Rapid Peptide Turnover and Inefficient Presentation of Exogenous Antigen Critically Limit the Activation of Self-Reactive CTL by Dendritic Cells[1]

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This study evaluated to what extent presentation of exogenously acquired self-Ags via MHC class I molecules on DC might contribute to the activation of self-reactive CTL and subsequent development of autoimmune disease. We show here by using the rat insulin promotor lymphocytic choriomeningitis virus glycoprotein model of autoimmune diabetes that the activation of self-reactive CTL by DC after uptake of exogenous Ag is very limited, first by the short half-life of MHC class I-associated peptides on DC in vitro and in vivo, and second by the rather inefficient MHC class I presentation of cell-associated self-Ags by DC. These two mechanisms are probably crucial in establishing high thresholds for the induction of self-reactive CTL that prevent autoimmune sequelae after release of sequestered and previously immunologically ignored tissue Ags. ***The Journal of Immunology,* 2001, 166: 3678–3687.**

Autoimmune diseases are characterized by vigorous immune responses against a set of self-Ags resulting in pathological damage in single organs or generalized in various tissues. It appears that the majority of self-Ags are immunologically ignored because they are either expressed at immunologically privileged sites (1–3) or they are not expressed above a certain threshold to achieve central or peripheral tolerance (4, 5). As a consequence, autoimmune reactions are most likely initiated when previously ignored Ags are presented in sufficient amounts and over a sufficiently long time in secondary lymphoid tissues. Transport of Ags to secondary lymphoid tissues is mediated by professional APCs, particularly dendritic cells (DC)[3] (6). Therefore, it is important to determine whether, how, and to what extent presentation of self-Ags by DC might contribute to the activation of either self-reactive helper or CTLs and subsequent development of autoimmune diseases.

Cellular debris, such as that resulting from cytopathic viral infections or traumatic tissue damage, may be taken up by DC and processed via the exogenous pathway to be presented as peptides associated with MHC class II molecules (7). Indeed, DC are highly efficient in acquisition of exogenous Ags and presentation of high levels of MHC class II-restricted peptides in vitro (8). DC also present pancreatic self-Ags via MHC class II during the development of autoimmune diabetes in nonobese diabetic mice (9). Furthermore, it has been shown in vitro that DC may present MHC class I-associated peptides after uptake of concentrated protein (10) or after uptake of virus-infected, apoptotic cells (11). MHC class I-restricted presentation of exogenous soluble, particulate, or cell-associated Ags has been termed cross-priming (12) and can be demonstrated in vivo in certain transgenic model situations where self-reactive CTL are peripherally deleted (13, 14). However, if truly ignored self-Ags could easily gain access to the exogenous MHC class I cross-presentation pathway, e.g., in the course of a cytopathic virus infection, self-reactive CTL could be activated and initiate a self-perpetuating circle of tissue destruction and subsequent increased release of self-Ags. Therefore, it is conceivable that a number of mechanisms exist that limit the induction of anti-self reactions, particularly in the CD8 compartment. These may be signals such as negative feedback stimulation via CTLA-4 during DC-T cell interaction (15). However, it is unclear and unexplained to what extent presentation of self-peptides via MHC class I, particularly after exogenous uptake of either peptides or whole proteinacious Ag by DC, influences the induction of autoimmunity.

In the present report, we investigated mechanisms that might contribute to limit the autoimmune sequelae after release of self-Ags and subsequent presentation by DC. We found that the duration of peptide presentation locally during DC-CTL interaction is critical for the induction of autoreactive CTL and that exogenous, cell-associated Ags are poorly immunogenic for CTL, even when presented by DC.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from the Institut für Labortierkunde (University of Zurich, Zurich, Switzerland). Mice expressing lymphocytic choriomeningitis virus (LCMV)-glycoprotein (gp) under the control of the rat insulin promotor (RIP)-gp (2), mice expressing the LCMV gp33 epitope ubiquitously (H8 mice; Ref. 16), and mice transgenic for a Vα2/Vβ8.1 TCR specific for H2-D^b and the major LCMV-gp epitope gp33–41 (gp33)

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Received for publication April 13, 2000. Accepted for publication January 4, 2001.

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[1] This work was supported by the Swiss National Science Foundation, the Deutsche Forschungsgemeinschaft (B.L.), Human Frontier Science Program (K.M.), and the Kanton Zürich.

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[3] Abbreviations used in this paper: DC, dendritic cells; LCMV, lymphocytic choriomeningitis virus; gp, glycoprotein; RIP, rat insulin promotor; MC-gp, methyl-collanthrene-induced fibrosarcoma cells expressing LCMV-gp; H8-DC, DC derived from H8 mice; HA, influenza hemagglutinin; NP, nucleoprotein; B6, C57BL/6.

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(17), or for a Vα2/Vβ8.3 TCR specific for I-A^b and the LCMV-gp peptide p13 (18) have been described previously. Experiments were conducted with age (8–16 wk)- and sex-matched animals.

Cell lines, Abs, and peptides

Methyl-collanthrene-induced fibrosarcoma cells expressing LCMV-gp (MC-gp) have been described previously (19). MC-gp were made apoptotic by cultivation at 43°C for 24 h; necrotic MC-gp were produced by three rounds of freeze-thawing. Apoptosis of MC-gp was tested by FACS analysis, and routinely 50–75% of the apoptotic MC-gp were propidium iodide negative and annexin V positive; necrotic MC-gp were always 100% trypan blue positive. EL-4 (H-2^b), a thymoma cell line, was used as target cell. LCMV-gp peptides KAVYNFATM (gp33), FQPQNGQFI (nucleoprotein (NP) 396), and GLNGPDIYKGVYQFKSVEFD (p13) were produced from Neosystem Laboratoire (Strasbourg, France).

Supernatants or purified Ig from the following mAb-producing hybridomas were used: rat anti-mouse CD4 (YTS191.1; Ref. 20), rat anti-mouse CD8 (YTS169.4.2; Ref. 20), rat anti-mouse CD45R (RA3-3A1/6.1; American Type Culture Collection (ATCC), Manassas, VA), rat anti-mouse I-A^b (B21-2; ATCC), anti-CD40 (21), anti-CTLA-4 (22), and anti-H2-D^b (141-51; Ref. 23).

Preparation and Ag pulse of DC

Generation of DC from B6 and H8 bone marrow cultures has been described previously (24). Both DC populations express high levels of the costimulatory molecules CD80, CD86, and CD40, and >50% of the cells express high levels of MHC class II Ags. Incubation of the DC with LPS leads to further up-regulation of these surface markers (not shown). For Ag pulsing, DC were resuspended in RPMI 1640/5% FCS at 10^6/ml and incubated with the indicated peptides for 60 min. Alternatively, DC were cocultivated with apoptotic or necrotic MC-gp at a ratio of 1:5 (DC:MC-gp) for 6 h at 37°C followed by metrizamide gradient centrifugation to separate the fibroblasts from the DC. DC were washed two times with balanced salt solution and i.v. injected in a volume of 0.5 ml.

Cytotoxicity assays

Spleen cells (4×10^6/well) from primed mice were restimulated for 5 days in 24-well tissue culture plates with 2×10^6 gp33-labeled, irradiated (1000 rad) spleen cells in IMDM supplemented with 10% FCS, penicillin/streptomycin, and 0.001 M 2-ME. Restimulated spleen effector cells from one well were resuspended in 1 ml MEM/2% FCS, and 3-fold serial dilutions were made (indicated as dilution of culture). For detection of primary ex vivo cytotoxicity, effector cell suspensions were prepared from spleens of immunized mice at the indicated time point after priming. EL-4 cells were pulsed with LCMV-gp33 or LCMV-NP394 (10^{-5} M, 1.5 h at 37°C) and used in a standard 5-h ^{51}Cr release assay or in an overnight (15 h) assay. Unlabeled EL-4 cells served as controls. The supernatant of the cytotoxicity cultures was counted in a Cobra II γ Counter (Canberra Packard, Downers Grove, IL). Spontaneous release was always below 20% for 5 h assays and below 30% for overnight assays.

