57BL/6 mice were adoptively transferred with 10^6 CFSE OT-I cells 1 d prior to immunization with 10^6 IR or F/T 4T1-OVA cells. At the indicated time points, DLNs were harvested and analyzed for CFSE dilution and expression of activation markers by FACS. (B) MFI was determined for CD25, $CD44^{high}$, and CD69 expression following immunization as indicated. (C) Differential expression of CD25 on CFSE-diluted and undiluted OT-I cells 3.5 d following immunization with IR or F/T 4T-1 OVA cells. Groups of C57BL/6 mice were adoptively transferred and treated according to panel A. Regions were established for CFSE-divided and undivided populations and the percentage of cells expressing CD25 was determined. Representative histograms are shown for IR and F/T groups from 3 experiments, and the average percentage of CD25 positive cells from a representative experiment is noted (upper right quadrant). (D) Expression of CD25 on OT-I cells that have diluted CSFE decreases from d 2 to d 3.5 following immunization with F/T cells. DLNs from mice immunized with IR or F/T 4T1-OVA cells were harvested and CD25 expression in the CFSE-diluted and undiluted populations was analyzed. Data are plotted as the percentage of divided OT-I T cells that express CD25. Mean values (±SD) of replicate samples from 2 independent experiments are shown. (E) Immunization with IR or F/T 4T1-OVA cells elicits OT-I cells capable of producing IFN-γ and TNF-α. Mice were adoptively transferred and immunized. Three days post-immunization, DLNs were harvested and stimulated *in vitro* with SIINFEKL peptide for 5 h. Intracellular cytokine staining was performed according to manufacturer's protocol as outlined in Materials and Methods. Percentage of OT-I cells expressing intracellular IFN-γ (left) or TNF-α (right) are plotted with error bars representing the SEM of replicate samples from 2 independent experiments.

blished (M1-M6) and the amount of cells within each gate was calculated. Similar percentages of OT-I T cells were observed to have undergone division following IR or F/T immunization and the percentage of cells within each respective gate were also similar (Figure 2C). These data are in apparent contrast to the data with the tumor model shown in Figure 1.

In order to characterize the fate of the primed and expanded OT-I cells, accumulation kinetics of OT-I T cells in the DLNs

was assessed at various time points following immunization with IR or F/T 4T1-OVA cells. Mice were treated as in Figure 2A and DLNs were harvested at the indicated time points (2-9 d post-immunization). The percentage of OT-I cells in the total CD8+ population was determined for each group at each time point. PBS-immunized mice maintained a baseline percentage of OT-I/CD8+ cells during the entire time course. Groups immunized with IR 4T1-OVA demonstrated robust expansion and accumulation peaking at ~4.5%, 6 d post-immunization, and returned to baseline levels by d 9. In contrast, mice that received F/T 4T1-OVA cell immunization initially accumulated OT-1 cells, but the accumulation peaked at d 4 at ~2.6% and returned to baseline 6 d post-immunization (Figure 2D). In order to more fully evaluate this phenomenon, this experiment was repeated with titrated numbers of IR or F/T 4T1-OVA cells, and the DLNs monitored for accumulation 6 d post-immunization; a dramatic difference between the IR and F/T cells was noted at every dose level tested (Figure 2E). Thus, although IR and F/T cells primed OT-1 cells to a similar degree (Figure 2, panels B and C), immunization with IR cells lead to a significantly higher and prolonged accumulation relative to F/T cells (Figure 2, panels D and E).

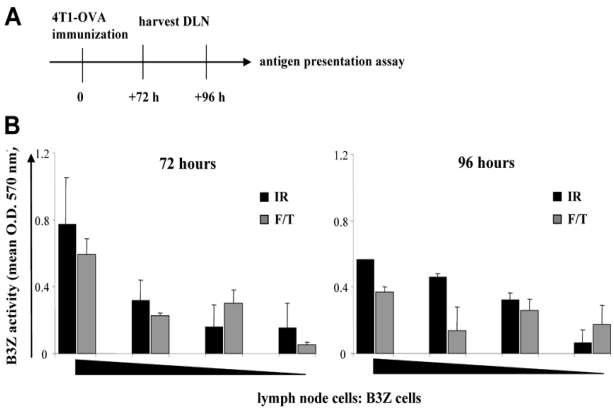
The T cells elicited by immunization with IR and F/T 4T1-OVA cells were evaluated for effector function using a cytotoxic T lymphocyte (CTL) assay *in vivo*, as described in Materials and Methods. C57BL/6 mice which had received adoptive transfer of OT-1 cells (as in Figure 2) were immunized with IR or F/T cells to permit priming and expansion. The ability of these primed T cells to recognize and lyse specific SIINFEKL-pulsed targets *in vivo* was tested. The immunized mice were recipients of a mixture of equal numbers of SIINFEKL-pulsed $CFSE^{high}$ and unpulsed $CFSE^{low}$ splenocytes on d 2, 3, 4, 6, 8, or 9 post-immunization (Figure 3A). Twenty hours after each transfer, the DLNs were evaluated for the percentage of specific killing of SIINFEKL-pulsed target cells, as described in Materials and Methods. Both IR and F/T immunizations elicited T cells that could recognize and kill SIINFEKL-pulsed targets specifically, albeit with significant differences (Figure 3B). In the mice immunized with IR 4T1-OVA cells, ~55% killing was observed on d 3, and this increased to a peak of 98% on d 6, followed by a gradual diminution of cytotoxicity. In the mice immunized with F/T 4T1-OVA cells, specific killing was also detected as early as d 3, but it was significantly lower (~28%) and it never accentuated on the following days. On d 8, the specific killing of pulsed targets in F/T immunized mice was close to baseline (Figure 3B). The possibility that T cells elicited by immunization with F/T 4T1-OVA cells had emigrated from the DLN to the spleen was tested, and the same experiment as in Figure 3B was performed, with two changes: the immunized mice were recipients of target cells on d 9 only, and cytotoxicity was evaluated in the spleen, in addition to in DLNs. The differences in specific killing by the T cells elicited by immunization with IR and F/T cells persisted in the spleen as well (Figure 3C).

