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Influenza virus and poly I:C inhibit MHC class I-restricted presentation of cell-associated antigens derived from infected dead cells captured by human dendritic cells

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Abstract

During viral infection dendritic cells (DCs) capture infected cells and present viral antigens to $CD8^+$ T cells. However, activated DCs might potentially present cell-associated antigens derived from captured dead cells. Here we find that human DCs that captured dead cells containing the Toll-like receptor 3 (TLR3) agonist poly I:C produced cytokines and underwent maturation, but failed to elicit autologous $CD8^+$ T cell responses against antigens of dead cells. Accordingly, DCs that captured dead cells containing poly I:C, or influenza virus, are unable to activate $CD8^+$ T cell clones specific to cell-associated antigens of captured dead cells. $CD4^+$ T cells are expanded with DCs that have captured poly I:C-containing dead cells, indicating the inhibition is specific for MHC class I-restricted cross-presentation. Furthermore, these DCs can expand naive allogeneic $CD8^+$ T cells. Finally, soluble or targeted antigen is presented when co-loaded onto DCs that have captured poly I:C-containing dead cells, indicating the inhibition is specific for dead cell cargo that is accompanied by viral or poly I:C stimulus. Thus, DCs have a mechanism that prevents MHC class I-restricted cross-presentation of cell-associated antigen when they have captured dead infected cells.

INTRODUCTION

Dendritic cells (DCs) have the ability to present exogenous antigens via MHC class I in a process called cross-presentation (1-3). This mechanism is thought to be particularly important for the generation of $CD8^+$ cytotoxic T lymphocyte (CTL) responses against certain viruses that do not infect DCs, or against tumors (4,5). In the context of viral infection, DCs can capture dead infected cells, cross-present viral antigen derived from infected dead cells and prime virus-specific CTLs. However, DCs that capture dying cells might also cross-present cell associated antigens of dead cells leading to generation of autoreactive T cells.

Conceivably, DCs regulate activation of cell associated antigen-specific T cells both through induction of T cell tolerance as well as regulating presentation of cell-associated antigenic peptides on MHC class I and II. The first concept stems from studies on the role of DCs in the maintenance of peripheral tolerance. There, it has been demonstrated that immature DCs can present antigen in a tolerogenic fashion (6-9). A hypothesis has been put forward that DCs induce peripheral tolerance to cellular antigens associated with dead non-infected cells

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generated during the normal tissue turnover (9-11). Consequently, the T cells have been tolerized in the steady-state, long before the DCs are called upon to initiate immunity to viral antigens. Such preemptive strategy would therefore lower the risk of activating autoreactive T cells. Inherent to this hypothesis is however an assumption that all and each of cell-associated antigens have been presented by DCs before the first viral infection of the host. Furthermore, new peptides might be generated from cell-associated antigens in the context of infection but not necessarily during the normal tissue turnover (12). Presentation of these peptides by DCs activated in the course of infection could lead to expansion of autoreactive T cells. Further studies demonstrated that activated DCs found a way to deal with the issue of antigens derived from captured dead cells by developing a capacity to activate and expand $CD4^+$ regulatory T cells (Tregs) (9-11,13). Thus, activated DCs could support Tregs that have been generated specifically to prevent autoreactive response (9-11,13). Nevertheless, this mechanism does not provide an explanation for how antigens presented by MHC class I are dealt with.

One such model for how DCs might regulate presentation of cell-associated antigen is based on the concept of phagosome-autonomy whereby antigen presentation to MHC class II-restricted $CD4^+$ T cells is enhanced when DCs captured dead cells which carry a TLR agonist, but not for concomitantly-captured dead cells without a TLR agonist (14). This model thus allows DCs to discriminate between cell-associated antigens and microbial antigens based on the TLR signals that accompany the specific cargo they engulf for presentation by MHC class II (14). However it remains to be determined how human DCs regulate presentation of captured antigens for MHC class I presentation. This is important because understanding of such mechanisms could offer therapeutic targets, for example a tool to enhance immunogenicity of DCs vaccines. Indeed, mouse studies showed enhanced cross-priming of transgenic T cells against OVA upon immunization with poly I:C- and OVA-expressing cells (15).

Here, we have taken advantage of our in vitro system in which loading human DCs with dead allogeneic melanoma cells permits generation of melanoma-specific CTLs (16-18). Using this strategy, we found that influenza virus or poly I:C within dead cells inhibits MHC class I-restricted presentation of cell-associated antigens by human DCs in vitro thereby not allowing generation of $CD8^+$ T cell immunity to these antigens.

MATERIALS AND METHODS

Cells

HLA-A*0201^+ Me290 melanoma cells were a gift from Drs. J.-C. Cerottini and D. Rimoldi (both from Ludwig Institute for Cancer Research, Lausanne, Switzerland). T2 cells were purchased from American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA), 1% L-glutamine, 1% penicillin/streptomycin, and 10% heat-inactivated FCS. HLA-A*0201-restricted $CD8^+$ CTL clones M26 (specific for MART-1 peptide 27-35; AAGIGILTV) and G154 clone (specific for gp100 peptide 154-162; KTWGQYWQV) were provided by Dr. Cassian Yee (Fred Hutchinson Cancer Center, Seattle, WA).

Reagents

GM-CSF was from Bayer HealthCare Pharmaceuticals (Seattle, WA) and IFN-α was from Schering Co. (Kenilworth, NJ). CD40L, IL-2, IL-7 and IL-4 were all purchased from R&D Systems (Minneapolis, MN). Betulinic acid (BA) was purchased from Sigma-Aldrich (St. Louis, MO). Poly I:C was purchased from InvivoGen (San Diego, CA). Antibodies used to assess DC maturation (anti-HLA-DR, HLA-ABC, CD40, CD80, and CD86) were purchased from BD Biosciences (Franklin Lakes, NJ). Peptides were synthesized by Biosynthesis Inc. (Lewisville, TX). Anti-type I IFN antibodies and influenza A virus M1 (FluM1) protein

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conjugated anti-DCIR (dendritic cell immunoreceptor) humanized IgG4 mAb were generated in house.

Preparation of dead cells

Poly I:C-bodies were prepared by electroporation of Me290 melanoma cells at 4×10^6 cells/mL with 100 μg/mL poly I:C in Opti-MEM media (Invitrogen) at 300V for 500 μs. Cells were then plated and after 24-h culture at 37°C, cultured at 42°C for 4 h. Cells were then treated with 10 μg/ml BA for an additional 24 h at 37°C and non-adherent dead cells were harvested the next day and washed several times with complete RPMI media+ 10% FCS. Mock-bodies were prepared using Me290 cells that were mock electroporated (no poly I:C).

Lipofected bodies were prepared by plating Me290 melanoma cells at $3\text{-}4\times10^5$ cells/well in a 24-well plate with 0.5mL Opti-MEM media. After 24 h at 37°C, cells were treated with a 100 μL mixture of 10 μg poly I:C in Lipofectamine 2000 reagent (Invitrogen) prepared according to manufacturer's instructions. After 24 h lipofection at 37°C, cells were heat-shocked and treated with BA as described above. Dead cells were then harvested and washed with complete RPMI media + 10% FCS. Mock-bodies were prepared from mock lipofected cells.

Bodies were analyzed for poly I:C content after preparation by immuno-fluorescent microscopy using K1 monoclonal antibody (English & Scientific Consulting Bt., Hungary) that recognizes large (over 40 bp) dsRNA.

Flu-infected bodies were prepared by infecting Me290 melanoma cells with influenza virus A/PR8/34 (Charles River Laboratories, Wilmingtaon, MA) at 10^4 hemagglutinin units/1 million cells. Control cells were left uninfected. Cells were then heat shocked and treated with BA as described above and then dead cells harvested. Both Flu-infected and control bodies were further irradiated for 20 min (6600 cGy) to prevent virus replication in the DCs.

Monocyte-derived DCs generation and loading

Monocytes were enriched by apheresis from HLA-A*0201$^+$ healthy volunteers and cultured in Cellgenix media (CellGenix, Germany) with GM-CSF (100 ng/ml), and IL-4 (10 ng/ml). Cells were fed with fresh cytokines at day 2 and 4 post-culture. For some experiments (e.g. peptide priming), 3 day old DCs prepared with GM-CSF (100 ng/mL) and IFNα (500 U/mL) were used. DCs were harvested and loaded with killed melanoma cells at a 2:1 ratio for 24 h at 37°C. After 24 h, cells were washed, harvested and used to prime autologous naive CD8$^+$ T cells. For some experiments, DCs were loaded with CFSE-labeled tumor bodies (1 μM CFSE for 10 min at RT; washed with complete RPMI + 10% FCS) followed by sorting of CD11c$^+$, CFSE$^+$ DCs. DCs were then pulsed with 10 μg/mL MART-1 peptide analog (ELAGIGILTV) and gp100 peptide (IMDQVPFSV), Influenza-M1 peptide (GILGFVFTL), or FluM1 protein conjugated to anti- DCIR IgG4 mAb, or soluble FluM1 protein.

Naive CD8$^+$ T cell purification and priming

Lymphocytes were enriched by apheresis from HLA-A*0201$^+$ healthy volunteers. Naive CD8$^+$ T cells were sorted as CD8$^+$, CD45RA$^+$, CCR7$^+$ or CD27$^+$, CD4/CD19/CD56$^-$ cells (>95% purity) and co-cultured with DCs at a 20:1 ratio in a 24-well plate with 2mL complete RPMI + 10% human AB serum + 200 ng/mL soluble CD40L and 5 ng/mL IL-7. At day 7 of co-culture, cells were split and fresh CD40L and IL-7 added along with 10 U/mL of IL-2. CD8$^+$ T cells were harvested for Cr^{51} release assay at day 10 of co-culture.