Proliferation assay

DC were preincubated with gp33 at the indicated concentration or left untreated and washed twice with balanced salt solution. Spleen cells from 318 TCR-transgenic mice recognizing the gp33 epitope were incubated in round-bottom 96-well plates with 3-fold serial dilutions of DC, starting with 2×10^4 DC per well. In blocking experiments, DC were incubated with the indicated concentration of anti-H2-D^b mAb 141-51 for 30 min before the addition of 318 responder cells. After incubation for 48 h at 37°C/5% CO_2, [^{3}H]thymidine (1 μCi/well) was added for a further incubation period of 12 h. Proliferation was determined as [^{3}H]thymidine incorporation by using a Microbeta scintillation counter (Wallac, Turku, Finland).

Cytofluorometry

Naive B6 mice were transfused i.v. with 5×10^5 syngeneic spleen cells from 318 mice (containing ~5×10^4 TCR-transgenic CTL) on day −1 (B6/318). To detect expansion of transgenic TCR-expressing T cells (25) after immunization with H8-DC, peripheral blood cells were stained for CD8 and transgenic Vα2 and Vβ8.1 using FITC-conjugated rat anti-mouse CD8, PE-conjugated rat anti-mouse Vα2 and biotinylated rat anti-mouse Vβ8.1, respectively (all obtained from BD PharMingen, San Diego, CA) followed by streptavidin-Tricolor (Caltag, South San Francisco, CA). Activation of gp33-specific CTL was detected by staining with biotinylated anti-CD44 (BD PharMingen) followed by incubation with streptavidin-Tricolor (Caltag). Tetrameric class I complexes of H-$2D^b$ and gp33–41 were produced as described previously (26) and used to identify gp33-specific $CD8^+$ T cells in spleen cell suspensions. Erythrocytes were lysed with FACS lysis solution (Becton Dickinson), and the cell suspensions were analyzed on a FACScan flow cytometer (Becton Dickinson) after gating on viable lymphocytes.

Immunohistology

Freshly removed organs were immersed in HBSS and snap-frozen in liquid nitrogen. Tissue sections of 5 μm thickness were cut in a cryostat and fixed in acetone for 10 min. Sections were incubated with anti-mouse mAb against $CD8^+$ cells (YTS169.4.2), and polyclonal guinea pig Abs against insulin (Dako, Glostrup, Denmark). Alkaline phosphatase-labeled, species-specific goat Abs (Tago Scientific, Burlingame, CA) were used as secondary reagents. The substrate for the red color reaction was AS-BI phosphate/New Fuchsin. Sections were counterstained with hemalum. Fifteen to 20 islets from two or three histological sections of each mouse were evaluated. The fluorescent dye CFSE (Molecular Probes, Eugene, OR) was used to label DC (24). A total of 2×10^5 CFSE-labeled DC were injected s.c. into the footpad of naive recipients. Draining popliteal lymph nodes were removed at different time points, immersed in HBSS, and snap-frozen in liquid nitrogen. Histological procedures were performed as above using rabbit anti-fluorescein Ab (Dako).

Measurement of blood glucose

The glucose concentration in blood obtained from a tail vein was measured by using an ELITE Haemoglucometer (Bayer, Wuppertal, Germany). Mice were considered diabetic with values >14 mM at two consecutive measurements.

Results

DC pulsed with exogenous peptide exhibit reduced ability to induce diabetes in RIP-gp mice

We have shown previously that DC derived from H8 mice (H8-DC) and constitutively expressing a viral neoself-peptide (the H-$2D^b$-restricted peptide gp33 derived from LCMV-gp) are able to induce autoimmune diabetes after adoptive transfer into RIP-gp mice (27). In RIP-gp mice, the viral transgene (LCMV-gp) is immunologically ignored such that both strong initial expansion and

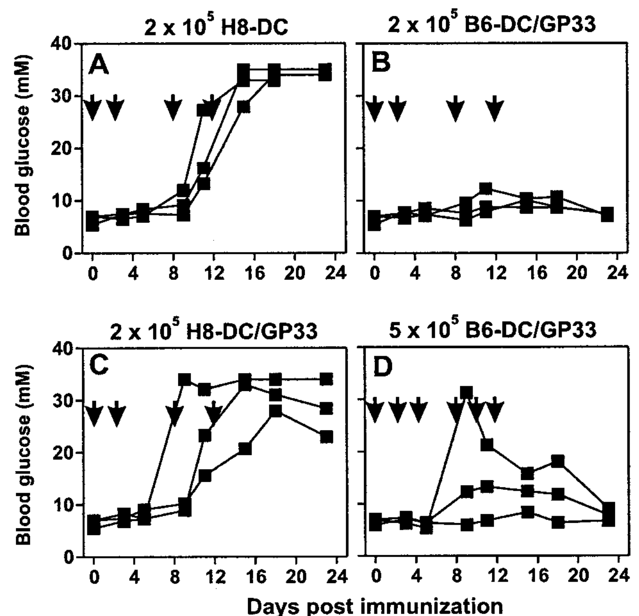

FIGURE 1. Blood glucose levels in RIP-gp mice after i.v. immunization with H8-DC- or gp33-pulsed B6-DC (B6-DC/gp33). Mice were repetitively immunized with 2×10^5 of (*A*) H8-DC (*n* = 16), (*B*) B6-DC/gp33 (*n* = 16), (*C*) H8-DC pulsed with gp33 (*n* = 6) on days 0, 2, 8, and 12, or (*D*) with 5×10^5 B6-DC/gp33 (*n* = 10) on days 0, 2, 4, 8, 10, and 12. Arrows indicate day of DC injection. Values of three representative mice per group are shown.

Table I. *Influence of peptide turnover and concomitant Th for the induction of diabetes in RIP-gp mice after DC immunization*

Immunization Protocol[a]	Additional Treatment[b]	Incidence of Diabetes[c]	Onset of Diabetes (day)
2×10^5 H8-DC (4×)	None	15/16	11 ± 2
2×10^5 B6-DC/gp33 (4×)	None	0/16	–
5×10^5 B6-DC/gp33 (6×)	None	6/14	12 ± 2
5×10^5 B6-DC (6×)	None	0/4	–
2×10^5 B6-DC/gp33 + p13 (4×)	10^7 Smarta i.v.	3/3	9 ± 1
2×10^5 B6-DC/gp33 + p13 (4×)	10^6 Smarta i.v.	10/10	10 ± 1
2×10^5 B6-DC/gp33 + p13 (4×)	10^5 Smarta i.v.	2/3	12, 13
2×10^5 B6-DC/p13 (4×)	10^7 Smarta i.v.	0/3	–
2×10^5 B6-DC/gp33 + p13 (4×)	None	0/6	–
2×10^5 B6-DC/gp33 (4×)	100 μg anti-CD40 i.v.	3/9	11 ± 2
2×10^5 B6-DC/gp33 (4×)	100 μg anti-CTLA-4 i.v.	0/6	–

[a] Mice were immunized i.v. repetitively with the indicated amount of DC (H8-DC; B6-DC/gp33, B6-DC pulsed with gp33 at 10^{-6} M; B6-DC/gp33 + p13, B6-DC pulsed with gp33 at 10^{-6} M and p13 at 100 μg/ml) four times on days 0, 2, 10, and 12 or six times on days 0, 2, 4, 8, 10, and 12.

[b] Mice received either the indicated number of splenocytes from TCR-transgenic Smarta mice on day −1 or 100 μg anti-CD40 mAb or anti-CTLA-4 mAb on days −1, 6, and 12.