Phenotype and cytokine secretion profiles of T cells elicited by apoptotic and necrotic cells

C57BL/6 mice were adoptively transferred with CFSE-loaded OT-I cells and immunized with IR or F/T 4T1-OVA cells as in Figure 2. DLNs were harvested at d 2 or 3.5 and analyzed for CD25, CD44, and CD69 phenotypes as outlined (Figure 4A). OT-I cells from mice immunized with IR or F/T 4T1-OVA cells express similar levels of CD69 and CD44 high activation markers as assessed by mean fluorescence intensity (MFI) (Figure 4B). In contrast, CD25 expression on OT-I cells was dramatically different between the IR and F/T 4T1-OVA immunized groups. This difference in expression is underscored by comparing the level of CD25 expression on CFSE-diluted and undiluted OT-I cells following IR and F/T immunization (Figure 4C). In agreement with Figure 2B, both IR and F/T immunization elicits similar priming and CFSE dilution of OT-I T cells. However, when CFSE-diluted cells were analyzed for CD25 expression, dramatic differences were observed. OT-I cells from mice that received IR immunization demonstrated ~52% of the CFSE-diluted population to be CD25-positive; even ~10% of the undivided population was positive. Significantly, only ~14% of the OT-I cells that had been induced to divide by F/T 4T1-OVA immunization expressed CD25 and > 3% of the undivided population was positive for this marker (Figure 4C). CD25 is known to be expressed early on activated T cells, and its expression is required for survival of antigen-experienced T cells (14, 15). The possibility that more OT-I cells primed by F/T 4T1-OVA cells express CD25 early (before the eventual loss of expression seen in Figure 4C) was investigated. Mice were treated as outlined in Figure 4A, but DLNs were harvested 2 d (as opposed to 3.5 d) post-immunization and analyzed for CD25 expression on CFSE-diluted OT-I cells. Mice that received IR 4T1-OVA immunization maintained similar percentages (~50%-55%) of divided OT-I cells that express CD25 on d 2 and d 3.5. In contrast, mice that received immunization with F/T 4T1-OVA had a larger percentage of CFSE-diluted cells that expressed CD25 on d 2 (~30%) than on d 3.5 (~14%) (Figure 4D).

Production of selected cytokines by T cells elicited by immunization with IR or F/T 4T1-OVA cells was examined. Groups of mice were treated as in Figure 4A, except that OT-I

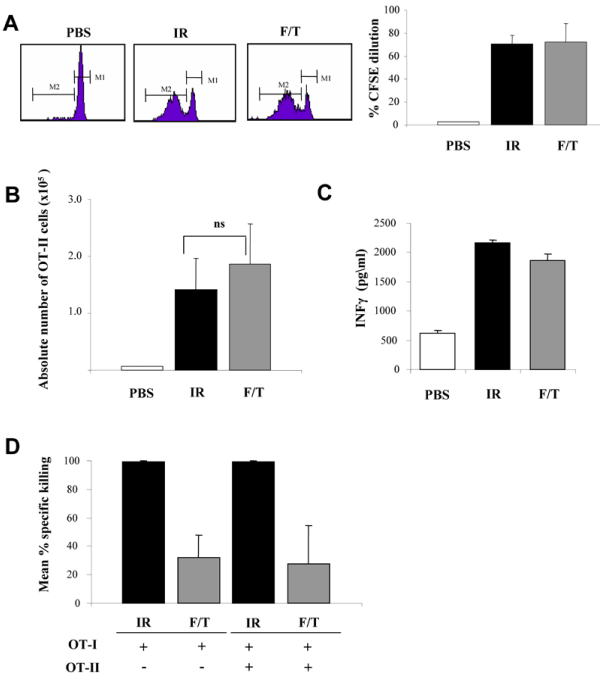
Figure 5



Immunization with IR or F/T 4T1-OVA cells elicits similar numbers of APCs capable of cross-presenting antigen. (A) Experimental design: groups of C57BL/6 mice were immunized with 10^6 IR or F/T 4T1-OVA cells. At the indicated time points post-immunization, DLN-derived cells were assayed for the ability to cross-present IR or F/T cell-derived antigen to B3Z cells. (B) Immunization with IR or F/T 4T1-OVA cells generates apparently similar amount of antigen-presenting capacity. Mice were immunized, and at 72 or 96 h post-immunization, DLNs were harvested and co-cultured in titrated numbers ($1x10^6$ - $1.25x10^5$) with 10^5 B3Z cells. CPRG assays were performed as described in Materials and Methods. Data are plotted as the average O.D. at 570 nm minus the values for PBS immunized groups at each concentration. Error bars are representative of the SD between values of triplicate wells. Results are representative of 3 similar experiments.