Cr^{51} release assay

Targets were labeled with $Na^{51}CrO_4$ for 1 h at 37°C. A 4-hr standard killing assay was performed as previously described. The mean of triplicate wells for each sample was calculated,

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and the percentage of specific ^{51}Cr release was determined according to the following formula: % specific ^{51}Cr release = 100 × (experimental ^{51}Cr release - spontaneous release)/ (maximum ^{51}Cr release - spontaneous release).

Tetramer binding

The iTAg MHC HLA-A*0201 tetramers, MART-1 (ELAGIGILTV), gp100 (IMDQVPFSV), and Influenza-M1 (GILGFVFTL), were purchased from Beckman Coulter (Fullerton, CA). Primed T cells were stained with PE-conjugated tetramer and with anti-CD8/anti-CD3 mAb for 45 min at room temperature. Tetramer binding to MHC HLA-A*0201 tetramer HIV gag (SLYNTVATL) was used as negative control.

$CD4^+$ and $CD8^+$ T cell proliferation

T cells were labeled with 1 μM CFSE for 10 min at room temperature and washed three times with complete RPMI + 10% human AB serum. For $CD4^+$ T cell proliferation, cell were co-cultured with tumor bodies-loaded DCs for 7 days at 37°C and CFSE dilution analyzed by FACS analysis. Naïve CFSE-labeled $CD8^+$ T cells were co-cultured with tumor bodies-loaded DCs in the presence of 5 ng/mL IL-7 and 200 ng/mL CD40L. $CD8^+$ T cell proliferation was read out at day 4, 5, and 7 of co-culture by FACS analysis.

Antigen presentation experiments with CTL clones

100,000 CTL clones were co-cultured with 5,000 tumor body-loaded DCs in a 96-well plate in 200 μL of complete RPMI media with 10% human AB serum and 200 ng/mL CD40L. After 8-16 h of co-culture, cells were centrifuged and supernatant harvested for assessment of IFNγ and IP-10 levels by Luminex assay.

Luminex analysis of cytokine

DC supernatant was collected 24 h after loading with tumor bodies. Supernatant from $CD4^+$ T cell-DC co-culture was collected at day 7. DC supernatant was analyzed for IL-1α, IL-1β, IL-3, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-15, TNFα, MCP-1, MIP-1α, IFNα, and IP-10 by Luminex. T cell co-culture supernatant was analyzed for IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, TNFα, IFNα, and IFNγ by Luminex.

Confocal microscopy assay

DCs were harvested, washed with PBS, and fixed overnight with 1% paraformaldehyde at 4° C. The next day, cells were permeabilized with 0.1% saponin/1% BSA/ PBS. Cells were incubated with mouse anti-human MART-1, gp100, tyrosinase mAbs for 30 min. After washing with 0.1% saponin/PBS, $Alexa_{568}$-conjugated goat anti-mouse IgG Ab was added for additional 30 min in 0.1% saponin/1% BSA/ PBS. After washing with 0.1% saponin/PBS, cells were stained with FITC-conjugated mouse anti-human HLA-DR for an additional 30 min in 0.1% saponin/1% BSA/ PBS. Cells were then washed with PBS and mounted onto Superfrost slides with DAPI/Vectashield. Leica TCS-NT SP confocal microscopy was applied with detection channels of FITC (510-550 nm) and $Alexa_{568}$ (580-660 nm). $HLA\text{-}DR^+$ DCs were then scored for intracellular melanoma antigen and data given as percentage of DCs that were positive for melanoma antigen.

RESULTS

DCs loaded with influenza virus-infected dead allogeneic tumor cells do not generate tumor-specific CTLs

Melanoma cells (Me290) were infected with influenza virus and then killed (Flubodies) by exposure to betulinic acid as described previously (18). Flu-bodies were further γ-irradiated

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for 20 minutes (corresponding to radiation dose of 6600 cGy) to prevent virus replication within DCs. The lack of viral replication was confirmed by the progressive decrease of Flu nuclear protein (NP) staining in Flu-bodies-loaded DCs over three days in culture (data not shown). Monocyte-derived DCs were loaded with either control non-infected bodies or with Flu-bodies and cultured at a 1:20 ratio with autologous purified naïve $CD8^+$ T cells. DC:T cell co-cultures were supplemented with CD40L, IL-7 and IL-2 and CTL generation was assessed at day 10 in a standard Cr^{51} release assay. As expected, control cultures generated CTLs able to kill Me290 melanoma cells (Fig. 1*A*). However, loading DCs with Flu-bodies resulted in the complete lack of Me290 cell killing (Fig. 1*A*). Importantly, the DCs were able to elicit FluM1 response as demonstrated by the expansion of FluM1-specific $CD8^+$ T cells (Fig. 1*B*). Thus, capture of influenza virus infected dead cells by DCs inhibits generation of $CD8^+$ T cells specific to cell associated-antigens derived from the dead cells. As we will demonstrate hereunder this striking observation is not an indicator of a complete blockade of DC capacity to generate T cell immunity.

DCs loaded with poly I:C-carrying dying allogeneic tumor cells do not generate tumor-specific CTLs

To determine whether dsRNA was involved, melanoma cells were transfected with synthetic dsRNA poly I:C (poly I:C-bodies) and loaded onto DCs which were then used in cultures with autologous naïve $CD8^+$ T cells. As expected, $CD8^+$ T cells from cultures with DCs loaded with mock transfected bodies (mock-bodies) efficiently killed Me290 cells (Fig. 1*C*). However, $CD8^+$ T cells recovered from cultures with poly I:C-bodies-loaded DCs were unable to kill Me290 cells (Fig. 1*C*). This result was highly reproducible and observed with at least 5 different donors (Fig. 1*D*). DCs loaded with 10-fold less tumor bodies (5×10^4 bodies per 1×10^6 DCs rather than 5×10^5 bodies per 1×10^6 DCs) produced the same results (Fig. 1*E*). There, even when DCs were loaded with poly I:C-bodies at levels comparable to mock-bodies (Fig. 1*F*), poly I:C-bodies-loaded DCs failed to prime CTLs that kill Me290 cells. This inhibition is not restricted to Me290 cells and can also be found when other melanoma cells are used (not shown).

In line with the absence of CTL function, autologous naive $CD8^+$ T cells exposed to poly I:C-bodies-loaded DCs did not dilute CFSE indicating the lack of $CD8^+$ T cell proliferation (Fig. 2). This was contrary to cultures with mock-bodies-loaded DCs, where CFSE dilution was detected already at day 4 and at day 7 of co-culture more than 50% of $CD8^+$ T cells diluted CFSE indicating their proliferation (Fig. 2). Thus, capture of poly I:C carrying-dead cells by DCs does not lead to induction of $CD8^+$ T cell responses against cell-associated antigens derived from the dead cells, including alloantigens.

This lack of CTL generation was not due to insufficient uptake of poly I:C-bodies (Fig. 3). In fact, DCs loaded with CFSE-labeled poly I:C-bodies showed significantly more CFSE staining by flow cytometry ($p<0.001$; Fig. 3, *A* and *B*). Fluorescence microscopy analysis confirmed the presence of CFSE-labeled bodies within the DCs (Fig. 3*C*). Therefore, reduced CTL generation is not a result of the lack of cell-associated antigens derived from dead cells captured by the DCs.

DCs loaded with poly I:C-carrying dying allogeneic tumor cells expand type 1 $CD4^+$ T cells

DCs loaded with control and poly I:C-bodies were then cultured with CFSE-labeled autologous $CD4^+$ T cells. At day 7, co-cultures of DCs loaded with poly I:C bodies displayed twice as many $CD4^+$ T cells that have diluted CFSE than co-cultures with DCs loaded with mock bodies (Fig. 4*A*). Enhanced autologous $CD4^+$ T cell proliferation was associated with the enhanced IFNγ secretion by $CD4^+$ T cells (Fig. 4*B*), consistent with $CD4^+$ T cell differentiation. Expansion of $CD4^+$ T cells was consistent with DC maturation (Supplementary Fig. 1, *A* and

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B) and their cytokine secretion pattern. Loading DCs with poly I:C-bodies preferentially induces IFNα secretion but no IL-12p70 secretion by DCs (Fig. 4*C*). This pattern was due to delivery of poly I:C within dead cells because soluble poly I:C treatment induces IL-12p70 secretion but very little IFNα secretion (Fig. 4*C*). Flu-bodies also fail to induce IL-12p70 secretion from DCs, but do induce IFNα production from DCs (Fig. 4*D*). Both cytokines have been implicated in Th1 skewing of helper $CD4^+$ T cells (19-22). Thus, DCs that captured poly I:C carrying-dead cells elicit proliferation and differentiation of $CD4^+$ T cells, indicating that activation of autologous $CD4^+$ T cells is not inhibited with poly I:C-bodies-loaded DCs as is activation of autologous $CD8^+$ T cells.

$CD4^+$ T cells and cytokines cannot rescue activation of $CD8^+$ T cells

To determine whether $CD4^+$ T cells could rescue $CD8^+$ responses against cell-associated antigens of captured dead cells (23,24), DCs loaded with control bodies, poly I:C- bodies or Flu-bodies were cultured with autologous T cells containing both $CD4^+$ and $CD8^+$ T cells. At day 10 of culture, $CD8^+$ T cells were purified and tested for their capacity to kill Me290 melanoma cells. Even in the presence of $CD4^+$ T cells, poly I:C-bodies-loaded DCs (Fig. 5*A*) or Flu-bodies- loaded DCs (Fig. 5*B*) did not activate $CD8^+$ T cells that kill Me290 cells.

