Immunomodulatory dendritic cells require autologous serum to circumvent non-specific immunosuppressive activity in vivo

Running title: Differential effects of FBS- versus NMS-derived DCs in vivo.

Claus Haase¹,³, Mette Ejrnaes², Amy E. Juedes², Tom Wolfe², Helle Markholst¹,³,*,** and Matthias G. von Herrath²,‡.

¹Hagedorn Research Institute, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark
²La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121, USA.
³Novo Nordisk A/S, ImmunoPharmacology, Novo Nordisk Park, DK-2760 Målov, Denmark

‡H.M and M.G.v.H. contributed equally to this work.

*Corresponding author:
Helle Markholst
Hagedorn Research Institute,
Niels Steensens Vej 6,
DK-2820 Gentofte,
hmar@hagedorn.dk
phone:  +45 4443 9766
fax:  +45 4443 8000

Editorial note:
Performed research: C.H., T.W.
Analysed data: C.H., H.M., M.G.v.H.
Wrote paper: C.H., H.M., M.G.v.H.

Research support:
Supported in part by the Danish Ministry of Science, Technology and Development (to C.H.) and institutional funds from Novo Nordisk. Hagedorn Research Institute is an independent basic research component of Novo Nordisk A/S. M.G.V.H. was supported by R01DK51091, AI44451 and U19 and P01 awards from NIAID.

Scientific heading/section: Immunobiology

Word count: Text – 4937 words (incl. Abstract); Abstract – 196 words.

Abbreviations:
BSA: bovine serum albumin; NP: nucleoprotein; LCMV: lymphocytic choriomeningitis virus; T1D: type 1 diabetes; FBS: fetal bovine serum; NMS: normal mouse serum; RIP: rat insulin promoter; ICCS: intracellular cytokine staining; OVA: ovalbumin.
Abstract

In immunotherapy, dendritic cells (DCs) can be used as powerful antigen-presenting cells to enhance or suppress antigen-specific immunity upon in vivo transfer in mice or humans. However, to generate sufficient numbers of DCs most protocols include an ex vivo culture step, wherein the cells are exposed to heterologous serum and/or antigenic stimuli.

In mouse models of virus infection and virus-induced autoimmunity, we tested how heterologous serum affects the immunomodulatory capacity of immature DCs generated in the presence of IL-10 by comparing FBS- or normal mouse serum (NMS)-supplemented DC-cultures.

We show that FBS-exposed DCs induce a systemic immune deviation characterized by reduction of virus-specific T cells, delayed viral clearance and enhanced systemic production of IL-4, IL-5 and IL-10 to FBS-derived antigens, including BSA.

By contrast, DCs generated in NMS-supplemented cultures modulated immunity and autoimmunity in an antigen-specific fashion. These cells did not induce systemic IL-4, IL-5 or IL-10 production and inhibited generation of virus-specific T-cells or autoimmunity only if pulsed with a viral antigen.

These data underscore the importance of using autologous serum-derived immature DCs in preclinical animal studies to accurately assess their immunomodulatory potential in future human therapeutic settings, where application of FBS is not feasible.
Introduction

Dendritic cells (DCs) are potent antigen-presenting cells important for induction of both tolerance and immunity\(^1\)\(^-\)\(^3\) and animal studies have sought to utilize these features to induce either antigen-specific immunity, antigen-specific tolerance or anti-tumor immunity.\(^4\)\(^,\)\(^5\) Methods have been developed to generate large numbers of DCs \textit{in vitro} from spleen or bone-marrow precursors in mice or from monocytes in humans.\(^6\)\(^-\)\(^9\) Most of these protocols involve the use of growth factors like granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4), which can specifically promote DC generation and other and less well characterized factors are provided by e.g. heterologous fetal bovine serum (FBS) which has been used extensively.

By nature, DCs sample their environment by antigen-uptake\(^10\)\(^,\)\(^11\) and antigens are subsequently presented on MHC-I and MHC-II molecules. Thus, in our opinion it is quite likely that DCs cultured in medium containing exogenous proteins will process and present not only an experimental antigen (e.g. an antigenic peptide), but also antigens derived from heterologous proteins, e.g. bovine serum albumin (BSA) from FBS. In addition, DCs rapidly respond to a variety of maturation and modulatory stimuli and it is not clear if and how a culture step in heterologous serum might affect subsequent DC function upon \textit{in vivo} transfer.

Nevertheless, DCs propagated or cultured in FBS-containing medium \textit{in vitro} have been used in a number of different \textit{in vivo}-protocols, including attempts to immunize against model antigens\(^12\), induction of anti-tumor immunity\(^13\), induction of and protection against autoimmunity\(^14\)\(^-\)\(^17\) and protection against allograft rejection.\(^18\) However, in some studies, protection by DC-therapy was antigen-independent and could be due to a skewing of the immune response rather than induction of antigen-specific tolerance.

