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Systemic Autoimmune Disease Induced by Dendritic Cells that have Captured Necrotic but not Apoptotic Cells in Susceptible Mouse Strains

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Abbreviations: DNA: deoxyribonucleic acid; Anti-dsDNA: antibody against double stranded DNA; DC: dendritic cells; Nec: necrotic cells; Apo: apoptotic cells; DC/nec: DC loaded with necrotic cells; DC/apo: DC loaded with apoptotic cells; ICs: immune complexes.

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disorder of a largely unknown aetiology. Anti-double stranded DNA antibodies (anti-dsDNA) are a classical hallmark of the disease, the mechanism underlying their induction remains unclear. We demonstrate here that, in lupus-prone and normal mouse strains, strong anti-dsDNA antibody responses can be induced by dendritic cells (DCs) that have ingested syngeneic necrotic (DC/nec), but not apoptotic (DC/apo), cells. Clinical manifestations of lupus were evident however only in susceptible mouse strains, which correlate with the ability of DC/nec to release IFN- γ and to induce the pathogenic IgG2a anti-dsDNA antibodies. Injection of DC/nec not only accelerated disease progression in the MRL/MpJ-*lpr/lpr* lupus-prone mice but also induced a lupus-like disease in the MRL/MpJ-*+/+* wild-type control strain. Immune complex deposition was readily detectable in the kidneys, and the mice developed proteinuria. Strikingly, female MRL/MpJ-*+/+* mice that had received DC/nec, but not DC/apo, developed a ‘butterfly’ facial lesion resembling a cardinal feature of human SLE. Our study therefore demonstrates that DC/nec inducing a Th1 type of responses, which are otherwise tightly regulated in a normal immune system, may play a pivotal role in SLE pathogenesis.

Introduction

SLE is a prototypic systemic autoimmune disease characterized by the production of a variety of autoantibodies which are directed predominantly against nuclear antigens [1]. In both SLE patients and the lupus mouse models, the presence of anti-DNA antibodies, especially anti-dsDNA antibodies, is closely correlated with clinical disease and hence of diagnostic and even prognostic value [2]. Purified native mammalian DNA is however a weak immunogen [3, 4]. It is not clear whether these anti-DNA responses are induced against somatic DNA, or represent cross-reactivity of the antibodies to pathogens – ‘molecular mimicry’ [5], or an ‘anti-idiotypic response’ [6]. A recent model proposes that some uncommon modifications or handling of self-tissue components, including nucleic acids, may explain such altered auto-antigenicity, and that the modified self-antigens are most likely to originate from dying cells [7-10]. The macrophage-mediated mechanism for removal of dying cells is defective in some autoimmune diseases including SLE [11]. As a consequence, dying cells (e.g. following infection or even due to normal tissue/cell turnover) accumulate and the cellular contents normally-shielded from the adaptive immune system may be released acting as the “auto-antigens”. A key question is still however “what is the process that renders DNA ‘immunogenic’?”.

DCs are potent antigen presenting cells (APC) playing a key role in the initiation of immune responses, and they may take up dying cells for antigen processing and presentation [12]. We report here that DCs loaded with necrotic cells (DC/nec) but not apoptotic cells (DC/apo) are powerful inducers of anti-DNA antibody response. Injection of syngeneic DC/nec not only accelerated disease progression in the MRL/MpJ-*lpr/lpr* (MRL/*lpr*) lupus-prone mice but also induced a lupus-like disease in the MRL/MpJ-+/+ (MRL/+) wild-type control strain. However, despite the fact that

high levels of anti-dsDNA antibodies were also detected in the DC/nec-treated C57BL/6 mice, these animals failed to develop clinical disease. We show further evidence indicating that the isotype of anti-dsDNA antibodies are also critical. The DC-induced pathological changes in susceptible mouse strains correlate closely with the ability of these cells to mediate a Th1 type of response. This includes the release of high levels of IFN- γ and reduced level of IL-10 *in vitro* and, more importantly, the preferential induction of pathogenic IgG2a anti-dsDNA autoantibodies that readily form renal immune complex deposits *in vivo*.

Results

Anti-DNA antibody responses.

To test the hypothesis that anti-DNA responses can be induced by DCs that had captured nuclear antigens in the form of dying cells leading to lupus disease, we generated DCs from mouse bone marrow. The cells were then allowed to phagocytose either necrotic or apoptotic splenocytes *in vitro*, before being injected intravenously into syngeneic young mice (*see Methods*). Three strains of mice were tested: the MRL/*lpr* strain which carries a defective *Fas* gene and develops a spontaneous lupus-like disease as they age [13, 14], the wild-type MRL/+ strain that express intact *Fas*, and a normal inbred C57BL/6 strain. After injection of the DCs pre-loaded with necrotic cells into young mice at 5-weeks of age, highly though transiently elevated serum anti-dsDNA was detected in the MRL/*lpr*, but not the MRL/+ (<30U/ml), strain (Data not shown). However, when DC/nec were injected into adult mice (11 weeks of age), strong and rapid anti-dsDNA responses were detected not only early in the MRL/*lpr* lupus mouse strain, but also in the wild-type MRL/+ as well as the C57BL/6 control strains (Fig. 1, filled circles). In particular, sera from the adult female MRL/+ mice 4 weeks after treatment with DC/nec contained anti-dsDNA antibodies at levels comparable with that of aged (>6-month) and terminally-ill MRL/*lpr* lupus mice (Fig. 1, histograms as indicated). In contrast, when the mice were injected with DCs loaded with apoptotic cells (Fig. 1, filled triangles, and histogram labelled DC/apo), levels of anti-dsDNA antibodies were significantly lower, similar to the mice treated with DCs alone (histogram labelled DC). DC may become activated by the handling process or they may take up some dying cells derived during DC generation accountable for the small amount of antibodies induced. Minimal amounts of serum anti-dsDNA antibodies were detected in the groups treated with

necrotic cells alone (Nec), apoptotic cells alone (Apo), PBS or untreated controls (Fig. 1, open circles and histograms indicated). In the MRL/*lpr* strain, however, the differences between DC/nec and DC/apo-treated groups or the treated groups over the untreated control group were less clear due to higher individual variability within the treated groups and the spontaneous age-dependent anti-DNA antibody production in the control group. Nevertheless, compared to the control MRL/*lpr* mice at some time points tested, the DC/nec-treated group displayed significantly higher serum anti-dsDNA levels, whereas the DC/apo-treated group displayed decreased levels (* Fig. 1, lower left panel, n = 5). The kinetics of DC-induced anti-DNA responses was similar in both female and male mice, but the levels were in general lower in the males (data not shown). Serum anti-single stranded DNA antibody (anti-ssDNA) were also highly elevated in the DC/nec-treated MRL/+ mice which correlated kinetically and proportionally with the anti-dsDNA levels (data not shown).

Lupus-like facial skin lesions.

One of the typical clinical manifestations of human lupus is the facial erythema, the so-called ‘butterfly rash’. In Latin, the term ‘lupus’ means wolf and it has been used since mediaeval time to describe the erythemic ulcerations which could ‘eat away’ the face [15]. We first noticed that adult female MRL/+ mice (wild-type control MRL strain) that had received 3 injections of DC/nec, uniformly developed ‘butterfly’ facial skin lesions resembling the human butterfly rash (Fig. 2a-b). The facial lesions started at around 3-months post-injection of DCs with hair loss, some presented with ear and eye lesions, and by 4-months all female mice in the group developed such lesions to different degrees (Fig. 2a, upper panel). By around 5-months post-injection, the lesions developed extensively extending above the eyebrow (Fig. 2b, F). Fig. 2c

shows results from a separate experiment in which female MRL/+ mice that had received 2 (instead of 3) injections of DC/nec also developed such typical facial lesions, though at much a later stage (Fig. 2c, 11-months post injection). In a total of 10 DC/nec-treated female MRL/+ mice from 3 separate experiments (n = 3, 3, 4; treatment started at the age of 11-week), 9 developed such lesions including 5 of them extensively (results from Exps. I and II are shown in Fig. 2). In contrast, no such lesion was observed in mice treated with DC/apo, DC, or PBS-treated control group (Fig. 2a, 2c), or in necrotic cells alone (Nec) or apoptotic cells alone (Apo) treated groups (data not shown). Neither male MRL/+ mice (Fig. 2b, M) nor C57BL/6 mice of either sex that received DC/nec or DC/apo developed the rash. Some localised facial lesions were also seen early on in the DC/nec-treated MRL/*lpr* mice similar to those observed in the IL-18-treated MRL/*lpr* mice reported by us previously [16]. However, the lesions were less extensive than those observed in the DC/nec-treated MRL/+ mice, probably because the MRL/*lpr* mice died soon after the treatment, before the lesions could fully develop.