[c] Mice were scored diabetic with blood glucose >14 mM on two consecutive measurements.

sustained activation of self-reactive CTL by repetitive immunization with DC (27) or by infection with LCMV (2) are required to mediate autoimmune disease. Here, we assessed whether DC exogenously loaded with gp33 are able to induce diabetes in RIP-gp mice. We chose a protocol where repetitive i.v. injection of 2×10^5 H8-DC led to manifest diabetes in > 90% of the RIP-gp mice (Fig. 1*A*). In contrast to the rapid hyperglycemia that developed after injection of H8-DC, RIP-gp mice remained normoglycemic when the same protocol was applied with nontransgenic DC derived from B6 mice (B6-DC) exogenously pulsed with gp33 (Fig. 1*B*). H8-DC exogenously loaded with gp33 also induced diabetes (Fig. 1*C*), indicating the endogenous production of self-peptide is responsible for the high autoimmune potential of the constitutively gp33-expressing H8-DC. Increasing the dose and frequency of application of exogenously peptide-loaded B6-DC partially compensated for the limited ability of peptide-pulsed B6-DC to elicit strong anti-self CTL responses in 6 of 14 mice (Fig. 1*D* and Table I), suggesting that the amount of Ag available for a certain time during interaction of DC with self-reactive CTL is probably a critical parameter for $CD8^+$-T cell-mediated autoimmune disease.

The histological analysis of pancreata from RIP-gp mice on day 14 after repetitive immunization with either H8-DC (Fig. 2, *A* and *E*), gp33-pulsed B6-DC/gp33 (Fig. 2, *B*, *C*, *F*, and *G*) or unpulsed control B6-DC (Fig. 2, *D* and *H*) revealed that activation of self-reactive CTL by H8-DC led to strong and destructive infiltration of insulin-positive pancreatic islets (Fig. 2*A*) with CD8-positive lymphocytes (Fig. 2*E*). Similarly, repetitive priming with a high dose of B6-DC/gp33 (six injections of 5×10^5) elicited a destructive insulitis (Fig. 2, *C* and *G*), whereas less frequent immunization with a lower dose of B6-DC/gp33 (four injections of 2×10^5) induced only a mild inflammation in the endocrine pancreas with peri-insular infiltration of $CD8^+$ T cells (Fig. 2, *B* and *F*). RIP-gp

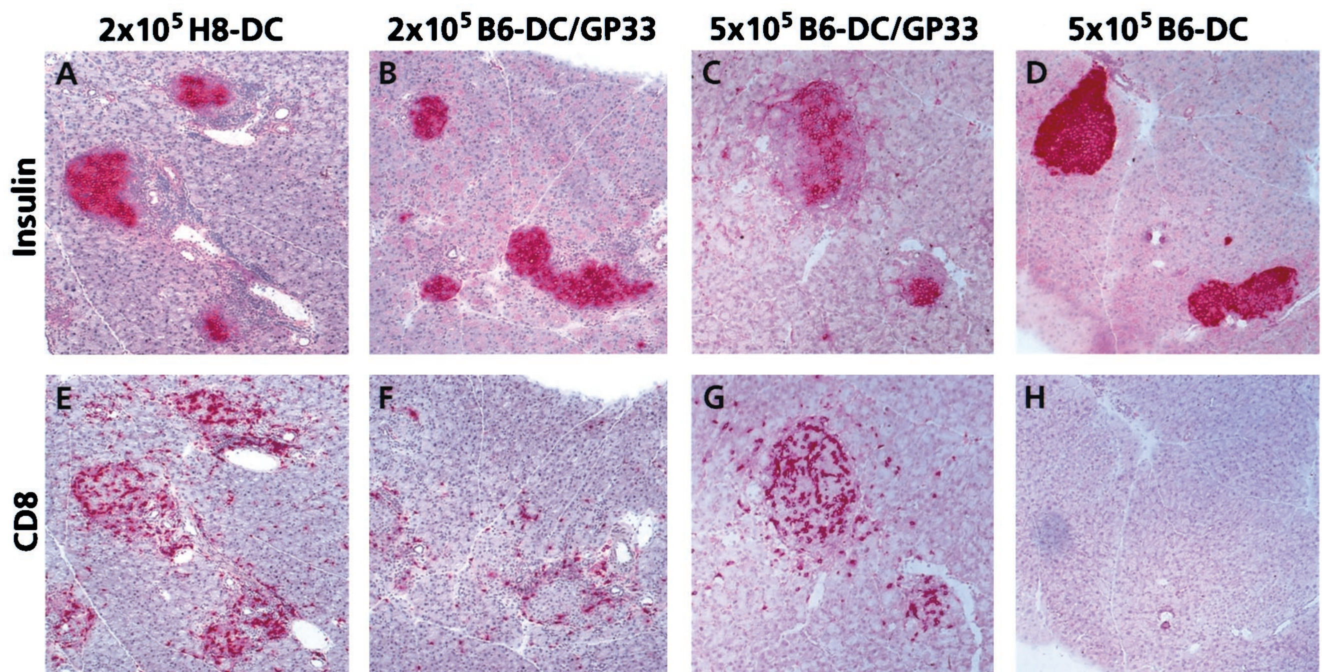

FIGURE 2. Immunohistological analysis of pancreatic islets in RIP-gp mice on day 14 after repetitive i.v. immunization with H8-DC or B6-DC/gp33. After adoptive transfer of 2×10^5 H8-DC (*A* and *E*), 2×10^5 B6-DC/gp33 on days 0, 2, 8, and 12 (*B* and *F*), 5×10^5 B6-DC/gp33 on days 0, 2, 4, 8, 10, and 12 (*C* and *G*), or 5×10^5 unpulsed control B6-DC on days 0, 2, 4, 8, 10, and 12 (*D* and *H*) pancreata were analyzed for the presence of insulin-producing islet cells (*A*–*D*) and $CD8^+$ T cells (*E*–*H*). Original magnification: ×80.

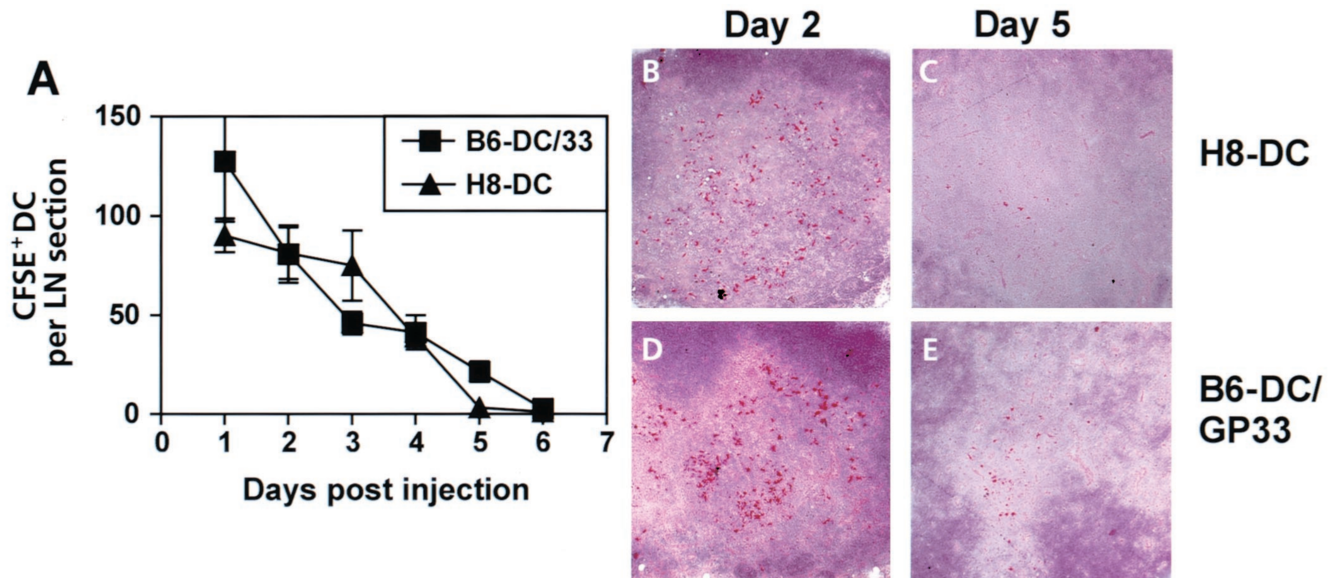

FIGURE 3. Persistence of DC in vivo. After labeling with CFSE, 2×10^5 H8-DC or B6-DC/gp33 were injected s.c. into the footpad of naive B6 recipients. At the indicated time points, the popliteal lymph nodes were removed and analyzed for the presence of CFSE-positive DC by immunohistochemistry. *A*, Number of CFSE$^+$ DC per lymph node section at the indicated time points postinjection. Values represent mean ± SD from six lymph nodes (two sections per lymph node) per time point. Representative microphotographs of T cell areas of popliteal lymph nodes showing CFSE$^+$ DC after injection of (*B* and *C*) H8-DC or (*D* and *E*) B6-DC/gp33.