Because autocrine IFNα has been implicated in inhibited DC-mediated cross-presentation of antigens from apoptotic bodies (25), we next set to determine its role by adding IFNα-neutralizing and IFN α/β receptor blocking antibodies at the time of DC loading and at the time of DC:T cell co-culture. $CD8^+$ T cells harvested from these cultures still failed to kill target Me290 cells (Fig. 5*C*). We confirmed that type I IFN activity was blocked by decreased IP-10 production which is produced in response to IFN stimulation (Supplementary Fig. 2). Furthermore, trans-well experiments showed that $CD8^+$ T cells expanded by mock-bodies-loaded DCs effectively kill Me290 target cells and this killing was somewhat enhanced when mock-bodies-loaded DCs were exposed to soluble factors produced by poly I:C-bodies-loaded DCs (Fig. 5*D*), possibly due to the soluble poly I:C that might have been released from poly I:C-bodies-loaded DCs and/or inflammatory cytokines produced by these DCs. These results indicated that inhibited responses against cell-associated antigens of captured dead cells by poly I:C-bodies-loaded DCs was not due to soluble factors acting on DCs in an autocrine manner.

Inhibition of $CD8^+$ T cell expansion is specific to antigens associated with captured dead cells

In order to determine the specificity of the observed inhibition we next analyzed whether DCs loaded with poly I:C-bodies could generate responses from naïve CD8+ T cells to antigens other then those associated with captured dying cells. First, loaded DCs were used to stimulate allogeneic naïve $CD8^+$ T cells in a mixed lymphocyte reaction. As shown in Fig. 6*A*, allogeneic $CD8^+$ T cells cultured with poly I:C bodies-loaded DCs showed CFSE dilution comparable to that of control cultures demonstrating that DCs can actually expand naïve $CD8^+$ T cells possibly due to pre-formed MHC class I/peptide complexes presented by the DCs. To further analyze the capacity of these DCs to present pre-processed antigens, in the second set of experiments the DCs loaded with the control or poly I:C-bodies were additionally pulsed with 9-10aa HLAA*0201-restricted peptides. To ensure that MHC class I-restricted presentation was being assessed with DCs that actually captured poly I:C-bodies, the DCs were loaded with CFSE-labeled tumor-bodies and then sorted as $CD11c^+CFSE^+$ cells. $CD11c^+CFSE^+$ DCs were then pulsed with MART-1 and gp100 peptides and used in cultures with autologous naïve $CD8^+$ T cells. As shown in Fig. 6*B* in a tetramer binding assay, poly I:C-bodies-loaded DCs that were co-loaded with MART-1 peptide, were able to expand MART-1 peptide-specific $CD8^+$ T cells. The frequency of MART-1 specific $CD8^+$ T cells in cultures with poly I:C- loaded DCs was comparable to that observed in cultures with control-bodies-loaded DCs (Fig. 6*B*).

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Furthermore, when the expansion of rare gp100-specific $CD8^+$ T cells was analyzed, the poly I:C-bodies-loaded DCs were far more efficient at expanding gp100-specific CTLs (Fig. 6*B*), thereby suggesting the adjuvant effect of poly I:C. Generated T cells were functional as demonstrated by their capacity to kill T2 cells pulsed with respective peptides (Supplementary Fig. 3). Similar results were obtained with poly I:C-bodies-loaded DCs pulsed with HIV gag151 peptide (data not shown).

Experiments with allogeneic $CD8^+$ T cells and with autologous $CD8^+$ T cells thus demonstrated that the overall capacity of DCs to generate $CD8^+$ T cell responses was not altered. However, since in both cases the peptides were pre-processed or MHC class I/peptide complexes pre-formed, these results did not exclude the possibility that cell-associated poly I:C actually inhibited antigen processing for or intracellular loading onto MHC class I. Generation of $CD4^+$ T cell responses shown above indicated that the global antigen processing capacity of DCs was not altered. Therefore, to determine whether poly I:C- bodies-loaded DCs were at all able to process antigens for MHC class I presentation we measured response to soluble proteins which require internalization and processing for presentation. To this end, sorted $CD11c^+$ $CFSE^+$ DCs were co-loaded with FluM1 protein targeted to DCIR on DCs by way of FluM1 protein conjugated to an anti-DCIR mAb (26,27) (Klechevsky et al. submitted). As shown in Fig. 6*C*, DCs co-loaded with poly I:C-bodies and targeted FluM1 protein expanded antigen-specific $CD8^+$ T cells comparable to DCs loaded with control bodies and targeted FluM1 protein. This suggested that the overall antigen processing and presentation capacity of poly I:C-bodies-loaded DCs is not inhibited. However, DCIR could target the antigen to a unique compartment, not affected by poly I:C. Therefore, we have further analyzed the presentation of soluble FluM1 protein delivered in the non-targeted fashion. To this end, DCs were loaded with CFSE-labeled mock-bodies or poly I:C-bodies along with soluble FluM1 protein. After 24 hr loading $CFSE^+$ DCs were sorted and used in co-cultures purified autologous $CD8^+$ T cells. No significant difference in the frequency of FluM1-tetramer binding $CD8^+$ T cells in cultures with DCs loaded with mock-bodies or with poly I:C-bodies could be observed (Fig. 6*D*). Importantly, adding soluble poly I:C to mock-bodies loaded-DCs significantly enhanced the expansion of FluM1-specifc $CD8^+$ T cells (Fig. 6*D*). Thus, the inhibition of $CD8^+$ T cell responses is specific for cell-associated antigens of captured dead cells that contain poly I:C.

DCs that captured poly I:C- or Flu-loaded dead tumor cells do not present cell-associated antigens of captured dead cells

The results thus far implied that the inhibited generation of $CD8^+$ T cell responses with poly I:C-bodies-loaded DCs may be due to a lack of presentation of cell-associated antigens of captured dead cells. In order to test this, we made use of a melanoma antigen-specific $CD8^+$ CTL clone. The HLA-A*0201-restricted G154 clone recognizes melanoma-associated gp100 peptide 154-162 (KTWGQYWQV)(28). IFNγ or IP-10 secretion by the G154 clone after 8-16 hr co-culture with DCs was used as a read-out of activation. Secretion of IFNγ by the gp100-specific CTL clone was observed when G154 cells were co-cultured with mock-bodies-loaded DCs (Fig. 7*A*). However, no IFNγ secretion was elicited with poly I:C-body-loaded DCs (Fig. 7*A*). Both poly I:C- and control-bodies-loaded DCs activated the gp100-specific CTL clone when the DCs were co-loaded with the respective gp100 peptide, suggesting that inhibition of cross-presentation is not due to DC dysfunction (Fig. 7*B*). The lack of melanoma-antigen presentation by poly I:C-bodies-loaded DCs did not appear to be due to altered kinetics of antigen digestion/processing in the DCs because when DCs were loaded with respective tumor bodies, and allowed an additional 48 hrs after loading to “digest” the tumor-body antigen, poly I:C-bodies-loaded DCs still failed to activate G154 cells (Fig. 7*C*). Similar results were obtained with the HLA-A*0201-restricted M26 $CD8^+$ CTL clone which recognizes MART-1 peptide (data not shown) (28). These results indicated the lack of presentation of cell-associated antigen when the dead cell is loaded with poly I:C. DCs from 3 different donors loaded with

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Flu-bodies also failed to present melanoma antigen to the G154 clone, whereas control bodies-loaded DCs presented endogenous cell-associated antigen to the T cell clone (Fig. 7*D*). Adding soluble poly I:C to DCs loaded with uninfected bodies did not inhibit antigen presentation (Fig. 7*E*) further attesting to a specific inhibition by cell bodies containing virus or poly I:C.

Finally, the lack of melanoma antigen presentation was not due to the lack of melanoma antigen. Indeed, immunofluorescence analysis demonstrated the presence of melanoma proteins in loaded DCs (Fig. 7*F*). As was the case with CFSE-labeled bodies (Fig. 3), a larger fraction of DCs showed staining demonstrating the capture of melanoma antigens upon loading with poly I:C bodies when compared to loading with mock-bodies (Fig. 7, *F* and *G*).

DISCUSSION

Here we found that DCs that captured dead cells infected with influenza virus, or carrying poly I:C, did not present cell-associated antigens derived from captured dead cells to autologous $CD8^+$ T cells. That resulted in the remarkable inhibition of generating $CD8^+$ T cell responses against these antigens. Interestingly, neither type 1 $CD4^+$ T cells nor CD40 signal could overcome the specific inhibition of $CD8^+$ T cell responses. Furthermore, it could not be rescued by cytokines or by innate effector cells such as NK cells (data not shown). Whereas the mechanism of this specific inhibition is yet to be determined, it is clear that the overall DC antigen-presenting capacity has not been altered. First, the capacity to expand $CD8^+$ T cells and generate specific responses is maintained in poly I:C- or Flu-body-loaded DCs when MHC class I/peptide complexes are pre-formed, i.e., DCs can expand naïve allogeneic $CD8^+$ T cells. DCs that captured poly I:C-bodies-loaded DCs can also expand autologous $CD8^+$ T cells if they are further pulsed with 9-10mer peptides that do not require processing. Finally, when poly I:C-bodies-loaded DCs are further given a secondary antigen in the form of soluble protein or receptor-targeting complex, $CD8^+$ T cells specific to secondary antigen are expanded thus suggesting that the secondary antigen is cross-presented. Furthermore, tumor-bodies-loaded DCs are capable of activating autologous $CD4^+$ T cells. Thus, DCs that have captured infected or poly I:C-containing dead cells are capable of processing antigen for cross-presentation, but there is a selective inhibition in the cross-presentation of antigens derived from dead cells to $CD8^+$ T cells.

Several observations suggest that this inhibition is DC-intrinsic First, intracellular analysis by confocal microscopy shows the presence of specific cell-associated tumor antigens within poly I:C-bodies-loaded DCs. Indeed, loading of DCs with poly I:C-bodies results in more total cellular antigen and specific tumor antigens than loading DCs with control bodies. Second, since activation of allogeneic $CD8^+$ T cells or autologous $CD8^+$ T cells specific for secondary antigens is maintained, the inhibition is not due to any direct effect on $CD8^+$ T cells, such as through inhibitory cytokines or negative costimulatory molecules.

