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Altered dendritic cells (DC) might be responsible for regulatory T cell imbalance and autoimmunity in nonobese diabetic (NOD) mice

Abstract

Nonobese diabetic (NOD) mice spontaneously develop diabetes, an auto-immune disease characterized by the destruction of insulin-secreting beta-cells by autoreactive T cells. Defects in development and/or functions of dendritic cells (DC) might be critical in eliciting the auto-immune reaction to beta cells in this model. In this paper, DC differentiation in NOD mice was investigated in vitro

using bone marrow-derived progenitors (BM-DC) in the presence of GM-CSF and IL-4 or spleen-derived progenitors in the presence of GM-CSF and early acting cytokines such as Flt-3L and IL-6 (SPL-DC). In both culture systems, the absolute number of NOD DC generated was strongly reduced as compared to control strains. In addition, both BM-DC and SPL-DC from NOD mice show defective differentiation into mature DC in conventional culture conditions as indicated by low expression of MHC class II and CD80 molecules among CD11c positive cells and low capacity to stimulate allogeneic T cells. However, DC achieved full maturation when exposed to LPS, except for MHC class II expression that remained decreased. Ex vivo analysis confirmed an unusual phenotype of NOD DC. Both sets of results are thus consistent with a specific defect of DC maturation in these mice.

Full text

ABBREVIATIONS

Ab: antibody
APC: antigen presenting cell
BB: biobreeding
BM-DC: bone marrow-derived dendritic cell
CSF-1: colony stimulating factor-1
DC: dendritic cell
FL: Flt3-L
GM-CSF: granulocyte macrophage colony stimulating factor
IDDM: insulin-dependent diabetes mellitus
IL: interleukin
LPS: lipopolysaccharide
mfi: mean fluorescence intensity
MHC: major histocompatibility complex
MLR: mixed lymphocyte reaction
NOD: nonobese diabetic mice
SD: standard deviation
SPL-DC: spleen-derived dendritic cell
TCR: T cell receptor

INTRODUCTION

Dendritic cells (DC) play a key role in negative selection of T cells in the thymus. In addition, DC are the most potent APC in the periphery where they can either activate or tolerize T cells [1, 2]. DC are heterogeneous as for their origin, phenotype and function. Two DC subclasses have been reported depending on expression of CD8alpha and are referred to as lymphoid DC ($CD8alpha^+$) and myeloid DC ($CD8alpha^-$), respectively [3, 4]. Shortman and coll. have suggested that $CD8alpha^+$ DC favor the development of peripheral tolerance by inducing Fas-mediated death of activated T cells, whereas $CD8alpha^-$ DC are conventional APC [5]. However, recent studies show that both subclasses are able to fully activate T cells. Lymphoid DC induce Th1-associated immune responses whereas myeloid DC induce Th2-associated immune responses in the mouse [6]. A defect in DC development might contribute to mechanisms leading to auto-immune diseases.

In insulin-dependent diabetes mellitus (IDDM), the destruction of pancreatic insulin-secreting beta-cells is mediated by T cells which show a Th1 cytokine secretion profile. The disease strongly associates with MHC class II susceptibility alleles, both in the human and in the nonobese diabetic (NOD) mouse [7, 8]. In NOD mice, defects in the selection or expansion of T cells [9] and in immune regulatory circuits that maintain peripheral tolerance to autoantigens possibly account for disease development [10-14]. Non-MHC linked defects may also contribute to the inability of NOD APC to support tolerance as well. Defective maturation and/or function of both macrophages and DC have been described. Activated macrophages in NOD mice produce low amounts of IL-1beta and GSH [15, 16]. NOD myeloid precursors display diminished proliferative responses to CSF-1 and disability to generate phenotypically and functionally mature macrophages indicating that this functional defect may take place early in hematopoiesis [15]. In type I diabetes patients and in asymptomatic IDDM relatives, blood monocyte-derived DC display an altered phenotype, *i.e.* reduced co-stimulatory function and T cell stimulation capacity [17, 18]. Similar disfunction has been reported in other autoimmune animal models. In the biobreeding (BB) rat, splenic DC display low expression level of MHC class II and co-stimulatory molecules, high phagocytic activity and are less efficient at generating $RT6^+$ and $CD8^+$ regulatory/suppressor T cells [19]. In NOD mice, phenotypic and/or functional defects of DC maturation have also been reported [20-23]. However, data have been conflicting regarding MHC class II and co-stimulatory molecule expression, casting doubts on the role of DC maturation defects in disease development. We report here a quantitative,

phenotypical and functional analysis of NOD DC using DC derived from hematopoietic progenitor cells - based on two independent culture systems - and DC isolated *ex vivo* from the spleen (*ex vivo* DC).

