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Optimal conditions required for influenza A infection enhanced cross-priming of CD8+ T cells specific to cell-associated antigens

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Abbreviations: T_{CD8+}, CD8+ T cells; TLR, toll-like receptors; OVA, chicken ovalbumin; IAV, influenza A virus; HA, IAV haemagglutinin; NA, IAV neuraminidase; NP, IAV nucleoprotein; PA, IAV polymerase 2 protein; SV40, Simian Virus 40; T-Ag, SV40 large T antigen; ICS, intracellular cytokine staining.

Abstract

Dendritic cells can take up exogenous tumor antigens and present their antigenic epitopes to CD8⁺ T cells, a process called cross-presentation. Cross-presentation is especially important in anti-tumor immunity because tumor cells, although carrying tumor antigens, do not activate naïve T cells efficiently due to a lack of co-stimulatory molecules. Our group has recently shown that influenza A virus infection of allogeneic cells lead to enhanced cross-priming of CD8⁺ T cells specific to cellular antigens. To develop this into a potential vaccine strategy, in this study, we have systematically investigated the numbers of allogeneic cells infected by IAV, IAV doses and their infectious activity, the length of *in vitro* infection, and other associated factors. We have defined the optimal immune enhancing conditions and we have also shown *in vivo* that such enhanced cross-priming did lead to enhanced tumor protection. The knowledge should be useful for developing more robust cancer vaccine.

INTRODUCTION

Dendritic cells (DCs) provide constant surveillance of their microenvironment by expressing various surface and intracellular toll-like receptors (TLRs) that specifically sense invading pathogens and diseased self components¹⁻³. The sensing of such molecules leads to the uptake of the exogenous materials and subsequent DC maturation marked by the up-regulation of surface costimulatory molecules required for priming cellular immune response. DCs not only have the capacity to uptake exogenous antigens from dying cells and their surrounding area but also further process these antigens and present the derived antigenic epitopes onto their own MHC class I molecules to antigen-specific CD8⁺ T cells (T_{CD8+}), a process termed cross-presentation. The initial activation of naïve antigen-specific T_{CD8+} in this manner is referred to as cross-priming⁴. Cross-priming is especially important in anti-tumor immunity because tumor cells, although carrying and presenting tumor antigens, do not express costimulatory molecules and therefore are not able to prime T cells⁵.

Many tumor antigens are also self antigens. The T cell repertoire specific to such antigens are often shaped by the negative selection in the thymus and other tolerizing mechanisms in the periphery^{6, 7}. In an effort to achieve greater cross-priming of anti-tumor immunity, various TLR ligands specifically recognized by TLR expressed on DCs are opted to enhance cross-priming⁸. In fact, synthetic TLR ligands have been used in cancer clinical trials⁹. On the other hand, natural TLR ligands have been shown to activate TLR and enhance cross-priming in murine models. For example, Schulz *et al* have demonstrated that Semiliki Forest Virus could enhance the cross-priming of chicken ovalbumin (OVA)-specific T_{CD8+} in a TLR3 dependant manner¹⁰. Our group also discovered that IAV infection of allogeneic cells carrying either Simian virus 40 (SV40) large T antigen or membrane-bound OVA significantly enhanced cross-priming of T_{CD8+} response to T-Ag₄₀₄ (large T antigen) and OVA₂₅₇ (OVA antigen) through TLR7-mediated sensing which involved the MyD88 and IFN α R signaling pathways, as well as possible interplay between

different DC subsets¹¹.

These findings lead us to believe that IAV could potentially serve as an excellent adjuvant in cancer immunotherapy. To test this hypothesis, we first wanted to explore the optimal conditions required to achieve maximum T_{CD8+} activation with this method. Studies have shown that the amount of antigen correlates with the extent of activation of antigen-specific T_{CD8+} both *in vitro*¹² and *in vivo*¹³. However, the amount of cell-associated antigens required for cross-presentation has not been well investigated. Shen *et al* demonstrated that OVA₂₅₇-specific T_{CD8+} could be cross-primed by injecting OVA-containing cellular lysates¹⁴. Diebold *et al* showed on the other hand that the quantity of IAV viral RNA can be important^{15 16}. However, it is not known whether IAV only augments T cell priming to cell bound antigen or it also enhances priming to antigens contained in cell lysates. With these and potentially other considerations in mind, we systematically investigated the number of IAV infected cells, IAV doses and their infectious activity, the length of *in vitro* infection time, and other important factors associated with IAV-enhanced cross-priming. Finally, we incorporated these findings into the design of a basic vaccination protocol targeted against melanoma and interrogated its anti-tumor efficacy.