mice immunized repetitively with a high dose of control B6-DC did not show inflammatory responses in the pancreas (Fig. 2, *D* and *G*). Taken together, it appears that there are significant qualitative differences in the autoimmune potential between DC constitutively expressing a self-peptide and DC that have acquired the peptide exogenously: H8-DC induced destructive insulitis and manifest hyperglycemia, whereas exogenously pulsed DC induced mild insulitis, and only when administered in high numbers and with high frequency did a hyperglycemia develop.

Rapid peptide turnover on DC limits their ability to activate CTL effectors

The above findings suggested that the duration of peptide presentation by DC in the microenvironment of lymphoid tissues might critically influence the activation of self-reactive CTL. In principle, two steps limiting peptide presentation by DC in these experiments may be envisaged: 1) short persistence of the exogenous peptide on DC, and/or 2) shorter life-span of peptide-pulsed DC. To test the latter possibility, B6-DC and H8-DC were labeled with CFSE and injected s.c. into the footpad of naive C57BL/6 recipients. One and 2 days after injection of 2×10^5 cells, similar numbers of both H8-DC or B6-DC/gp33 reached the draining popliteal lymph node (Fig. 3, *A*, *B*, and *D*). From day 2 on, the number of DC decreased continuously and both H8-DC and B6-DC/gp33 were hardly detectable by day 5 after injection (Fig. 3, *A*, *C*, and *E*). As the persistence of H8-DC and B6-DC/gp33 appeared to be comparable, it appears more likely that differences in the persistence of peptide may account for the profound differences in the autoimmune potential of exogenously peptide-pulsed vs constitutively peptide-expressing DC.

Therefore, we evaluated the persistence of gp33 on DC in vitro and in vivo. For the in vitro assays, B6-DC were pulsed with gp33 for 60 min at 37°C and either tested immediately by cocultivation with TCR-transgenic T cells recognizing gp33 in the context of H-2D^b (318 cells; Ref. 17 and Fig. 4*A*) or first cultured in the absence of peptide for 1 (Fig. 4*B*) or 2 days (Fig. 4*C*) before they were used as stimulators. H8-DC and unpulsed B6-DC were processed in parallel and served as controls. B6-DC pulsed with gp33 at high concentrations, i.e., at 10^{-6} M or 10^{-8} M, maintained the capacity to stimulate naive CTL in vitro for >48 h (Fig. 4*C*). After pulsing of the DC at the low concentration of 10^{-10} M, the stimulatory capacity of the DC was strongly reduced after 1 day (Fig.

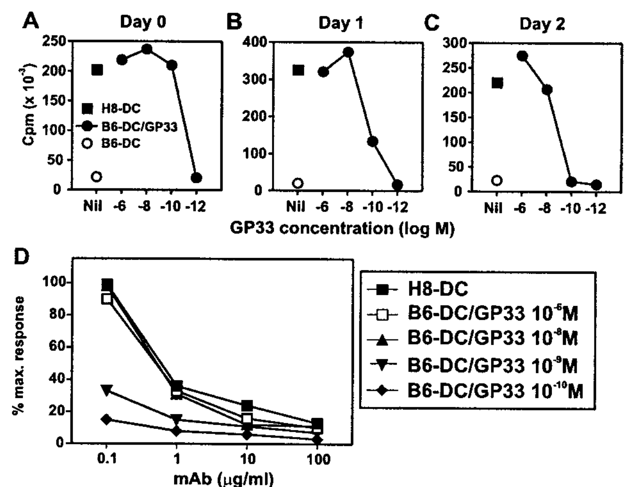

FIGURE 4. Persistence of MHC class I-associated peptides on DC in vitro. DC were pulsed with gp33 at the indicated concentration for 60 min at 37°C, washed, and either (*A*) tested immediately on day 0 or recultured for (*B*) 24 h or (*C*) 48 h before being tested for their ability to stimulate naive gp33-specific 318 spleen cells. H8-DC and untreated B6-DC served as controls. The data show the [^{3}H]thymidine incorporation at a DC:spleen cell ratio of 1:10. One of two experiments with comparable results is shown. *D*, Effect of anti-H2-D^b mAb on DC-induced proliferation of 318 spleen cells. H8-DC or B6-DC pulsed with gp33 at the indicated concentration were preincubated with the blocking mAb 141-51 at the indicated concentration for 30 min before addition of 318 responder cells. [^{3}H]Thymidine incorporation was determined after 48 h. Results are expressed as the percent of [^{3}H]thymidine incorporation considering the maximal proliferation in each condition in the absence of blocking mAb as 100%.