METHODS

Mice

NOD (H-2^{g7}) and AB/H Biozzi (K^d I-A^{g7} D^q) mice were bred in our animal facilities under standard pathogen-free conditions and checked every 6 months for bacterial, viral, and parasitic infections. The spontaneous incidence of diabetes in our colony reaches 85% in females and 45% in males by postnatal week 35. DBA/2 (H-2^d), CBA (H-2^k) and C57BL/6 (H-2^b) mice were purchased from Iffa Credo (l'Arbresle, France). All experiments were performed in 7 to 8 weeks old NOD female mice.

Generation of DC from hematopoietic progenitors

BM-DC were obtained as described [24, 25] with slight modifications. Briefly, BM suspensions from 7 to 8 weeks old female mice were depleted from lymphocytes with a cocktail of Ab (anti-mouse CD4- clone RL172-, anti-mouse CD8 -clone TIB 105- and anti-mouse B cell -clone B220-, kindly provided by Dr. Laurence Zitvogel), and rabbit complement (Sigma). After overnight culture at 37° C in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mug/ml streptomycin, essential amino acids and sodium pyruvate), non adherent cells were recovered and cultured for 7 days in complete RPMI medium with 1 ng/ml murine rGM-CSF (R&D System) in combination with 100 U/ml rIL-4 (TEBU, Le Perray en Yvelines, France). DC were also derived from spleen progenitors (SPL-DC) as described [26]. Briefly, after ammonium chloride treatment to remove erythrocytes, unfractionated nucleated splenocytes were cultured in complete Iscove's modified Dulbecco's medium (IMDM) (supplemented with 12.5% heat inactivated FCS, 100 U/ml penicillin, 100 mug/ml streptomycin, essential amino acids and sodium pyruvate) with recombinant human FL (TEBU, France), murine rGM-CSF (R&D System) and recombinant human IL-6 (TEBU). From day 0 to day 6, splenocytes were cultured with rGM-CSF 1 ng/ml + FL 50 ng/ml + IL-6 25 ng/ml at 7.5 x 10^5 cell/ml. Beyond day 6, cells were reseeded every 4 to 5 days at 3-6 x 10^5 cell/ml, with rGM-CSF 1 ng/ml + FL 30 ng/ml. DC were recovered after a 3 week culture. Both BM-DC and

SPL-DC were purified on metrizamide gradient. The average purity of DC ($CD11c^+$ cells) was > 90% and viability (propidium iodide negative) was > 95%.

*Purification of splenic DC (*ex vivo *DC)*

For splenic DC isolation, spleen cells were digested with collagenase, further dissociated in $Ca2^+$-free medium in the presence of EDTA. After enzymatic treatment, splenocytes were resuspended in complete RPMI medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mug/ml streptomycin and sodium pyruvate). After 2 hours of culture at 37° C, lymphocytes and erythrocytes were removed by washing. Upon overnight culture [27], the non-adherent cell fraction was recovered and 2 ml of the cell suspension was layered on 2 ml 14.5% Nycodenz (Nycodenz Pharma AS, Oslo, Norway) and centrifugated for 20 min at 530 g. The average purity of DC ($CD11c^+$ cells) was 80 to 95% and viability (propidium iodide negative) was > 95%. To avoid confusion with SPL-DC, splenic DC isolated with this procedure will be referred to as *ex vivo* DC.

Immunostaining and flow cytometric analysis

Cells were harvested and washed twice in PBS 3% FCS. After incubation with unlabelled anti-FcRIIgamma Ab (clone 2.4G2, PharMingen, San Diego, CA) to avoid non specific binding to Fc-receptors, cells were directly incubated with biotinylated anti-I-$A^{b\&d}$ (clone 28-16-8S, Caltag, Burlingame, CA) or anti-I-$A^{k\&g7}$ (clone 10-3-6, PharMingen) or anti H-2^k (clone SF1-1.1, PharMingen) Ab + Streptavidin-PE (Caltag). Anti-CD11c (clone HL3, PharMingen), anti-CD80 (clone RMMP-2, Caltag), anti-CD86 (clone RMMP-1, Caltag), anti-CD40 (clone 3/23, Caltag) and anti-CD8alpha (clone CT-CD8alpha, Caltag) FITC or PE conjugated Ab were used for double staining. Cell viability was determined using propidium iodide. Flow cytometric analysis were performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest software.