cells were not CFSE-labeled prior to adoptive transfer. At 3.5 d post-immunization, OT-I cells were analyzed for intracellular expression of IFN-γ and TNF-α following 5 h stimulation *in vitro* with SIINFEKL peptide. PBS-immunized mice demonstrated a low percentage of OT-I cells expressing either cytokine, while mice immunized with IR or F/T 4T1-OVA demonstrated a significant and similar percentage of cells producing intracellular IFN-γ and TNF-α (Figure 4E). This observation argues that the OT-I cells elicited by either immunization are potentially capable of full functional activation, even though only the cells elicited by immunization with IR cells achieve that status *in vivo* (Figure 3B).

Figure 6



Immunization with IR or F/T 4T1-OVA cells elicits comparable cross-presentation of antigen to CD4+ T cells. (A) C57BL/6 mice were adoptively transferred with 10^6 CFSE-labeled OT-II T cells 1 d prior to immunization with IR or F/T 4T1-OVA cells. Four days post-immunization, DLNs were harvested and the average percentage (±SD) of OT-II cells that had diluted CFSE was determined and plotted in panel A (bar graph). Representative histograms are shown for immunization with PBS, IR, and F/T cells. (B) IR or F/T 4T1-OVA immunization elicits OT-II cell accumulation in DLNs. C57BL/6 mice were adoptively transferred with 10^6 OT-II cells and immunized. DLNs were harvested 6 d post-immunization and the total number of OT-II cells was determined. Data are presented as the mean (±SD) number of OT-II T cells in the DLNs of triplicate samples per group. (C) Immunization with IR or F/T 4T1-OVA cells generates OT-II cells capable of producing IFN-γ. C57BL/6 mice were adoptively transferred with 10^6 OT-II T cells 1 d prior to immunization with IR or F/T 4T1-OVA cells. At d 6, DLNs were harvested and stimulated with OT-II specific peptide (OVA peptide 323-339, sequence ISQAVHAAHAEINEAGR) for 11 h. Supernatants were harvested and samples analyzed for the presence of IFN-γ. Error bars represent SD of triplicate samples. (D) Increasing precursor frequency of OT-II T cells does not lead to greater CTL activity *in vivo* in immunized mice. Groups of C57BL/6 mice were adoptively transferred with OT-I, OT-II, or a mixture of OT-I and OT-II cells (5:1 ratio) 1 d prior to immunization with IR or F/T 4T1-OVA cells. Six days post-immunization, CTL activity *in vivo* was determined as described in Figure 3. Data are presented as the mean percent (±SD) specific killing 20 h post target transfer of triplicate samples.

Characterization of APCs cross-presenting apoptotic or necrotic materials

We used the 4T1-OVA cells of the *d* haplotype to immunize C57BL/6 mice of the *b* haplotype. Elicitation of a T cell response was therefore strictly dependent on involvement of an APC. Groups of C57BL/6 mice were immunized with PBS, IR, or F/T 4T1-OVA cells. Forty-eight and 96 h post-immunization, DLNs were harvested and stained for expression of CD11c, CD40, CD86, and MHC II. Flow cytometric analysis demonstrated no significant differences in the median fluorescence intensity (MFI) of the bulk CD11c population for any of the markers tested following immunization with IR or F/T cells, or with PBS (data not shown). As expected, analysis of the bulk CD11c population in the DLNs, which includes a large number of resident APCs, may not be sensitive enough to distinguish changes in the level of maturation markers on antigen-experienced APCs (ae-APCs) immigrating from the immunization site.

The ability of DLN-derived ae-APCs elicited by immunization with IR or F/T 4T1-OVA cells to stimulate the B3Z cells (T cell hybridoma that produces β-galactosidase in response to engagement of MHC I/SIINFEKL complexes) (16) was examined. Groups of mice were treated as outlined in Figure 5A and DLNs were harvested at 72 or 96 h post-immunization. The total population was serially diluted (1:1) in 96-well plates outlined as in Materials and Methods and $5x10^4$ -$1x10^5$ B3Z cells were added. The cells were co-cultured for 20 h and assayed for β-galactosidase activity. DLNs that were harvested following immunization with IR or F/T 4T1-OVA cells demonstrated significant and titratable B3Z activity at both (72 and 96 h) time points. B3Z activity between the immunization groups was generally indistinguishable at either time point (Figure 5B), indicating that similar numbers of cross-presenting ae-APCs are generated from the site of immunization with IR or F/T 4T1-OVA cells.