Histological examination of the affected skin showed a focal lymphohistiocytic infiltrate in the interstitium of the papillary dermis and around the bulbs of hair follicles (Fig. 2d [ii], [iii]). Detailed examination of the alopecic skin revealed a significant increase in the number of catagen, and decrease in anagen, hair follicles, as evidenced by scattered apoptotic bodies in the outer root sheath, disappearance of the inner root sheath and progressive thickening, hyalinization and corrugation of the vitreous membrane (Fig. 2d[v], and histograms). Anagen and catagen are two types of hair follicles at different phases of hair cycling. In normal skin, majority of hair follicles are anagens (~90%) which are in the phase of active hair production, while

catagens are in the involutionary stage of the hair cycle characterized by the loss of mitotic activity being short lasting and development of apoptotic cell death in the outer root sheath[17, 18]. The present findings indicate that a process of catagenization within the skin with mild peri-bulbar follicular inflammation can be induced following such abrupt but transient insults of DC/nec injection. The DC/nec-induced histopathology, although not exactly the same as the typical chronic lupus dermatitis in human disease, is more in keeping with a non-scarring alopecia such as the allied autoimmune skin disease of alopecia areata also observed clinically in lupus patients [17, 18].

Proteinuria and renal histopathology.

Glomerulonephritis, the renal involvement caused by immune complex deposition and complement activation, is a severe complication of lupus disease and represents the major cause of death [19]. We next assessed the renal pathological changes in the DC-treated mice. MRL/*lpr* lupus mice develop age-dependent spontaneous and progressive proteinuria, which is not seen in untreated MRL/+ or C57BL/6 mice up to 7 months of age (Fig. 3a, right panel). Following injection of DCs, however, high levels of proteinuria were observed in the DC/nec-treated MRL/+ mice (Fig. 3a, left panel), at levels similar to those seen in aged MRL/*lpr* mice (5-6 months, female, right panel) with overt clinical disease, but not in the similarly treated C57BL/6 mice. In contrast, significantly lower levels of proteinuria were detected in MRL/+ mice treated with DC/apo, DCs alone (*DC*), apoptotic (*Apo*) or necrotic (*Nec*) cells alone. Acceleration in proteinuria development was also observed in MRL/*lpr* mice following injection of DC/nec. However, unlike the MRL/+ mice which gradually recovered after cessation of treatment, the MRL/*lpr* strain developed progressively

severe proteinuria and died early. Light microscopy showed cellular proliferation in the glomeruli (glomerular hypercellularity) of female MRL/+ mice treated with DC/nec but not with DC/apo (data not shown). Immunostaining for IgG revealed substantial immune complex (IC) deposition in kidneys of the DC/nec-treated, but not the DC/apo-treated, female MRL/+ mice (Fig. 3b). Such deposits were found mainly within the affected glomeruli, some were focalised around the hilar region (Fig. 3b[i]) while others were more diffusely distributed (Fig. 3b[ii]). This was further confirmed by electron microscopy showing large electron dense deposits in the mesangial areas, with expansion of the mesangium (Fig. 3c, arrows). The DC/nec-induced renal pathology and proteinuria were generally more prominent and appeared earlier in female than male MRL/+ mice, but such gender differences were less clear cut for the MRL/*lpr* strain. No lymph node enlargement or splenomegaly was found in treated or untreated MRL/+ and C57BL/6 mice. No gross pathology was detected in the joints, lungs, heart and liver of these mice.

Anti-dsDNA antibody isotype and DC cytokine expression profile.

The lupus-like disease induced in the wild type control MRL/+ strain following treatment with DC/nec can be first explained by the strong anti-dsDNA responses, which reached levels similar to those seen in terminally ill MRL/*lpr* lupus mice (Fig. 1 histograms). However, the failure to induce clinical disease (skin lesions, proteinuria and renal pathology) in C57BL/6 mice by DC/nec or DC/apo, despite the presence of considerable levels of anti-DNA antibodies (Fig. 1 upper right panel), prompted us to analyse the potential contribution of the anti-dsDNA antibody isotypes so-induced in disease induction, and the DC functional activities upon interactions with different types of dying cells.

To investigate the mechanisms underlining these different effects observed above, DC functional phenotypes and their abilities to produce cytokines including the Type I (IFN- γ , IL-12, IL-23, IL-18) and Type II (IL-4, IL-5, IL-10) related cytokines following ingestion of dying cells were examined. After triggering by necrotic cells, DCs showed marginal up-regulation of MHC class II, CD80, CD86 and CD40 (data not shown). Although these DCs did not spontaneously secrete significant amount of most of the cytokines mentioned above (Fig. 4a open bars), their responsiveness to further stimulation altered dramatically. Shortly after stimulation with LPS (16h), DC/nec but not DC/apo or control DCs (*DC*) from MRL/+ mice secreted large amounts of IFN- γ , producing up to 53 folds more IFN- γ than the similarly stimulated control DCs, together with a moderately increased IL-10 activity (Fig. 4a left panels, filled bars). In contrast, whereas control DCs from C57BL/6 mice produced some levels of IFN- γ , DC/nec from this mouse strain displayed a suppressed IFN- γ activity, yet the production of IL-10 instead was much enhanced (Fig. 4a right panels, filled bars). No or only background levels of IFN- γ and IL-10 were detected in cultures of either necrotic or apoptotic cells alone, in the presence (Fig. 4b) or absence (data not shown) of LPS. Kinetic study indicated that the differences in IFN- γ level did not correlate with differences in IL-12 activity, and the early phase of IFN- γ release by MRL/+ DC/nec in particular was independent of IL-12 activity (Fig. 4b). Fig. 4b also shows that IL-12 p70 production by DC/nec and DC/apo from both MRL/+ and C57/BL6 strains of mice is significantly suppressed instead when compared to that of DCs alone (*DC*). In addition, the production of IFN- γ did not correlate with IL-12/IL-23 (p40) and IL-18 activities either, and only low levels of IL-4 and IL-5 were detected in the DC cultures tested (data not shown).

We next studied the *in vivo* relevance of the above findings. Isotypic analysis of the serum anti-dsDNA antibodies revealed that certain isotypes of these autoantibodies were differentially induced in the mouse strains following treatment with DC/nec, DC/apo or DC. As shown in Fig. 5a, anti-dsDNA antibodies of the IgG1 isotype were readily detectable in the treated mice of both MRL/+ and C57BL/6 strains, as well as the IgM and IgG2b isotypes in the treated C57BL/6 mice. However, the levels of these antibodies were generally comparable among the three differently treated groups of either strain. In contrast, in the MRL/+ mice, serum levels IgG2a anti-dsDNA antibody levels were significantly higher in the DC/nec-treated mice (Fig. 5a, IgG2a, left panel), compared to that of DC/apo or DC alone treated groups. Such enhanced IgG2a anti-dsDNA response was however not seen in similarly treated (DC/nec) C57BL/6 mice (Fig. 5a, IgG2a, right panel). This was further confirmed by using a monoclonal antibody (Clone R19-15, *BD Biosciences, San Jose*) which was raised against C57BL/6 IgG2a antibody subclass (data not shown). The close correlation of the heightened IFN- γ activity (Fig. 4), enhanced anti-dsDNA IgG2a antibody production (Fig. 5a), and the disease development (Figs. 2, 3) induced by DC/nec in MRL/+ mice suggests a potential role of Th1 type of response in lupus pathogenesis, since IFN- γ is the prototypic Th1 cytokine important for IgG2a antibody isotype switching [20].

We then also carried out detailed assessments of the renal ICs using IgG1 and IgG2a isotype-specific antibodies. This revealed that ICs containing IgG1 and IgG2a were both present in the kidneys of DC/nec-treated MRL/+ mice. There was no significant difference in the frequency of IgG1 ICs between the mice treated with DC/nec and

with DC/apo. However, kidney sections from the DC/nec-treated female MRL/+ disease mice exhibited strong IgG2a positive glomerular deposits with a frequency significantly higher than those from mice treated with DC/apo or untreated control mice (Fig. 5b, histograms). More importantly, whereas the IgG1 ICs were generally weaker and more diffusely distributed, the IgG2a ICs were much stronger and focalised around glomerular hilar regions (Fig. 5b photograph, in red), a pattern similar to that seen in aged MRL/*lpr* mice. By comparison, minimal IC deposits for both IgG1 and IgG2a were detected in the DC/nec and DC/apo-treated C57BL/6 mice. Similar results were obtained by using the isotypic (IgG2a) specific polyclonal (Fig. 5b) and the R19-15 monoclonal (data not shown) antibodies.

Discussion

Our present findings show that systemic autoimmune responses including anti-dsDNA antibodies and tissue immunopathology can be induced *in vivo* by DCs that have acquired nuclear antigens derived from somatic dying cells, especially of the necrotic form. This is intriguing because necrotic cell death is usually associated with inflammation or infection, and many autoimmune diseases are indeed accompanied or induced by chronic infections [21]. The maturation and activation state of DCs is known to be critical in determining their immuno-adjuvantivity, which can be enhanced by pro-inflammatory stimuli such as bacterial products (e.g. LPS) as well as cellular contents released by necrotic cells (“endogenous danger signals” [22-25]. In contrast, apoptotic cell death is generally associated with normal tissue turnover or removal of virally infected, tumour or other unwanted cells in a controlled manner [26, 27]. During such programmed cell death, toxic cellular contents are largely degraded and normally prevented from extensive exposure to the immune system. In agreement with these notions, uptake of necrotic, but not apoptotic, tumour cells by DCs has been shown to trigger DC maturation resulting in up-regulation of co-stimulatory molecules and antigen-presenting activity of the cells [22, 28], and to enhance the antitumor immunity [29]. Injection of apoptotic thymocytes or DCs primed with apoptotic thymocytes failed to induce clinical disease in non-autoimmune mice, despite an anti-DNA response being elicited [9, 30]. Apoptotic cells may develop to become secondary necrotic if not removed timely [31]. It is possible that the limited responses of DCs loaded with apoptotic cells observed here and in these previous studies are due to secondary necrotic changes in cells resulted from handling processes, and that have exceeded the dying cell removal threshold. Another possibility is that the apoptotic and necrotic cell preparations may contain different

amounts of nuclear antigens contributing to the observed differential induction of anti-DNA responses. However, total DNA purified from the two types of dying cells was found to be comparable following incubation at 37°C for 3 hrs (before co-culturing with DCs). After an overnight incubation that was at the time immediately before injection, the amount of DNA recoverable from the cultured necrotic cells (10.06±1.16 µg, per 5 million cells of origin) was no more than that of apoptotic cells (14.38±0.96 µg). Nevertheless, we provide direct evidence here that, unlike MRL DCs that have been conditioned by necrotic cells, apoptotic cell-loaded DCs induce only low level of anti-DNA antibodies, especially of the IgG2a isotype, *in vivo* and are insufficient to cause systemic disease in the mouse models studied.