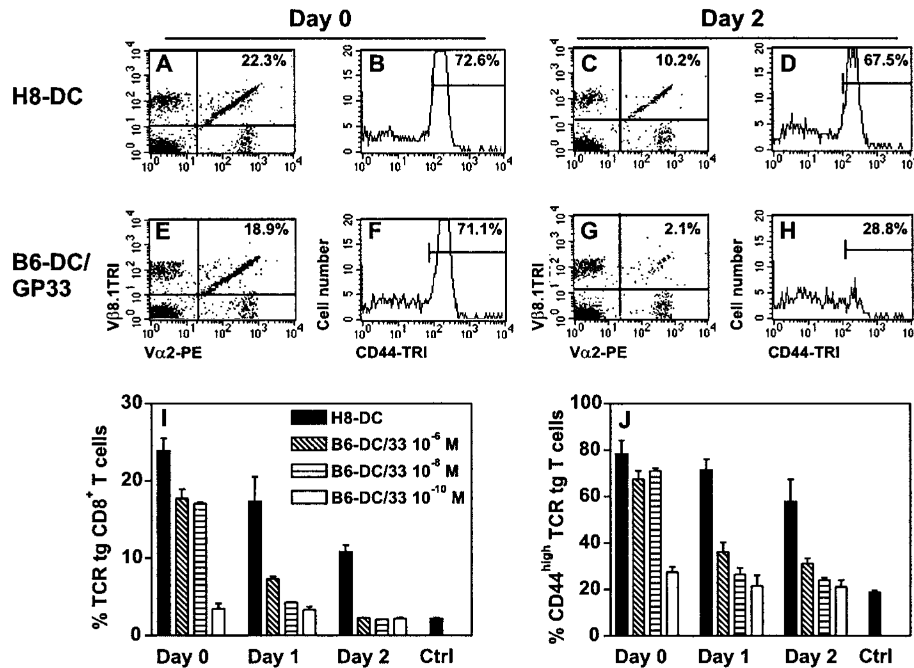

FIGURE 5. In vivo persistence of MHC class I-associated peptides on DC. Expansion and activation of TCR-transgenic CTL after adoptive transfer of (*A--D*) H8-DC- or (*E–H*) gp33-pulsed B6-DC in naive B6 recipients. A total of 2×10^5 H8-DC or B6-DC/gp33 were either cotransferred on the same day with 5×10^5 spleen cells from TCR-transgenic 318 mice (*A*, *B*, *E*, and *F*) or injected 2 days before transfer of the transgenic 318 spleen cells (*C*, *D*, *G*, and *H*). Expansion of the TCR-transgenic T cells (*A*, *C*, *E*, and *G*) was analyzed on day 4 by three-color FACS analysis and is indicated as percentage of $V\alpha2^+V\beta8.1^+$ cells in the CD8 compartment. The activation of TCR-transgenic CTL after DC immunization (*B*, *D*, *F*, and *H*) was monitored using up-regulation of CD44 on TCR-transgenic CTL as a marker. The values indicate the percentage of $CD44^{high}$ cells after gating on $CD8^+V\alpha2^+$ cells. Expansion (*I*), depicted as percentage of $V\alpha2^+V\beta8.1^+$ cells after gating on $CD8^+$ lymphocytes, and activation (*J*) of 318 cells, depicted as percentage of $CD44^{high}$ TCR-transgenic CTL, after transfer of H8-DC or B6-DC pulsed with gp33 at the different concentrations. B6 mice that received 5×10^5 318 spleen cells without DC immunization served as controls. Representative data from one of two experiments are shown.

4*B*) and lost after 2 days (Fig. 4*C*), indicating that B6-DC were efficiently pulsed with gp33 and presented MHC class I-associated peptides only for a limited period of time when pulsed with low doses of gp33. Next, we evaluated the availability of gp33 on H8-DC and B6-DC by blocking with an Ab specific for H2-D^b (141-51; Ref. 23). H8-DC or B6-DC/gp33 were incubated with increasing concentrations of the blocking Ab and subsequently cocultured with 318 spleen cells. The anti-MHC class I Ab inhibited the proliferative response induced by H8-DC and B6-DC pulsed with 10^{-6} and 10^{-8} M gp33 in a similar extent, whereas proliferation induced by B6-DC pulsed with 10^{-10} M gp33 was completely inhibited (Fig. 4*D*). These data suggest that the relative density of the gp33 Ag on H8-DC and B6-DC pulsed with gp33 at high concentrations is, at least initially, equivalent.

To determine the turnover of gp33 on DC in vivo, H8-DC (Fig. 5, *A–D*) or B6-DC/gp33 (Fig. 5, *E–H*) were adoptively transferred into naive recipients, and TCR-transgenic 318 spleen cells were cotransferred either on the same day (Fig. 5, *A*, *B*, *E*, and *F*) or 2 days later (Fig. 5, *C*, *D*, *G*, and *H*). Four days after the transfer of TCR-transgenic T cells, the expansion of gp33-specific CTL was determined by staining for the transgenic TCRs Vα2 and Vβ8.1. In addition, the activation status of the cells was assessed by staining for the activation marker CD44. H8-DC transferred and "parked" in vivo for 48 h were still able to significantly expand and activate naive CTL (Fig. 5, *C* and *D*), compared with H8-DC cotransferred with 318 cells on the same day (Fig. 5, *A* and *B*). Peptide-pulsed B6-DC showed a similar efficiency to activate naive CTL (Fig. 5, *E* and *F*) when cotransferred with 318 cells on the same day. However, B6-DC pulsed with gp33 at 10^{-6} M had lost their ability to expand and activate naive 318 cells after 48 h (Fig. 5, *G* and *H*).

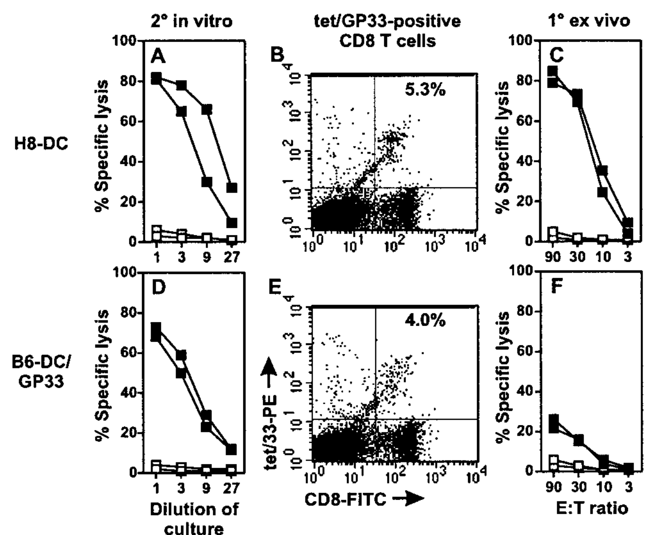

FIGURE 6. CTL expansion and activity after priming with H8-DC or B6-DC exogenously loaded with gp33. B6 mice were immunized i.v. with (*A–C*) 2×10^5 H8-DC or (*D–F*) with 2×10^5 B6-DC/gp33. Eight days later, spleen cells were either (*A* and *D*) restimulated in vitro for 5 days with peptide-labeled, irradiated spleen cells and then tested in a standard 5-h ^{51}Cr release assay, (*B* and *E*) stained with gp33-tetramer vs CD8-FITC and analyzed by flow cytometry, or (*C* and *F*) analyzed immediately for direct ex vivo CTL activity in a 15-h ^{51}Cr release assay. Numbers in B and E indicate percentage of gp33-tetramer-positive cells in the CD8 compartment (mean of three mice per group). Specific lysis was measured on gp33-labeled EL-4 target cells (■) or on EL-4 cells without peptide (□). Spontaneous release was <12% in the 5-h assay and < 28% after 15 h. Representative data from one of three experiments.