A possible explanation is that dead cells containing viral stimulus are differentially compartmentalized within DCs. It is important to note that TLR3 which recognizes double-stranded RNA is localized within endocytic vesicles (29,30). In our system, TLR3 signaling within an endocytic compartment may target the accompanying antigen to a compartment that precludes cross-presentation. Similar to the work of Blander et al., we found that DCs loaded with dead cells containing a TLR stimulus display enhanced activation of $CD4^+$ T cells (14). Blander et al. attributed this enhanced MHC class II-restricted presentation to enhanced “phagosomal maturation” that occurs in a phagosome-autonomous manner, i.e. antigen accompanied by a TLR stimulus targets to $LAMP\text{-}1^+$ lysosomal compartments with greater efficiency than antigen not accompanied by a TLR agonist, as well as activation of MHC class II loading components (31,32). Recent work by Burgdorf et al. has indicated that loading of antigen for $CD4^+$ T cell activation and cross-presentation to $CD8^+$ T cells occur in distinct

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intracellular compartments (33,34). The former occurs in LAMP-1^+ lysosomal compartment, while the latter takes place in early endosomal compartments distinct from lysosomes (33, 34). Therefore, it is conceivable that in our studies the presence of a TLR stimulus within dead cells stimulates targeting of the dead cells antigenic material to LAMP-1^+ lysosomes for potentiated MHC class II-restricted presentation, but not to a compartment amenable for presentation via MHC class I to $CD8^+$ T cells. Many groups report that cross-presentation requires transport of antigen from endosomes to the cytosol, therefore the presence of poly I:C within dead cells may inhibit this transport (3). Further work is required to understand mechanistically how poly I:C or influenza virus within dead cells prevent cross-presentation of antigens associated to captured dead cells.

Our in vitro finding with poly I:C-bodies in the human system appears in contrast with the in vivo data in mice. There, Schulz et al analyzed responses against OVA that is over-expressed in cells used for immunization and found enhanced cross-priming of transgenic T cells against OVA upon immunization with poly I:C- and OVA-expressing cells (15). In our model the antigens naturally expressed by melanoma cells are analyzed. It is possible that in vivo there is leakage of antigen so that not all of it is cell-associated and accompanied by a TLR stimulus. It is worth noting in our system that concomitant loading of DCs with tumor bodies and treatment with soluble poly I:C does not inhibit cross-presentation, i.e. inhibition requires the poly I:C be associated with the dead cell. This is also indicated by our experiments with poly I:C-bodies-loaded DCs further loaded with a different soluble or targeted antigen. Similar reasoning applies to observations by Cui et al. where repeated vaccination of mice with the freeze-thaw lysates of tumor cells that were transduced with poly I:C led to enhanced tumor rejection (35,36). Thus, when the antigen is provided in vivo in excess (Schulz et al.) or in the form of soluble protein (Cui et al. and FluM1 protein herein) it can escape inhibition. Additionally, we might be faced with consequences of inherent discrepancies between the murine and human systems. Indeed the human counterpart of murine $CD8\alpha_+$ DCs (15,37-39), which appear responsible for enhanced CTL cross-priming in the work described by Schulz et al., remains to be identified.

A lack of cross-presentation of cell-associated antigen by human DCs that have captured infected dead cells has a direct implication on activation of both viral-specific and self-antigen specific $CD8^+$ T cells. It is conceivable such inhibited cross-presentation would prevent activation of destructive self-antigen specific $CD8^+$ T cells in the context of viral infection, however it raises the inquiry of how virus-specific $CD8^+$ T cells are maintained. One possibility is that abundant amounts of viral-derived peptides are generated in infected cells that are more readily loaded onto recycling surface MHC class I as dead cells are captured. Changes in self-peptide processing have been described in infected cells due to autocrine type I IFN which may prevent availability of self-peptides for MHC class I presentation (12). Another possibility is that DCs that capture infected dead cells pass on antigenic material for cross-presentation to secondary DCs. Carbone et al. have shown that in the context of skin HSV infection, migratory DCs such as Langerhans cells do not cross-present HSV antigen but rather transfer the antigen to lymph node resident DCs (40). Recent work by Randolph et al. has indicated a similar antigen transfer with human DCs that capture influenza-infected dead cells (personal communication). At this stage our results cannot be interpreted as differential cross-presentation of self and foreign antigens by DCs that captured dead infected cells.

Lastly, the inhibition of cell-associated antigen cross-presentation has significant implication for the use of DCs in cancer immunotherapy. Since generation of tumor-specific $CD8^+$ T cell responses is one of the goals of such immunotherapy, our work highlights issues that arise when using whole dead tumor cells as a source of antigen.

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Supplementary Material

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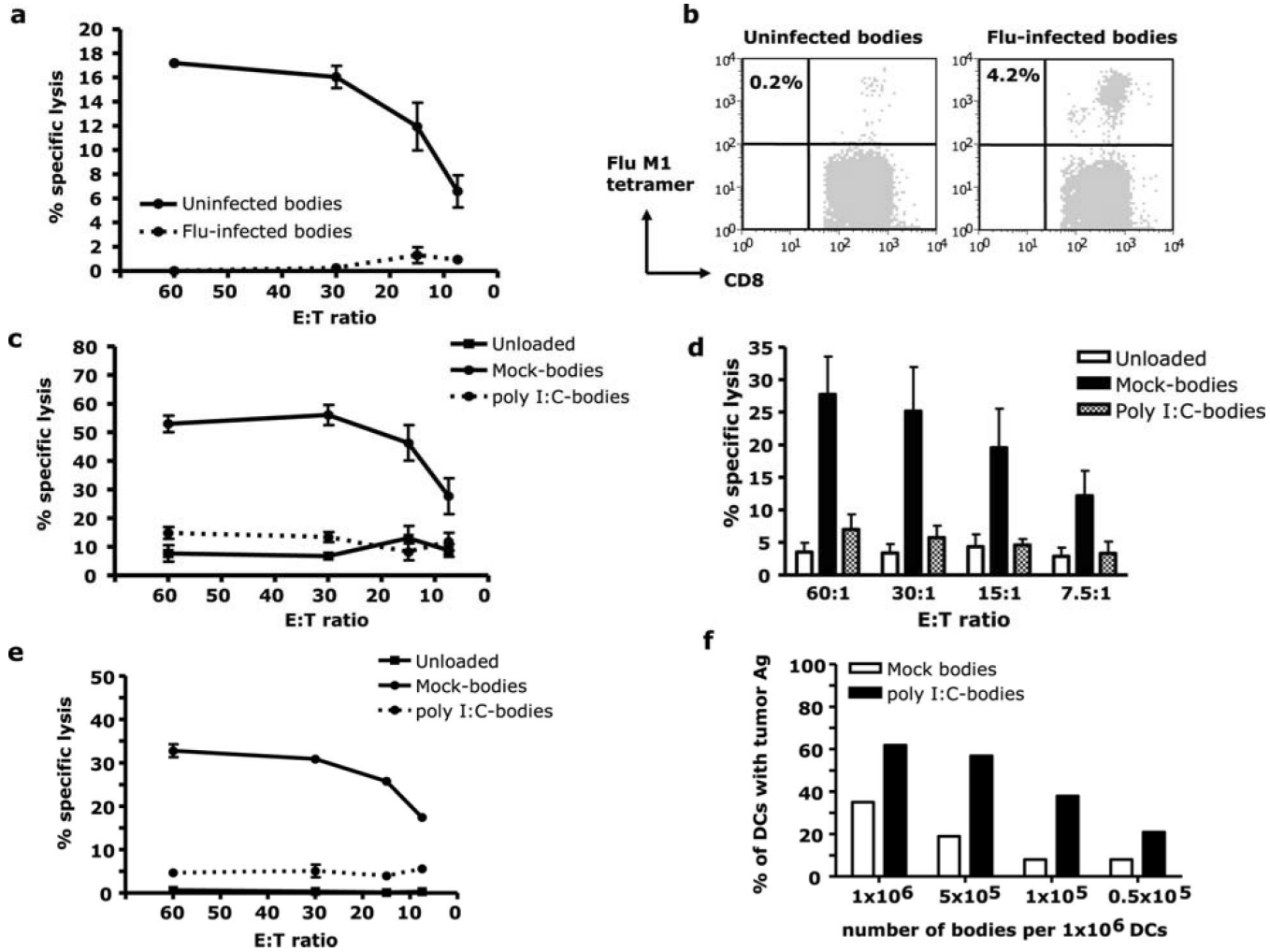

Figure 1. Inhibited CTL generation with Flu-bodies/poly I:C-bodies-loaded DCs

A) Cr^{51} release from HLA-A*0201$^+$ Me290 cells after 4 hr co-culture with primed HLAA*0201$^+$ CD8$^+$ T cells. Purified CD8$^+$ were stimulated with autologous DCs loaded with uninfected bodies (closed circles; solid line) or Flu-infected bodies (closed circle; dashed line). IL-7 and CD40L were added at start of all DC-T cell co-cultures with IL-2 added at day 7 and CTL activity read-out at day 10 with a standard Cr^{51} release assay. Percent of specific lysis is shown with mean and SD. *B*) Binding of CD8$^+$ T cells from A to HLA-A*0201$^+$/FluM1 peptide tetramer. Percent of CD8$^+$ T cells specific for HLAA*0201$^+$/FluM1 peptide tetramer is indicated. *C*) Cr^{51} release assay with naïve CD8+ T cells stimulated with autologous DCs loaded with mock-bodies (closed circles; solid line), loaded with poly I:C-bodies (closed circles; dashed line), or unloaded DCs (closed squares). Results are shown for 6 different experiments (5 different donors) with mean and standard deviation (Fig. 1*D*). *E*) Cr^{51} release assay as described in *C*, except stimulation was performed by DCs loaded with tumor bodies at a ratio of 20 DCs per 1 tumor body (as opposed to 2:1). *F*) Loading of tumor antigen in DCs with dose titration of tumor bodies. DC loading is assessed by immunofluorescence microscopy as described in Fig. 7.