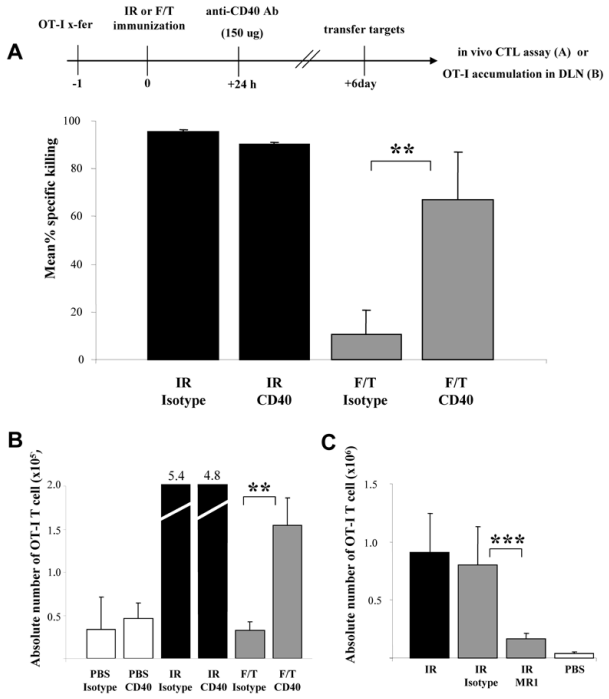
Priming, accumulation, and effector function of CD4+ T cells elicited by apoptotic and necrotic cells

C57BL/6 mice were adoptively transferred with CFSE-loaded OT-II cells 1 d prior to immunization with IR or F/T 4T1-OVA cells. DLNs were harvested 4 d post-immunization and analyzed for CFSE dilution. DLNs from mice that received PBS immunization demonstrated baseline dilution. DLNs from mice immunized with IR 4T1-OVA cells exhibited CFSE dilution in ~75% of the transferred OT-II cells. A similar percentage (~78%) of OT-II cells exhibited CFSE dilution following immunization with F/T 4T1-OVA cells (Figure 6A). Accumulation in the DLNs following immunization with IR and F/T 4T1-OVA was tested. In contrast to the diminished OT-I response observed at d 6 post-immunization with F/T, as compared to IR cells (Figure 2, panels D and E), immunization with IR or F/T cells resulted in significant and equivalent accumulation of OT-II cells in the DLNs (Figure 6B). To assess the functionality of the primed OT-II cells, IFN-γ production was determined 6 d post-immunization with IR or F/T 4T1-OVA cells. C57BL/6 mice were adoptively transferred with 10^6 OT-II cells 1 d prior to immunization. Six days post-immunization, DLNs were harvested and $30x10^6$ total lymph node cells per treatment group were stimulated with ISQAVHAAHAEINEAGR peptide (recognized by OT-II cells)

for 11 h, at which time cultures were spun down and supernatants were tested for the presence of IFN-γ. OT-II cells from mice immunized with IR or F/T 4T1-OVA cells produced significant and similar amounts of IFN-γ (~1900-2100 pg/ml) (Figure 6C).

These data show that unlike the diminished CD8+ response elicited by F/T (as compared to IR) cells, CD4+ cells divide, accumulate, and produce cytokines to similar levels following immunization with IR and F/T cells. The possibility that the diminished CD8+ activity elicited by F/T cells could be restored to the levels seen following immunization with IR cells, by increasing the precursor frequency of CD4+ cells, was considered. C57BL/6 mice were adoptively transferred with OT-I cells alone or with a 4:1 mixture of OT-I and OT-II cells 1 d prior to immunization with IR or F/T 4T1-OVA cells. The immunized mice were recipients of a mixture of SIINFEKL-pulsed and unpulsed splenocytes on d 6 post-immunization, as described previously. Twenty hours after transfer, the DLNs were evaluated for the percentage of specific killing of SIINFEKL-pulsed target cells. DLNs from mice that were adoptively transferred with OT-I cells only demonstrated specific killing ability consistent to that observed in Figure 3; mice immunized with IR cells showed 100% specific cytotoxicity, while those immunized with F/T cells showed poor (~25%) cytotoxicity. This pattern of cytotoxicity was not altered by the inclusion of OT-II cells in the system (Figure 6D).

Figure 7



Activating anti-CD40 antibody partially mitigates the deficit in CTL generation and accumulation elicited by immunization with F/T 4T1-OVA cells. (A) Groups of C57BL/6 mice were adoptively transferred with $2.5x10^5$ OT-I T cells 1 d prior to immunization with IR or F/T 4T1-OVA cells. Twenty-four hours post-immunization, mice received an injection of 150 μg of activating anti-CD40 antibody or isotype control antibody. Six days following immunization, all mice were adoptively transferred with target cells and a CTL assay *in vivo* was performed. Data are shown as the mean specific killing 20 h post-immunization. One of three experiments is shown and error bars represent SD of triplicate samples. (B) Immunization with F/T 4T1-OVA cells followed by anti-CD40 antibody injection increases OT-I T cell accumulation in the DLNs. Mice were treated according to panel A. Six days post-immunization, DLNs were harvested and the absolute number of OT-I cells was determined. Data is shown as the mean (±SD) of triplicate samples. (C) Blocking CD40/CD40L interactions diminishes OT-I accumulation elicited by immunization with IR 4T1-OVA cells. Groups of C57BL/6 mice received 10^6 OT-I cells 1 d prior to immunization with PBS or IR 4T1-OVA cells. At the indicated time points post-immunization, groups were treated i.v. with 200 μg of MR1 (anti-CD154, CD40L) blocking antibody. Six days post-immunization DLNs were analyzed for OT-I accumulation.