In immunotherapy, immature DCs are thought to be capable of inducing antigen-specific tolerance\(^3\)\(^,\)\(^19\)\(^,\)\(^20\) and IL-10 is an immunoinhibitory cytokine which prevent DC matura-
tion thereby keeping the cells in an immature state\textsuperscript{21}. Use of immature DCs or DCs grown \textit{in vitro} in the presence of IL-10 is of high clinical interest, since such cells can induce antigen-specific tolerance, if they have been pulsed with a cognate antigen prior to \textit{in vivo} injection.\textsuperscript{22-24} Antigen-specific tolerance is the ultimate clinical goal to prevent or treat autoimmune diseases and enhance graft acceptance.

Based on these considerations, we reasoned that in protocols where immature DCs are used to induce tolerance, exposure to heterologous proteins (e.g. FBS) could result in systemic immune deviation or modulation of immunity rather than targeted induction of antigen-specific tolerance or unresponsiveness. This would make extrapolation of such observations to human settings, where ultimately no serum or autologous serum is used, quite problematic.\textsuperscript{25,26} Such potential problems could be avoided if immature DCs could be generated with normal mouse serum (NMS) instead of FBS.

We therefore tested how bone-marrow derived DCs grown in the presence of IL-10 in either FBS- or NMS-supplemented culture medium would influence immune responses in three models; healthy normal mice receiving un-pulsed syngeneic DCs, mice infected with lymphocytic choriomeningitis virus (LCMV) and the RIP-LCMV model of virus-induced type 1 diabetes (T1D) where disease is initiated by viral infection.\textsuperscript{27-29}

Our data show that FBS-exposed DCs induce systemic and antigen-independent immune deviation due to T-cell responses to FBS-antigens, whereas NMS-derived DCs only could modulate immunity if pulsed with a relevant antigen \textit{in vitro}. Based on these data, we conclude that immunomodulatory DCs cultured in the presence of heterologous serum strongly interfere with the systemic generation of immune responses and mask antigen-specific effects – which at least in part is due to responses to FBS antigens themselves. We would therefore suggest that heterologous serum should be replaced by autologous serum in preclinical proof-of-concept studies involving the \textit{in vivo} use of \textit{in vitro}-generated DCs.
Materials and Methods

Mice and virus stocks

BALB/c, C57BL/6, MHC-II KO mice (ABBN12M) and OT-I mice were from TaconicM&B (Ry, Denmark) and Jackson Labs (Maine, USA). Generation and characterization of RIP-LCMV transgenic mice that develop T1D after LCMV infection has been described.\textsuperscript{27,28} RIP-NP (H-2\textsuperscript{d}) transgenic mice expressing the viral nucleoprotein from LCMV strain Armstrong in the β-cells of their islets\textsuperscript{29} were bred at La Jolla Institute for Allergy and Immunology (LIAI). All mice were housed under specific pathogen-free conditions. Virus stock consisted of LCMV-Armstrong (clone 53b) and was plaque-purified three times on Vero cells and prepared by a single passage on BHK-21 cells. Mice were infected with a single dose of 10\textsuperscript{5} pfu i.p. Analysis of virus titer was done by plaque assay as described.\textsuperscript{30} All animal studies have been approved by either the Danish Animal Experimentation Inspectorate or the review committee at LIAI.

Generation of DCs and adoptive transfer

DCs were generated largely as described.\textsuperscript{6,31,32} Briefly, bone marrow cells were harvested from the femur and tibiae and washed in ice-cold Hanks’ balanced salt solution (HBSS) following lysis of red blood cells. T cells were depleted using baby rabbit complement (Harlan Sera Labs, Leicestershire, UK) and hybridoma antibodies against mouse CD4 (RL172.4), CD8 (31M) and Thy1 (AT83). B-cells were not depleted as these disappeared during culture (C.H. and H.M., unpublished data). Remaining cells were washed extensively and plated in RPMI-1640 (Invitrogen) with either 10%, 5% or 1.5% FBS (Invitrogen or Hyclone) or 1.5% normal mouse serum (TaconicM&B) with 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES and 50 μM 2-mercaptoethanol at 4-5x10\textsuperscript{6} cells/well in a 6-well plate (Nunc,
Denmark). Three different batches of FBS from two different suppliers were tested, and DCs grown in all three batches display similar responses in vivo. Fresh medium was added every other day. BMDCs developed in the presence of GM-CSF (Pharmlingen, 20 ng/ml days 0-4 or 10ng/ml days 5-8) and 20 ng/ml IL-10 or IL-4 (Pharmlingen) when indicated (days 0-8). Cells were pulsed with 10 µg/ml NP-118 peptide (RPQASGVYM), 10% FBS (for NMS-DCs and splenic DCs) and activated by 10 ng/ml IFN-γ and/or 1 µg/ml LPS on day 7 as indicated and harvested on day 8. Splenic DCs were isolated using CD11c-microbeads and AutoMACS (Miltenyi) and were MHC-II^hi, CD11c^hi. For adoptive transfer, cells were washed extensively in PBS and 1x10^6 cells were injected i.p.