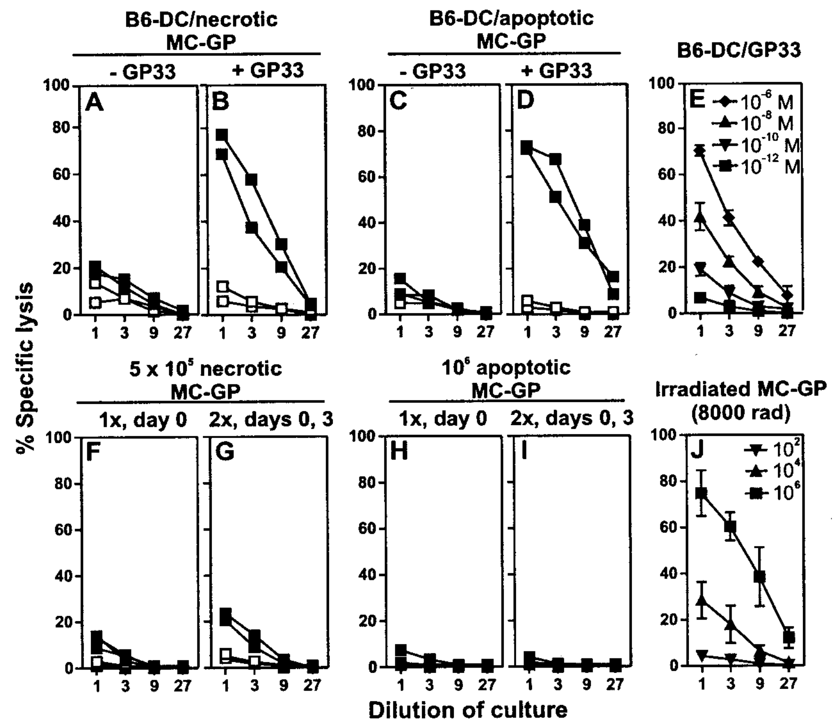

FIGURE 7. Priming of CTL with necrotic or apoptotic fibroblasts. B6-DC were coincubated with either (*A* and *B*) necrotic or (*C* and *D*) apoptitic LCMV-gp expressing MC-gp cells for 6 h a 37°C at a DC: MC-gp ratio of 1:5. As a control for DC function gp33 peptide (final concentration 10^{-6} M) was added for the last 60 min of the incubation period (*B* and *D*). Live DC were recovered by metrizamide gradient centrifugation, washed, and 2×10^5 DC were i.v. injected into naive B6 mice. *E*, Titration of gp33 on B6-DC and induction of gp33-specific CTL after adoptive transfer of 2×10^5 gp33-pulsed DC. Necrotic (*F* and *G*) or apoptotic (*H* and *I*) MC-gp were injected directly into the spleen of naive B6 mice either once on day 0 (*F* and *H*) or twice on days 0 and 3 (*G* and *I*). *J*, Induction of gp33-specific CTL after intrasplenic injection of graded numbers of nonreplicating, irradiated MC-gp. Eight or 10 days after injection of DC or MC-gp, spleen cells were restimulated in vitro for 5 days with peptide-labeled, irradiated spleen cells and tested in a 5-h ^{51}Cr release assay on gp33-labeled EL-4 target cells (closed symbols) or on EL-4 cells pulsed with the irrelevant NP394 peptide (open symbols). Spontaneous release was <12%. Data are from one of two experiments with similar results.

Fig. 5, *I* and *J* summarize these experiments and show that B6-DC pulsed with gp33 at 10^{-6} M or 10^{-8} M, but not with 10^{-10} M, efficiently expanded and activated naive CTL after transfer on day 0. Importantly, after only 1 day, gp33-pulsed B6-DC had lost most of their in vivo-stimulatory capacity for gp33-specific CTL (Fig. 5, *I* and *J*, day 1), indicating that the turnover of gp33 on B6-DC in vivo is rapid and might be a limiting factor for the activation of naive CTL.

Although the persistence of peptide on DC in vivo appeared to be rather short, gp33-pulsed B6-DC still mediated an efficient expansion and up-regulation of activation markers on gp33-specific CTL when cotransferred simultaneously with the TCR-transgenic CTL. Similarly, H8-DC (Fig. 6*A*) and B6-DC/gp33 (Fig. 6*D*) induced comparable polyclonal CTL responses as determined in a ^{51}Cr release assay after secondary restimulation in vitro or by staining with tetrameric MHC class I complexes (Fig. 6, *B* and *E*). In contrast, primary ex vivo cytotoxicity was significantly stronger after priming with H8-DC (Fig. 6*C*) compared with priming with B6-DC/gp33 (Fig. 6*F*), indicating that the short persistence of gp33 on B6-DC limits the differentiation into effector CTL. Collectively, these data suggest that DC presenting exogenously acquired self-peptides can efficiently prime self-reactive CTL. However, the autoimmune potential of these self-reactive CTL is reduced because the differentiation into CTL with effector function is limited by the rather short half-life of the peptides on DC in vivo.

Concomitant induction of T help is required to compensate for the limited activation of self-reactive CTL by DC presenting exogenous peptide

We next examined whether activation of cognate CD4$^+$ Th cells or nonspecific activation signals might influence the response of gp33-specific CTL. This was achieved by either cotransferring TCR-transgenic Th cells specific for the LCMV-gp epitope p13 (Smarta cells; Ref. 18), by nonspecific activation of APC via CD40 (28), or by blocking potential negative regulatory signals, such as inhibition via CTLA-4 (15). After injection of Smarta splenocytes and activation of both Smarta Th cells and gp33-specific CTL by peptide-pulsed DC, RIP-gp mice rapidly developed diabetes, comparable to the kinetics after transfer of constitutively gp33-expressing H8-DC (Table I). Only 1×10^5 DC-activated Smarta splenocytes containing $\sim$1–2×10^4 TCR-transgenic Th cells were sufficient to compensate for the limited activation of gp33-specific CTL by peptide-pulsed DC (Table I). In contrast, in the absence of additional TCR-transgenic Th cells, B6-DC pulsed with gp33 and p13 did not induce diabetes (Table I). Similarly, DC pulsed with p13 alone and coinjected with 1×10^7 Smarta splenocytes failed to elicit a diabetogenic response (Table I), indicating that both islet-specific CTL and relatively large numbers of specific Th cells had to be activated by peptide-pulsed DC to induce a strong autoimmune response. A similar effect was achieved when unspecific "Th-like" signals were provided by repeated injection of a cross-linking anti-CD40 Ab; 33% of the RIP-gp mice treated with gp33-pulsed DC and anti-CD40 developed diabetes (Table I). Interestingly, blocking of CTLA-4 did not lower the threshold for activation of diabetogenic CTL after stimulation with gp33-pulsed B6-DC (Table I). Thus, rather strong signals, provided either by concomitantly activated transgenic Th cells or via stimulation of APC via CD40 were required to convert a weak anti-self response induced by DC presenting exogenously acquired self-peptides into clinically manifest autoimmune disease.

Inefficient presentation of MHC class I-associated peptides by DC after acquisition of exogenous Ags

It has been suggested that acquisition of exogenous Ags, e.g., released from either necrotic or apoptotic cells, by bone marrow-derived APC might be a mechanism for induction of CTL responses (29). To evaluate the role of exogenously acquired self-Ags by DC for the induction of autoimmune responses, we tested the activation of gp33-specific CTL with either apoptotic or necrotic LCMV-gp-expressing fibroblasts (MC-gp). The specific CTL response after adoptive transfer of B6-DC pulsed with necrotic MC-gp was negative (Fig. 7*A*). Similarly, B6-DC exposed to apoptotic MC-gp for 6 h did not elicit a measurable CTL response

Table II. *Induction of autoimmune diabetes or insulitis by apoptotic or necrotic cells in RIP-gp mice*

Immunization Protocol[a]	Additional Treatment[b]	Incidence of Diabetes[c]	Severity of Insulitis[d]
2×10^5 H8-DC s.c. (4×)	None	4/4	+++
10^7 irr. MC-gp s.c. (6×)	None	1/6	+ or ++
10^7 irr. MC-gp s.c. (6×)	10^7 Smarta i.v.	3/6	++ or +++
10^7 apopt. MC-gp s.c. (6×)	None	0/4	–
10^7 necr. MC-gp s.c. (6×)	None	0/6	–
10^7 necr. MC-gp s.c. (6×)	10^7 Smarta i.v.	0/4	+

[a] Mice were immunized s.c. either repetitively with 2×10^5 DC on days 0, 2, and 10 or with 10^7 irradiated MC-gp (irr. MC-gp, 8000 rad), 10^7 apoptotic MC-gp (apopt. MC-gp, 24 h at 43°C), or 10^7 necrotic MC-gp (necr. MC-gp, three freeze-thaw cycles) on days 0, 2, 4, 8, 10, and 12.

[b] Mice received the indicated number of splenocytes from TCR-transgenic Smarta mice on day −1 or were left untreated.

[c] Mice were scored diabetic with blood glucose >14 mM on two consecutive measurements.

[d] Insulitis was classified on day 14 by immunohistochemistry as strong (+++) with severe destructive infiltration in >90% of the islets, moderate (++) with destructive infiltration in 50–90% of the islets, and weak (+) with <50% destructive infiltration and mainly periinsular and perivascular infiltration; (–) no insulitis. Two to four mice were evaluated per group. Fifteen to 20 islets were evaluated from two to three histological sections per mouse.

after adoptive transfer into naive recipients (Fig. 7*C*). However, when the DC were pulsed with gp33 for the last 60 min of the incubation period, a strong CTL response was generated (Fig. 7, *B* and *D*), indicating that the DC were not damaged by the cellular debris and were still capable of activating gp33-specific CTL. The minimal amount of peptide that DC had to be exposed to to induce a minimal CTL response was 10^{-10} M (Fig. 7*E*).