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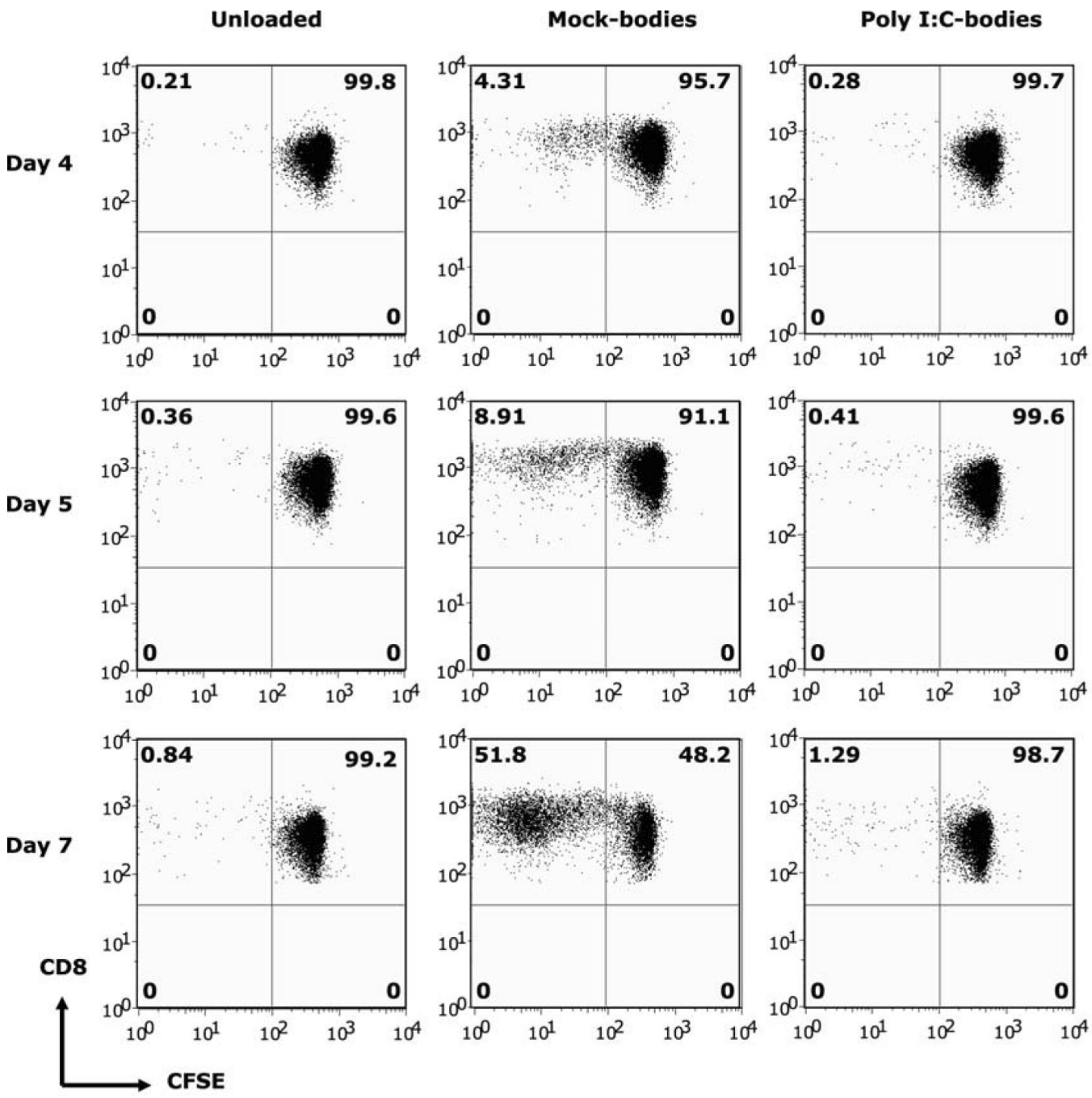

Figure 2. Inhibited CD8$^+$ T cell proliferation with poly I:C-bodies-loaded DCs

Purified naïve CD8$^+$ T cells were CFSE-labeled and co-cultured with autologous DCs loaded with mock-bodies, poly I:C-bodies, or with unloaded DCs. Percent of CFSE^{low}, CD8$^+$ T cells is indicated at days 4, 5, and 7 of co-culture. Results are representative of 3 independent experiments.

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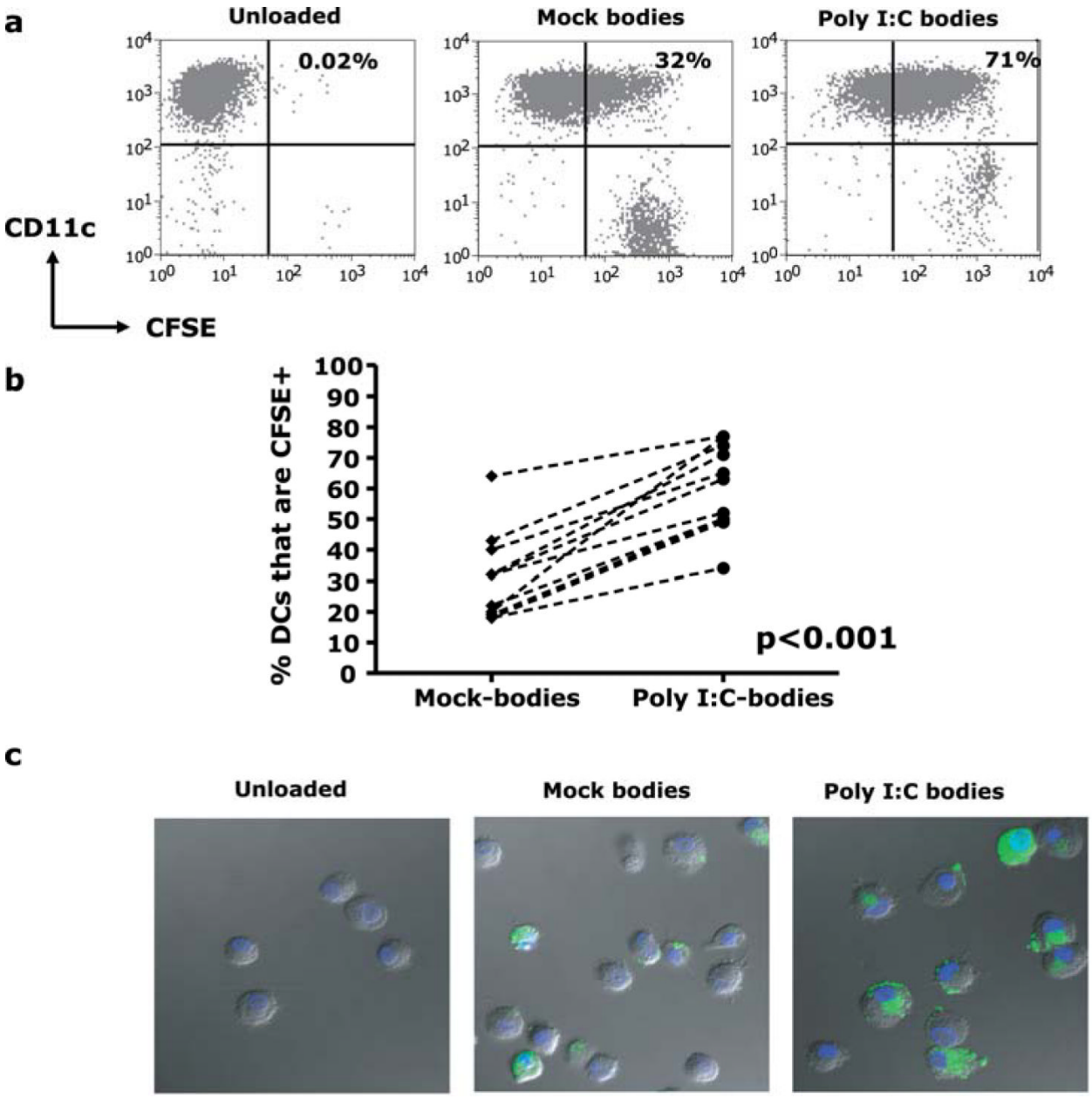

Figure 3. DCs more effectively capture dying cells containing poly I:C

A) DCs were loaded with CFSE-labeled mock-bodies or poly I:C-bodies and analyzed by flow cytometry. Double positive $CD11c^+$ $CFSE^+$ cells are considered as loaded DCs. Single CSFE positive events represent uncaptured tumor bodies. *B*) Fraction of loaded DCs as per tumor body condition. Indicated is percentage of $CD11c^+$ DCs that are positive for CFSE. *C*) Confocal microscopy of DCs showing internalized $CFSE^+$ tumor bodies.