Role of CD40

CD40-CD40L interaction between APCs and CD4+ T cells plays a critical role in licensing APCs to promote full effector function of CD8+ cells (17-19). Mice that had received an adoptive transfer of OT-I cells the previous day, and had been immunized with IR or F/T 4T1-OVA cells, were injected with an activating anti-CD40 antibody or isotype control 24 h post-immunization. Six days post-immunization, the mice were recipients of a mixture of SIINFEKL-pulsed and unpulsed splenocytes, as described previously. Twenty hours after transfer, the DLNs were evaluated for the percentage of specific killing of SIINFEKL-pulsed target cells (Figure 7A). DLNs from mice immunized with IR 4T1-OVA cells and injected with activating CD40 antibody (or control antibody) demonstrated > 90% specific cytotoxicity. While DLNs from mice immunized with F/T 4T1-OVA cells and injected with isotype control antibody demonstrated the consistently low level of cytotoxicity observed in previous experiments (~20%), those from mice immunized with F/T 4T1-OVA cells and injected with activating CD40 antibody demonstrated a significant increase in specific killing ability (~70%) (Figure 7A). Accumulation of OT-I cells in the DLNs, under the same conditions, was tested. Mice were treated as in Figure 3A, and at d 6 post-immunization, cells from the DLNs of all treatment groups were analyzed for OT-I accumulation. DLNs from mice that received PBS immunization and were injected with anti-CD40 or isotype control antibody, demonstrated baseline accumulation of absolute numbers of OT-I cells in DLNs. Robust accumulation of OT-I cells in the DLNs was observed in the IR-immunized mice that received anti-CD40 or isotype control antibody. In the mice immunized with F/T cells, however, there was a significant and substantial increase in the absolute number of OT-I in the DLNs following anti-CD40 antibody injection (Figure 7B).

Due to the ability of activating anti-CD40 antibody to mediate enhancement of accumulation and *in vivo* CTL effector function of OT-I cells in mice immunized with F/T 4T1-OVA cells, the requirement of CD40/CD40L interaction following immunization with IR 4T1-OVA cells was investigated. C57BL/6 mice were treated as in Figure 7A with the following change: all groups of mice were adoptively transferred with 10^6 OT-I cells 1 d prior to immunization with 10^6 IR 4T1-OVA cells. The immunized mice were recipients of 200 μg of the MR1 (anti-CD154 blocking antibody) or isotype control antibody, on d 0, 1, 2, 3, and 4 post-immunization. DLNs were harvested on d 6 post-immunization and analyzed for OT-I accumulation. DLNs from PBS-treated mice demonstrated baseline accumulation of OT-I cells, whereas mice that were immunized with IR 4T1-OVA cells and not treated, or that received control antibody,

demonstrated robust accumulation of OT-I cells in the DLNs consistent with that observed in Figure 2, panels D and E. In contrast, mice that received treatment with MR1 antibody failed to elicit accumulation of OT-I cells in the DLNs ($p < 0.001$) (Figure 7C).

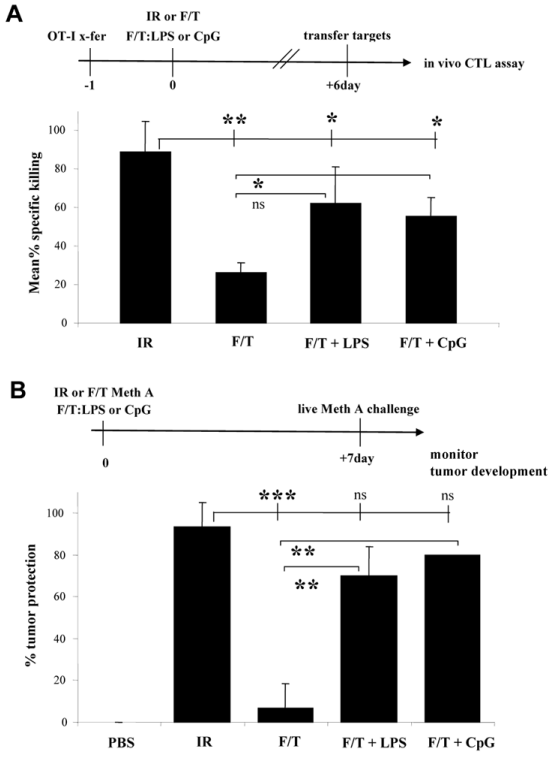
Effect of lipopolysaccharides and CpG oligonucleotides