**Blood glucose measurements**

Blood glucose concentration was determined on tail vein blood using the OneTouch Ultra system (Johnson & Johnson). Mice with measurements >300 mg/dl were considered diabetic.

**Mixed leukocyte reaction**

BALB/c DCs were harvested on day 8, washed, counted and irradiated and placed in U-bottom 96 well plates. 1x10^5 allogeneic T cells were enriched from C57BL/6 mouse spleen using T-cell enrichment columns (R&D Systems, 85-90% CD3^+ cells) and added to the wells. After 96 hrs of cells were pulsed with 0.5 µCi of ³H-thymidine and the plates incubated for another 18 hrs. Finally, T-cell proliferation was evaluated by measuring the incorporation of radioactivity by scintillation counting as described.³¹
In vitro stimulation, ELISA and flow cytometry

Spleens were harvested from uninfected or LCMV-infected mice at the indicated times. Single-cell suspensions were obtained by disrupting tissue through a 100 μm cell strainer (Falcon) and lysis of erythrocytes. 1x10^6 cells/well were cultured in complete FBS- or NMS-medium (see above) or with 1 μg/ml BSA (Sigma-Aldrich), 2.5 μg/ml conA, 10 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 in 1.5% NMS. Where indicated, samples were depleted of CD4^+, CD8^+ or CD19^+ cells before culture, using microbeads and the AutoMACS system (Miltenyi). Quantification of cytokine levels in cell culture supernatants after in vitro stimulation was performed after 72 hrs in a sandwich ELISA, using capture and detection antibody pairs (OptEIA) from BD Pharmingen. For intracellular staining, spleen cells were restimulated for 4½ hrs with 10 μg/ml NP-118 peptide in the presence of 1 μg/ml brefeldin A (Sigma-Aldrich). Cells were stained for surface expression of CD4 and CD8, fixed, permeabilized, and stained for intracellular IFN-γ. Samples were acquired using a FACSCalibur™ (Becton Dickinson). For stimulation of OT-I cells, mesenteric lymph node cells (5x10^5) were labeled with CFSE and co-cultured with 5x10^3 NMS-IL10 DCs or NMS-IL4 DCs matured with LPS or IFN-γ, pre-pulsed for 24 hrs with 10 μg/ml of the K^b-restricted OVA257-264-peptide SIINFEKL in 96U wells. Where indicated, 50 ng/ml mIL-2 was added directly to the co-cultures. Samples were stained for expression of CD8 combined with either Annexin V and 7-AAD (Pharmingen) or the K^b-OVA257-264-tetramer which was generated as previously described.33,34

Immunohistochemistry

Tissues were immersed in Tissue-Tek OCT (Bayer) and quick frozen on dry ice. Using a cryomicrotome and sialin-coated Superfrost Plus slides (Fisher Scientific), 6 μm tissue sections
were cut. Sections were fixed with 95% ethanol at –20°C, and after washing in PBS, an avidin/biotin-blocking step was included (Vector Laboratories). Primary and biotinylated secondary antibodies (Vector) were incubated with the sections for 60 min each, and color reaction was obtained by sequential incubation with avidin–peroxidase conjugate (Vector) and diaminobenzidine–hydrogen peroxide. Primary antibodies used were rat anti–mouse CD8a (Ly2) and rat anti–mouse CD4 (L3T4; Pharmingen) and were diluted in PBS with 10% FBS.

Flow cytometric analysis

DCs were harvested, washed in PBS and stained with the following antibodies: CD11b-PE (M1/70), CD11c-APC (HL3), CD40-PE (3/23), CD86-FITC (GL1), H2Kb-FITC (AF6-88.5), I-A/E-PE (M5/114.15.2), all from Pharmingen. Cells were analyzed on a FACS-Calibur (BD). 10,000-20,000 events were acquired and live cells were gated based on forward/side scatter properties. Data was analyzed with CellQuest software (BD).

Statistics

Statistical analysis was carried out using the Student t-test (equal or un-equal variance was evaluated using the F-test) and Fischer’s exact test.
Results

Phenotype of DCs generated in FBS- and NMS-supplemented cultures

Our goal was to generate immature DCs capable of preventing the expansion of virus-specific T cells after injection into naïve mice. Consequently, immature DCs were generated from bone-marrow precursors both in the presence and in the absence of the immunomodulatory cytokine IL-10.21,24,31

We compared DCs generated in the presence of FBS versus NMS, yielding a total of four different DC subsets. Flow cytometric analyses demonstrated that all four subsets of DCs were CD11b<sup>hi</sup>CD11c<sup>hi</sup> (fig. 1A, top panels) and MHC-I<sup>+</sup>. FBS-generated DCs were MHC-II<sup>int</sup>-hi whereas NMS-generated DCs were MHC-II<sup>lo</sup> (fig 1A, bottom panels). To further investigate the maturation level of the DCs, all four subsets were matured with LPS for 24 hrs and expression of CD40 and CD86 was analyzed. Both FBS-generated subsets (with or without IL-10) could be matured in the presence of LPS, resulting in upregulation of CD40 and CD86. In contrast, both NMS-generated subsets only upregulated CD40 moderately in response to LPS and the CD86 expression remained very low after maturation (fig 1B). This was not due to an intrinsic failure of NMS-derived DCs to mature, since as shown in fig.1D, a considerable subset of DCs generated in NMS supplemented with the DC maturation factor IL-4, expressed high levels of both CD40 and CD86.

