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CUTTING EDGE

Cutting Edge: Dissociation Between Autoimmune Response and Clinical Disease After Vaccination with Dendritic Cells

Attilio Bondanza,* Valérie S. Zimmermann,* Giacomo Dell'Antonio,[†] Elena Dal Cin,[†] Annalisa Capobianco,* Maria Grazia Sabbadini,*[††] Angelo A. Manfredi,* and Patrizia Rovere-Querini*

Autoimmunity represents a caveat to the use of dendritic cells (DCs) as adjuvant for human vaccines. We derived DCs from normal BALB/c mice or from mice prone to autoimmunity (NZB × NZW) F_1. We allowed DCs to phagocytose apoptotic thymocytes and vaccinated syngeneic animals. All mice developed anti-nuclear and anti-dsDNA Abs. Autoantibodies in normal mice were transient, without clinical or histological features of autoimmunity or tissue involvement. In contrast, autoimmunity was maintained in susceptible mice, which underwent renal failure and precociously died. The data suggest that DC vaccination consistently triggers autoimmune responses. However, clinical autoimmunity develops in susceptible subjects only. The Journal of Immunology, 2003, 170: 24–27.

T he identification of tumor-associated Ags prompted an effort toward the development of immunotherapeutic strategies. Dendritic cells (DCs)[3] loaded with tumor Ags have been used in patients with melanoma, renal cell carcinoma, colon, breast and ovarian cancer, lymphoma and prostate cancer, with evidence of enhanced T cell immunity and, in some cases, clinical benefit. Whole dying tumor cells represent a source of tumor Ags. DCs that phagocytosed apoptotic tumor cells activate tumor-specific T cells in vitro and in vivo (1–14). Dying tumor cells contain a whole array of tumor Ags in a physical form that ensures their optimal uptake by DCs and the simultaneous activation of MHC class I- and II-restricted T cells. DCs "resurrecting" Ags from dying cells have been suggested to play a role in the initiation of autoimmune diseases. This may impose limitations on antitumor therapies since normal cells and their transformed counterparts share most Ags (15, 16). DC vaccination is accompanied by the activation of autoreactive MHC class I-restricted cytotoxic T cells, with destruction of tissues expressing relevant Ags (15). Different factors, including the turnover of MHC/peptides complexes and the inefficient processing of cell-associated Ags contribute to limit the activation of autoreactive T lymphocytes and to quench the phenomenon (17). In this study, we show that DCs that phagocytosed dying cells are sufficient to initiate systemic autoimmunity in vivo, leading to the development of anti-nuclear Abs (ANA) and anti-dsDNA Abs. However, in normal animals, the autoimmune response is only transient. The genetic predisposition of the recipient determines whether the break of tolerance is sustained, leading to clinical and histological evidence of life-threatening autoimmune disease.

Materials and Methods

Mice

BALB/c female mice (Charles River Breeding Laboratories, Calco, Italy) and the offspring of New Zealand Black and New Zealand White mice (NZBW F_1; The Jackson Laboratory, Bar Harbor, ME) were housed in a pathogen-free animal facility in accordance with European Community guidelines.

Cell death

Thymocytes were retrieved from 6- to 8-wk-old BALB/c or NZBW F_1 mice and gamma-irradiated (600 rad). We used FITC-labeled annexin V (Bender MedSystems,Vienna, Austria) to assess the exposure of phosphatidylserine and propidium iodide (PI) to evaluate plasma membrane integrity. Cells were analyzed at 0, 4, 9, and 18 h after irradiation by flow cytometry (BD Biosciences, Mountain View, CA).

Dendritic cells

Bone marrow precursors were propagated for 7 days in medium containing recombinant murine GM-CSF (1000 U/ml) and IL-4 (5 ng/ml) (BD PharMingen, San Diego, CA).

Phagocytosis

DCs were coincubated with gamma-irradiated apoptotic cells (5:1 ratio) at 37°C or at 4°C for 3 h. Cell suspensions were treated with trypsin/EDTA for 5 min at 37°C and DCs were separated from unengulfed apoptotic cells and retrieved by magnetic sorting, using beads conjugated with anti-CD11c Abs (MiniMacs; Miltenyi Biotec, Bologna, Italy). Phagocytosis was evaluated by flow cytometry (1).