RESULTS:

The cross-priming of T-Ag-specific T_{CD8+} was enhanced by IAV in intact cells, but not cell lysates, in a dose-dependent manner

We showed previously that IAV enhanced the cross-presentation of cell associated antigens¹¹. To show whether the immunizing antigen dose could affect the response significantly, four D2.SV cell numbers were chosen (10^6 , 3×10^6 , 5×10^6 and 10^7) and their ability to elicit T_{CD8+} responses was investigated. The T-Ag₄₀₄-specific T_{CD8+} responses did not differ significantly in mice immunized with increasing uninfected D2.SV cells. The percentage of responding T-Ag₄₀₄-specific T_{CD8+} remained around 1% in the spleen (Figure 1A) and 10% in the peritoneal cavity (Figure 1B). In contrast, several differences were observed when mice were immunized with increasing D2.SV/PR8 cells. While the percentage of NP₃₆₆-specific

T_{CD8+} was constant at around 2% in the spleen and 5% in the peritoneal cavity (Figure 1A, B), the percentage of PA₂₂₄-specific T_{CD8+} increased gradually (Figure 1A, B). However, the most dramatic increase was seen for the T-Ag₄₀₄-specific T_{CD8+} (Figure 1C, D). Cross-priming reached statistical significance when mice were immunized with $\geq 3 \times 10^6$ D2.SV/PR8 cells and the enhancement was the highest when mice were immunized with $> 5 \times 10^6$ D2.SV/PR8 cells, which supported the previously reported results^{11, 17}. The response reached a plateau when 10^7 cells were injected. 5×10^6 cells was therefore selected for subsequent experiments.

Several studies showed that cross-priming of soluble OVA antigen was enhanced by TLR ligands^{8, 18}. Moreover, cellular lysates¹⁴, including tumor lysates have been used in human cancer clinical trials¹⁹⁻²¹. We therefore investigated whether IAV could enhance cross-presentation of antigen derived from cellular lysates. We homogenized D2.SV or D2.SV/PR8 cells to generate cellular lysates and injected them into B6 mice and compared their responses using whole cells as controls. We found that cellular lysates barely induced detectable T-Ag₄₀₄-specific T_{CD8+} in the absence of IAV in the peritoneal cavity or spleen (Figure 1C, D). T_{CD8+} response primed by lysates from D2.SV/PR8 was only at a similar level to that induced by D2.SV whole cell immunization (Figure 1C, D).

The cross-priming of T-Ag-specific T_{CD8+} was enhanced by IAV in a viral dose- and infection time-dependant manner

The MOI for *in vitro* IAV infection of cell lines used in the above experiments was 10. We investigated whether different doses of PR8 affected the enhancement of cross-priming. Four different PR8 doses were chosen for infecting D2.SV cells (5, 10, 50 and 100 MOI). Mice immunized with D2.SV cells infected with the lowest dose of PR8 (5 MOI) did not significantly enhance cross-priming of T-Ag₄₀₄-specific T_{CD8+} in the spleen, although a significantly increased response was observed within the peritoneal cavity (Figure 2A, B). At 10 MOI, PR8 significantly enhanced cross-priming in both the spleen and the peritoneal cavity. The enhancement reached a plateau when mice were immunized with D2.SV infected with either 50 or 100 MOI

of PR8 (Figure 2A, B).

Interestingly, as the dose of PR8 increased, the percentage of PA₂₂₄-specific T_{CD8+} increased and it did not seem to have reached a plateau at 100 MOI. In contrast, the percentage of NP₃₆₆-specific T_{CD8+} reached its plateau at even the lowest MOI (Figure 2A, B). These results are similar to those acquired after increased IAV injection intraperitoneally²². From these data, 50 MOI was deemed a most suitable dose and was used for subsequent experiments.