Mixed lymphocyte reaction (MLR)

Irradiated (3,000 rad) BM-DC or SPL-DC were co-cultured with 1×10^5 allogeneic splenocytes per well in 96 well culture plates. DC were cultured under standard conditions (unstimulated, see above) or stimulated with 1 mug/ml lipopolysaccharide (LPS) (*E. coli*, Sigma, Saint Louis, MO, USA) 48 hours before

the MLR. BM-DC and SPL-DC were stimulated with LPS from day 6 to day 8 and from day 18 to day 20 respectively. The DC/allogeneic splenocyte ratio was 1/5, 1/25, 1/125, 1/625 and 1/3125. T cell proliferation was measured by ^{3}H-Tdr incorporation (0.1 muCi/well) after 3 days of culture.

IL-12p70 ELISA

Day 6 BM-DC or day 12 SPL-DC were stimulated with 1 mug/ml LPS (*E. coli*, Sigma, Saint Louis, MO, USA), and/or 1 mug/ml anti-CD40 Ab (clone 3/23, PharMingen, San Diego, CA, USA), for 48 hours. IL-12p70 production was determined using a mouse IL-12p70 ELISA kit (PharMingen, San Diego, CA, USA) according to manufacturer's recommendations. Briefly, 100 mul of standard cytokine or culture DC supernatant was incubated in anti-mouse IL-12p70 coated well for 2 hours, then incubated with 100 mul of biotinylated antibody combined with HRP for 1 hour, and revealed with TMB substrate solution. Absorbance was measured at 450 nm using an ELISA reader.

RESULTS

Low number of DC in NOD mice

The growth and the phenotype of cells recovered from BM progenitors cultured in the presence of GM-CSF + IL-4 were determined in NOD and control strains. As previously described [20-22], the growth of progenitor cells was reduced in NOD cultures, yielding lower number of CD11c^{+}/MHC class II^{+} cells than in control cultures (Table 1) ($p < 0.05$). We then investigated whether an alternative protocol shown to produce high number of DC in control mice, could improve the yield of DC in NOD culture. Unfractionated splenic progenitors cultured over 3 weeks with GM-CSF + FL in combination with IL-6 during the first week of culture (GM-CSF + FL (+IL-6)) gave rise to high numbers of DC which exhibited a more mature phenotype than BM-DC [26]. In the DBA/2 strain, 1×10^{7} splenocytes led to the development of more than 10×10^{7} DC after a 3 week culture, whereas less than 5×10^{6} SPL-DC were recovered from NOD mice under the same culture conditions (Table 1). The growth of NOD SPL-DC was slow and the reduced growth shown at day 21 was observed at all time points analyzed (day 13, 16, 18, 23) up to day 23 culture (data not shown). Optimized procedure using low-density fraction of collagenase-digested splenocytes after overnight culture, allow the isolation of mature DC. Using this procedure, 5 to 7×10^{5} CD11c^{+}/MHC

class II^+ cells/per spleen (normalized to 10^8 splenocytes) were recovered from control mice, whereas this yield only reached 2×10^5 in NOD mice (Table 1) ($p < 0.05$).

Our finding indicate that the reduced growth of NOD hematopoietic progenitor cells to GM-CSF cannot be restored by addition of early acting cytokines such as FL and IL-6. In addition, our results show that the number of *ex vivo* DC is significantly lower in NOD mice than in control mice, indicating that the quantitative defects described *in vitro* has significant relevance *in vivo*.

Low MHC class II expression on NOD DC

Although low yields of DC in NOD mice have been described by different authors (and the current report), conflicting results have been reported referring to the phenotype of DC generated *in vitro* in the NOD mouse [20-22]. To analyse in more details the MHC expression on NOD DC, BM-DC and SPL-DC were double stained with anti-CD11c and anti-I-A Ab. The mean fluorescence intensity (mfi) of I-A staining on CD11 positive cells was lower in NOD than in DBA/2, both on BM-DC ($mfi^{DBA/2} = 1,293 \pm 109$, $mfi^{NOD} = 194 \pm 66$, $n = 3$, $p < 0.01$) (Figures 1a, d) and SPL-DC ($mfi^{DBA/2} = 2,562 \pm 1,122$, $mfi^{NOD} = 497 \pm 390$, $n = 3$, $p < 0.05$) (Figures 1b, e). The level of MHC class II was higher on SPL-DC than BM-DC, but remained lower in the NOD as compared to DBA/2 strain regardless of culture duration. Data obtained with CBA, C3H and C57BL/6 strains were similar to those obtained with DBA/2.