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[3] Abbreviations used in this paper: DC, dendritic cell; ANA, anti-nuclear Ab; PI, propidium iodide.
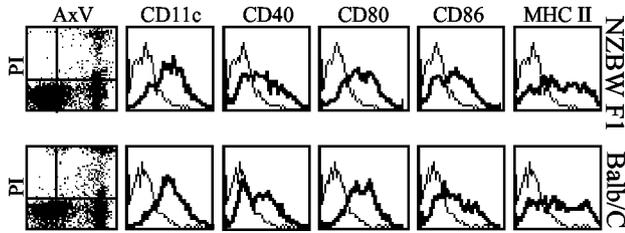

FIGURE 1. DC vaccination. Thymocytes from NZBW F_1 or BALB/c mice were committed to apoptosis by gamma irradiation and analyzed by flow cytometry (*left plots*) after staining with PI (*y*-axis) and FITC-annexin V (*x*-axis). DCs were derived from bone marrow precursors, allowed to phagocytose syngeneic apoptotic cells and injected in vivo (see *Materials and Methods*). Histograms show the expression of membrane molecules from NZBW F_1 and BALB/c DCs, evaluated by flow cytometry immediately before in vivo injection.

Autoantibodies

ANA were assessed by immunofluorescence. Murine TSA cells were used as a substrate. Intensity of the fluorescence was assessed based on the lightmeter's estimated required exposure time. Samples >120 s were considered negative. Values for samples <120 s were calculated dividing 120 by the estimated required exposure time. Anti-dsDNA Abs were assessed by the *Crithidiae luciliae* assay (Immunoconcepts, Sacramento, CA).

Clinical and pathological assessment

Mice were studied from 2 to 12 mo of age or until cohorts were sacrificed. Serum and urine samples were collected every 14 days. Histological assessment was performed at 24 wk of age and included heart, lungs, liver, brain, and kidneys. Organs were fixed in 4% paraformaldehyde and included in paraffin. Sections were stained with H&E. Ig deposits were assessed on tissues frozen in OCT (Tissue Tek-Miles, Elkart, IN). Kidneys for electron microscopy were fixed in 2% gluteraldehyde, embedded in Epon resin, and stained with uranyl acetate and lead citrate. An expert pathologist (G.D.A.) evaluated 297 blinded sections by optical, fluorescence, and electron microscopy and scored each one of them, singularly, from zero to four (normal to increasing severity).