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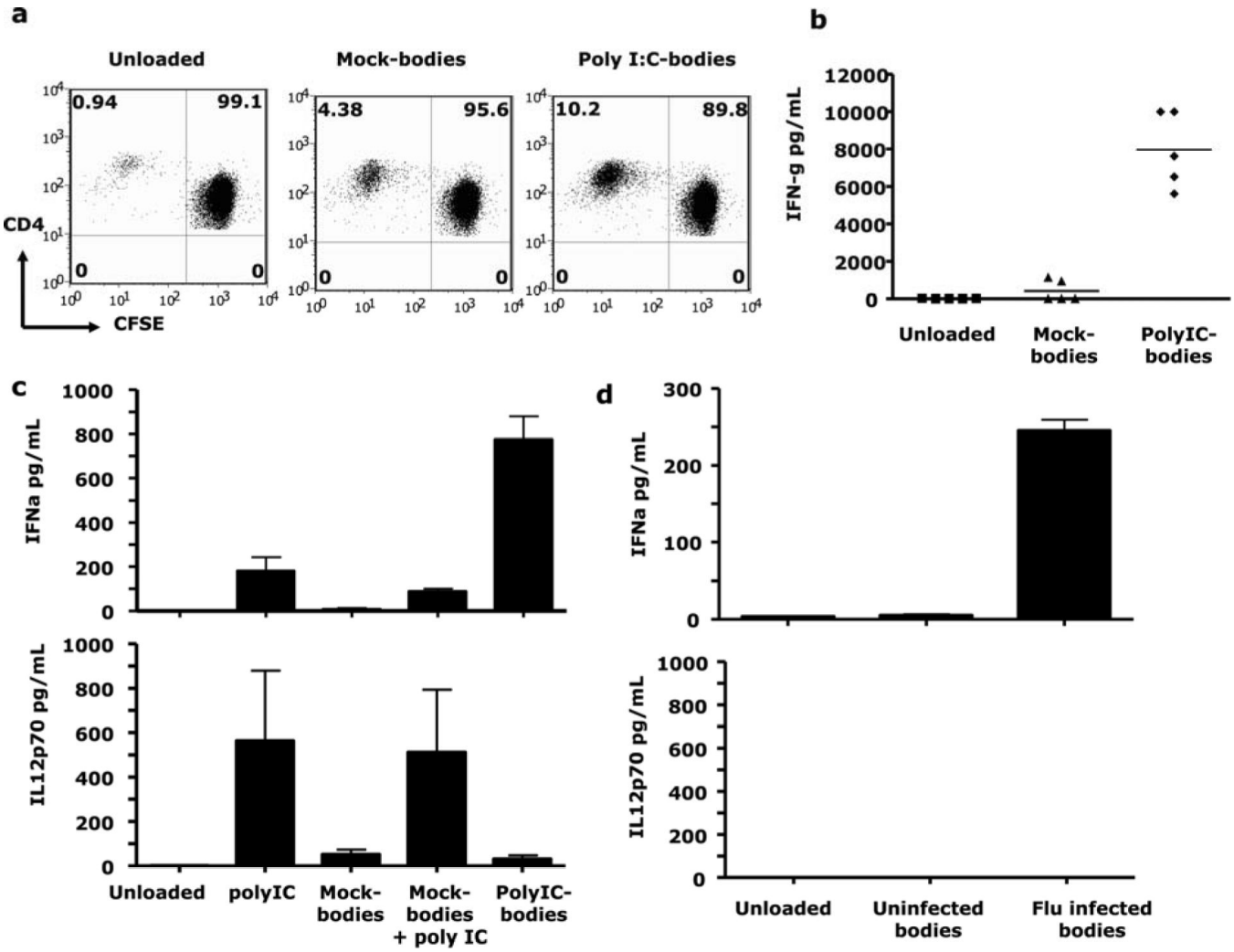

Figure 4. Expansion of IFNγ-secreting CD4$^+$ T cells by DCs loaded with poly I:C-bodies

A) CFSE-labeled naïve CD4$^+$ T cells were co-cultured with autologous DCs loaded with mock-bodies, poly I:C-bodies, or unloaded DCs. Percent of CFSE^{low}, CD4$^+$ T cells is shown at day 7 of co-culture. *B*) IFNγ levels in culture supernatant of CD4$^+$ T cells primed for 7 days with autologous DCs loaded with mock-bodies, poly I:C-bodies, or with unloaded DCs. Shown are the results for five independent experiments with mean and SD given. *C*) IFNα and IL-12p70 levels from supernatant of DCs loaded with mock-bodies, poly I:C-bodies, 10 μg/mL soluble poly I:C, mock-bodies + 10 μg/mL soluble poly I:C, and unloaded DCs. *D*) IFNα and IL-12p70 levels from supernatant of DCs loaded with uninfected bodies, Flu-infected bodies, and unloaded DCs. Results are representative of at least 3 independent experiments with mean and SD given.

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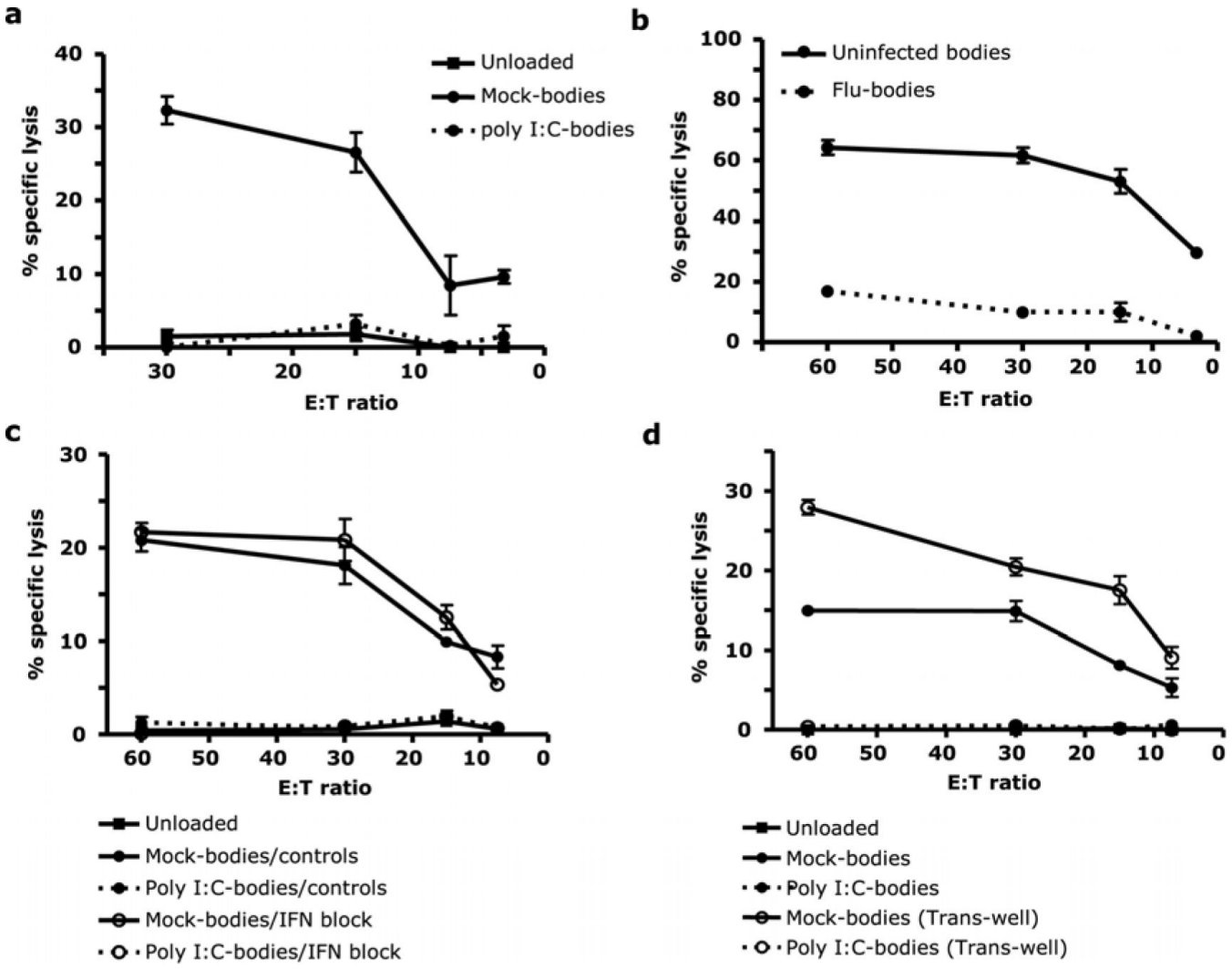

Figure 5. CD4$^+$ T cells and cytokines cannot rescue generation of CD8$^+$ T cell responses

A) CD4$^+$ and CD8$^+$ T cells were stimulated with autologous DCs loaded with mock-bodies (closed circles; solid line) or poly I:C-bodies (closed circles; dashed line). IL-7 and CD40L were added at start of DC-T cell co-cultures with IL-2 added at day 7. At day 10 of co-culture, CD8$^+$ T cells were purified from each co-culture condition and used for the Cr^{51} release assay. Results are representative of five independent experiments. *B*) Same as in *A* with DCs being loaded with control uninfected bodies or Flu-bodies. *C*) DCs were loaded with mock- or poly I:C-bodies in the presence of anti-IFNα + anti-IFNα/β receptor blocking antibodies or control IgG. Loaded DCs were then co-cultured with naïve autologous CD8$^+$ T cells also in the presence of IFN blocking antibodies or control IgG. IL-7 and CD40L were added at start of DC-T cell co-cultures with IL-2 added at day 7 and CTL activity read-out at day 10 with a standard Cr^{51} release assay. Percent of specific lysis is shown with mean and SD. Results are representative of three independent experiments. *D*) DCs were loaded in trans-well plates with poly I:C-bodies-loaded DCs in upper well and mock-bodies-loaded DCs in lower wells. Control conditions consisted of same tumor bodies in both upper and lower wells. DCs were then co-cultured with naïve autologous CD8$^+$ T cells and CTL activity read out as described in *A*.