To exclude that low MHC class II expression was due to artificial conditions *in vitro*, we analysed *ex vivo* DC. Our finding confirmed that the level of MHC class II expression was lower on NOD DC ($mfi^{NOD} = 157 \pm 90$, $n = 3$) than on DBA/2 DC ($mfi^{DBA/2} = 956 \pm 247$, $n = 3$) ($p < 0.05$) (Figures 1c, f). The low level of class II expression on NOD DC was not due to low affinity of the 10-3-6 Ab for the $I\text{-}A^{g7}$ allele since AB/H Biozzi ($I\text{-}A^{g7}$) *ex vivo* DC displayed high level of I-A staining ($mfi^{AB/H} = 888 \pm 141$, $n = 3$) which is significantly higher than I-A staining on NOD DC ($p < 0.05$) but similar to I-A staining on DBA/2 DC ($p > 0.05$) (Figure 1g). Furthermore, we did not observed defective expression of class I molecule, since similar levels of K^d expression were observed in NOD and DBA/2 mice (Figures 1h, i).

The spontaneous maturation of mouse DC *in vitro* leads to an upregulation of class II expression. Our results clearly indicate that it does not occur on NOD DC and is not related to the I-A^{g7} allele.

In vitro *and* ex vivo *expression of costimulatory molecules on NOD DC*

BM-DC and SPL-DC were double stained with anti-CD11c and Ab specific for co-stimulatory molecules. As shown in Figure 2 the proportion of $CD11c^+$ cell expressing high level of CD80 was reduced in NOD mice as compared to DBA/2, both on BM-DC (55% ± 10 in DBA/2 and 31% ± 5 in NOD, $n = 3$, $p < 0.05$) and on SPL-DC (91% ± 6 in DBA/2 and 64% ± 8 in NOD, $n = 3$, $p < 0.05$). Kinetic analysis showed that upregulation of CD80 is reduced on NOD DC at each time point (data not shown). In contrast, no difference was seen referring to the level of CD86 expression. The level of CD40 expression was similar or slightly increased on NOD cells as compared to DBA/2 cells. However, this difference was weak and not consistent throughout experiments, in contrast with data reported by others on BM-DC [20, 22]. Thus, our finding clearly show that, in NOD mice, both BM-DC and SPL-DC show defective or delayed upregulation of CD80 expression. When *ex vivo* DC were analysed, the situation was different. The majority of NOD DC expressed similar or slightly reduced level of CD80 than DBA/2 DC and similar level of CD86. However, the level of CD40 expression was higher than on control DC, as described by others for BM-DC [20, 22].

NOD DC derived from hematopoietic progenitors show a disability to upregulate CD80, which is not counterbalanced by increased expression of CD86. *Ex vivo* DC have similar levels of CD80 and CD86 but lower levels of class II and higher level of CD40 expression than control mice. Thus, *in vitro* and *ex vivo* phenotypes suggest profound alterations of DC development in NOD mice.

Functional analysis of NOD DC in MLR

The ability of BM-DC and SPL-DC from NOD and control strain to stimulate MLR was then investigated. As shown in Figures 3a, b, unstimulated DBA/2 BM-DC were highly efficient at inducing allogeneic T cell proliferation. By contrast, unstimulated NOD BM-DC were unable to stimulate allogeneic T cells regardless of the number of stimulatory cells used. Unstimulated SPL-DC were more potent than BM-DC to stimulate allogeneic T cells, in agreement with their higher level of MHC class II and higher proportion of cells with high level of CD80 (Figures 1,

2). However, unstimulated NOD SPL-DC induced lower T cell proliferation than their DBA/2 counterpart. These data showed that both BM-DC and SPL-DC from NOD mice have low capacity to stimulate naive allogeneic T cells. These observations are not related to MHC haplotype or genetic background differences, such as Mls, between NOD and DBA/2, since similar results were obtained using DBA/2 splenocytes as responder cells and C57BL/6 DC as stimulating cells (data not shown).

NOD DC response to LPS

To investigate whether the deficient maturation of dendritic progenitors in the NOD could be reversed by activation, we studied the maturation of BM-DC and SPL-DC in the presence of LPS [28]. Under LPS stimulation, in both NOD and control strain, most cells expressed MHC class II molecules at high levels (Figure 4). However, class II expression on BM-DC and SPL-DC remained lower in NOD than in DBA/2 mice. Expression of CD80, CD86 and CD40 was enhanced in NOD mice reaching similar level as in DBA/2. This indicates that upregulation of co-stimulatory molecules occurs in NOD DC in the presence of inflammatory stimuli.When activated with LPS, BM-DC and SPL-DC from NOD mice were as efficient as DBA/2 to stimulate T cell proliferation (Figures 3c, d).