Vaccination

Mice were vaccinated s.c. at 6 wk of age with 200,000 syngeneic DCs, which phagocytosed or not apoptotic cells, or with PBS. Vaccination was repeated after 7 days (age: 7 wk), after 14 days (age: 8 wk), and after 126 days (age: 24 wk).
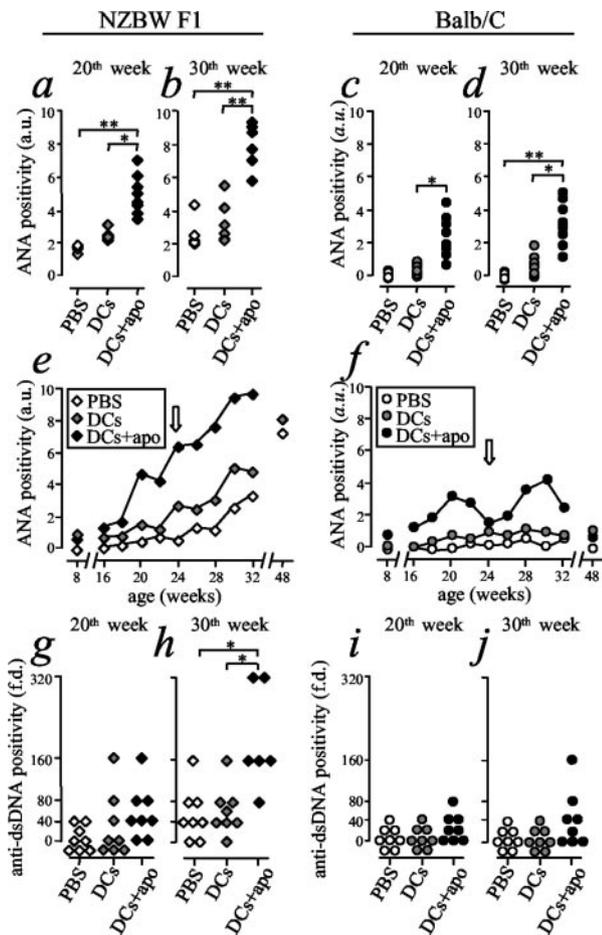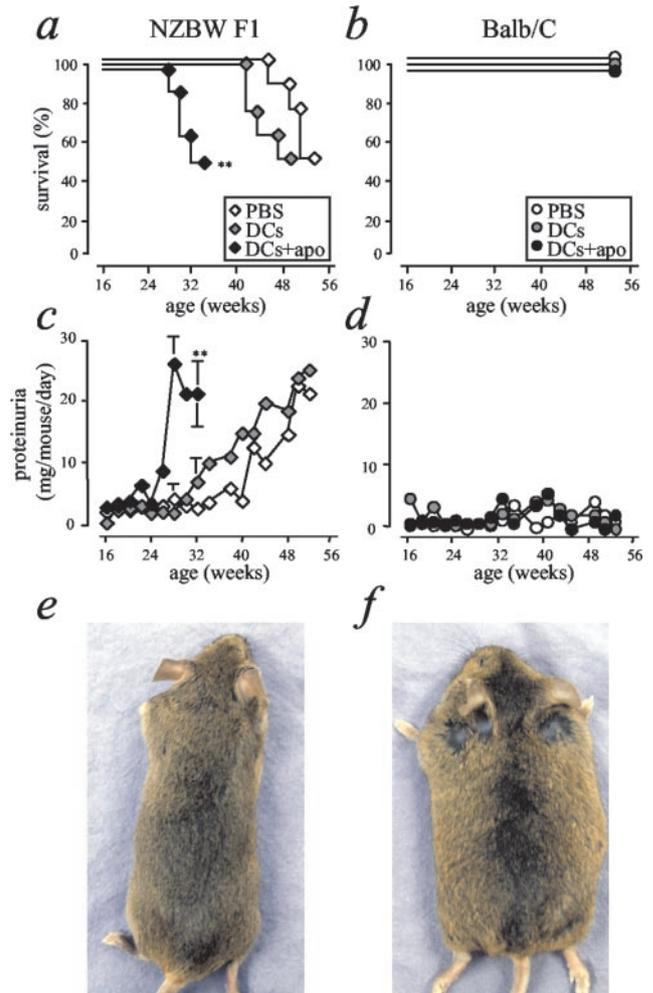

FIGURE 2. Autoantibodies. ANA (arbitrary units) and anti-dsDNA (fold dilutions) Abs were assessed after vaccination of NZBW F_1 mice (diamonds) or BALB/c (circles) with DCs that phagocytosed syngeneic irradiated cells (filled symbols), with DCs alone (gray symbols) or PBS (empty symbols). Data refer to mice at 20 (NZBW F_1, *a* and *g*; BALB/c, *c* and *i*) or 30 wk of age (NZBW F_1, *b* and *h*; BALB/c, *d* and *j*). *e* and *f*, ANA kinetics from representative NZBW F_1 and BALB/c mice. Student's *t* test for ANA and χ^2 test results for anti-dsDNA are: *, $p < 0.05$ and **, $p < 0.01$.