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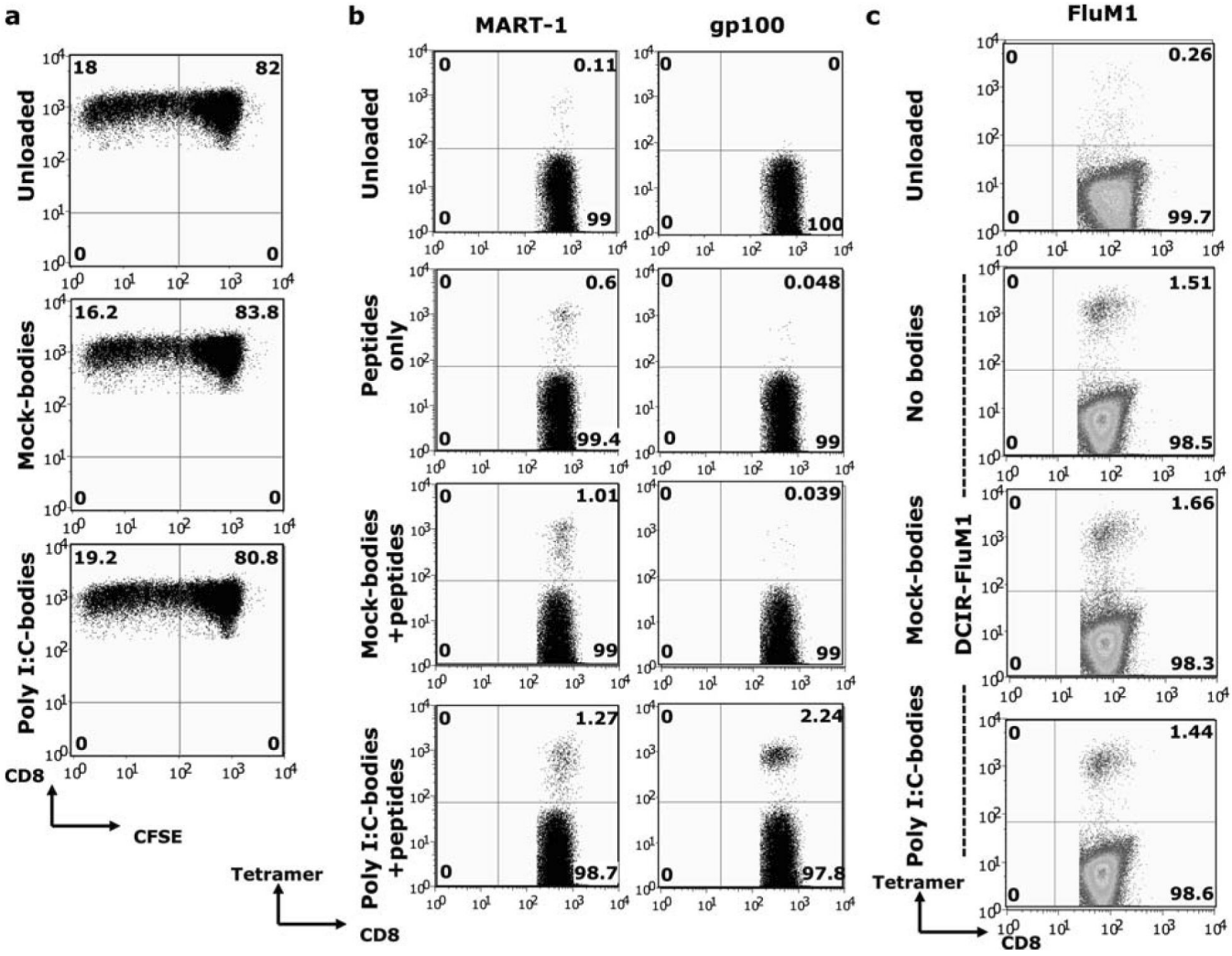

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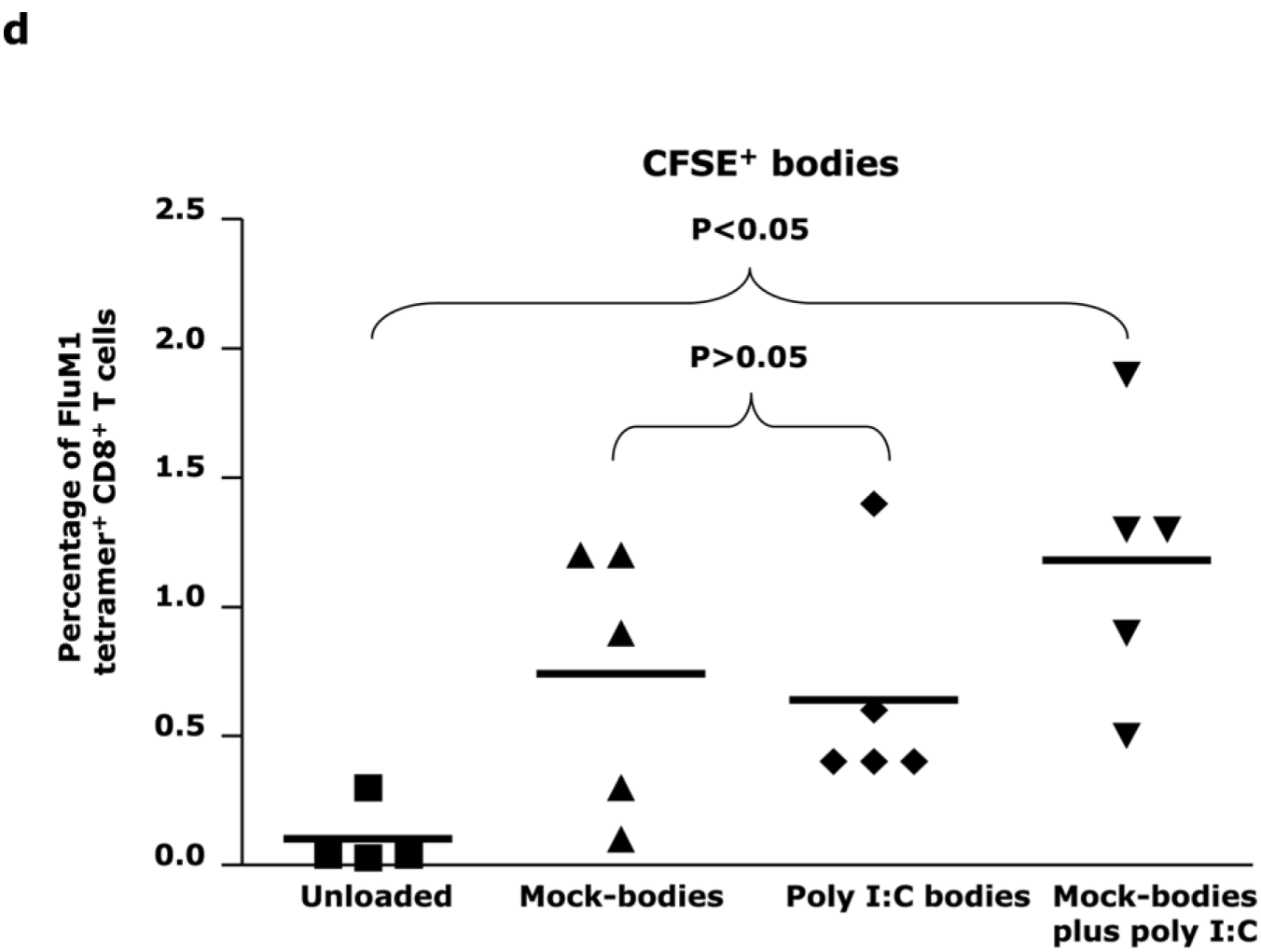

Figure 6. Inhibition of CD8$^+$ T cell responses is specific to antigens associated with captured dead cells

A) Purified CD8$^+$ T cells were CFSE-labeled and co-cultured with allogeneic DCs loaded with mock-bodies, poly I:C-bodies, or with unloaded DCs. Percent of CFSE^{low}, CD8$^+$ T cells is shown at day 7 of co-culture. *B*) Tetramer binding assay. DCs were loaded with CFSE-labeled mock-bodies or poly I:C-bodies and after 24 hr loading CFSE$^+$ DCs were sorted. All DCs from unloaded control were also sorted. Sorted DCs were pulsed with 10 μg/mL of MART-1 and gp100 peptides and then co-cultured with purified autologous CD8$^+$ T cells. IL-7 and CD40L were added at start of DC-T cell co-cultures with IL-2 added at day 7. After day 10 of co-culture, binding of CD8+ T cells to HLAA*0201$^+$/MART-1 peptide and HLA-A*0201$^+$/gp100 peptide tetramers was assessed by FACS. *C*) CFSE$^+$ DCs (loaded with tumor bodies) were treated with 10 μg/mL of FluM1 protein conjugated to human anti-human anti-DCIR mAb (IgG4). DCs were then co-cultured with purified autologous CD8$^+$ T cells. IL-7 and CD40L were added at start of DC-T cell co-cultures with IL-2 added at day 7. After day 10 of co-culture, binding of CD8$^+$ T cells to HLA-A*0201+/FluM1 peptide tetramer was assessed by FACS. *D*) DCs were loaded with CFSE-labeled mock-bodies or poly I:C-bodies along with 10 μg/mL soluble FluM1 protein. After 24 hr loading CFSE$^+$ DCs were sorted. DCs from unloaded control were also sorted. Sorted DCs were co-cultured with purified autologous CD8$^+$ T cells. IL-7 and CD40L were added at start of DC-T cell co-cultures with IL-2 added at day 7. After day 10 of co-culture, binding of CD8$^+$ T cells to HLAA*0201+/FluM1 tetramers

was assessed by FACS. Results of five independent experiments showing the percentage of tetramer-positive $CD8^+$ T cells elicited with $CFSE^+$ DCs. Anova and post analysis of significance.