This indicate that the inflammatory stimuli from LPS can induce the maturation of NOD DC. MHC class II expression, however, remains decreased in all experimental conditions tested.

Production of IL-12p70 by NOD DC

DC maturation is associated with an initial elevation in production of IL-12p70, followed by a gradual decrease at later stages of DC maturation [29, 30]. To evaluate the production of IL-12p70, BM-DC from different mice (NOD, DBA/2, CBA, AB/H Biozzi) were stimulated at day 6 for 48 hours with either LPS and anti-CD40 Ab. A significant amount of IL-12p70 was produced with DC from all strains including the NOD (Table 2 and data not shown). In addition, the level reached with NOD BM-DC was 3.5 times that obtained with DBA/2 (Table 2) ($p = 0.035$). However, IL-12 production by SPL-DC stimulated at day 12 was not different between NOD and DBA/2 strains (Table 2) indicating that the capacity of NOD DC to produce higher amounts of IL-12 than control DC is dependent on the culture conditions.

DISCUSSION

As specialized in activating naive T lymphocytes, DC are likely to be central in the activation of T cells in auto-immune diseases. They are the first cell type that invade the islets at initiation of the auto-immune reaction to beta cells in diabetes [31]. In the NOD mouse, DC possibly interact with T cell subsets endowed with different functions in disease development. As $CD4^+$ T cells either protect against diabetes [32-36] or initiate the immune reaction that leads to beta cell destruction in the NOD mouse [37, 38], DC may control either suppression or induction of the auto-immune process. In order to characterize more extensively DC in the NOD mouse, we performed a phenotypical and functional analysis of NOD DC propagated from hematopoietic progenitors using two different culture systems. DC were propagated from BM and SPL progenitors in the presence of GM-CSF + IL-4 or GM-CSF + FL (+IL-6), respectively [26]. In both culture systems, NOD progenitors showed defective growth and led to lower yields of DC than in DBA/2, CBA, C57BL/6 or AB/H Biozzi control mice. A reduction in the yield of NOD BM-DC has previously been reported [20-22]. Our finding indicate that this reduced growth of DC is not dependent of the culture system and cannot be restored by the use of FL, although this cytokine has been shown to induce dramatic increase in the number of all DC subsets when injected *in vivo* [39]. The number of strains that we tested as controls, including the AB/H Biozzi strain which shares the $I\text{-}A^{g7}$ allele with the NOD, likely identifies defective DC growth as associated to the development of autoimmunity in the NOD rather than to peculiar features of the $I\text{-}A^{g7}$ molecule. Our culture conditions possibly point to a defective response of DC progenitors to GM-CSF. This inability of NOD mice progenitors to generate DC in response to GM-CSF is in keeping with the general defect of myelopoiesis in this strain [15, 40, 41]. Broad defects of myeloid progenitor response to IL-3 [41], and CSF-1 [15] have been reported in the NOD mouse. Defect in DC number have also been reported in the BB rat [19] and in human diabetes [17, 18]. Interestingly, we show that the number of DC per spleen is significantly reduced in NOD mice, indicating that defective growth of DC may occur *in vivo*. Such a defect possibly plays role in disease development by interfering with either the selection of autoreactive or regulatory T cells within the thymus or by contributing to presentation of autoantigens in the periphery.

Following their expansion from BM as well SPL progenitors, NOD DC show low levels of MHC class II expression and CD80 expression. It has been reported that

NOD DC derived from BM progenitor cells cultured with GM-CSF alone display an immature phenotype, with low level of MHC class II, CD80, CD86 and CD40 expression [20, 21, 42]. However, conflicting results have been reported when progenitor cells were cultured with GM-CSF + IL-4. In agreement with our data, Lee *et al.* [21] showed that NOD DC maintain an immature phenotype, even in the presence of IL-4. In contrast, others reported a more mature phenotype with higher expression of CD80, CD86 CD40 and CD54 markers [20, 22] after 4 days of culture. In our hands, as in Lee *et al.*, cultures of BM progenitors with GM-CSF yield few mature DC and mostly proliferating immature cells, the differentiation of which is promoted by further reseeding [25, 43]. It is likely that in NOD mice, day 4 BM-DC are enriched in a subset of DC with high level of costimulatory and activation molecules, that is no longer detectable in day 6/7 culture.