FIGURE 3. Clinical assessment. NZBW F_1 mice (diamonds) or BALB/c (circles) vaccinated with DCs that phagocytosed syngeneic irradiated cells (filled symbols), with DCs alone (gray symbols) or with PBS (empty symbols) were studied. *a* and *b* contain Kaplan-Meyer plots of survival and *c* and *d* proteinuria ± SEM (milligrams per mouse per day, *y*-axis) over time (weeks of age, *x*-axis) from NZBW F_1 and BALB/c, respectively. Fisher's exact and Student's *t* tests results for survival and proteinuria of mice vaccinated with DCs that phagocytosed apoptotic cells compared with controls are: **, $p < 0.01$. *e* and *f*, Pictures of representative 32-wk-old NZBW F_1 mice vaccinated with untreated DCs or DCs that phagocytosed irradiated syngeneic cells, respectively.
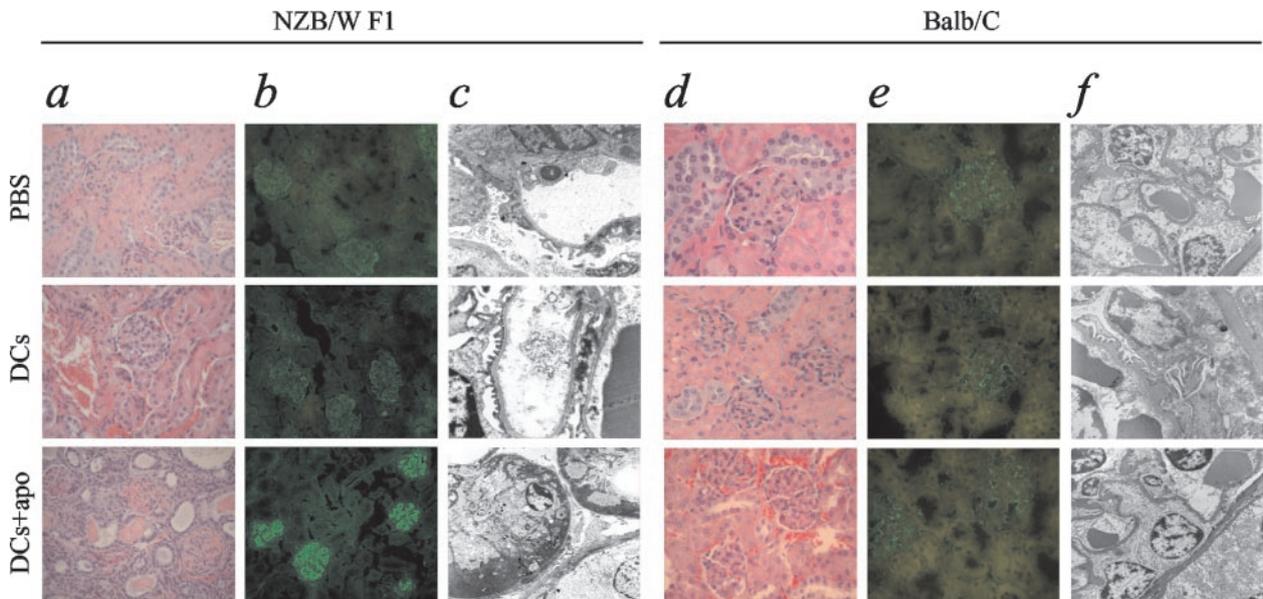
FIGURE 4. Kidney involvement. Optical microscopy sections (H&E) from NZBW F_1 (*a*) and BALB/c (*d*) mice vaccinated with PBS, untreated DCs, or DCs that phagocytosed irradiated cells. BALB/c and NZBW F_1 mice vaccinated with PBS or DCs have normal glomeruli. Diffuse proliferative glomerulonephritis with enlarged mesangium, proliferation in the Bowman's space, and fibrinoid necrosis (original magnifications: ×250) is evident in NZBW F_1 vaccinated with DCs that phagocytosed irradiated cells. Fluorescence microscopy sections (FITC-conjugated anti-mouse IgG staining) from NZBW F_1 (*b*) and BALB/c mice (*e*). NZBW F_1 mice vaccinated with DCs that phagocytosed irradiated cells have extensive mesangial and capillary immune deposits without glomerular basal membrane staining (original magnifications: ×400). Electron microscopy (uranyl acetate and lead citrate staining) from NZBW F_1 (*c*) and BALB/c (*f*). Large electron dense deposits are present in the mesangial and subendothelial regions of glomeruli from NZBW F_1 mice vaccinated with DCs that phagocytosed irradiated cells (original magnifications: ×12,000). Optical, fluorescence, and electron microscopy analysis was performed on all vaccinated mice sacrificed at 32 wk of age (see Table I).