We next investigated whether 5 hrs is the optimum *in vitro* infection time for enhancing cross-priming. D2.SV cells were therefore infected with PR8 *in vitro* for a period of 5 min, 1 hr, 3 hrs, 5 hrs or 7 hrs before intraperitoneal injection. D2.SV cells infected for a mere 5 min prior to immunization did not enhance the cross-priming of T-Ag₄₀₄-specific T_{CD8+} (Figure 2C, D). However, D2.SV cells infected with PR8 for longer periods clearly led to enhanced cross-priming, in fact the longer the infection time (up to 7 hrs) the bigger the enhancement of T-Ag₄₀₄-specific T_{CD8+} response (Figure 2C, D). Like the previous experiments, the PA₂₂₄-specific T_{CD8+} increased when mice were immunized with D2.SV cells infected with PR8 for prolonged infection while the NP₃₆₆-specific T_{CD8+} remained constant (Figure 2C, D). Thus, to achieve the optimum enhancement, D2.SV cells need to be infected with PR8 for 5 or more hours.

Inactivated, but not killed IAV could effectively enhance the cross-priming of T-Ag-specific T_{CD8+}

If IAV were to be used as an adjuvant in anti-tumor immunity, patient safety would be a priority concern. We thus assess whether inactivated virus retained the capacity to enhance cross-priming. IAV can readily be inactivated by heat or UV irradiation²³. Indeed, D2.SV cells incubated with PR8 virus inactivated by 56°C heat treatment for 30 min (D2.SV/PR8-56°C) or UV irradiation for 2 hr (D2.SV/PR8-UV) had significantly less infectious virus in them (6×10^7 PFU per 10^6 cells in D2.SV/PR8 to 4×10^3 and $<2 \times 10^3$ PFU per 10^6 cells in D2.SV/PR8-56°C and D2.SV/PR8-UV, respectively) (Figure 3A). When D2.SV cells were incubated with PR8 killed at 65°C

(D2.SV/PR8-65°C), there were no detectable residual infectious viral particles within cell lysates (Figure 3A).

The expression of HA molecules on infected cell surface is a good indication of IAV attachment, entry into the infected cells and subsequent de novo IAV replication. Therefore, HA expression on the surface of infected D2.SV was monitored by specific antibody at 5 min, 10 min, 30 min, 1 hr and 3 hrs after incubation and three washes. Within 5 to 10 min, viral HA molecules could be easily detected on the surface of D2.SV cells (Figure 3B, broken line) indicating immediate viral attachment. Between 30 min and 1 hr, the detectable surface HA molecules on infected D2.SV cells decreased (Figure 3B, thin and dotted line), indicating viral entry into the cells. By 3 hrs post viral infection, a population of D2.SV cells expressed a much higher level of HA, indicating de novo IAV replication (Figure 3B, thick line).

As expected, D2.SV cells incubated with inactivated PR8-56°C showed highest HA level at 5 min and the level remained relatively high during the 3hrs (Figure 3C). On the other hand, the HA level on the D2.SV cells incubated with UV-inactivated PR8 was significantly lower at all time points (Figure 3D) indicating that heat- and UV-inactivation affected IAV cell attachment and entry differently.

We next examined the ability of inactivated IAV to induce enhanced cross-priming. Interestingly, heat inactivated PR8-56°C was not only able to enhance cross-priming of T-Ag₄₀₄-specific T_{CD8+}, the enhancement was even larger reaching approximately 6 fold in both the spleen and the peritoneal cavity (Figure 4A, B, dotted bars). In contrast, heat killed PR8-65°C was unable to enhance cross-priming (Figure 4A, B, stripped bars). Additionally, UV-inactivated PR8 could enhance cross-priming of T-Ag₄₀₄-specific T_{CD8+} to a magnitude similar to that induced by live PR8 (Figure 4C, D, gray bars).