We show that NOD DC carry low expression of MHC class II that does not relate with I-Ag^7 instability [44], since it is not observed with the Biozzi control strain which express the I-A^{g7} molecule. In agreement with our data, it has been reported that congenic NOD. H-2^{h4} mice (K^k, I-A^k, I-E^k) exhibit low MHC class II expression indicating that this defect does not map to the MHC [42]. The fact that spleen DC maturing *in vivo* also display low MHC class II expression suggests that NOD DC could allow escape of T cells with high affinity for beta cell autoantigens as well as defective selection of regulatory T cells expressing low affinity TCR [44-46]. In addition, because normal level of MHC class I is expressed on NOD DC [23] (and ours data), NOD DC remain able to efficiently cross-prime class I-restricted cytotoxic T lymphocytes, which are involved in initiating the diabetes auto-immune process [38, 47, 48].

The level of CD80 expression is also decreased on NOD DC. Similar observations have been reported on DC derived from BM progenitors cultured in the presence of GM-CSF alone [21, 42]. Our observations show that the use of IL-4 or FL in culture conditions known to give rise to mature functional DC does not restore CD80 expression. The fact that *ex vivo* splenic DC display normal level of CD80 does not exclude that CD80 expression is altered on DC from other tissues or organs. This inability of NOD DC propagated *in vitro* with GM-CSF to upregulate CD80, with normal level of CD86, might have pathological relevance. Treatment with anti-CD86 Ab inhibits diabetes development, whereas treatment with anti-CD80 Ab or a combination of anti-CD80 and anti-CD86 Ab accelerates diabetes [49]. In addition, B7- and CD28-deficient NOD mice show altered development of $CD4^+CD25^+$ regulatory T cells [50]. It is thus possible that DC defects in diabetes

contribute to abnormal recruitment of immunoregulatory T cells. Indeed, in NOD mice, defective T cell suppressor activity seems to be related to defective APC functions rather than the absence of an immunoregulatory T cell subset [14]. We show that strong inflammatory stimulus known to circumvent diabetes development [51] upregulate MHC class II and co-stimulatory molecules on NOD DC. Interestingly, addition of LPS *in vitro* restore the capacity of NOD cells to achieve maturation, normal expression of CD80, CD86 and MHC class II molecules. This is remarkable in light of the role of environmental factors on the incidence of diabetes in NOD colonies [51].

NOD BM-DC produce higher IL-12p70 than control strains when stimulated with LPS with or without anti-CD40 triggering. Increased IL-12p70 secretion by NOD DC has been reported as a consequence of defective NF-kappaB regulation [52]. Our data could appear conflicting with those of Feili-Hariri *et al.* showing lower amounts of IL-12p70 production by NOD BM-DC [22]. However, the DC used were isolated in different culture conditions. The fact that, in our hands, IL-12p70 production by SPL-DC stimulated at day 12 was similar between NOD and DBA/2 clearly indicate that NOD DC are not intrinsically defective in IL-12 production.

Our finding finally show that *ex vivo* DC from unmanipulated 8 week old female NOD mice express low MHC class II and high CD40 levels. Data on NOD DC isolated from peripheral tissues are scare and conflicting, probably due to the low yields of DC obtained. It has been reported that NOD splenic DC exhibit a normal phenotype, and induce prolonged proliferation of syngeneic T cells probably due to defective apoptosis induction [23], but without studying and/or comparing CD80, CD40 and class II expression on NOD DC to control strains. Other reports show low CD86 expression [53]. We are currently investigating whether DC functional defects are associated with the low MHC class II and high CD40 expression. Preliminary results show that the relative proportion of CD8alpha$^+$ DC *versus* CD8alpha$^-$ DC is enhanced in NOD spleen and that splenic DC are more potent to induce proliferation of allogeneic T cells than C57BL/6 DC. Altogether, these data strongly suggest that splenic DC with low MHC class II and high CD40 expression could be responsible for defect in T cell regulation in the NOD. This DC subset, could be the *in vivo* counterpart of the activated BM-DC described by others [20, 22].

CONCLUSION

In conclusion, it is unlikely that a unique defect is responsible for the development of autoimmunity in the NOD. Defective growth and function of DC identified *in vitro* and *ex vivo* should be considered as one factor involved. We hypothesize that low MHC class II expression on NOD DC could be responsible for altered T cell repertoire selection. In addition, NOD DC with defective or delayed maturation could either fail to activate regulatory T cells and/or enhance Th1 response through high IL-12p70 production.