MC-gp express significant amounts of LCMV-gp and are able to directly trigger CTL responses without cross-priming being involved when they reach organized secondary lymphoid organs (19, 30). To assess the immunogenicity of MC-gp that cannot directly activate CTL any longer, we directly introduced necrotic (Fig. 7, *F* and *G*) or apoptotic MC-gp (Fig. 7, *H* and *I*) into the spleen, where splenic DC and other professional host APC could take up and process the cellular proteins and induce gp33-specific CTL. Repeated intrasplenic injection of necrotic MC-gp induced a minimal CTL response (Fig. 7*G*), whereas after single injection of 5×10^5 MC-gp, CTL activation was barely detectable (Fig. 7*F*). Neither single (Fig. 7*H*) nor repeated (Fig. 7*I*) injection of 10^6 apoptotic MC-gp elicited a significant CTL response. The number of necrotic cells had to be reduced, because the cellular debris formed clumps that caused death of the animal when $>5 \times 10^5$ cells were injected directly into the spleen. As mentioned above, MC-gp express considerable amounts of LCMV-gp; therefore, the failure of apoptotic or necrotic MC-gp to elicit a significant CTL response is not likely to be a result of insufficient Ag amount, because only 1×10^4 irradiated, i.e., nonreplicating, MC-gp induced a CTL response after intrasplenic injection (Fig. 7*J*).

We next determined whether repeated delivery of apoptotic or necrotic MC-gp via the s.c. route, where immature dermal and epidermal DC might efficiently pick up and transport the cellular Ag to secondary lymphoid organs, elicits a significant anti-self response in RIP-gp mice. As a control, 2×10^5 H8-DC were repetitively administered s.c. Similar to the i.v. route, s.c. application of H8-DC induced severe insulitis and long-lasting hyperglycemia (Table II). Peripheral s.c. injection of high doses (10^7) of irradiated MC-gp that migrate to secondary lymphoid organs and directly induce CTL responses (19, 30) elicited a weak anti-islet response with mild insulitis and diabetes in one of six mice. Importantly, repetitive application of high doses of necrotic or apoptotic MC-gp did not suffice to induce diabetes or insulitis (Table II). To test whether concomitant delivery of LCMV-gp-specific Th might convert the weak anti-self response after priming with MC-gp into an autoimmune response with pathological consequences, 10^7 Smarta spleen cells were adoptively transferred 1 day before the start of the treatment with MC-gp cells. In the presence of high numbers of islet-specific Th cells, repetitive priming with irradiated MC-gp induced diabetes in 50% of the RIP-gp mice and exacerbated the insulitis in these mice (Table II). Similarly, in RIP-gp mice treated with necrotic MC-gp, the islet-specific Th cells contributed to an aggravation of the islet infiltration leading to mild insulitis (Table II).

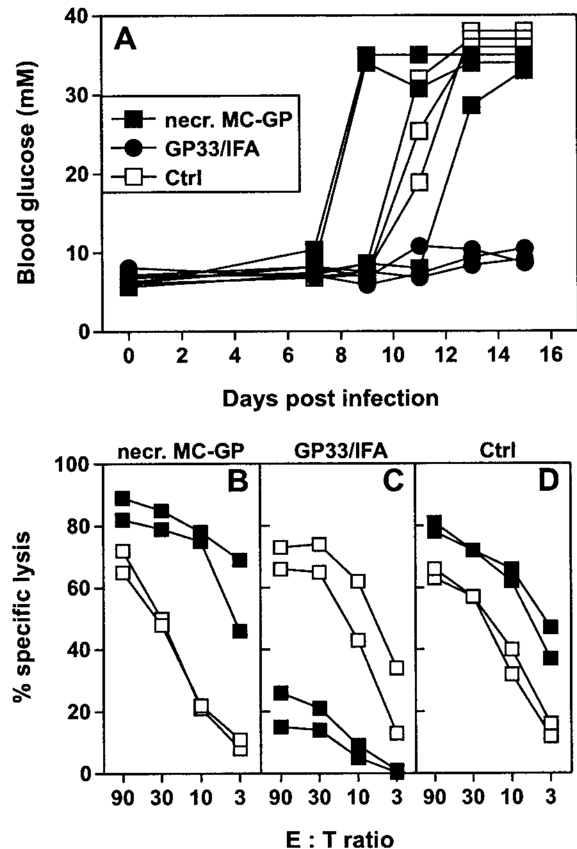

FIGURE 8. Repetitive priming with necrotic MC-gp does not tolerize gp33-specifc CTL. *A*, RIP-gp mice were immunized either 5 times s.c. on days −10, −8, −6, −4, and −2 with 10^7 necrotic MC-gp (necr. MC-gp), three times i.p. on days −10, −6, and −2 with 100 μg of gp33 in IFA (gp33/IFA), or left untreated (Ctrl). On day 0, mice were infected with 200 PFU of LCMV i.v. and blood glucose values were tested on the indicated days. B6 were treated as above with either necrotic MC-gp (*B*), gp33 in IFA (*C*), or left untreated (*D*) and infected with 200 PFU of LCMV i.v. Eight days later, direct ex vivo cytotoxicity was determined in a 5-h ^{51}Cr release assay on gp33-pulsed (closed symbols) or NP396-pulsed (open symbols) targets. Specific release for unpulsed targets was <10%. Spontaneous release was <15%.

One might argue that the failure to detect any autoimmunity after repetitive priming with either high doses of apoptotic or necrotic MC-gp is attributable to induction of tolerance after presentation of the exogenous Ag ("cross-tolerance"; Ref. 29). To test this, RIP-gp (Fig. 8*A*) or B6 (Fig. 8*B*) mice were repetitively immunized s.c. with 10^7 necrotic MC-gp and subsequently challenged with LCMV. Similar to untreated controls, RIP-gp mice primed with necrotic MC-gp developed diabetes between days 8 and 12 (Fig. 8*A*). In addition, B6 mice primed with necrotic MC-gp (Fig. 8*B*) showed a strong CTL reactivity against gp33 as well as the nucleoprotein epitope 396 (NP396) on day 8 after infection with LCMV, comparable to untreated controls (Fig. 8*D*). In contrast, repetitive i.p. administration of 100 μg of gp33 in the mild adjuvant IFA tolerized gp33-reactive CTL, but not NP394-reactive CTL (Fig. 8*C*) and therefore prevented LCMV-induced diabetes in RIP-gp mice (Fig. 8*A*). Taken together, these data clearly show that, in our model system, MHC class I presentation of cell-associated Ags derived from cellular debris is rather inefficient and did not lead to tolerization of self-reactive CTL. Importantly, even the presence of high numbers of islet-specific Th cells did not convert the weak anti-self CTL activity after priming with necrotic MC-gp into a self-perpetuating and clinically manifest autoimmune response.

Discussion

Our study shows that stringent thresholds limit the induction of self-reactive CTL by DC after acquisition of exogenous peptides or cell-associated Ag. Importantly, our data indicate a clear hierarchy for the induction of autoimmunity by DC: priming with DC constitutively expressing a self-peptide elicited strong anti-self CTL responses and clinically manifest autoimmune disease, whereas the rapid turnover of exogenously loaded, MHC class I-associated peptides on DC allowed expansion of self-reactive CTL but limited differentiation of CTL into effectors and thereby restricted the development of autoimmunity. In addition, the rather low, if at all measurable, efficiency of DC to present exogenously acquired Ag via MHC class I is probably of prime importance to avoid self-perpetuating, CTL-mediated autoimmune responses.