IAV-specific T_{CD8+} responses correlated with the residual viral titers obtained by the plaque assays (Figure 3A). Although D2.SV/PR8-56°C was able to prime NP₃₆₆- and PA₂₂₄-specific T_{CD8+}, the responses were significantly lower. D2.SV/PR8-65°C (Figure 4A, B, stripped bars) and D2.SV/PR8-UV immunization (Figure 4C, D, gray bars) did not induce detectable NP₃₆₆- and PA₂₂₄-specific T_{CD8+}.

The lack of priming of IAV-specific T_{CD8+} when mice were immunized with D2.SV/PR8-UV in the face of enhanced cross-priming of T-Ag₄₀₄-specific T_{CD8+} was perplexing. We expected the injected D2.SV/PR8-UV cells to carry abundant IAV-derived NP even in the absence of viral replication as NP is part of the viral particle²⁴. To confirm that D2.SV/PR8-UV cells did contain NP, a Western blot was performed to reveal both SV40 large T-Ag and NP in these cells after D2.SV cells were either infected with live PR8 or incubated with UV-irradiated PR8 for 5 hrs. Unsurprisingly, the T-Ag expression in infected D2.SV cells decreased as a result of reduced host mRNA levels after IAV infection²⁵. Interestingly, D2.SV/PR8-UV and D2.SV/PR8 cells contained abundant NP at similar amount (Supplementary Figure 1). Taken together, inactivated IAV are as effective as live IAV in inducing enhanced T_{CD8+} priming to cell-associated tumor antigen.

Excess IAV particles abolished enhanced cross-priming

Initially we were unable to achieve enhanced cross-priming in a reproducible fashion. We discovered that extensive washing of the infected cells was necessary. When D2.SV/PR8 cells were only washed once after infection, the enhancement effect was abolished (Figure 5-striped bars). This was possibly due to the presence of excess IAV particles. To test this hypothesis, we co-injected D2.SV/PR8 cells that had been washed thrice with 50 μ L (the calculated residual volume after each wash) of the supernatant from the first wash (D2.SV/PR8 + s/n). Indeed, the enhanced cross-priming of T-Ag₄₀₄-specific T_{CD8+} was abolished (Figure 5, dotted bars). This amount was calculated to contain approximately 10^5 PFU of PR8. We then co-injected extensively washed D2.SV/PR8 cells with 10^5 PFU of PR8 (D2.SV/PR8 + free PR8) to confirm that the enhanced cross-priming (Figure 5, grey bars) was again abolished. Therefore, free IAV could hinder the enhanced cross-priming of cell-associated antigen in our system.

IAV-infected cells could enhance cross-priming of T_{CD8+} specific to cell-associated antigens on bystanders

The above experiments show enhanced cross-priming of T_{CD8+} to cell-associated antigens derived from IAV-infected cells. However, whether IAV could achieve the same effect for uninfected cells in the vicinity was not clear. We addressed this by co-immunizing mice with D2.SV/PR8 and uninfected Dap.OVA or vice versa. Mice immunized with uninfected D2.SV + Dap.OVA induced unaltered T-Ag₄₀₄- and OVA₂₅₇-specific T_{CD8+} responses in both the spleen and peritoneal cavity (Figure 6, white bars). Mice immunized with either D2.SV/PR8 + Dap.OVA or D2.SV + Dap.OVA/PR8 induced enhanced cross-priming to T_{CD8+} specific for both antigens (Figure 6, black and dotted bars) although there was detectable bias towards the antigen carried by the infected cells.

Vaccination of mice with IAV-infected tumor cells impeded tumor progression

The overarching aim of this study was to optimize IAV-enhanced cross-priming and implement the procedure to assist in tumor control. A standard prime/boost regime was utilized to test the efficacy of this potential vaccination protocol in a well-established B16 melanoma model. Two different IAV strains, possessing different HA and neuraminidase (PR8: H1N1; X31: H3N2) were used to avoid potential neutralizing antibody. Mice were immunized 30 days apart with either two injections of B16.mOVA or B16.mOVA.PR8 followed by B16.mOVA.X31. Seven days later, vaccinated mice as well as control-unimmunized mice were inoculated subcutaneously with B16.mOVA tumor cells and tumor growth was assessed. Antitumor effects were clearly present in the cohort of mice receiving the B16.mOVA.IAV vaccine, whereas the cohort receiving B16.mOVA alone fared almost identical to control-unimmunized mice (Figure 7).