To show that all DC subsets could present antigen in the context of MHC-I in vitro, we pulsed DCs overnight with the dominant H-2<sup>d</sup>-restricted epitope of the LCMV nucleoprotein, the NP118 peptide and used them as APCs for antigen-experienced splenocytes from memory-LCMV infected mice (35 days after virus inoculation) in an in vitro recall assay. This confirmed that all DC subsets could present the NP118 peptide to T cells to a similar degree as measured by IFN-γ production (fig. 1C). Finally, to test the ability of the DC subsets
to activate naïve T cells, we performed a mixed leukocyte reaction using allogeneic T cells as responders. This experiment demonstrated that whereas FBS-DCs were more potent than NMS-DCs, both DC subsets grown in the presence of IL-10 were less efficient than DCs grown without IL-10 in activating naïve, allogeneic T cells (fig. 1E).

In conclusion, DCs were generated under all four culture conditions and as expected, presence of IL-10 during DC differentiation resulted in a fairly immature (or at least not fully mature) DC phenotype.

**T-cell stimulation in vivo by DC subsets**

We and others\textsuperscript{10,17,35} have noted that syngeneic, unpulsed FBS-derived DCs upon adoptive transfer into naïve mice can influence immunity, irrespective of \textit{in vitro} antigen-pulsing.

We tested if this could be due to priming to FBS-antigens by immunizing naïve mice two times with syngeneic, unpulsed DCs. Splenocytes were subsequently analyzed \textit{in vitro} for cytokine production. Strikingly, as demonstrated in fig. 2A, splenocytes from animals treated with FBS-DCs secreted high amounts of the Th2 cytokines IL-4, IL-5 and IL-10 when cultured in FBS-containing medium \textit{in vitro}. This cytokine production correlated with proliferation of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (as evidenced by CFSE-dilution, fig. 2C) and was dependent on the presence of FBS \textit{in vitro} since no cytokines or proliferation was observed when splenocytes were cultured in NMS-medium (fig. 2A). Furthermore, part of the response was due to priming against BSA present in serum, since splenocytes from FBS-DC treated mice proliferated and produced IL-4, IL-5 and IL-10 in response to BSA in a dose-dependent manner (fig. 2B+C, only IL-5 is shown). Moreover, the production of cytokines to FBS antigens was systemic and not dependent on the BALB/c background, since cells isolated from both mesenteric and pancreatic lymph nodes responded to FBS in a similar manner as splenocytes.
and since similar results were obtained in C57BL/6 and NOD mice (fig. 3). We tested whether induction of a FBS-specific type 2 immune response by FBS-DCs was due to the lack of IL-12 production by using LPS or LPS+IFN-γ activated DCs. These treatments induce low and high IL-12p70 production in DCs, respectively. However, even LPS+IFN-γ activated FBS-DCs induce a type 2 response to FBS, although this was less potent in some animals (see supplementary figure S1).

In an attempt to identify the source of cytokine production, we depleted splenocytes from FBS-DC treated mice of CD4+, CD8+ and CD19+ cells. As shown in figure 2D, only depletion of CD4+ cells was able to completely remove cytokine production in vitro. This, together with the demonstration that cytokines could be induced by anti-CD3/anti-CD28 or conA stimulation (fig. 3), suggests that the source of cytokine production was indeed CD4+ T cells.

In a similar assay, we then tested the other DC subsets. In contrast to the results above, splenocytes from animals treated with NMS or NMS+IL10 DCs did not produce IL-4 or IL-10 whereas FBS-IL10 DCs had a similar effect as FBS-DCs (fig. 4 top). Again, the production of cytokines was dependent on the presence of FBS in vitro, since no cytokines was produced when splenocytes from either of the four DC-treated groups were cultured in NMS-containing medium (fig. 4 bottom). In addition, the effect of FBS-derived DCs was not due to the high concentration of FBS used, since DCs grown in 1.5% FBS had a similar effect (fig. 3). Furthermore, it was evident that any DC exposed to FBS could induce a FBS-specific type 2 response in vivo, since both FBS-pulsed NMS-DCs and MACS-purified splenic DCs induced type 2 cytokines (fig. 3).