Statistical analysis

Statistical analyses were performed using two-tailed Student's, χ^2 (Microsoft Excel), or Fisher's exact (GraphPad Prism) tests. Results were considered statistically significant for $p < 0.05$.

Results

We propagated DCs from bone marrow precursors of BALB/c and NZBW F_1. We irradiated thymocytes and verified by flow cytometry that most cells exposed anionic phospholipids, indicating that they underwent apoptosis (Fig. 1). Furthermore, they excluded PI, indicating that they were still in the early phase of the process. The extent and kinetics of apoptosis of cells from BALB/c or NZBW F_1 mice were similar. We allowed DCs to phagocytose apoptotic cells and retrieved them by magnetic bead sorting of CD11c$^+$ cells. In these conditions, most

DCs (consistently >70%) internalized apoptotic thymocytes. Phagocytosis abated at 4°C, i.e., a temperature that does not allow the reorganization of the actin-based cytoskeleton (63 ± 11% at 37°C vs 7 ± 2% at 4°C, $p < 0.01$).

DCs from BALB/c and NZBW F_1 mice expressed before or after phagocytosis similar levels of molecules involved in T cell activation and costimulation, including CD40, CD80, CD86, and MHC class II molecules (Fig. 1 and data not shown). We injected BALB/c or NZBW F_1 mice with DCs that phagocytosed syngeneic apoptotic cells and assessed serological, clinical, or pathologic evidences of autoimmunity. As a control we injected mice with DCs alone or with PBS.

All animals injected with DCs that phagocytosed apoptotic cells developed high titers of autoantibodies (Fig. 2). They developed statistically significant higher titers of ANA than mice immunized with untreated DCs or PBS (NZBW F_1: wk 20 of age, $p < 0.05$ vs DCs and $p < 0.01$ vs PBS-injected animals; wk 30 of age, $p < 0.01$ vs both DC- or PBS-injected animals, Fig. 2, *a* and *b*; BALB/c: wk 20, $p < 0.05$ vs both DC- or PBS-injected animals; wk 30, $p < 0.05$ vs DCs and $p < 0.01$ vs PBS-injected animals, Fig. 2, *c* and *d*). NZBW F_1 mice also developed higher titers of anti-dsDNA (wk 30, $p < 0.05$ vs both DC PBS-injected animals, Fig. 2, *g* and *h*). Fig. 2 also shows that vaccination with DCs that did not phagocytose irradiated cells did not influence ANA and anti-dsDNA Ab titers.

Titers of autoantibodies were similar in 20-wk-old injected BALB/c or NZBW F_1 mice (Fig. 2, *a* and *c*). At wk 30 of age (Fig. 2, *b* and *d*), NZBW F_1 mice titers of ANA and anti-dsDNA Abs were higher than those of BALB/c (Fig. 2, $p < 0.05$). Fig. 2, *e* and *f*, depicts results obtained in representative BALB/c and NZBW F_1 mice. NZBW F_1 mice ANA titers remained higher than control animals and raised steadily in

Table I. *Pathological kidney score*

	OM^a	FM^b	EM^c
NZBW F1			
PBS	1/8	2/8	1/8
DCs	1/6	3/6	1/6
DCs + apo^d	6/7**	7/7**	5/7*
BALB/c			
PBS	0/4	0/4	0/4
DCs	0/4	0/4	0/4
DCs + apo	0/4	0/4	1/4

^{a} No. of mice showing an optical microscopy (OM) score ≥2/total analyzed.
^{b} No. of mice showing an optical microscopy (FM) score ≥2/total analyzed.
^{c} No. of mice showing an optical microscopy (EM) score ≥2/total analyzed.
^{d} apo, apoptotic cells.
*, $p < 0.05$ (χ^2 test results for DCs that phagocytosed irradiated cells compared to controls).
**, $p < 0.01$ (χ^2 test results for DCs that phagocytosed irradiated cells compared to controls).

boosted animals. In contrast, BALB/c ANA titers dropped to baseline levels. A second self-limiting Ab response developed in BALB/c mice further vaccinated at 24 wk of age.