The uptake of necrotic cells that provides both the nuclear antigens and the endogenous signals for DC maturation may account for their ability to induce anti-DNA antibody response *in vivo*. We also show here that MRL DCs after being primed by necrotic cells are hypersensitive to LPS stimulation to produce high levels of IFN- γ *in vitro*. IFN- γ is a Th1 cytokine known to be crucial for IgG2 antibody isotype switching in mouse. Therefore, both endogenous and exogenous signals appear to be required for the DCs to mediate such a Th1 type response, and this may explain the ability of these cells to induce preferentially IgG2a anti-dsDNA antibodies *in vivo*. However, the direct *in vivo* relevance of LPS to the present experimental model is still yet to be established, which may be associated with the role of environmental pathogens. All the animals on experimental schedule in the present study shown were housed in conventional animal facility, and it will be interesting to compare them with those kept under specific pathogen free or even germ-free condition in future studies.

IgG2 anti-DNA antibodies are thought to be more pathogenic than other subclasses for many of their immunophysiological features [13, 32-34]. A switch from IgM to IgG2a and IgG2b subclasses has been previously shown to herald the onset of severe disease in the NZB/W lupus-prone mouse model [35] and, more recently, these IgG2 anti-dsDNA antibodies were found to be more frequently present in lupus patients with renal disease [36]. The lack of clinical manifestations in the DC/nec-treated C57BL/6 mice despite significant serum anti-dsDNA total IgG (Fig. 1) levels, which is also in agreement with a recent report [37], may therefore be explained by differences in the quality of the antibody responses induced (Fig. 5). The unique induction of IgG2a anti-dsDNA response exclusively in the DC/nec-treated mouse group of the MRL/+ but not C57BL/6 strain (Fig. 5a), which is consistent with the very antibody isotype associated with the immune complex deposits readily detectable in the affected kidneys (Fig. 5b), indicates that a Th1 type of response induced *in vivo* may be essential for the development of clinical disease. We have shown previously that systemic injection of the Th1-related, pro-inflammatory cytokines IL-12 α /and IL-18, accelerated lupus disease progression in MRL/*lpr* mice [16, 38]. IL-12 and IL-18 are generally thought to act by stimulating T cells and NK cells to produce IFN- γ , but recent evidence indicates that, apart from T (Th1) and NK cells, DC may also be a direct source of IFN- γ [39]. Here we show that DC/nec from MRL/+ mice may release directly high levels of IFN- γ independent of IL-12 and IL-18 activities. IFN- γ may also contribute directly to the lupus pathology by activating macrophages to produce elevated level of nitric oxide which has also been strongly implicated in the lupus pathogenesis [38]. Furthermore, type I interferons (IFN- α/β) are known to play important roles in SLE pathogenesis[40]. In particular, IFN-alpha has been shown to induce DC differentiation driving autoimmune response in SLE [41]. It will therefore

be interesting to determine whether the expression of these cytokines may also be modulated in DCs upon interactions with dying cells.

In a normal immune system, the expression of the pro-inflammatory cytokines is under tightly controlled regulatory mechanisms. IL-10 is a potent immunosuppressive cytokine controlling Th1 responses. Elevated serum IL-10 levels have been associated with lupus disease activity [42]. This may reflect a desperate yet failed attempt of the immune system to down-regulate the autoimmune responses. We show here that, in response to LPS stimulation, DC/nec from C57BL/6 mice produce much elevated levels of IL-10. The differences between MRL/+ and C57BL/+ strains of mice in the levels of IL-10 activity induced (Fig. 4) suggest that such feedback regulatory mechanism may be lacking in the MRL mice. Taken together, these findings suggest that, upon triggering by inflammatory stimuli, DC/nec from MRL/+, but not C57BL/6, mice produce high levels of IFN- γ mediating a Th1-biased response. Consequently, this may result in elevated IgG2a auto-antibody synthesis leading to the lupus-like pathological changes. The failure to induce a Th1-biased anti-DNA response in the C57BL/6 mice may explain the absence of clinical disease in this mouse strain, possibly reflecting a strong negative feedback regulatory mechanism depending at least in part on IL-10 activity. In addition and in agreement with what is commonly seen in autoimmunity, the gender differences (anti-DNA response, skin and renal pathology) observed in the present study indicate that the sex hormones also play a role in the DC-induced autoimmune responses.

The genetic basis of SLE is complex, involving contributions from multiple genes [43]. The MRL/*lpr* mouse strain is a widely used classical animal model for human

SLE. *Lpr*, identified as a mutation in *Fas* (CD95), interferes with T cell maturation and normal cell renewal process, allowing the release of auto-reactive T cells into the periphery and possibly the generation of 'bad' cell death [10]. Homozygosity for *lpr* results in a severe lupus-like disease in these mice [14]. However, other *lpr* congenic strains of mice that possess the *lpr* mutation develop lymphoproliferation but not always the lupus disease, depending on their genetic background [44]. At least two additional susceptibility loci in the MRL background have been mapped which are associated with nephritis [45]. We show here that anti-DNA responses followed by a lupus-like disease can be induced in the susceptible MRL/+ mouse strain independent of the *Fas* defect, and without the associated lymphoproliferation. Our present findings are based on the murine models and how these may be translated into the human disease is an important issue remains to be addressed. Our observations also suggest that while it is possible to induce autoimmunity by "DC vaccination" in susceptible individuals, this is insufficient to sustain a chronic disease and that in the absence of continuous "antigen stimulation" disease progression eventually subsides.

Materials and Methods

Mice

MRL/MpJ-*lpr/lpr* (MRL/*lpr*), MRL/MpJ-*+/+* (MRL/*+*) and C57BL/6 strains of mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and set up as breeding pairs in the Specific Pathogen Free Laboratory Animal Unit, University of Hong Kong Medical School. Animals undergoing experimental procedures were kept in a conventional animal facility. All experiments involving live animals were carried out strictly according to the protocol under a licence approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 550-01), the University of Hong Kong.

DC generation and uptake of dying cells *in vitro*

DCs were propagated from mouse bone marrow precursors for 7 days in medium containing murine GM-CSF (2% culture supernatant of the X63-Ag8 cell line kindly provided by Professor A Neil Barclay and Mr. MJ Puklavec, the MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford) and IL-4 (4 ng/ml, *PeptoTech EC Ltd*, London). Necrotic cells were generated by freezing (-80 °C, 12 minutes) and thawing (37 °C) syngeneic splenocytes (optimised to obtain maximum cell death without cell lysis) and necrosis confirmed by trypan blue staining. Apoptotic cells were prepared by UV irradiation (200 mJ/cm²) followed by 3 hours culturing at 37 °C, and cell death assessed by propidium iodide incorporation and annexin-V staining (*PharMingen*). The generation of apoptotic and necrotic cells was optimized by varying the dose of UV light for apoptotic cells and the numbers of freeze-thaw cycles as well as freezing time for necrotic cells. The optimised final conditions used for the study were: for apoptotic cells by UV irradiation at 200

mJ/cm²/s, followed by 3h culture in serum free medium; and for necrotic cells by one cycle of freezing at -80⁰C in fixed volume aliquots (0.5ml) and thawing in water bath at -37⁰C. Bone marrow DCs were then co-cultured overnight with the dying splenocytes (1:5 DC:dying cells) *in vitro*. Typically 60~70% of DCs (MHC class II⁺ and CD11c⁺) took up either necrotic or apoptotic cells (assessed by pre-labelling of the cells with CFSE) with similar efficiency as determined by flow cytometry (data not shown). DCs were then enriched by a single step density separation (*NycoPrep 1.068, Oslo, Norway*). For some experiments, DCs were further purified by using *MACS* bead-conjugated antibodies specific for mouse CD11c (*Miltenyi Biotec, UK*). DC purity and functional phenotypes were assessed by flow cytometry using antibodies to DC surface markers (MHC class II, CD11c, CD80, CD86 and CD40), and by their cytokine expression profiles (see below). To quantify DNA contents of the dying cells after overnight culture at 37⁰C, the apoptotic and necrotic cells were sampled, lysed and the total DNA purified using a commercial DNA purification kit (*QIAamp, QIAGEN, Valencia, USA*) before the DNA concentrations were measured at 260nm on a UV spectrophotometer.