Engagement of pathogen associated molecular pattern receptors by their ligands has been demonstrated to activate APCs and to influence downstream T cell responses (20-23). The effect of co-injection of various Toll-like receptor (TLR) agonists on the accumulation of OT-I cells following immunization with F/T was tested. C57BL/6 mice were adoptively transferred with OT-I cells 1 d prior to immunization with IR or F/T 4T1-OVA cells alone, or F/T 4T1-OVA cells mixed with agonists for TLRs 2, 3, 4, 5, 7, 7/8, or 9, as described in Materials and Methods. Six days post-immunization, DLNs were harvested and the absolute numbers of OT-I cell were determined. Co-immunization of F/T cells with any of the TLR agonists did not elicit significantly greater accumulation of OT-I cells as compared to that seen in mice immunized with F/T cells alone (data not shown); a small, but statistically insignificant increase was noted in mice co-immunized with CpG. The effect of co-immunization with CpG or LPS was explored further. C57BL/6 mice were transferred 1 d prior to immunization with IR or F/T 4T1-OVA cells or F/T 4T1-OVA cells mixed with 100 μg of LPS or 20 μg of CpG. Six days post-immunization, all groups of mice were recipients of a mixture of SIINFEKL-pulsed and unpulsed splenocytes, as described previously. Twenty hours after transfer, the DLNs were evaluated for the percentage of specific killing of SIINFEKL-pulsed target cells. Co-immunization with LPS or CpG was able to increase the specific cytotoxic activity elicited by F/T cells alone. The increase was statistically significant for CpG ($p = 0.031$) and approached significance for LPS ($p = 0.085$), however it did not achieve the cytotoxicity seen in mice immunized with IR cells alone (LPS, $p = 0.026$; CpG, $p = 0.034$) (Figure 8A). The ability of LPS and CpG to enhance the immune response elicited by F/T cells was tested in a tumor rejection model. Groups of mice were immunized with IR or F/T Meth A cells, or with F/T Meth A cells mixed with LPS or CpG on d 0, as described above. All groups were challenged with live Meth A cells 7 d post-immunization and monitored for tumor development. Mice that received immunization with IR cells demonstrated 100% protection, whereas mice immunized with F/T Meth A cells were minimally protected (~10%), as seen previously in Figure 1 and Figure 7. Groups of mice that were co-injected with F/T Meth A and LPS demonstrated significant (70%) tumor protection as did the groups of mice that received co-injection with CpG (80%) (Figure 8B). Thus, LPS and CpG enhanced the immunogenicity of F/T cells comparably, in both models tested.

Necrotic cells are not immunosuppressive nor tolerogenic

C57BL/6 mice were adoptively transferred with OT-I cells 1 d prior to immunization. Groups of mice were immunized with IR or F/T 4T1-OVA cells, or with a 1:1 mixture of IR and F/T 4T1-OVA cells. Six days post-immunization, DLNs were harvested and analyzed for OT-I accumulation by determining the percentage of OT-I cells in the total CD8+ population as outlined in Figure 2, panels D and E. DLNs from mice immunized with PBS demonstrated baseline accumulation. DLNs from mice immunized with IR or F/T 4T1-OVA cells alone demonstrated percentages of accumulation consistent with those seen previously (~4.2% and ~0.5%, respectively), however when mice received co-immunization with IR and F/T 4T1-OVA cells, the percentage of accumulation was indistinguishable from that elicited by IR cells (Figure 9A). Thus, immunization with F/T cells is not acutely immunosuppressive. This possibility was additionally tested in a mechanistically different manner in the Meth A model. BALB/c mice were immunized with PBS, IR, or F/T Meth A cells. Seven days post-immunization, a second immunization (as indicated) was given. Seven days following the second immunization, all mice were challenged with live Meth A cells and monitored for tumor development (Figure 9B). Mice immunized with PBS, IR, or F/T cells demonstrated characteristic levels of tumor protection as seen in other experiments (Figure 8B). Mice immunized with IR cells followed by immunization with F/T cells, or mice immunized in the reverse order, showed the same level of protection as mice immunized once with IR cells alone (Figure 9B).

Figure 8



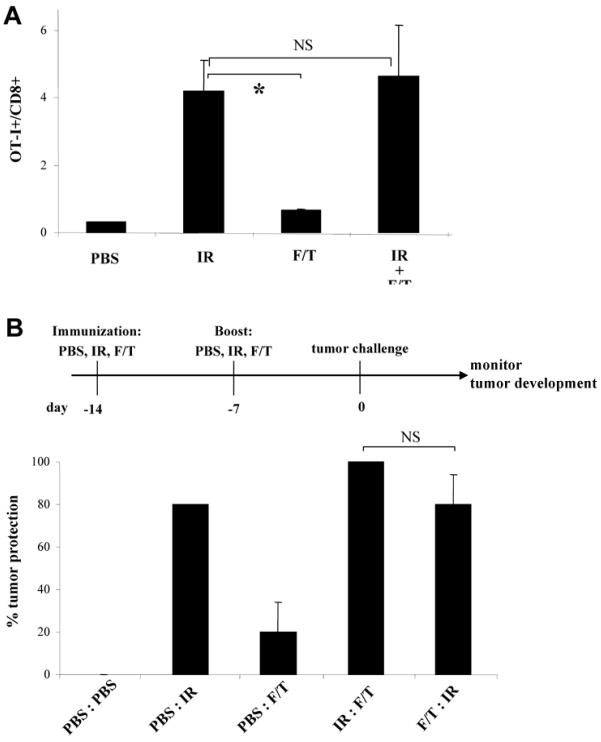
Co-immunization of F/T cells with TLR4 and TLR9 ligands causes modest enhancement in CTL activity and tumor-protective capacity. (A) Co-immunization of F/T 4T1-OVA cells with LPS or CpG increases CTL activity *in vivo*. C57BL/6 mice were adoptively transferred with $2.5x10^5$ OT-I cells 1 d prior to co-immunization with 10^6 F/T 4T1-OVA cells mixed with 20 μg of CpG or 100 μg LPS. Six days post-immunization, a CTL assay *in vivo* was performed. Data are shown as the mean percent specific killing (±SD) of replicate samples. Combined data from 3 independent experiments are shown. (B) Co-immunization of F/T 4T1-OVA cells with LPS or CpG increases tumor-protective capacity against Meth A challenge. BALB/c mice were immunized on the left flank with PBS, IR, F/T, or F/T supplemented with 20 μg of CpG or 100 μg of LPS. Seven days post-immunization, mice were challenged with 10^5 live Meth A cells on the right flank, and monitored for tumor growth. Data are presented as the average percent tumor protection (±SD) for each treatment group from 3 independent experiments.