DISCUSSION

From the results shown in this study, it was apparent that the quantity of antigen carried by cells was less important than the quality and quantity of the adjuvant, in our case the TLR ligands derived from IAV. This was shown by the lack of dramatic increase in T-Ag₄₀₄-specific T_{CD8+} when we increased the number of

1 immunizing D2.SV cells in the absence of IAV (Figure 1) yet the quantity and quality
2 of IAV had greater effect on the magnitude of the response (Figure 1, 2).

3 Furthermore, IAV was shown to enhance cross-priming of cell-associated
4 antigen in a dose dependent manner, and the cells required incubation with IAV for a
5 sufficient amount of time *in vitro* (Figure 2). The requirement for sufficient incubation
6 time could potentially mean two things: 1) acquiring the maximum number of viral
7 particles by the co-incubated cells; and/or 2) requirement of viral replication to
8 enhance cross-priming. However, the latter explanation was unlikely as cross-priming
9 was also enhanced by heat- and UV-inactivated IAV (Figure 4).

10 Heat inactivation at 56°C damages the NA molecules of IAV, but not HA
11 molecules; and UV inactivation damages the viral genome but not viral proteins. In
12 both cases, HA molecules should remain functional allowing the viral particles to
13 attach to cells. In contrast, heat treatment at 65°C denatures both HA and NA
14 molecules²⁶ which should prevent viral attachment and subsequent cell entry. This
15 may explain why T-Ag₄₀₄-specific T_{CD8+} response was not enhanced by heat-killed
16 IAV. These observations indicate that viral attachment and cell entry¹¹, but not viral
17 replication is required for IAV to enhance cross-priming.

18 Viral NA protein cleaves cellular sialic acids to release virus from infected
19 cells²⁷. Rendering viral NA protein dysfunctional by heat inactivation further
20 enhanced cross-priming (Figure 4A, B), which was unexpected and not easily
21 explained. However, it has been shown that heat-inactivated (56°C) IAV was able to
22 stimulate larger amount of IFN α production by DCs¹⁵ than live IAV. We have
23 previously shown enhanced cross-priming in our system was dependent on type I IFN
24¹¹; therefore, it is likely that increased production of IFN α may further enhance
25 cross-priming.

26 Unlike the enhanced cross-priming to cell-associated antigen, PR8-specific
27 responses were reduced in mice immunized with D2.SV/PR8-56°C or drastically
28 reduced in mice immunized with D2.SV/PR8-UV. Lysates of D2.SV/PR8-UV cells
29 did contain residual infectious PR8 (4000 pfu per 10⁶ incubated cells), which could
30 explain the detected NP₃₆₆- and PA₂₂₄-specific T_{CD8+} (Figure 4A). However,

D2.SV/PR8-UV showed a further >50% PFU reduction in our plaque assays (Figure 3A) and failed to prime T_{CD8+} specific to either NP₃₆₆ or PA₂₂₄ despite carrying large amount of viral NP (Supplementary Figure 1). This finding prompted us to postulate that the NP antigen from D2.SV/PR8-UV was likely taken up into a subcellular location where the antigen could not efficiently access the cross-presentation pathways. Taken together, the capacity of inactivated IAV to enhance cross-priming of T_{CD8+} makes our approach potentially more useful when it is applied as an adjuvant for cancer vaccine due to minimizing any potential risks associated with live IAV infection.

Although it has been well demonstrated in the literature that TLR stimulation could lead to enhanced cross-priming of soluble OVA⁸, tumor antigen contained in tumor cell lysates in our system were poorly cross-presented even in the presence of IAV (Figure 1). This result is in agreement with the data published by Shen *et al*, demonstrating particulate antigens were better cross-presented than soluble antigens²⁸. It is envisaged, in our system, that homogenized cell lysates may be taken up by macropinocytosis (for particles <1 μ M)²⁹, instead by phagocytosis (for particles >1 μ M)²⁸. Such different uptaking mechanisms are expected to affect the efficiency of cross-presentation and cross-priming. It seems the case that the IAV sensing mechanisms (TLR7 at least in our system¹¹) were not drastically affected as shown by the proportionally enhanced cross-priming for both whole cells and cell lysates (Figure 1C, 1D). However, more experiments are warranted to confirm these hypotheses.