Cell adoptive transfer

One million DCs loaded with either necrotic or apoptotic cells, or DCs alone; or 5 million apoptotic or necrotic cells alone, were injected in PBS intravenously into each mouse. Three or two injections were given at 14 days intervals starting at the age of 4 to 5-weeks (young) or of 11-weeks (adult).

Detection of anti-DNA responses

The mice were bled every two weeks by retro-orbital puncture under halothane anesthesia and serial dilutions of the sera tested for anti-DNA antibodies using standard ELISA protocols [46] for the detection of anti-dsDNA and anti-ssDNA antibodies. DNA antigen: Type I calf thymus DNA (*Sigma*, D-1501). For anti-dsDNA, the DNA-coated plates were treated with S1 nuclease (3 units/ml, *Invitrogen*, 18001-016) for 90 minutes at RT. Isotype-specific secondary antibodies for murine IgM, IgG, IgG1, IgG2a and IgG2b, conjugated with either horseredish peroxidase (HRP-goat anti-mouse IgM or IgG; *Sigma*, *Saint Louis*, *USA*) or alkaline phosphatase (AP-rabbit anti-mouse IgG1, IgG2a or IgG2b; *Zymed*, *San Francisco*), were used at optimum concentrations as determined individually by pre-titration. Serum anti-DNA levels were titrated (serial 4 fold dilutions) and presented as arbitrary units determined by using pooled sera from terminally ill aged (5-6 months) MRL/*lpr* mice as the standard, which was set as (units per ml): 1,000 (total IgG) and 100 (IgG1, IgG2a, IgG2b or IgM) for anti-dsDNA; and 10,000 for anti-ssDNA (total IgG). The standard sera were stored at -80 °C in aliquots and used as an internal quantitative reference throughout the study.

Cytokine measurements

Cytokine production by DCs *in vitro* with or without dying cell treatment (as described above), both spontaneously or in response to LPS stimulation (5µg per ml added 4 hours after co-culture with dying cells), was determined by using commercial ELISA kits for murine IL-12 (p70), IL-12/IL-23 (p40), IL-18, IFN-γ, IL-10, IL-4 and IL-5 (*OptEIA™ sets*; *BD Biosciences*, *San Diego*, *CA*, *USA*). Samples (culture

supernatants) were collected from replicated cultures at different time intervals (3, 16, 24, 48 and 72 hours after LPS stimulation) and measured without dilution.

Clinical and pathological assessments

Proteinuria and hematuria were assessed using a commercially available kit (*Multistix*, Bayer Diagnostic, South Wales, UK), graded according to manufacturer's instructions and shown as mg/dL: 0 (negative), 10 (trace), 30 (+), 100 (++), 300 (+++) and 1000 (++++). At the end point of each experiment, the mice were sacrificed and tissues collected including skin, kidneys, spleen, lymph nodes, liver, joints, hearts and lungs. Tissues were fixed in 4% buffered formaldehyde, embedded in paraffin, and sections stained with H&E for histological examinations. Glomerular cellularity was analysed by a computer-assisted program: a sector of renal cortex showing about 40 glomeruli was randomly selected from each kidney section. The four largest glomeruli within a selected sector were individually photographed using a digital photomicrographic system (*Nikon Digital Camera System DXM1200*). Images were captured at a resolution of 2560x2048 pixels. Glomerular size was measured by manual tracing of the capsular basement membrane on the computer image of a glomerulus using a mouse device under ImageJ v1.30 (*Wayne Rusbund, National Institutes of Health, U.S.A.*). Glomerular cellularity was assessed by counting the number of nuclei within a defined unit area. Renal Ig deposits were assessed on frozen kidney sections by immunostaining using either FITC-conjugated anti-mouse IgG (Sigma, USA), or AP-conjugated rabbit anti-mouse IgG1 or IgG2a (Zymed, San Francisco) followed by the alkaline phosphate substrate, new fuchsin, naphthol AS-B1 and 2-amino-2-methylpropan-1,3 diol with levamisole to block the endogenous alkaline phosphatase activity. For electron microscopy (*Philips CM100*), tissues were fixed in 2.5%

gluteraldehyde, embedded in Epoxy resin, stained with uranyl acetate and lead citrate. For immunocytochemistry, the tissues were snap frozen and embedded in OCT compound (*Tissue Tek, Sakura Finetek, USA*). For lymphocyte phenotypic analysis, splenocytes and lymph node cells were stained with antibodies to T (CD3, CD4, CD8) and B (CD19) cell markers (*BD PharMingen*) and analysed by flow cytometry.

Statistics: *Student's T test* was used throughout for statistical analysis, and considered p value ≤ 0.05 (*) being significant and ≤ 0.01 (**) highly significant.

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Figure legends

Figure 1 **Anti-dsDNA responses induced *in vivo* following adoptive transfer of DCs in young and adult mice.** Adult (11-weeks of age) female mice of 3 different strains were injected 3 times (arrows) at 14 days intervals with one million DCs that had been fed with either necrotic (DC/nec) or apoptotic (DC/apo) cells, or with DCs alone (DC), necrotic cells alone (Nec), apoptotic cells alone (Apo), PBS only (PBS) or without treatment (Control). Serum samples were taken at Week 0 then bi-weekly intervals thereafter the first injection, and tested by ELISA for anti-dsDNA antibodies (IgG). Data shown are means \pm SEM calculated from individual mice of each group to illustrate changes in time kinetics (curves, $n = 3\sim 5$). For graphic clarity, data for DC alone, dying cell alone and PBS treated groups (MRL/+ strain) only at 4-week-post-injection are shown in comparison (histograms, data combined from 2 experiments, $n = 6$). Antibody titres in sera from aged-terminally-ill female MRL/lpr mice ($n = >20$) are shown for comparison. * or * $p \leq 0.05$, ** or ** $p \leq 0.01$; * depicts significant difference between DC/nec and DC/apo-treated groups, and * depicts significant difference between the indicated treated and other treated or untreated control groups as determined by the *Student's T test*. (†) depicts the time point of >50% mortality observed.

Figure 2 **Facial lesions manifested in female MRL/+ mice following treatment with DC/nec.** 11-week female (*a*, *c*, *d*; & *b* labelled **F**) and male (*b*, labelled **M**) MRL/+ mice were injected for 3 times (*a*, *b*, *d*), or 2 times (*c*), with DC/nec, DC/apo, DC as described in Fig. 1. *a*. 18-weeks after injection, lupus-like facial skin lesions were observed in all ($n = 3$) female mice injected with DC/nec (upper panel), but not DC/apo, DC or PBS-treated (Control) mice; *b*. At 22-weeks

post-treatment, all 3 female (F) MRL/+ mice treated with DC/nec developed full-blown facial skin lesions (Exp. I), but similarly treated male (M) mice were all free of such skin lesion. *c.* In a separate experiment (Exp. II), female MRL/+ mice were injected as described above but for 2 times (instead of 3). The facial skin lesions were observed again in all of the 3 mice treated with DC/nec (2 of which extensively at 50-weeks post-treatment), but not in any of the DC/apo-treated mice. *d.* Skin histology (H&E x40): [i][iv] Normal non-alopecic control mouse facial skin showing vertical [i] and transverse [iv] sections of normal non-inflamed anagen hair follicles, with inset in [iv] highlighting normal majority anagen follicles (x100); [ii] focal lymphohistiocytic infiltrate (circled) in interstitium of papillary dermis of a DC/nec-treated mouse; [iii] alopecic facial skin showing two anagen hair follicles with a moderate peribulbar follicular lymphohistiocytic infiltrate with inset showing higher power (x100) of an affected follicle; [v] alopecic facial skin showing numerous catagen hair follicles with three catagen follicles highlighted in inset (arrows, x100). *Histograms:* statistical assessment of hair follicles in skin of different treatment groups (number of catagens in 100 hair follicles counted blindly, *Mean* \pm *SEM*, *n* = 3, **p* \leq 0.05, ** *p* \leq 0.01, *Student's T test*).

Figure 3 Proteinuria and glomerular histopathological changes induced by DC/nec. *a. Proteinuria. Left panel:* Female MRL/+ and C57BL/6 mice were injected with syngeneic DC/nec, DC/apo, DC alone (*DC*), necrotic (*Nec*) or apoptotic (*Apo*) cells alone, or PBS (*Control*) (*n* = 3~6). *Right panel:* levels of the spontaneous proteinuria developed in untreated MRL/*lpr* mice at different age (*n* = 13-25) in comparison with that of the MRL/+ and C57BL/6 strains (all females). Proteinuria was monitored bi-weekly (*see Methods*). Data shown (mg/dL) are means \pm *SEM*,

calculated from individual mice of each group. *ND*: not done. **b.** *Immunostaining for total IgG* (fluorescent microscopy): Ig deposits were assessed on frozen kidney sections after staining with a FITC-conjugated goat-anti-mouse IgG antibody, and examined by confocal microscopy. Both focal [i] and global [ii] staining patterns were seen in the affected glomeruli of the DC/nec-treated female MRL/+ mice. **c.** *Electron microscopy* confirmed large electron dense deposits present in the glomerular mesangial regions only in the DC/nec-treated mice (arrows, scale bar = 2 μ m). * $p \leq 0.05$, ** $p \leq 0.01$ (*Student's T test*).