Finally, whereas only FBS and FBS-IL10 DCs induced Th2 cytokines, IFN-γ production was detectable in splenocytes from both PBS-treated mice and mice treated with any of the four DC subsets but no difference was found (C.H. and H.M, unpublished data).
All together, our data show that FBS-exposed DCs prime CD4^+ T cells \textit{in vivo} towards a FBS-specific Th2 response, whereas NMS-derived DCs do not.

\textit{Pre-treatment of naïve mice with DCs before virus infection strongly influences expansion of virus-specific T cells.}

We wanted to use immature DCs to reduce virus-specific T cells in an antigen-specific manner. Based on our results, we chose to study the more immature FBS-IL10 and NMS-IL10 (see fig. 1E) subsets. For comparison, we also included the FBS DC subset. In an assay similar to above, we treated naïve mice two times with NP118-pulsed or unpulsed DCs before infection with LCMV and examined the NP-specific immune response on day 8 after infection.

Strikingly, mice treated with FBS or FBS-IL10 DCs had a highly significant reduction in the number of NP-specific T cells independent of antigen-pulsing of the DCs \textit{in vitro}. In contrast to this, NMS-IL10 DCs could only reduce the number of NP-specific T cells if pulsed with the antigenic peptide \textit{in vitro} whereas unpulsed NMS-IL10 DCs had no effect (fig. 5A+B). The reduction in virus-specific T cells in FBS and FBS-IL10-treated mice was both due to an impairment of the expansion of spleen cell numbers after infection, as well as a reduction in overall CD8^+ T-cell numbers and the percentage of NP-specific IFN-\gamma^+CD8^+ T cells. In contrast, NMS-IL10 DCs reduced the number of virus-specific T cells by reducing overall CD8^+ T cell percentage and the percentage of NP-specific IFN-\gamma^+CD8^+ T cells whereas total spleen cell number was similar to PBS-treated controls (see supplementary figure S2). These data suggest that splenocytes in FBS-DC or FBS-IL10 DC treated mice were impaired in their expansion in response to the viral infection, most likely due to the high levels of Th2 cytokines in these animals.
On average, peptide-pulsed NMS-IL10 DCs were not as efficient as FBS or FBS-IL10 DCs in inhibiting expansion of NP-specific T cells (fig. 5B) but were capable of reducing the number of NP-specific T cells to on average <40% of PBS-treated controls.

We also analyzed the expression level of CD8 on CD8⁺ splenocytes in treated animals. The CD8-coreceptor is down-regulated on CD8⁺ T cells after LCMV infection, since >90% of CD8⁺ T cells were CD8^{high} in uninfected control mice whereas only approximately 30% were CD8^{high} in LCMV-infected control mice (fig. 5C+D). However, in all four FBS-DC treated groups, the mean percentage of CD8^{high} cells was between 50-70% indicating that a smaller fraction of CD8⁺ T cells had been activated than in the PBS-treated mice. Importantly, animals treated with unpulsed NMS-IL10 DCs had the same percentage of CD8^{high} cells as the PBS-treated animals whereas cells from animals treated with NMS-IL10 DCs pulsed with the relevant NP-antigen displayed a higher percentage of CD8^{high} cells (fig. 5D), suggesting that fewer CD8 cells had become properly activated.

Altogether, these data demonstrate that only DCs generated without the use of FBS reduce the number of NP-specific T cells in LCMV-infected mice in an antigen-dependent manner in vivo.

FBS-derived DCs induce a systemic Th2 response and impair viral clearance in LCMV-infected mice

Immunity to LCMV is characterized by a type 1 response with generation of high numbers of IFN-γ producing cytotoxic CD8⁺ T cells. In general, IFN-γ induction is inhibited by type 2 cytokines like IL-4 and IL-10 and it was therefore possible that the impaired number of NP-specific T cells seen in the FBS and FBS-IL10 DC-treated animals was due to the systemic IL-4 and IL-10 production we had observed before (see fig. 2). Furthermore, the reduced
number of virus-specific T cells could influence viral clearance and we therefore tested spleen cell production of IL-4 and IL-10 of LCMV-infected mice, and virus titer in liver and kidney on day 8.

Indeed, splenocytes from all FBS and FBS-IL10 DC-treated LCMV-infected animals produced significant amounts of both IL-4 and IL-10 independently of addition of NP-peptide antigen in vitro (fig. 6A and B). Furthermore, several animals treated with FBS or FBS-IL10 DCs still contained infectious viral particles in liver and/or kidney on day 8 (fig. 6C). In contrast, spleen cells from PBS-injected control animals as well as NMS-IL10 DC-treated animals did not produce detectable levels of IL-4 or IL-10 and no virus was detectable on day 8 after infection. The impairment in viral clearance in FBS- or FBS-IL10 DC treated animals was only transient, since no virus could be detected on day 30 after infection (C.H., T.W., and M.G.v.H., unpublished data). In contrast, although animals treated with NP-pulsed NMS-IL10 DCs had a reduction in the number of virus-specific T cells (fig. 5B), these mice did not have detectable viral particles on day 8 (fig. 6C), suggesting that the reduced number of NP-specific T-cells was still sufficient to clear the virus.