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Figures

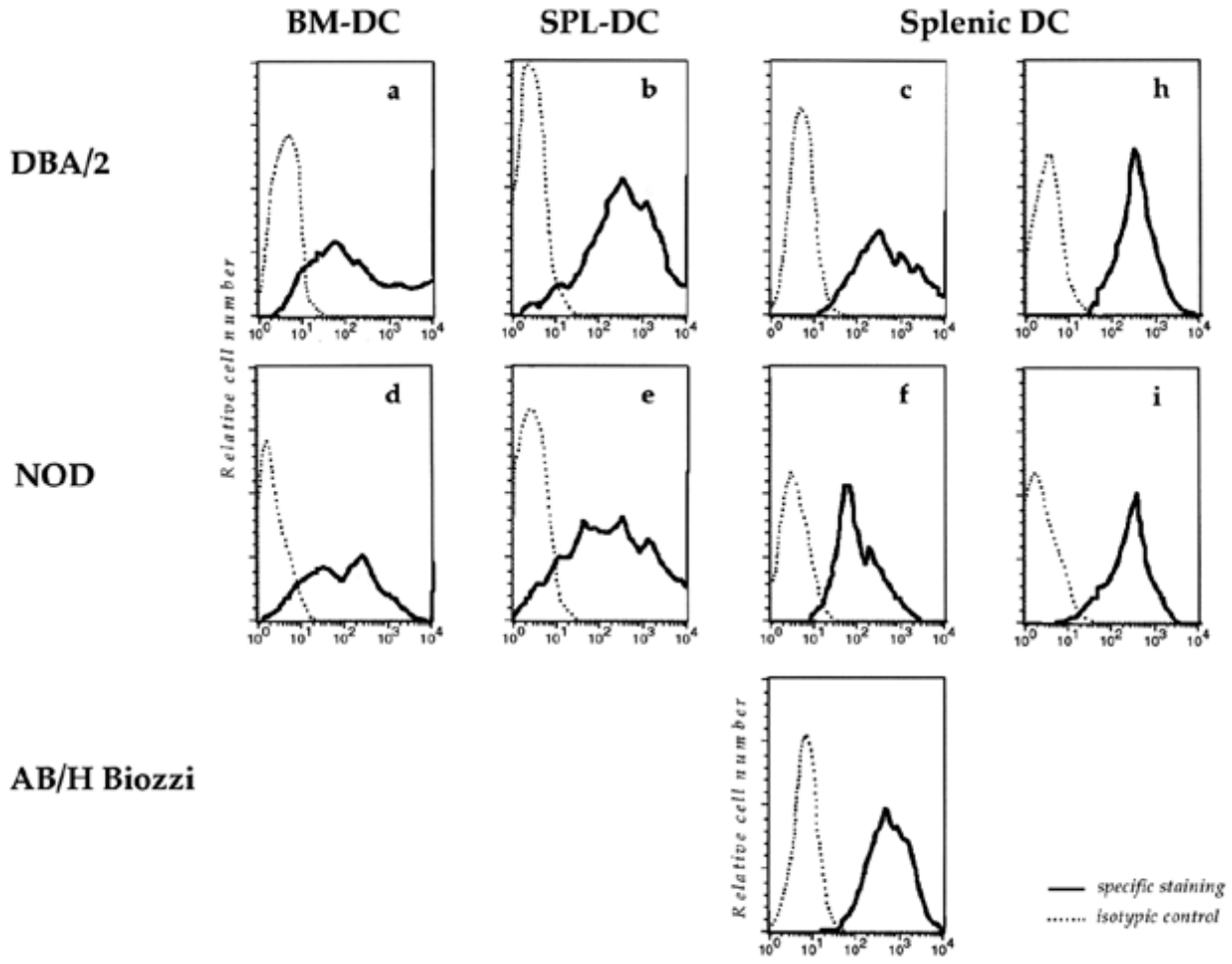


Figure 1

Low MHC class II expression on NOD DC. BM-DC (a, d), SPL-DC (b, e) and *ex vivo* splenic DC (c, f, g, h, i) from 8 week old female mice were double stained with anti-CD11c and anti-I-A Ab. *Ex vivo* splenic DC were also double stained with anti-CD11c and anti-H-2K^d Ab (h, i). Cells were analyzed by flow cytometry. Histograms represent I-A staining (a to g) or H-2K^d staining on the CD11c positive

cells and are representative of 3 independent experiments, each consisting of 3 cultures of BM-DC and SPL-DC or 3 suspensions of *ex vivo* DC from individual mice.