Figure 4 Comparison of the cytokine expression profiles of DC/nec from MRL/+ and from C57BL/6 mice. **a.** *Differential IFN- γ and IL-10 production by DC/nec from MRL/+ and C57/BL6 mice in response to LPS.* DCs were generated *in vitro* from mouse bone marrow precursors, enriched by density separation (*NycoPrep 1.068*) and co-cultured with necrotic (DC/nec) or apoptotic (DC/apo) cells as described previously (see Methods). Culture supernatants were collected from the DC/nec, DC/apo and control DC alone (*DC*) cultures, with (filled bars) or without (open bars) LPS stimulation (5ug/ml, added 4 h after cell co-culturing) for 16 hours at 37 $^{\circ}$ C. IFN- γ , and IL-10 production was determined by ELISA. **b.** *Kinetics of cytokine production.* Culture supernatants were collected at time intervals (16, 48, 72 and 96 hrs) from DC/nec, DC/apo, control DC alone (*DC*), necrotic cells alone (Nec) or apoptotic cells alone (Apo) after LPS stimulation. IFN- γ , IL-10, IL-12 (p70) and IL-12/23 (p40) production was determined by ELISAs. No or only background levels of the cytokines were detected in cultures of necrotic or apoptotic cells alone with LPS. Data shown are means \pm standard deviations (*SD*). * $p \leq 0.05$, ** $p \leq 0.01$ (*Student's T test*). The experiments were repeated for more than 3 times with consistent results.

Figure 5 **Isotypic analysis of the serum anti-dsDNA antibodies and renal immune complex deposits in the DC-treated MRL/+ and C57BL/6 mice.** *a. Serum anti-dsDNA antibody isotypes.* Serum samples were taken from adult female MRL/+ and C57BL/6 mice four weeks-post-injection of DC/nec, DC/apo or DC alone, and from age-sex-strain-matched untreated mice (*Control*). Anti-dsDNA antibodies of IgG1, IgG2a, IgG2b and IgM isotypes were determined by ELISA using murine isotypic specific antibodies (see *Methods*). Data presented are means \pm SEM of titration units from individual mice of each group (n = 3). *b. Isotypic analysis of renal ICs.* Renal Ig deposits were assessed by immunostaining using AP-conjugated rabbit anti-mouse IgG1 or rabbit anti-IgG2a (positivity shown in red, light microscopy, X200). For comparison, ICs positive for IgG1 and IgG2a on kidney sections from aged MRL/*lpr* mice with spontaneous disease are shown in the bottom micrographs. *NRS*: normal rabbit serum (negative control). For statistical analysis (histograms), the slides were randomly labelled and scored blindly for numbers of glomeruli positively stained for IgG1, IgG2a and total IgG (as indicated). Data shown are means \pm SEM calculated from individual mice of the same group (n = 3). * $p \leq 0.05$, ** $p \leq 0.01$ (*Student's T test*). The experiments were repeated for 3 times with consistent results.