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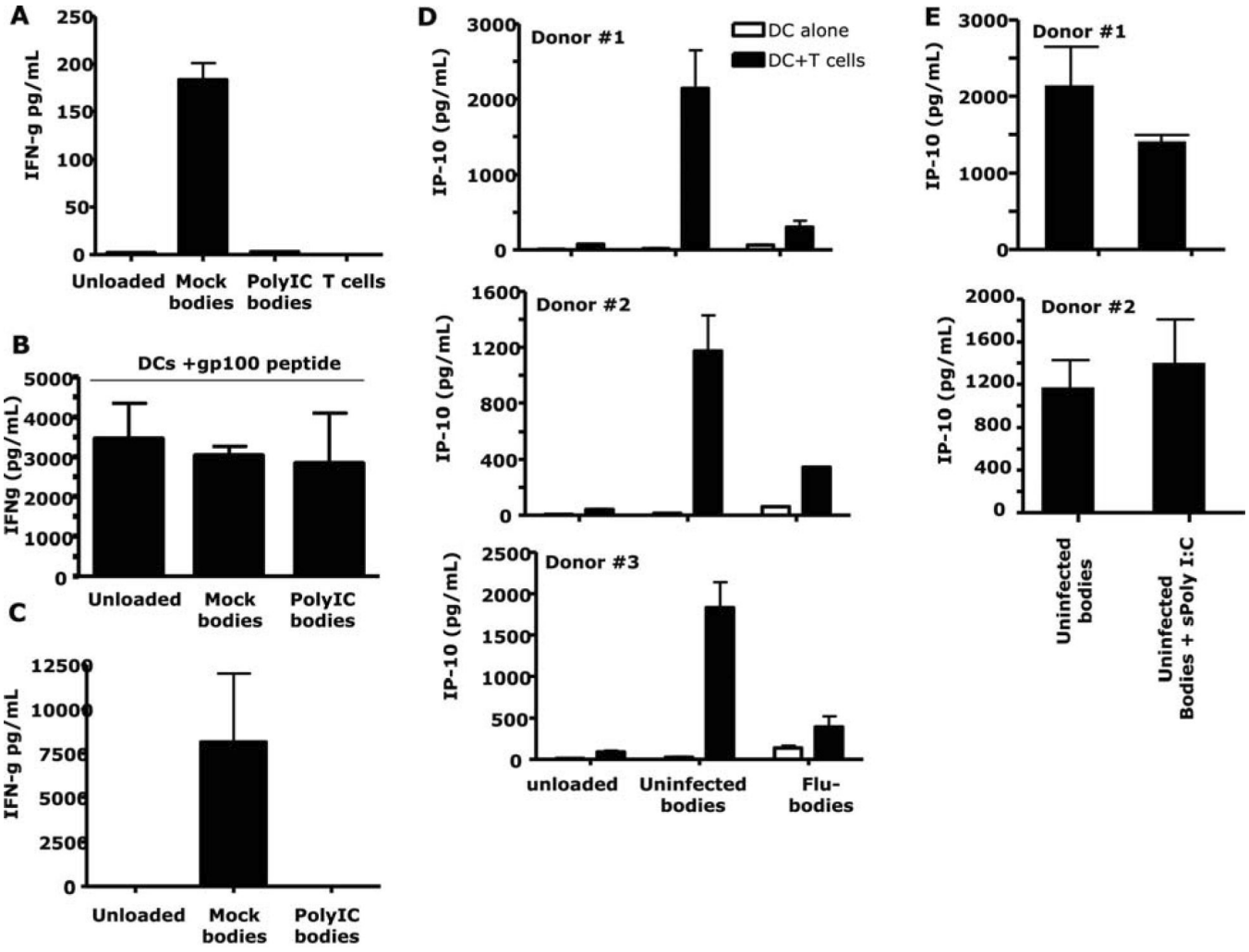

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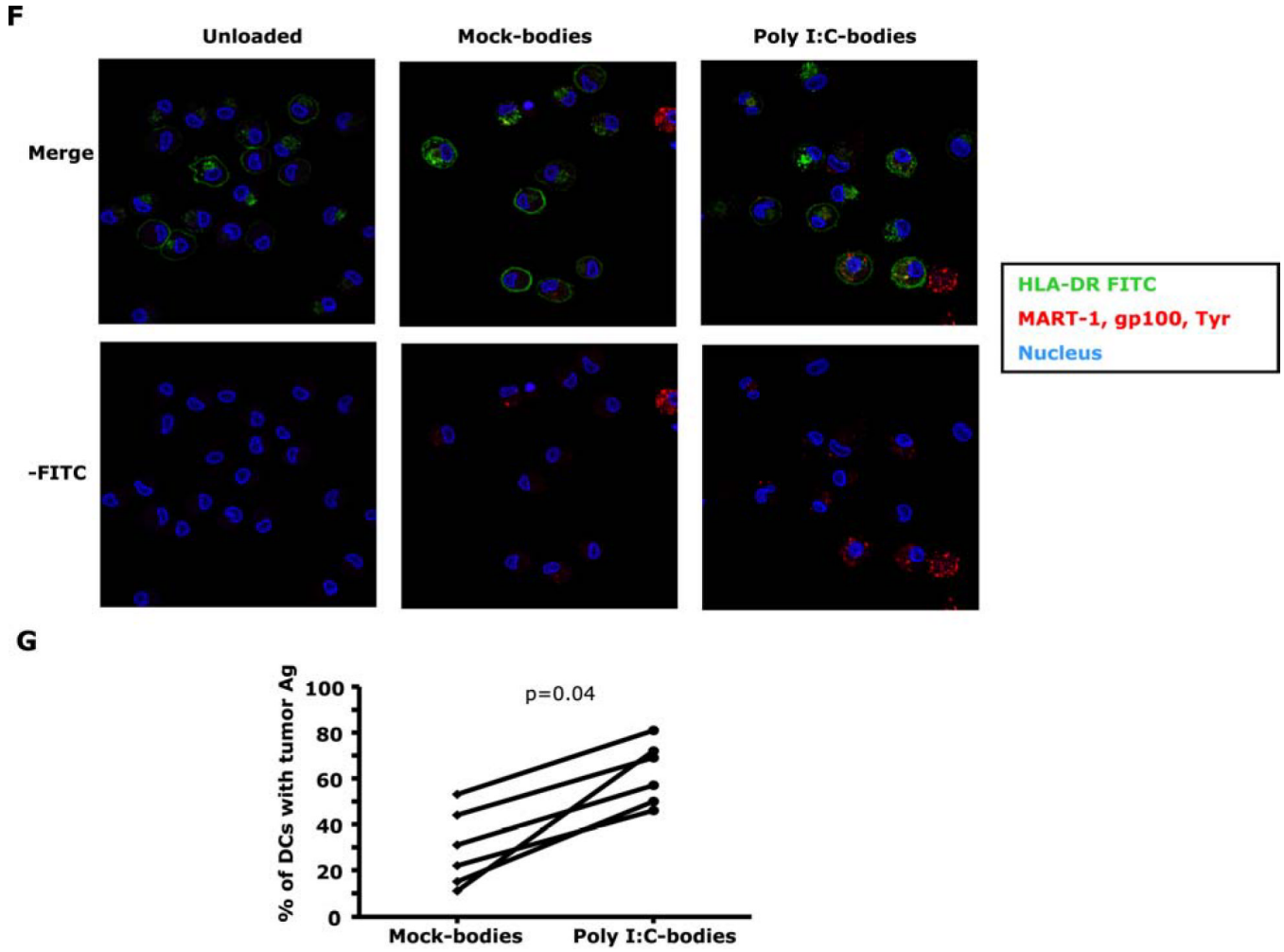

Figure 7. Poly I:C-bodies specifically inhibit presentation of endogenous antigens of captured dead cells by DCs

A) Gp100-specific CTL clone G154 was co-cultured with mock-bodies-, poly I:C-bodies-loaded, or unloaded DCs in the presence of CD40L stimulation. After 16 hrs, CTL activation was assessed by determining IFNγ levels in culture supernatant. Data is given as the mean of triplicate co-cultures with SD. Results are representative of at least 3 independent experiments. CTL clones alone and DC alone controls produced <1 pg/mL IFNγ. *B*) DCs were loaded overnight with mock-bodies or poly I:C-bodies and then with 10μg/mL gp100 melanoma peptide for 3 hrs. DCs were then washed twice with media to remove excess peptdie and co-cultured with the gp100-specific $CD8^+$ CTL clone G154 in the presence of CD40L stimulation. After 16 hrs, CTL activation was assessed by determining IFNγ levels in culture supernatant. Data is given as the mean of triplicate co-cultures with SD given. CTL clones alone and DC alone controls produced <1 pg/mL IFNγ. The same experiment as in *A*). *C)* DCs were loaded with mock-bodies or poly I:C-bodies. After loading, DCs were further incubated for additional 48 hrs to allow tumor body antigen to be processed. DCs were then co-cultured with the gp100-specific $CD8^+$ CTL clone G154 in the presence of CD40L stimulation. After 16 hrs, CTL activation was assessed by determining IFNγ levels in culture supernatant. Data is given as the mean of triplicate co-cultures with SD given. Results are representative of at least 3 independent experiments. CTL clones alone and DC alone controls produced <1 pg/mL IFNγ. *D*) Gp100-specific CTL clone G154 was co-cultured with uninfected-bodies-, Flu-bodies-loaded, or unloaded DCs in the presence of CD40L stimulation. After 16 hrs, CTL activation was assessed

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by determining IFNγ and IP-10 levels in culture supernatant. Data is given as the mean of triplicate co-cultures with SD. IP-10 results are shown for 3 separate donors. *E*) Gp100-specific CTL clone G154 was co-cultured with uninfected-bodies-, or DCs loaded with uninfected bodies and concurrently stimulated with 10μg/mL soluble poly I:C (non-cell-associated), all in the presence of CD40L stimulation. After 16 hrs, CTL activation was assessed by determining IP-10 levels in culture supernatant. Data is given as the mean of triplicate co-cultures with SD given. Results are shown for 2 separate donors. *F*) DCs were loaded with mock-bodies or poly I:C-bodies and 24 hrs after loading were stained to assess intracellular levels of tumor antigen by confocal microscopy. Upper panels show all fluorescent channels, lower panels have the FITC channel removed in order to better distinguish cells that contain (red) tumor antigen. *G*) Scoring of DCs for tumor antigen. Indicated is percentage of HLA-DR^+ DCs that are positive for tumor antigen per loading condition.