The removal of excess infectious IAV particles prior to immunizations was an important observation for the IAV-assisted enhancement of cross-priming. We had previously shown that DC cross-presentation was drastically inhibited *in vivo* when mice were pre-injected with CpG prior to antigen exposure³⁰. Similar to CpG, which stimulates DCs through TLR9, IAV infection activates DCs through TLR3, TLR7, inflammasome and other innate molecules^{15, 31-33}. It is therefore very likely that the free IAV particles induced DC maturation prior to optimal phagocytosis of the injected tumor cells, which might have limited antigen uptake by DCs as we have

1 shown recently that the enhanced cross-priming was DC-dependent ¹¹.

2 We further showed that the adjuvant potential of IAV went beyond the infected
3 cells as IAV-infection enhanced cross-priming to antigen carried by bystander cells
4 although the enhanced T_{CD8+} response directed against the antigen carried by
5 bystander cells was not as high as that against antigen carried by the directly infected
6 cells. This finding suggests that either a soluble factor was involved in the
7 enhancement process or DCs were encouraged to uptake antigen and viral RNA from
8 infected cells more so than the bystander cells.

9 The overarching goal of this study was to provide the first steps in
10 implementing this approach in a clinical setting to treat people burdened with cancer.
11 We applied this assay as a basic vaccination regime in mice, involving a prime boost
12 scenario, in order to test its efficacy against a tumor challenge. Our results
13 demonstrated that this approach was successful in delaying an aggressive tumor.
14 Combined with recent promising adjuvant therapies promoting prolonged anti-tumor
15 T cell immunity, such as anti-CTLA-4 ³⁴ and -PD-1 ³⁵, such a setting could
16 significantly hamper tumor survival and persistence.

17 Taken together, understanding the fine details relating to how to enhance
18 cross-priming of T_{CD8+} specific to cellular antigens using IAV as a potential adjuvant
19 may allow us to fine tune a potential future vaccine approach. For example, for
20 vaccines that utilize autologous tumor cells to provide antigen source, it might be best
21 to use UV-inactivated IAV to treat these tumor cells, not tumor cell lysates, *in vitro* for
22 a few hours before they are reintroduced back into the same patient. The
23 UV-treatment of IAV would eliminate any risk related to viral infection. Our approach
24 might be much simpler and less traumatic to the antigen carrying cells compared to
25 introducing other innate ligands, such as CpG and PolyI:C. Furthermore, our approach
26 may also provide a novel system for studying the involvement of various DC subsets,
27 CD4+ T helper and maybe even other innate cells in cross-priming of T_{CD8+} response
28 specific to various tumor or viral antigens.

29
30 **METHODS**

Mice

C57Bl/6 mice were purchased from Walter Eliza Hall Institute of Medical Research (Kew, Melbourne, Australia). The mice were housed under SPF condition. Mice were used at 6-12 weeks of age.

Peptide and Antibodies

IAV NP₃₆₆₋₃₇₄ (ASNENMETM, H-2D^b), PA₂₂₄₋₂₃₃ (SSLENFRAYV, H-2D^b), SV-40 T-Ag₂₀₆₋₂₁₅ (SAINNYAQKL, H-2D^b), and T-Ag₄₀₄₋₄₁₁ (VVYDFLKC, H-2K^b) were gifts from Drs. Yewdell and Bennink (NIAID, NIH, Bethesda, MD). FITC-labeled anti-interferon- γ (IFN- γ) and Cy-chrome labeled anti-CD8 α were purchased from Becton Dickinson (North Ryde, Australia).

Viruses and virus inactivation

Influenza A virus PR8 (Puerto Rico/8/34, H1N1) and X31 (HKx31, H3N2) were grown in 10-day embryonic chicken eggs and used as infectious allantoic fluid. For heat inactivation, the water bath was pre-heated to 56°C or 65°C. PR8 was then incubated for 30 min. For ultra-violet (UV) inactivation, PR8 stock was aliquoted into 6-well Falcon tissue culture plates and exposed to UVC for 2 hrs at close proximity (2-3 cm from the UVC source).