Finally, to study the mechanism of CD8\(^+\) T cell tolerance induced by NMS-IL10 DCs, we tested the activation of OVA-specific OT-I T cells by NMS-IL10 DC pulsed with OVA-peptide. Interestingly, NMS-IL10 DCs induced initial activation of OT-I T cells, followed by apoptosis, resulting in almost complete absence of OVA-specific T cells after 8 days of culture. This was in contrast to OT-I T cells stimulated by mature DCs (NMS-IL4 DCs stimulated with LPS+IFN-\(\gamma\)) or by NMS-IL10 DCs in the presence of IL-2 (see supplementary figure S3). Thus, these data suggest that NMS-IL10 DCs induce tolerance by abortive activation of CD8\(^+\) T cells followed by apoptosis and deletion.
Influence of DCs on diabetes development in the RIP-LCMV mouse model of type 1 diabetes.

To test whether FBS-DCs could influence virus-induced autoimmunity, we treated RIP-LCMV mice with FBS-DCs before viral infection. Only 16% of the mice treated with unpulsed FBS-DCs developed diabetes and no mice treated with NP-peptide pulsed FBS-DCs became diabetic (fig. 7). These observed frequencies were significantly different (p<0.01 and p<0.005, Fischer’s exact test) from the high diabetes-frequency of 87% observed in the PBS-treated control group. Thus, FBS-DC injection changed the normal diabetes incidence dramatically, yet independent of NP-pulsing in vitro.

Since NMS-IL10 DCs were capable of lowering the number of virus-specific T cells (fig. 5) in a manner dependent of antigen-pulsing in vitro, we tested whether NP-pulsed NMS-IL10 DCs could prevent autoimmunity. Although not all animals were protected from disease, disease development was inhibited as compared to controls, since only 45% of the animals developed diabetes (fig. 7A, p<0.05 compared to control). Importantly, unpulsed NMS-IL10 DCs did not affect disease development.

Immunohistochemistry on pancreatic sections showed that islets in PBS-treated control mice and in mice treated with unpulsed NMS-IL10 DCs were completely destroyed with infiltrates of both CD4+ and CD8+ cells (fig. 7B, panels a-d). Sections from non-diabetic mice treated with NP-pulsed NMS-IL10 DCs demonstrated the presence of many islets with both peri- and intra-islet insulitis (fig. 7B, panels e-f), however, it was striking that in these mice many islets were completely free of infiltration, thus offering a possible explanation why these mice remained normoglycemic (fig. 7B, panels g-h). In mice treated with either NP-pulsed or unpulsed FBS DCs nearly all islets were almost completely free of insulitis (fig. 7B, panels i-l). Thus, NMS-IL10 DCs are capable of inhibiting viral immunity and autoimmunity in an antigen-specific and selective fashion, whereas FBS-exposed DCs exert systemic effects, many of which cannot be attributed to the antigen used for in vitro pulsing.
Discussion

One of our principal new discoveries here is that FBS-exposed DCs induce a FBS-specific Th2 immune response upon injection in naïve mice. In contrast, NMS-derived DCs only influenced subsequent immune responses if pulsed with a relevant antigen *in vitro* and would thus appear to be better suited for antigen-specific immunotherapy. Induction of a FBS-specific Th2 response was not only caused by FBS DCs (MHC II<sup>int</sup>), but also by NMS DCs (MHC II<sup>lo</sup>) and by magnetic bead purified splenic DCs (MHC II<sup>hi</sup>) pulsed with FBS. The divergent MHC II levels on these various DCs would also suggest that the induced Th2 response is not directly related to the different MHC II expression level.

The finding that FBS-exposed DCs can prime T cells against FBS-derived antigens *in vivo* is important although conceptually not surprising but it was unexpected that the priming against FBS-derived antigens could exert such a strong influence on the development of a LCMV-specific immune response and virus-induced autoimmunity. We and others have shown that the injection of antigen-pulsed FBS-derived DCs can indeed induce antigen-specific immune response *in vivo,* but the *in vitro* recall assay must be performed in NMS-supplemented cultures instead of FBS-supplemented cultures. Collectively, these studies and our study demonstrate that FBS-exposed DCs are likely to prime against FBS-derived antigens. Depending on the particular assay this may not always be of concern, but in proof-of-concept protocols involving prevention or treatment of autoimmunity and/or tolerance induction, this might overestimate the tolerogenic potential of *in vitro* generated DCs.

In our hands, bone-marrow derived DCs induce a Th2 response, irrespective of IL-12 production or maturation state and splenic DCs also induce a Th2 response if exposed to FBS *in vitro.* This shows that, although the DC phenotype clearly can determine the nature of the subsequent immune response in other systems, the nature and amount of antigen is also im-
important in this decision and this is in agreement with other reports.\textsuperscript{38,39} Thus, all FBS-exposed 
DCs tested in this study induced a vigorous Th2 response.