Figure 1

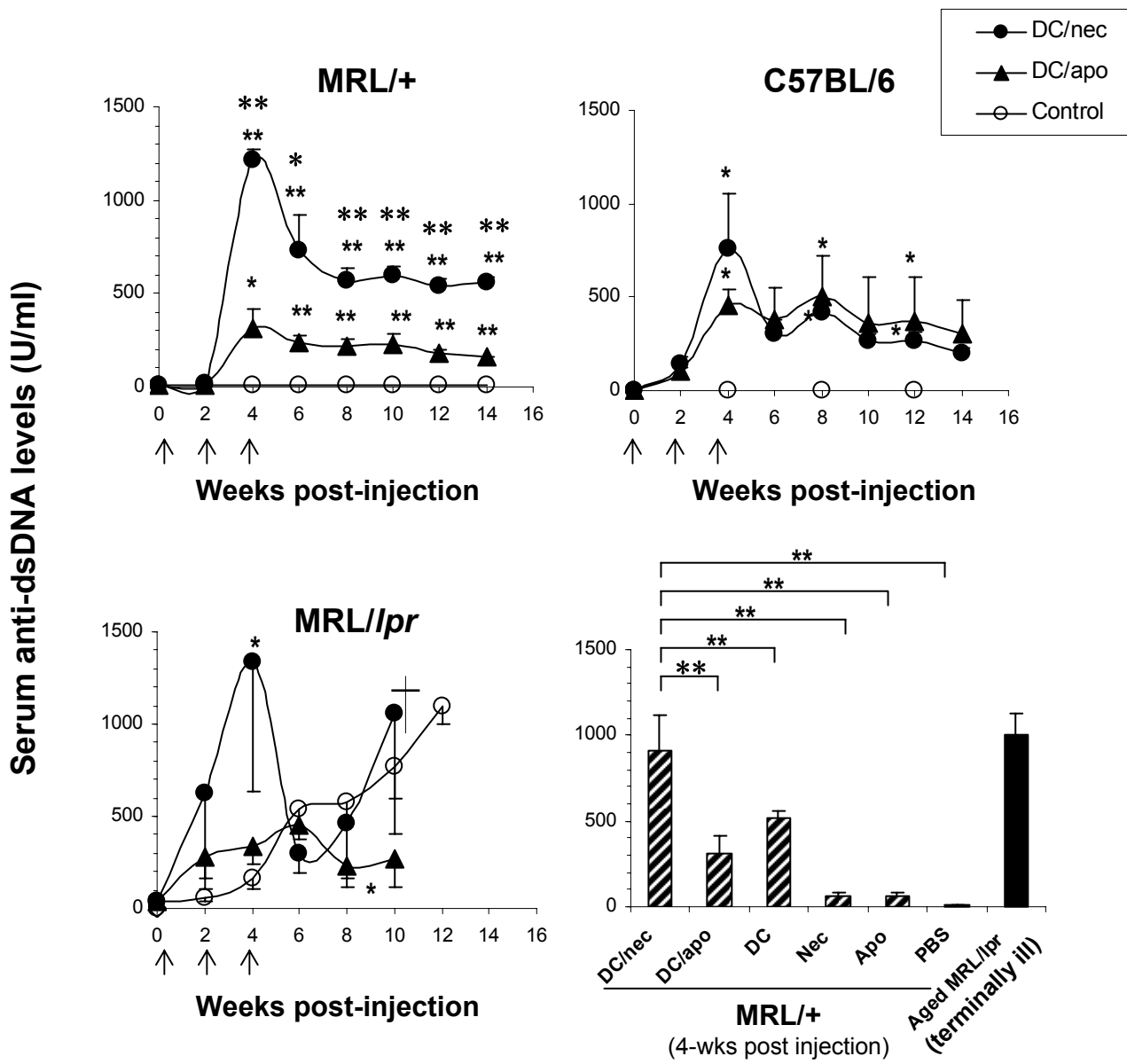


Figure 2

a

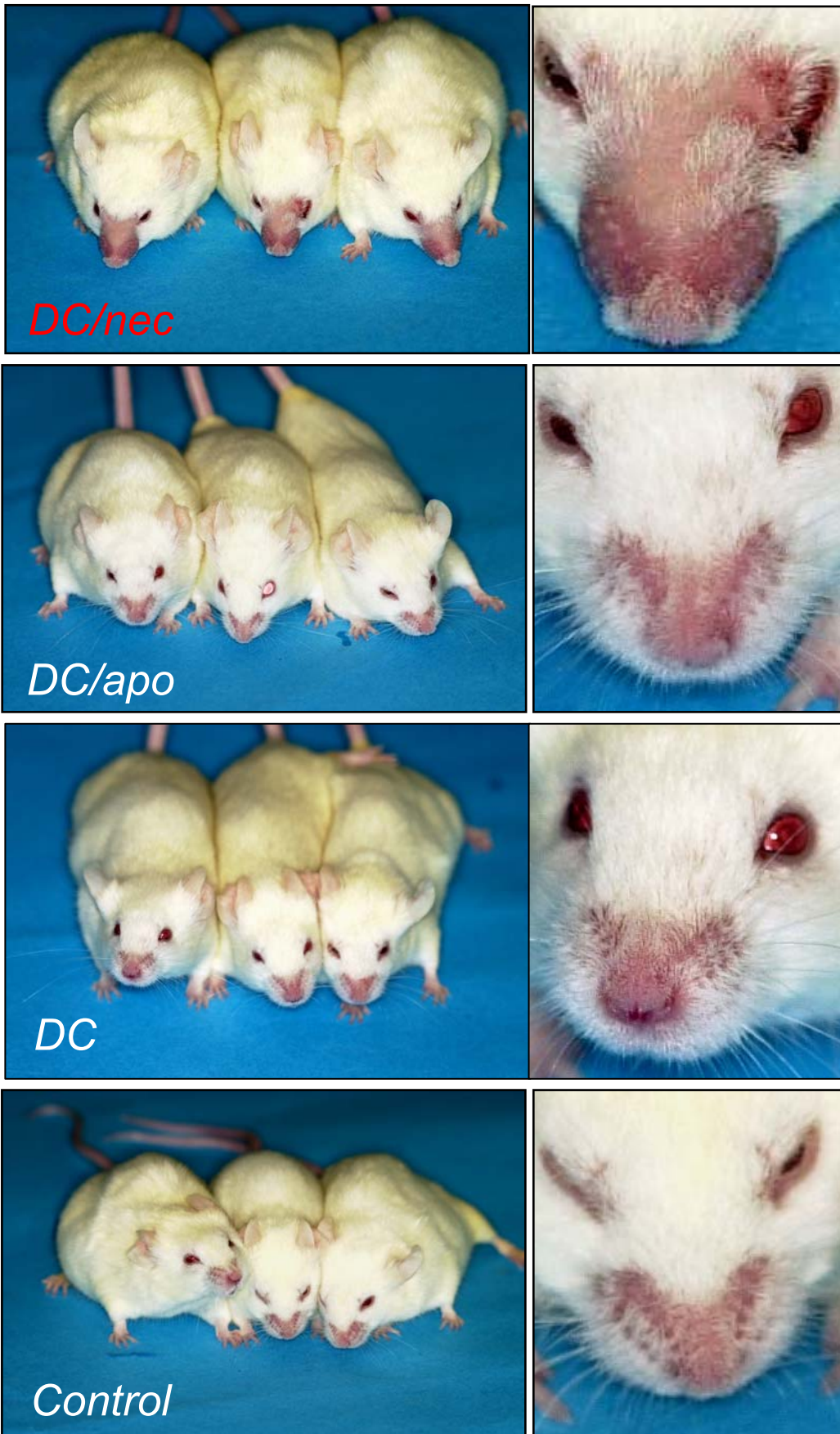


Figure 2

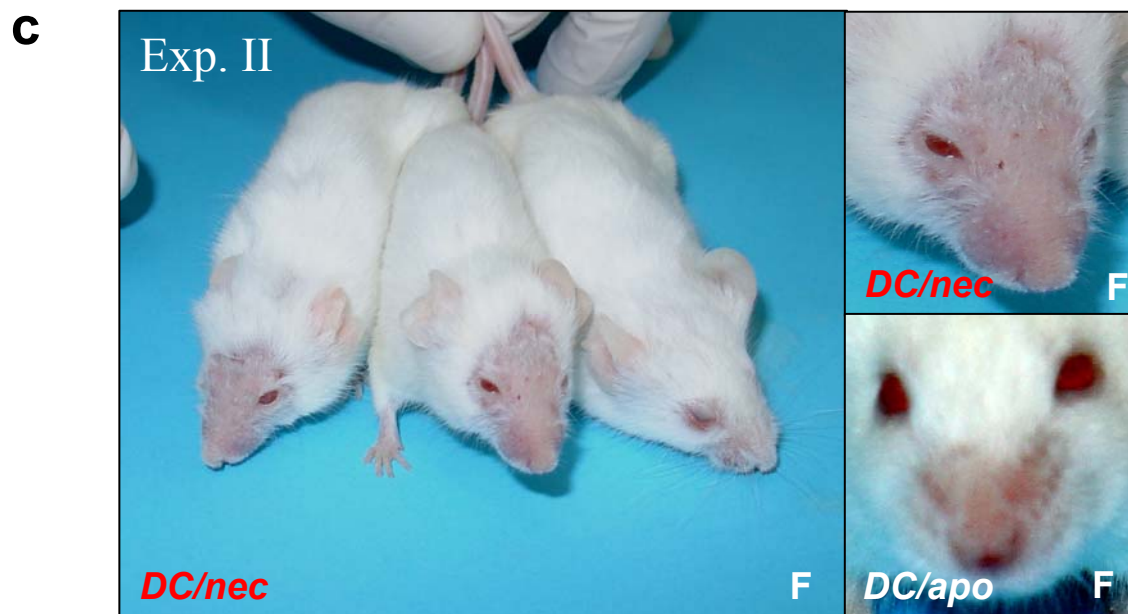
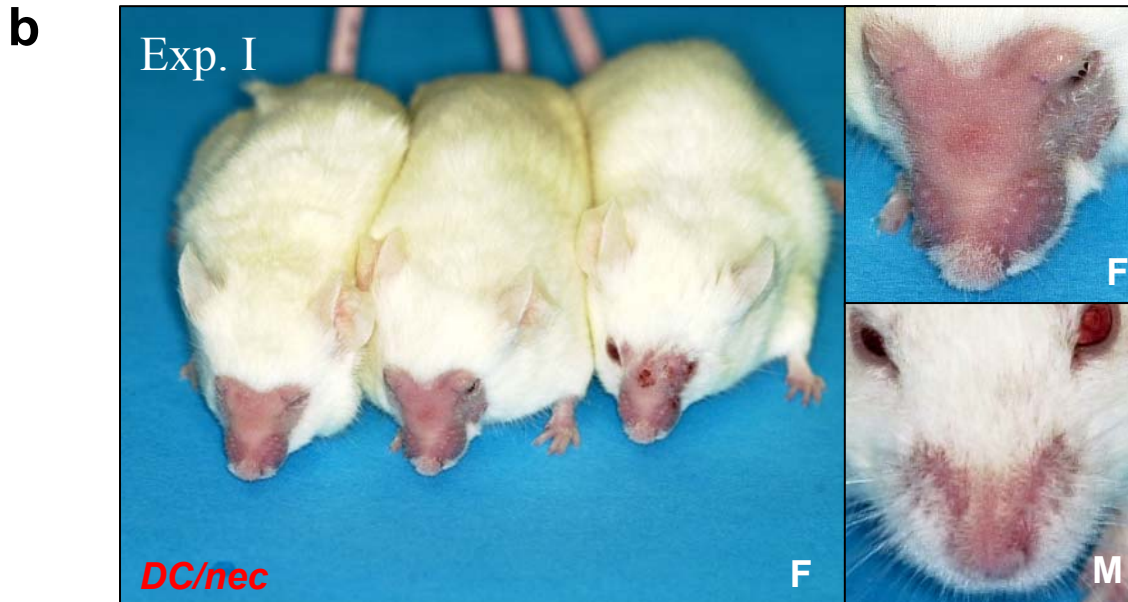