Cell culture

D2.SV¹⁷ cells were cultured in RPMI-1640 containing 10% FCS, 50 μ M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (RF-10). Dap.OVA cell line was a kind gift from Prof. Kenneth Rock (University of Massachusetts Medical School, Worcester, MA)¹⁴ and cultured in RF-10 supplemented with 1 mg/mL of zeocin (sigma). B16.mOVA (H-2^b) cells were generated from a B16-F1 clone transduced with a construct containing a membrane-bound form of ovalbumin and eGFP separated by an internal ribosome entry site. B16.mOVA cells were maintained in RF-10.

Generation of D2.SV/PR8, Dap.OVA/PR8, or B16.mOVA/PR8 cells

D2.SV, Dap.OVA or B16.mOVA cells were incubated with 50 MOI of PR8 (unless otherwise stated in the text) in FCS-free, acidified RPMI-1640 media for 1 hour (unless otherwise stated in the text) at 37°C. 10 mL of RF-10 was added and the

mixture was incubated for a further 4 hours (unless otherwise stated in the text) at 37°C. The infected cells were then harvested and washed thrice with PBS and irradiated at 10,000 Rads before use.

Generation of cell lysates

D2.SV or D2.SV/PR8 cells were suspended in PBS (10^7 cells/mL) and homogenized with a tissue grinder (Corning, Gaithersburg, MD, USA).

Immunization

Mice were immunized with irradiated D2.SV, Dap.OVA, B16.mOVA or D2.SV/PR8, Dap.OVA/PR8 or B16.mOVA/PR8 at 5×10^6 cells per mouse intraperitoneally (unless otherwise stated in the text).

***Ex vivo* enumeration of antigen-specific T_{CD8+}**

Peritoneal exudates and spleen cells were collected in RF-10 7 days post immunization. 1×10^6 cells were plated into a 96 well (U-bottom) and 1 μ M of antigenic peptide was added. The cells were incubated for 2 hours before Brefeldin A (BFA) addition (5 μ g/mL) and further 4 hours before ICS¹⁷.

Intracellular and surface staining

Cells were stained with Cy-Chrome anti-CD8 α mAb (Pharmingen, USA) or mouse anti-HA supernatant (clone H17-L2, gift from Drs Yewdell and Bennink, NIH, Bethesda, MD) on ice for 30 min, washed and fixed with 1% paraformaldehyde in PBS at RT for 20 min. Anti-HA stained cells were further stained with FITC labeled anti-mouse F'ab (Pharmingen, USA). For ICS, the cells were then further stained with fluorescein-anti-IFN- γ in PBS containing 0.2% saponin (Sigma). Stained cells were acquired on a FACSCanto II (BD) and analyzed using Flowjo software (TreeStar, Ashland, OR)

***In vivo* vaccination experiments**

Mice were untreated (Group 1), immunized twice with B16.mOVA cells (Group 2) or immunized with B16.mOVA/PR8 before re-challenge with B16.mOVA/X31 30 days later (Group 3). 7 days post the second immunization, mice were challenged subcutaneously with 5×10^4 B16.mOVA cells. Tumors were measured three times per week, and mice were euthanized when tumor exceeded 1000 mm³ (tumor volume

1 calculated as $([\text{larger diameter} \times (\text{short diameter})^2]/2)$.

2

3

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FIGURE LEGENDS

FIGURE 1: *The cross-priming of T-Ag-specific T_{CD8+} is enhanced by IAV in intact cells, but not cell lysates, in a dose-dependent manner.* In A and B, B6 mice were immunized i.p. with various numbers of either irradiated D2.SV or D2.SV/PR8 cells. In C and D, B6 mice were immunized i.p. with irradiated 5×10^6 intact D2.SV (open bars) or 5×10^6 intact, D2.SV cells being PR8-infected for 5 hrs (D2.SV/PR8, filled bars); or lysates equivalent to 5×10^6 D2.SV (dotted bars) or lysates equivalent to 5×10^6 D2.SV/PR8 cells (hatched bars). Seven day later, splenic (A, C) and peritoneal (B, D) Ag-specific T_{CD8+} were enumerated using synthetic peptides (NP₃₆₆₋₃₇₄, PA₂₂₄₋₂₃₃, T-Ag₂₀₆₋₂₁₅, and T-Ag₄₀₄₋₄₁₁) in an ICS for IFN- γ production. These graphs are plotted as mean $\pm$ SD of 3 mice. Data are a representative of three separate experiments.