Our data support the hypothesis that immunosuppression mediated by FBS-exposed 
DCs is due to the induction of FBS-specific CD$^+$ T cells producing Th2 cytokines. By con-
trast, no Th2 cytokines were detectable in mice treated with NMS-IL10 DCs. In this case, 
immunosuppression appears to be by a different mechanism – presumably by abortive activa-
tion and subsequent deletion of NP-118 specific T cells by NMS-IL10 DCs since we show 
that OT-1 T cells stimulated with OVA-pulsed NMS-IL10 DCs undergo initial activation fol-
lowed by apoptosis and deletion \textit{in vitro}. This would be in accordance with other studies 
showing that immature DCs can induce CD$^+$ T cell tolerance by a similar mechanism.\textsuperscript{40-42}

Importantly, spleen cells from mice treated with syngeneic FBS-derived DCs pro-
duce Th2 cytokines, even when measured after infection with LCMV. This correlated with an 
impairment to generate sufficient numbers of NP-118 specific T cells, and with a delay in vi-
ral clearance. This was surprising, since LCMV normally drives strong Tc1 and Th1 re-
sponses.\textsuperscript{43} However, the priming of T cells towards FBS-derived antigens and the subsequent 
skewing towards a Th2 response characterized by IL-4, IL-5 and IL-10 production might im-
pair development of virus-specific IFN-$\gamma$ T cells and indeed in other studies, Th2 cytokines 
have been shown to prevent the generation of a normal type 1 response.\textsuperscript{44,45} One tempting im-
plication of this is that the murine immune system is incapable of developing a robust type 1 
response when the majority of the T cells - at the same time - are being primed to a type 2 fate 
due to a systemic response to another antigen - in this case FBS. In some cases this can be un-
favorable as seen by the delayed clearance of viral particles but in other cases beneficial as 
seen by a protection against development of autoimmune diabetes. The latter is also exempli-
fied in infectious tolerance when orally induced T cells can use IL-4 and IL-10 to educate 
DCs, which subsequently can relay this information to naïve cells.\textsuperscript{46}
Our data show that only NMS-IL10 DCs were capable of significantly reducing the number of NP-specific T cells in a manner which was dependent on in vitro antigen-pulsing and to a level where virus is still cleared but where autoimmunity is nevertheless prevented. Such a correlation between the number of antigen-specific T cells and the development of autoimmune diabetes is in correlation with other studies, showing that autoimmunity in this model could be prevented by lowering the number of virus-specific T cells or by inducing a type 2 response.47,48

Although based on murine studies, our observations have important implications for the translation of findings with FBS-exposed murine DCs to the human clinical setting, where not FBS but autologous or no serum will be applied. Since many animal studies involving DC therapy are carried out using FBS-exposed DCs, some of the admittedly striking reports of protection against autoimmunity or transplant rejection after DC treatment might be due to systemic immune deviation rather than antigen-specific immunosuppression. In our opinion, this makes a translational interpretation of such data more difficult. One example is the demonstration that FBS-derived DCs could protect against diabetes development in the NOD mouse model of T1D15 in a manner that was independent of antigen-pulsing of DCs in vitro.17 Subsequent analysis of the protected NOD mice revealed a significant induction of the type 2 cytokines upon culturing of splenocytes in vitro.16 We find it likely that the observed protection from diabetes is due to systemic immunomodulation towards a type 2 response, similar to what we have demonstrated in this study and not due to antigen-specific immunosuppression.

Taken together, our data demonstrate that the results should be interpreted cautiously when using FBS-derived DCs as a means to induce antigen-specific unresponsiveness in vivo. It is possible that the protective effects of immature DCs in disease models in vivo in some cases should be re-evaluated, since the observed effects may be due to systemic immune deviation rather than antigen-specific immunosuppression. Still, our results support the concept
that immature DCs may reduce immunity in an antigen-specific manner, provided that these cells are generated in FBS-free culture systems. The issue of the \textit{in vivo} use of DCs is important, since DCs are now being used in pre-clinical proof-of-concept studies conducted for translational purposes. In this respect, studies involving DC-treatment in experimental animal models should be done in FBS-free culture systems and results from animal model systems using \textit{in vitro}-generated DCs grown in heterologous serum should be interpreted with caution.
Acknowledgements

The authors wish to thank Trine Larsen and the employees of our Animal Unit for excellent technical assistance. Dr. Urs Christen is thanked for help with preparing the OVA-Kb-tetramers.
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Figure legends

**Figure 1: Phenotypic characterization of DC subsets**

A+B: DCs were propagated from bone-marrow precursors under the conditions indicated with or without IL-10 in FBS- or NMS-supplemented cultures. On day 8, cells were harvested and analyzed for surface markers either unstimulated (A) or stimulated with 1 µg/ml LPS for the last 24 hrs (B). Histograms shown are representative of n=3-7 observations. C: DCs were pulsed or not with 10 µg/ml NP-118 peptide for the last 24 hrs of culture and used as APCs for splenocytes from LCMV-infected syngeneic mice and analyzed by ICCS as described. As positive control, peptide was added directly to splenocytes or splenocytes were left unstimulated. D: Phenotype of NMS-DCs grown with or without IL-4. E: DCs were used as stimulator cells in an allogeneic MLR using 10^5 C57BL/6-derived T cells as responders. T-cell activation was measured by ^3H-thymidine incorporation after 5 days.