Duration of self-Ag presentation by DC is a critical parameter for autoimmune responses

During their ontogeny, DC undergo characteristic functional changes that allow Ag acquisition in peripheral nonlymphoid organs during the immature stage and, after maturation, efficient Ag presentation in secondary lymphoid tissues. The turnover of MHC class II-associated peptides in vitro is high on immature DC, whereas inflammatory stimuli leading to DC maturation significantly enhance the half-life of MHC class II molecules and prolong Ag presentation (8). Similarly, the turnover of MHC class I-associated peptides on human DC in vitro appears to be regulated by maturation stimuli such as virus infections (31). Our comparison of exogenously peptide-pulsed DC with DC constitutively expressing the respective peptides allowed evaluation of the significance of peptide persistence on DC in vitro and in vivo. We found that the half-life of gp33 on nontransgenic DC is short and thereby limits the activation of gp33-specific CTL. This is most likely attributable to the spatial distribution and the initially rather rare cognate interaction between adoptively transferred DC and the responding CTL. In vivo, DC and responding CTL firstly have to home to secondary lymphoid organs and, secondly, most probably have to go through a number of noncognate interactions before meeting the correct partner.

Our data clearly show that the amount of self-peptides on DC and the duration of peptide presentation by DC in secondary lymphoid tissues is of prime importance for the induction of anti-self CTL responses and the degree of tissue destruction. Constitutively gp33-presenting DC efficiently expanded the gp33-specific CTL pool and induced differentiation of the CTL into effectors. In contrast, although the initial expansion of gp33-specific CTL precursors was comparable, exogenously peptide-pulsed DC could not mediate complete differentiation of gp33-specific CTL. Therefore, it appears that the prolonged presentation of peptide by DC for 2–4 days is important to mediate both expansion and differentiation of CTL. Extended presence and/or high doses of Ag in local proliferation/differentiation clusters of APC with CTL might be a prerequisite for optimal induction of T cell responses as suggested by Mitchinson and O'Malley (32). Accordingly, gp33-pulsed DC had to be adoptively transferred repetitively over several days and in higher doses to achieve diabetogenic CTL responses in our model situation.

The failure to induce autoimmunity because of the limited persistence of exogenous peptide on DC could be compensated for by simultaneous induction of strong T help provided by simultaneously activated TCR-transgenic Th cells. The observed effect of Th may be a result of various mechanisms: bystander help to limited CTL responses, activation of IFN-γ or TNF effector pathways, or enhanced maturation or activation of DC, perhaps by stimulation via CD40 (28, 33). Indeed, injection of an activating anti-CD40 mAb was able to partially compensate for the reduced immunogenicity of peptide-pulsed DC. However, the addition and activation of specific Th cells was more efficient than CD40 ligation in lowering the threshold for obtaining autoimmunity in response to DC presenting exogenous self-peptides. It has been shown that DC also mature rapidly during cognate interaction with CTL, even in the absence of CD40 (34), indicating that a number of Th cell signals are required to mediate complete CTL activation in the three-cell interaction between DC, Th cells, and CTL. CTLA-4 has been suggested to be important for the maintenance of peripheral tolerance by inhibiting T cells that received only low-level costimulation for brief periods (35). However, inhibition of T cell activation via CTLA-4 does not appear to be involved in the present system as blockade of CTLA-4 ligation was not able to increase the ability of DC presenting exogenous self-Ag to mediate autoimmunity. The presented system of DC-induced autoimmune disease appears to be particularly well suited to further investigate the Ag-specific interactions of the three cell types and their importance of CTL and Th cell activation and subsequent induction of an autoimmune disease.

A role for cross-presentation by DC in the induction of autoimmunity?

The genetically regulated cognate interaction between target cells and activated CTL resulting in target cell lysis has been termed "MHC restriction" (36). However, it has also been shown that exogenous, cell-associated proteins may gain access to the MHC class I presentation pathway via "cross presentation" (12). In addition, cross-presentation has been demonstrated in transgenic situations, where model Ags such as OVA (37) or influenza hemagglutinin (HA; Ref. 14) are expressed in peripheral nonlymphoid tissues. In OVA-transgenic mice, the peripheral Ag is presented via MHC class I in draining secondary lymphoid organs by bone marrow-derived APC (37). However, it appears that whenever the expression of a self-Ag is high and widespread, central (thymic) tolerance eliminates self-reactive, high-avidity T cells (37, 38). Local and intermediate expression of self-Ags, such as HA in RIP-HA mice, may also lead to elimination of high-avidity anti-self CTL by deletional mechanisms possibly involving cross-presentation via MHC class I (14, 39). However, complete deletion of

self-reactive CTL via cross-priming in the "intermediate expressor" situation of RIP-HA mice appears to be rather inefficient, because >100 days were needed for the elimination of only 10^4 self-reactive, high-avidity CTL (14).

Spontaneous autoimmunity that is mediated by cross-presentation can only be detected in "high expresser" situations with widespread expression of transgenic OVA, when central tolerance mechanisms are bypassed by transfer of TCR-transgenic CTL (13). Similarly, peripheral tolerance mechanisms that prevent spontaneous autoimmunity, e.g., in RIP-HA mice (39), can be circumvented by crossing with TCR-transgenic animals, thereby providing great numbers of self-reactive T cells that can either not be tolerized by the peripheral tolerizing mechanism (40, 41), or perhaps may be accidentally activated too frequently when compared with a "normal" low-frequency CTL precursor situation. In contrast, in transgenic situations with lower and more restricted expression of the model self-Ag, e.g., OVA (5) or LCMV-gp (2) in pancreatic islet cells, the presence of high numbers of specific TCR-transgenic T cells does not induce autoimmunity, despite potential release of self-Ag from pancreatic islets and subsequent cross-presentation in local lymph nodes (5). Therefore, it appears that self-perpetuating autoimmunity attributable to cross-presentation is a phenomenon that appears in certain transgenic constellations where T cell frequencies are extremely high and/or central tolerance mechanisms are bypassed. The data presented in this report strongly support this interpretation because not even a minimal autoimmune response was detected in RIP-gp when large amounts of self-Ag in the form of apoptotic or necrotic cells were repetitively injected. Only in the presence of high numbers of specific TCR-transgenic Th cells did a mild insulitis develop after injection of necrotic LCMV-gp expressing fibroblasts. However, in this situation as well, a self-perpetuating and progressive autoimmune response did not develop and the mice stayed normoglycemic. Taken together, it appears that high threshold levels for presentation of exogenous Ags via MHC class I prevent self-perpetuating autoimmune disease.

Use of exogenously peptide- or tumor protein-pulsed DC in antitumor vaccination

The migratory function and strong Ag-presenting properties make DC excellent vehicles for transport of tumor Ags into secondary lymphoid organs to facilitate induction of antitumor immunity. Indeed, DC are used currently in many tumor treatment programs as a means to generate primary antitumor immune responses or to enhance the existing tumor immunity (42, 43). However, Ag-specific tumor immunotherapy can be only successful if the tumor Ag has been immunologically ignored previously, i.e., specific, high-avidity T cells have not been centrally or peripherally deleted, and antitumor T cells can be induced by appropriate immunization. Therefore, it is possible to apply the conclusions from the present report also for the induction of tumor immunity with DC. First, tumor-specific CTL are probably most efficiently induced when a previously ignored tumor Ag is constitutively expressed by the DC. A number of approaches have been described to achieve in vitro transfection of DC with tumor Ags (44, 45). Second, exogenous pulsing of DC with tumor peptides is less efficient, because the rapid in vivo turnover of MHC class I-associated peptides limits the induction of specific CTL or requires additional exogenous stimuli, such as activation of APC via CD40. Finally, exogenous pulsing of DC with whole tumor proteins is probably very inefficient. It has been shown that either large amounts of pure protein or, alternatively, special delivery vehicles such as microbeads are required to overcome this inefficiency of exogenous loading of MHC class I molecules by DC (46).

In conclusion, we have demonstrated that the rapid turnover of MHC class I-associated peptides and the rather inefficient presentation of exogenous cellular self-Ags are likely to be basic and important mechanisms to limit the autoimmune consequences of self-Ag presentation by DC.

Acknowledgments

We thank Lenka Vlk and Anne Henzelin for expert technical assistance and Norbert Wey and Ida Schmieder for excellent photography.

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