Fig. 3a shows that 12.5% of NZBW F_1 vaccinated with DCs that phagocytosed irradiated cells were dead after wk 28 of age and 50% of animals died by wk 32. At this time point we sacrificed surviving animals and controls. Surviving animals had overt clinical involvement, with a significant increase of the body weight, consistent with anasarcatic state due to end-stage renal disease (average weight $\pm$ SD: mice injected with DCs that phagocytosed irradiated cells 43.7 $\pm$ 9.4 g, mice injected with DCs alone 31.2 $\pm$ 4.3 g, mice injected with PBS 28.4 $\pm$ 3.2 g, $p < 0.05$ vs both DC- or PBS-injected animals, Fig. 3, e and f). BALB/c mice were unaffected (Fig. 3, b and d). Proteinuria in vaccinated NZBW F_1 mice was the most strikingly affected clinical parameter (Fig. 3c). We failed to detect any acceleration in kidney involvement in mice injected with syngeneic DCs alone, which behaved as PBS-injected mice.

BALB/c tissues were healthy. The brain, heart, lung, and liver of NZBW F_1 injected with PBS, DCs alone, and DCs that internalized apoptotic cell were also normal (data not shown). The kidney of NZBW F_1 mice vaccinated with DCs that internalized apoptotic cells exhibited diffuse proliferative glomerulonephritis, with mesangial and capillary hypercellularity, cellular crescents, and fibrinoid necrosis (Fig. 4a). Kidney involvement and deposition of immune complexes were confirmed by immunofluorescence and electron microscopy (Fig. 4, b and c). Healthy NZBW F_1 mice injected with PBS or DCs alone, or BALB/c mice, regardless of the treatment they received, revealed no significant glomerular disease (Fig. 4 and Table I).

Discussion

Stringent constrains regulate the ability of T cells to cause autoimmunity. Two linked events are limiting: 1) the amount and the duration of MHC-restricted epitope presentation in lymph nodes; 2) the ability of DC to process soluble Ags for MHC class I presentation (16, 17). Immature DCs efficiently process cell bodies, presenting derived epitopes in association with MHC molecules. Vaccination of rodents with DCs that phagocytosed in vitro dying tumor cells leads to the recruitment of long-lasting immune responses, endowed with memory and specificity (1, 2, 10, 12). Conversely, DCs that phagocytosed human dying cancer cells prime antineoplastic T lymphocytes in vitro (3, 6, 13, 14). Although the mode of cell death (apoptosis vs necrosis) influences the efficiency of the response in different systems, the cross-presentation of epitopes of dead tumor cells has been substantiated by virtually all studies.

Dead tumor cells share most Ags with their nontransformed counterparts. Immunization of patients with DCs that phagocytosed apoptotic tumor cells is likely to elicit autoimmune responses (18). Our results are consistent with this hypothesis. We vaccinated healthy mice, predisposed or not to the development of autoimmunity, with DCs that phagocytosed apoptotic thymocytes. All vaccinated mice developed autoantibodies. Autoantibodies in normal mice progressively disappeared and did not cause autoimmune disease or tissue damage. In contrast, animals predisposed to autoimmunity developed progressive and eventually lethal organ involvement.

Thymocytes represent a source of syngeneic cells, for which the response to apoptotic stimuli is well-characterized. We used a more stringent vaccination schedule compared with previous

reports (1, 10). However, we cannot exclude that different routes or schedule of vaccination could be more efficient in eliciting autoimmunity. Furthermore, the altered gene expression in tumor cells committed to apoptosis and the disease-related immune depression might also influence the outcome of DC vaccination.

Our data are consistent with the lack of clinically evident autoimmunity after vaccination with DCs. In the absence of clinical disease, transient autoantibody responses in vaccinated patients may go undetected. Therefore, the use of DCs that phagocytosed dying tumor cells should be relatively safe. However, extreme caution should be used in the treatment of neoplastic patients with a predisposition to the development of autoimmunity, as suggested by the familial and personal clinical history and by the demonstration of clinical and laboratory features of ongoing autoimmunity.

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