Discussion

Our results show that form is of the essence for immunogenicity. Specifically, we show in a number of diverse models that apoptotic cells are significantly more immunogenic than necrotic cells, even though both innocula are identical in the sum total of their antigenic content, qualitatively and quantitatively. The necrotic form is not immunosuppressive or tolerogenic. Both forms of antigen appear to be taken up by APCs in an apparently equivalent manner, as seen by a quantitative comparison of the ability of DLN-derived APCs to stimulate the B3Z hybridoma. The priming of a naïve T cell response is also apparently equivalent. It is from this point on that the responses elicited by the two forms begin to diverge. The CD8+ T cells elicited by the apoptotic form continue to expand post-priming, where they accumulate and express full effector function. In contrast, the CD8+ T cells primed by the necrotic form do not accumulate, appear to "crash", and a relatively much smaller number of them achieve functional status. This dichotomy between the immunogenic potentials of the two forms does not extend to CD4+ T cells. Both forms elicit equivalent CD4+ priming, accumulation, and function. The dichotomy between the CD8+ responses elicited by the apoptotic and necrotic cells is attributable to engagement of CD40 by the former and the lack of such engagement by the latter. Considering that the CD4+ responses elicited by apoptotic and necrotic cells are equivalent in all parameters tested, we consider it unlikely that the difference in engagement of CD40 between the apoptotic and necrotic cells can be attributed to differences in CD4+ responses elicited. Instead, we favor the possibility that a ligand on the apoptotic cell, not yet identified by us, engages the APC in a manner that permits CD40 engagement. The immunizing cells used in our study are themselves devoid of CD154 (data not shown).

Our results unify a number of disparate observations and ideas on the immunogenicity of necrotic versus apoptotic cells. They confirm and extend mechanistically a previous study (8, 10) and dispel the notion that apoptotic death is an immunologically silent death; they confirm *in vivo* the studies *in vitro*, reported by Albert and colleagues, that apoptotic cell-derived antigens are cross-presented by DCs (3, 24, 25). The idea that necrotic cell death is highly inflammatory was based on a convergence of a number of factors. There was the intuitively appealing idea that exposure of the immune system to intracellular cellular contents should signal danger (1, 26). There were the observations that exposure of immature DCs to necrotic lysates caused maturation of DCs and exposure to apoptotic cells did not cause maturation (4-6). There were specific examples of cell lysate constituents that mediate maturation of DCs (6, 27-29). Regardless of these attractive components, it is clear in hindsight that the arguments and the assays *in vitro* did not reflect *in vivo veritas*. Apoptotic cells appear to transfer antigen for cross-presentation as efficiently as necrotic lysates, and the APCs that take up the apoptotic cells are able to present the antigens to naïve T cells as effectively as those that take up the necrotic cells. What then of the fact that apoptotic death is a normal physiological process and that a very large number of cells are always dying without an autoimmune pathology? Our data suggest that such normally apoptosing cells are perfectly capable of eliciting an immune response. We posit that they do not do so simply because they lack antigenic "non-self" material. The apoptosing cell contains the elements to create the innate environment needed for a functional immune response. We do not have a good understanding of what those elements are; regardless, the idea that necrosis and the release of intracellular contents are necessary for providing those elements appears unsubstantiated. In this context, the task of avoiding autoimmune pathology comes to rest on the multiple mechanisms of tolerance, and on the do-not-eat-me signals on live non-apoptotic cells, such as CD47 or CD31 (30, 31).

Figure 9



F/T 4T1-OVA cells are not suppressive or tolerogenic. (A) Groups of C57BL/6 mice were adoptively transferred with 10^6 OT-I cells 1 d prior to vaccination with 10^6 IR, 10^6 F/T, or 10^6 F/T + 10^6 IR cells. Six days post-immunization, DLNs were analyzed for accumulation of OT-I cells. Data are presented as the mean percentage (±SD) of OT-I cells/total CD8+ population of replicate samples. (B) Immunization with F/T does not inhibit a response to a second immunization. Groups of BALB/c mice were immunized with PBS, IR, or F/T-treated Meth A cells (10^6 cells/immunization). Seven days post-immunization, mice received a second immunization of either: PBS, IR, or F/T. Seven days following the boost immunization, all groups were challenged with 10^5 live Meth A cells and monitored for tumor growth. Data are presented as the average percentage of tumor protection for each treatment group (±SD) of replicate samples from 2 independent experiments.

Abbreviations

IR, irradiation; F/T, freeze-thaw; OVA, ovalbumin; DLN, draining lymph node; APC, antigen-presenting cell

Acknowledgements

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Materials and methods

Mice

Female BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory. CD45.1^{+} RAG$^{-/-}$ OT-I TCR Tg mice (OT-I) and CD45.1^{+} RAG$^{-/-}$ OT-II TCR Tg mice (OT-II) were bred and housed in barrier facilities maintained by the Center for Laboratory Animal Care (CLAC). Animal work was performed with permission from the Institutional Animal Care and Use Committee of the University of Connecticut Health Center (Farmington, CT), and was in compliance with established guidelines.