Figure 2

d

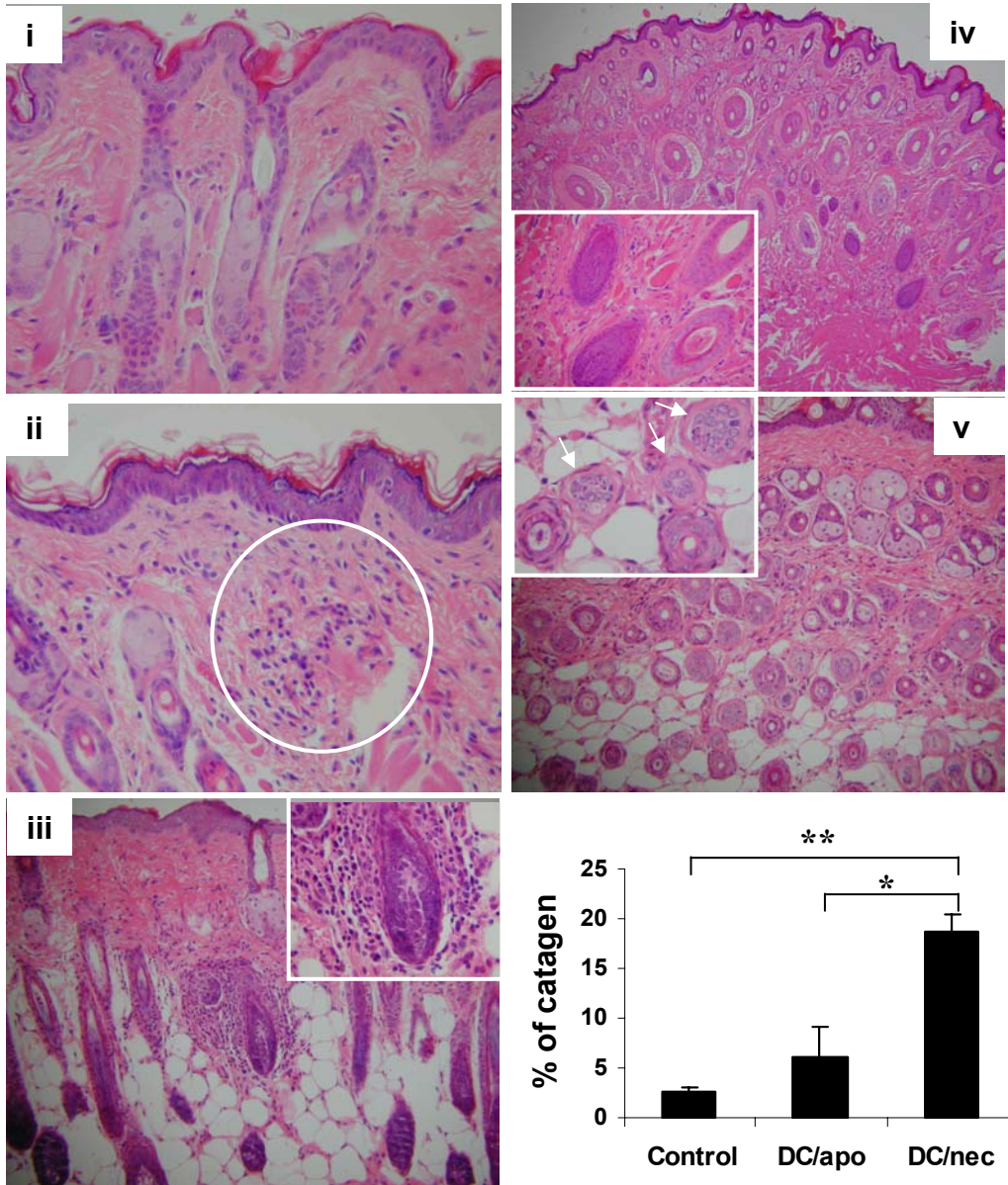


Figure 3

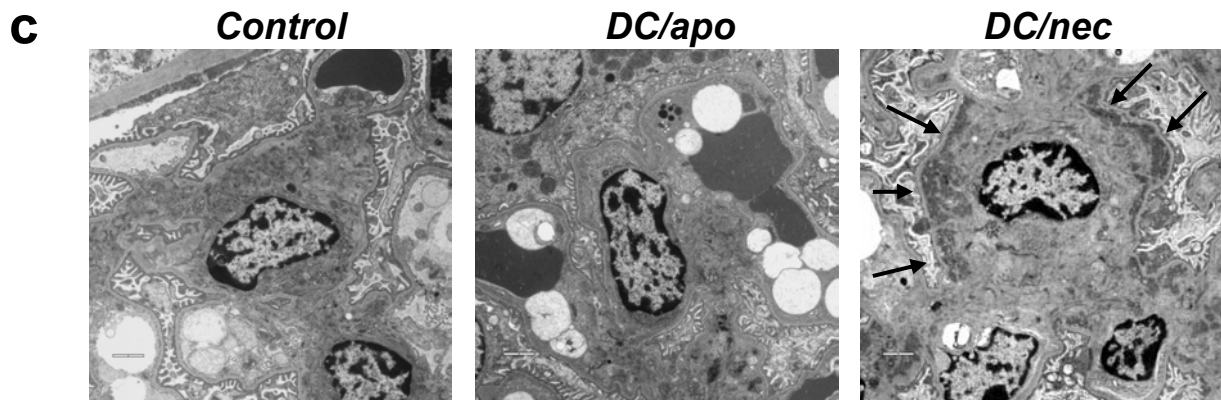
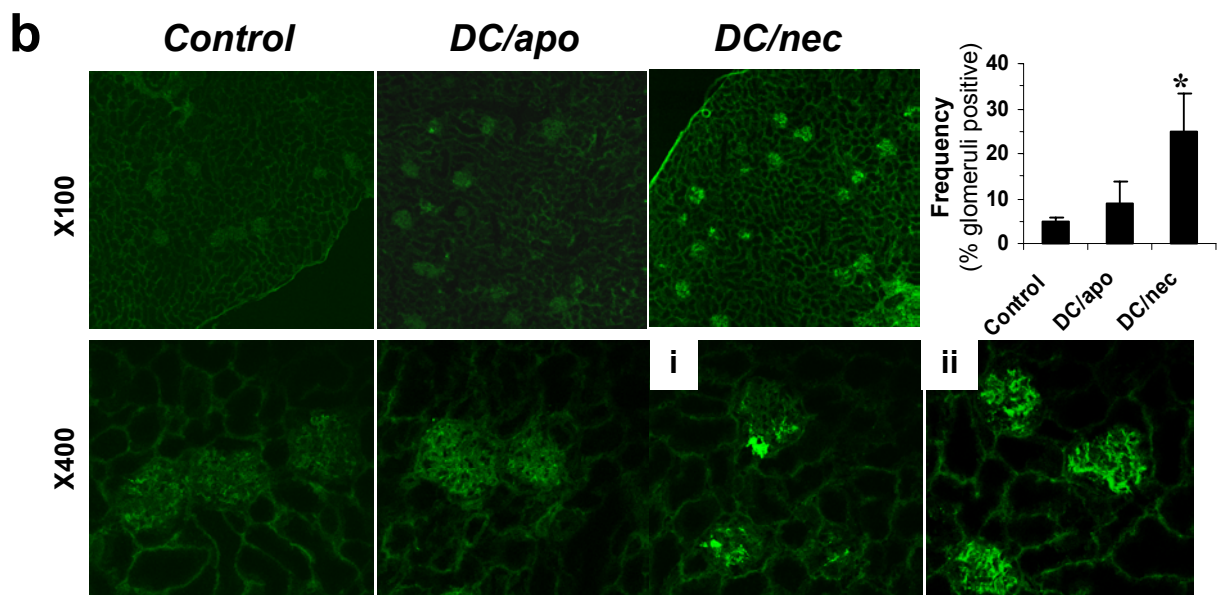
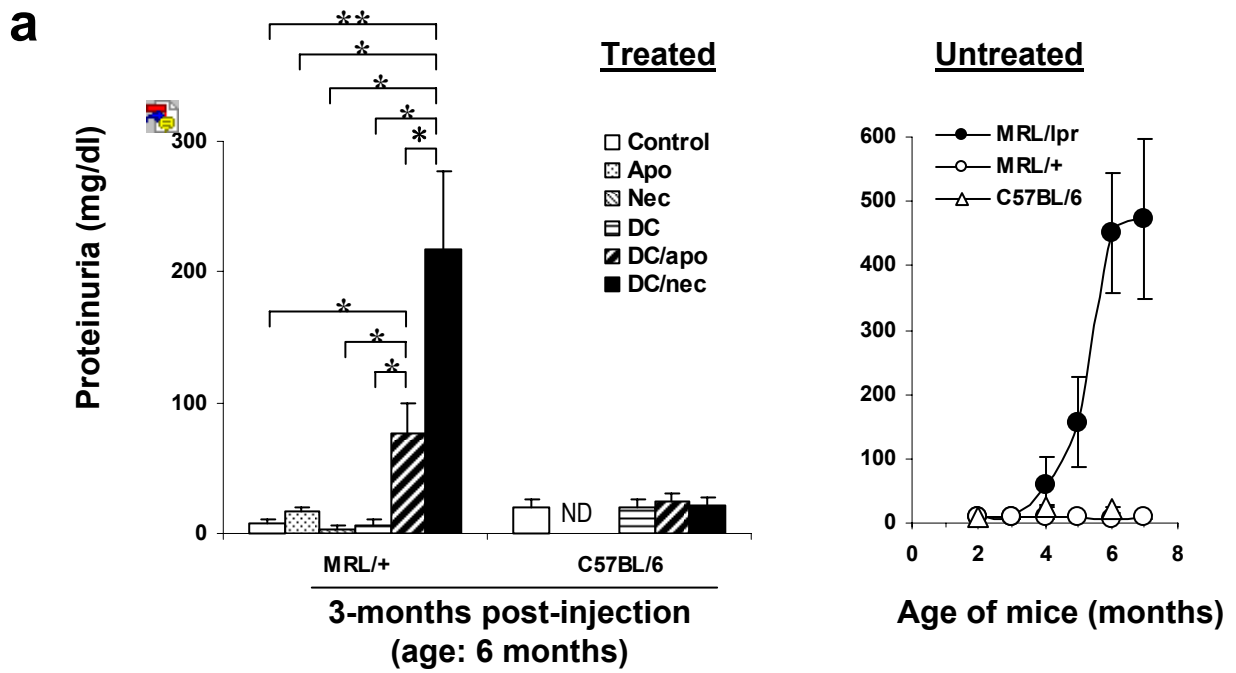


Figure 4

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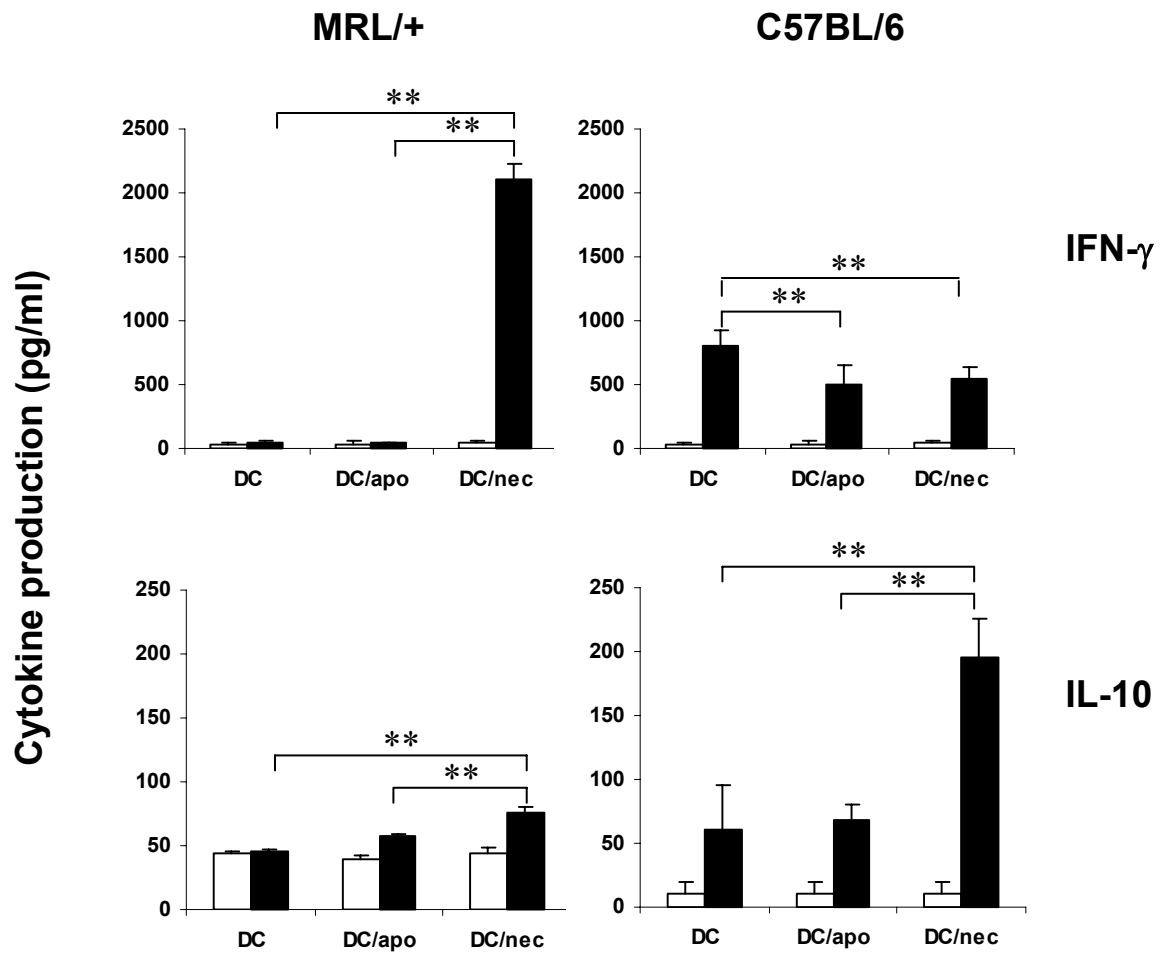