**Figure 2: T cell activation in vivo by FBS-DCs.**

A: BALB/c mice were treated with 10^6 syngeneic FBS-DCs on day -14 and -7. Splenocytes were harvested on day 0 and 10^6 cells were cultured in medium containing 10% FBS or 1.5% NMS for 72 hrs and cytokine production was analyzed. Each dot represents an individual mouse. B: As A, but cells were cultured in 1.5% NMS medium with the indicated concentrations of BSA and cytokine production was measured. C: As A, but cells were labeled with CFSE before culture and analyzed by flow cytometry. Cells are gated on live CD4^+ and CD8^+ cells. D: As A, except that cells from FBS-DC treated animals were depleted of the indicated subsets before culture. Figures are representative of two-four independent experiments.
Figure 3: Cytokine production by splenocytes after immunization of naïve mice with DCs.

Mice were immunized twice with the indicated DC subset as described in the legend to figure 2 and splenocyte production of cytokines was analyzed by ELISA. mesLN and panLN indicate mesenteric and pancreatic lymph nodes, respectively. conA is concanavalin A. Signal below detection limit is indicated by the gray box. NT: not tested. NMS-DC + FBS: NMS-DCs pulsed with 10% FBS. SpDC: MACS-purified splenic DCs. Note that SpDCs pulsed with NMS induced some cytokines which was probably due to the presence of BSA in the buffer used for purification of the cells. However, the levels induced by NMS pulsed SpDCs was still 10-fold lower than the levels induced by FBS-pulsed SpDCs.

Figure 4: T cell activation in vivo by DC subsets.

BALB/c mice were treated with $10^6$ syngeneic DCs on day -14 and -7. Splenocytes were harvested on day 0 and $10^6$ cells were cultured in medium containing 10% FBS (top) or 1.5% NMS (bottom) for 72 hrs and cytokine production was analyzed. Each dot represents an individual mouse.

Figure 5: Effect of DC treatment of LCMV-specific immunity in vivo.

Naïve BALB/c mice were injected i.p. with $10^6$ of the indicated DC subset on day -10 and -3, followed by LCMV-infection ($10^5$ pfu) on day 0. The LCMV-specific T-cell response was analyzed by restimulation of splenocytes in vitro with 10 µg/ml NP-118 peptide for 4½ hrs and analyzed by ICCS as described. A: Representative FACS-diagrams of the numbers shown in B. B: Total number of NP-118 reactive IFN-γ+CD8+ splenocytes after ICCS. C: Representative FACS-diagrams of CD8 expression as shown in D. D: Quantification of the percent
CD8\textsuperscript{lo} and CD8\textsuperscript{hi} spleen cells. In B and D, each dot represents an individual mouse. *: p<0.05; **: p<0.01 and ***: p<0.005 compared to PBS-group.

Figure 6: Cytokine production and virus titer after DC treatment and LCMV infection

Naïve BALB/c mice were injected i.p. with 10\textsuperscript{6} of the indicated DC subset on day -10 and -3, followed by LCMV-infection (10\textsuperscript{5} pfu) on day 0. The LCMV-specific T-cell response was analyzed on day 8 after infection. A+B: Spleen cell production of IL-4 (A) and IL-10 (B) was analyzed by ELISA after 72 hrs of restimulation with or without NP-118. C: LCMV viral titer in kidney and liver was analyzed by plaque assay. Lower detection limit of the assay (2000 pfu/g tissue) is indicated by a dotted line. Each dot represents an individual mouse and n=3-6 in each group.

Figure 7: Diabetes development in RIP-LCMV mice after DC treatment and LCMV infection

RIP-LCMV mice were injected i.p. with 10\textsuperscript{6} of the indicated DC subset on day -10 and -3, followed by LCMV-infection (10\textsuperscript{5} pfu) on day 0. A: Diabetes development was followed by measuring blood glucose. B: Pancreatic sections were stained for CD4 and CD8. Images are representative of 3-6 individual animals in each group. The data shown are pooled from two independent experiments.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Immunomodulatory dendritic cells require autologous serum to circumvent non-specific immunosuppressive activity *in vivo*

Claus Haase, Mette Ejrnaes, Amy E Juedes, Tom Wolfe, Helle Markholst and Matthias G von Herrath

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