Cells, peptides, chemicals, and antibodies

Meth A (H-2^{d}) fibrosarcoma cells are maintained as ascites in syngenic mice. 4T1 (H-2^{d}) mammary carcinoma cells were purchased from American Type Culture Collection (ATCC). B3Z T cell hybridomas were kindly provided by Nilabh Shastri (University of California, Berkeley, CA). β-galactosidase was detected by cleavage of chlorophenol red β-pyranoside (CPRG), purchased from Roche. OT-I (SIINFEKL) and OT-II (ISQAVHAAHAEINEAGR) reactive peptides were synthesized at Genemed Synthesis Inc., (San Antonio, TX) to a purity of > 95%. Chicken ovalbumin (OVA) was purchased from Sigma (Grade IV). Endotoxin was removed by Detoxi-Gel Endotoxin Removing Gel (Pierce Biotechnology, Rockford, IL) according to manufacturer's protocol. OVA protein was centrifuged at 100,000 x *g* for 90 min to remove aggregates. LPS-free and aggregate-free protein was used in all assays. Activating anti-CD40 antibody (clone FGK4.5) and IgG2a isotype control were purchased from Bio X Cell (West Lebanon, NH). CD154 blocking antibody (clone MR1) was received from Dr. Stephen Schoenberger and used as indicated in the text. LPS (*E. coli* 026:B6) was purchased from Sigma and CpG oligonucleotide (ODN 2395) was purchased from InvivoGen (San Diego, CA).

4T1-OVA cell generation

4T1 cells were loaded with OVA (4T1-OVA) by harvesting, washing 2x with PBS and 1x with electroporation buffer (Cyto Pulse Sciences, Inc., Glen Burnie, MD), and resuspended at 8x10^{6}/ml. Aggregate and LPS-free OVA was added to the cell suspension in a volume that resulted in 5 mg of OVA/4x10^{6} cells. 500 µl of this suspension was aliquoted into sterile 4 mm electroporation cuvettes (Bio-Rad Laboratories, Hercules, CA). Cuvettes were electroporated using a PulseAgile PA-4000 (Cyto Pulse Sciences, Inc.). Cuvettes received 2 pulses at 600 volts for 0.2 ms duration with a 0.5 s interval between pulses. Cuvettes were placed on ice for 10 min, after which cells were removed from cuvettes and cultured for 18-22 h before being harvested and used in assays. This method is a modification of (30) and has been demonstrated in our laboratory to result in ~5 fg of OVA per cell.

Apoptosis and necrosis induction

Cell lines were induced to initiate apoptosis by exposure to 7500 rads of γ-radiation using a Gammacell 1000 irradiator (Atomic Energy of Canada Limited) which contains a ^{137}cesium radioactive core. Cells were exposed for 35 min at a dose rate of 217 rads/min. Apoptosis of irradiated tumor cells was analyzed by several methods, including: Annexin V binding (Vybrant Apoptosis Assay Kit, Invitrogen), JC-1 (Sigma) incorporation into mitochondria, and morphologically by scanning electron microscopy. Necrosis was induced by serial cycles of freezing (liquid nitrogen) and thawing (37°C water bath). Necrosis was confirmed by loss of membrane integrity to vital dyes, failure to clonally expand in culture, and scanning electron microscopy.

Assessing T cell cytotoxicity *in vivo*

CTL assays *in vivo* were performed by adoptively transferring 2.5x10^{5} OT-I T cells into C57BL/6 mice 1 d prior to immunization. At the indicated time points post-immunization, mice were transferred i.v. with a 1:1 mixture of SIINFEKL-pulsed splenocytes labeled with 2.5 µM CFSE^{high}, and non-peptide pulsed splenocytes labeled with 0.25 µM CFSE^{low}. Twenty hours following transfer of target cells, spleens or DLNs were harvested and analyzed by FACS. The percentage of specific killing was determined by comparing the ratio of CFSE^{high}: CFSE^{low} cells recovered from animals immunized with IR or F/T cells and normalized to the ratio obtained from mice immunized with PBS as previously described (31).

Comparing APCs cross-presenting IR or F/T-derived material *in vivo*

Mice were immunized with 10^{6} IR or F/T 4T1-OVA cells. At the indicated time points, DLNs were harvested and digested at 37°C for 30 min in 2 ml of RPMI supplemented with 1 mg/ml of type II and IV collagenase also containing 50 Kunitz units/ml of DNAse (all from Sigma). Following incubation, wells were treated with EDTA (5 mM final concentration) and incubated for an additional 5 min. Cells were collected, washed 1x with digestion wash buffer (1% FBS in PBS with 2.5 mM EDTA and 25 Kunitz units/ml DNAse), and resuspended in staining media (1% FBS in PBS) and counted. Samples were resuspended at 10^{7} cells/ml and serially diluted with 5% RPMI (complete) at 100 µl volumes in 96-well round bottom plates. B3Z cells were added (10^{5} cells/well) and co-cultured for 20 h. Plates were centrifuged, washed 1x with PBS (200 µl/well), and completely resuspended in ~130 µl of substrate solution (0.125% NP40 and 0.15 mM CPRG in PBS). This mixture was transferred to 96-well flat bottom plates and allowed to incubate overnight in a humidified chamber at 37°C. Absorbance at 570 nm was measured using a Benchmark microplate reader (Bio-Rad Laboratories).

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