Figure 4

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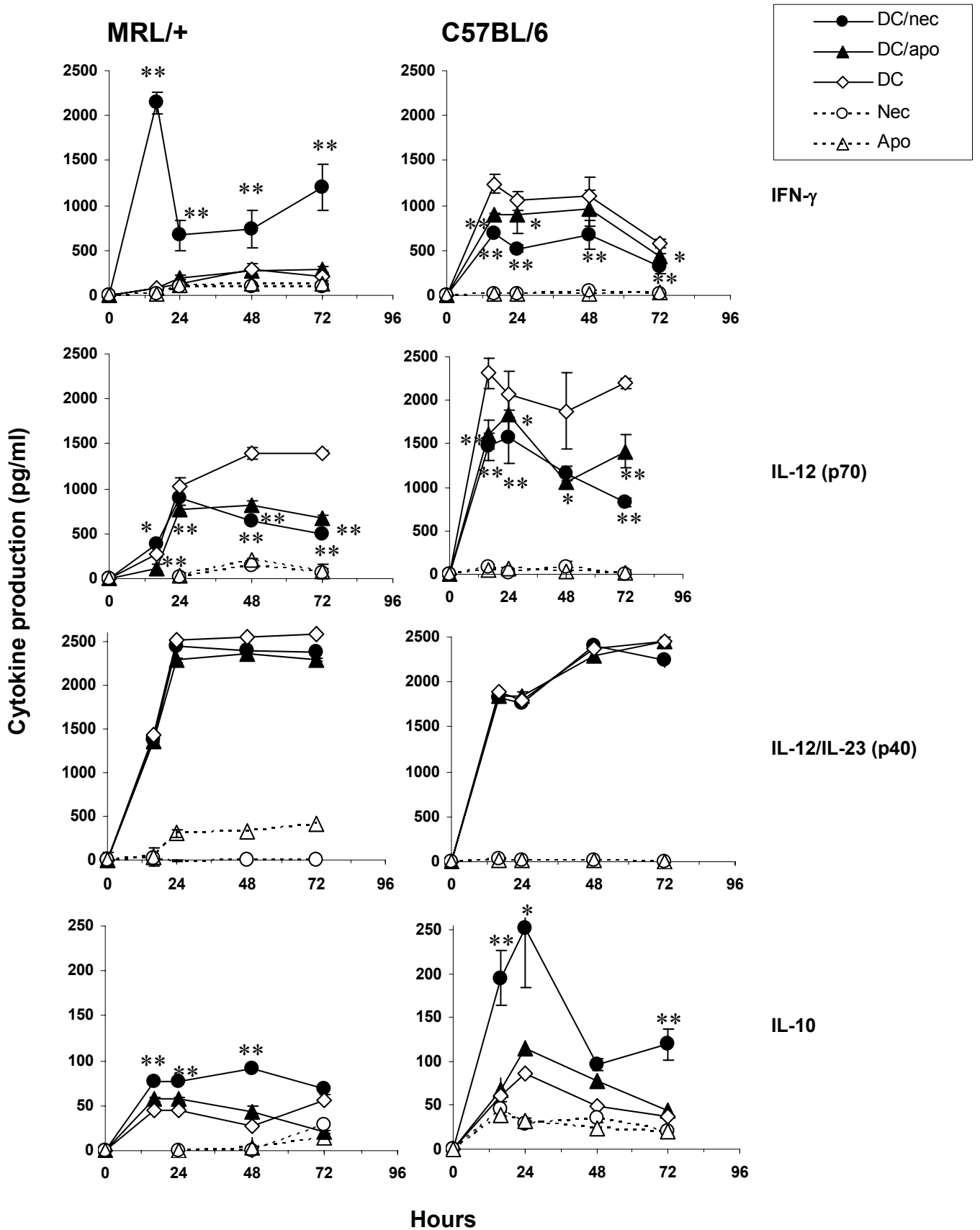


Figure 5

a

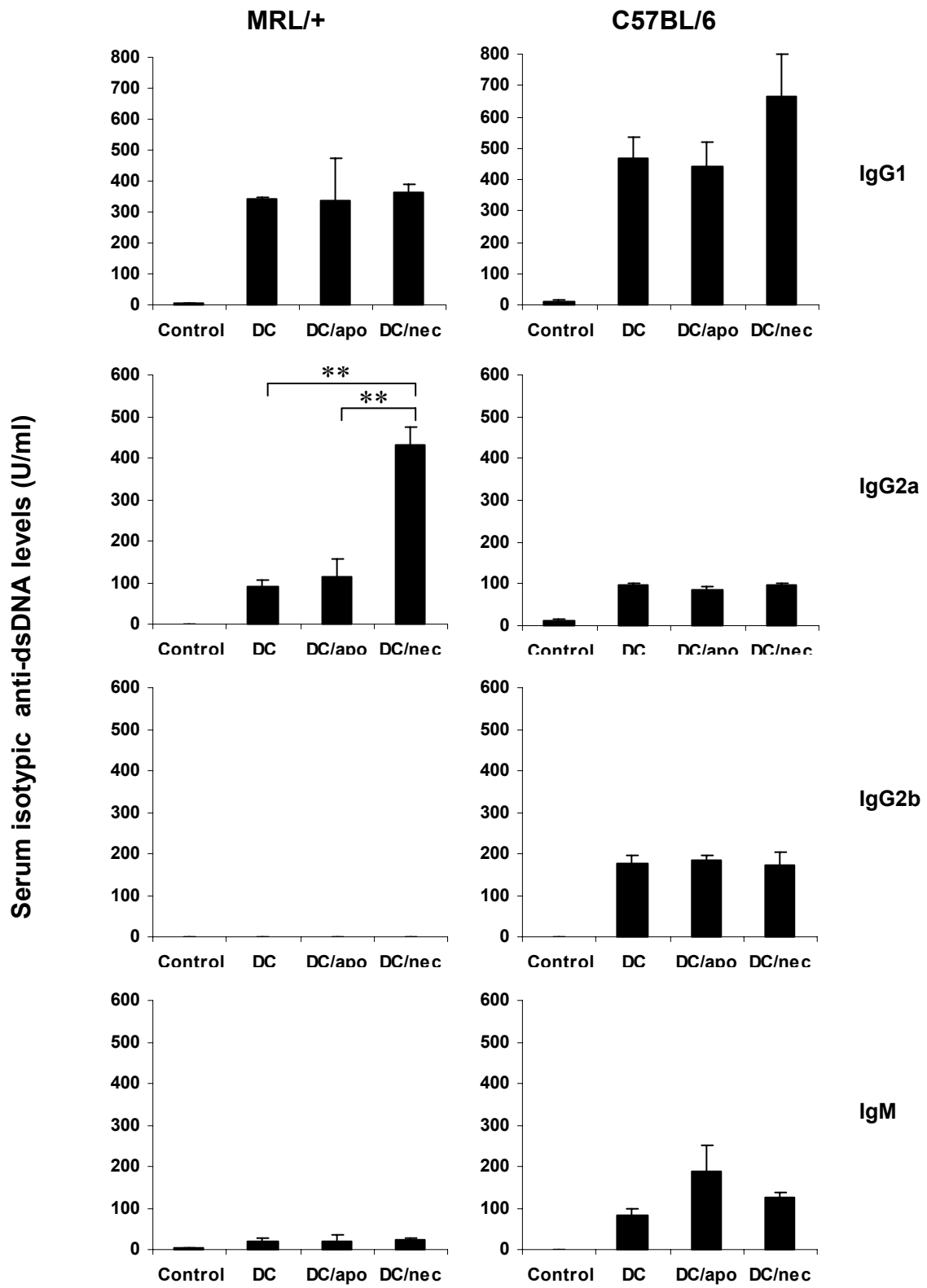


Figure 5

b