FIGURE 2: *The cross-priming of T-Ag-specific T_{CD8+} is enhanced by IAV in a viral dose- and infection time-dependant manner.* In A and B, mice were immunized with 5×10^6 D2.SV infected with various MOI of PR8. In C and D, mice were immunized with 5×10^6 D2.SV cells infected with PR8 for various times. Seven day later, splenic (A, C) and peritoneal (B, D) Ag-specific T_{CD8+} were enumerated as stated in legend to Figure 1. These graphs are plotted as mean $\pm$ SD of 3 mice. This Figure is a representative of three separate experiments.

FIGURE 3: *Heat and UV inactivation of IAV.* PR8 was treated with heat or UV as stated in the Material and Method. The treated and untreated viruses of equivalent 50 MOI PR8 were co-incubated with 5×10^6 D2.SV cells for 5 hrs, lysed by freezing-thawing cycles and the residual infectious viruses were assessed using an MDCK plaque assay (A). 10^6 D2.SV cells were infected with live PR8 at 50 MOI (B), or similar amount of heat-treated (C) or UV-inactivated (D) PR8 for various times. The infected cells were then stained for their cell surface HA expression. The results are a representative of three separate experiments.

FIGURE 4: *Inactivated, but not killed PR8 effectively enhance the cross-priming of T-Ag-specific T_{CD8+} .* B6 mice were immunized with 5×10^6 D2.SV cells infected with live PR8 at 50 MOI or inactivated PR8 at equivalent dose for 5 hrs before being irradiated. Seven days later, Ag-specific T_{CD8+} were enumerated by ICS for the splenic (A) and peritoneal (B) response to heat-treated PR8; and splenic (C) and peritoneal (D) response to UV-treated PR8, respectively. These graphs are plotted as mean $\pm$ SD of three mice for each group. The data are a representative of two separate experiments.

FIGURE 5: *Excess PR8 viral particles abolished enhanced cross-priming*

B6 mice were immunized i.p. with 5×10^6 of either D2.SV (open bars) or D2.SV/PR8 cells that were washed 3 times (filled bars), washed only once (hatched bars); or washed 3 times then with 50 μ l of supernatant from the first wash was added back to the cells (dotted bars); or washed 3 times then with 10^5 PFU of PR8 added back to the cells. Two separate experiments were conducted with similar results.

FIGURE 6: *PR8-infected cells, as bystanders, can enhance cross-priming of T_{CD8+} specific to cell-associated antigen.*

B6 mice were immunized i.p. with irradiated 2.5×10^6 D2.SV plus 2.5×10^6 Dap.OVA (open bars); or 2.5×10^6 D2.SV/PR8 plus 2.5×10^6 Dap.OVA (filled bars); or 2.5×10^6 D2.SV/PR8 plus 2.5×10^6 Dap.OVA/PR8 (hatched bars); or 2.5×10^6 D2.SV plus 2.5×10^6 Dap.OVA/PR8 (dotted bars). The PR8 infection *in vitro* was 5hrs. 7 days later, Ag-specific T cells in the spleen (A) and peritoneum (B) were enumerated by ICS. These graphs were plotted as mean $\pm$ SD of three mice per group. Data are a representative of two separate experiments.

Figure 7: *Vaccination with IAV-infected tumor cells enhances the survival of mice inoculated with subcutaneous melanoma.*

B6 mice were untreated (circles), immunized twice with B16.mOVA cells (squares) or immunized with B16.mOVA/PR8 + B16.mOVA/X31 (triangles) as described in the Materials and Methods. All mice from each group received 5×10^4 B16.mOVA cells

- 1 seven days post the second immunization.

Figure 1

Figure 2

Figure 3

A

Surface HA expression

B

C

D

Figure 4

Figure 5

Figure 6

Figure 7