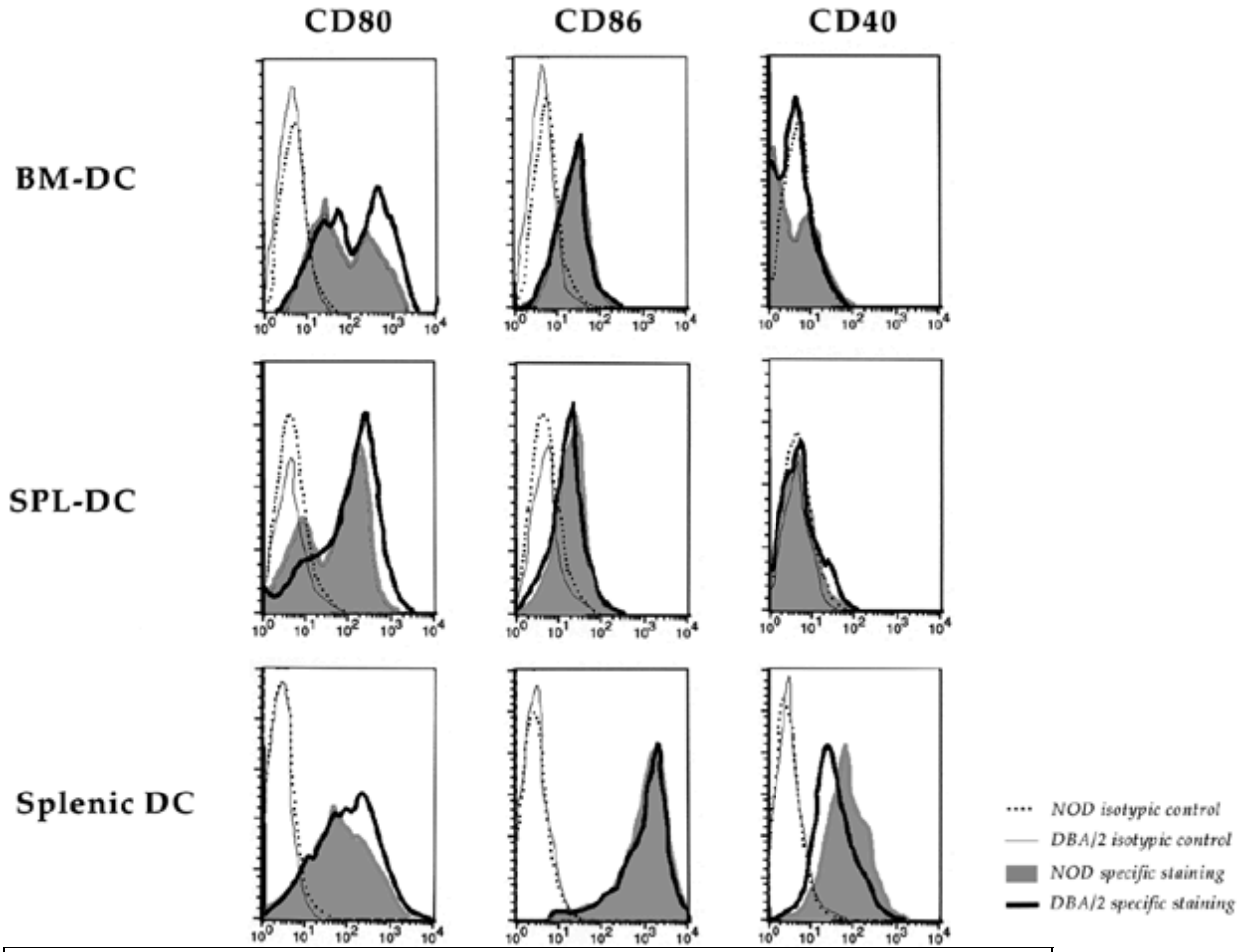


Figure 2

Expression of co-stimulatory molecules on NOD DC.

BM-DC, SPL-DC and *ex vivo* splenic DC from 8 week old female mice were double stained with anti-CD11c and the indicated Ab and were analyzed by flow cytometry. Histograms represent CD80, CD86 and CD40 staining on the CD11c positive cells and are representative of 3 independent experiments, each consisting of 3 cultures of BM-DC and SPL-DC or 3 suspensions of *ex vivo* DC from individual mice.

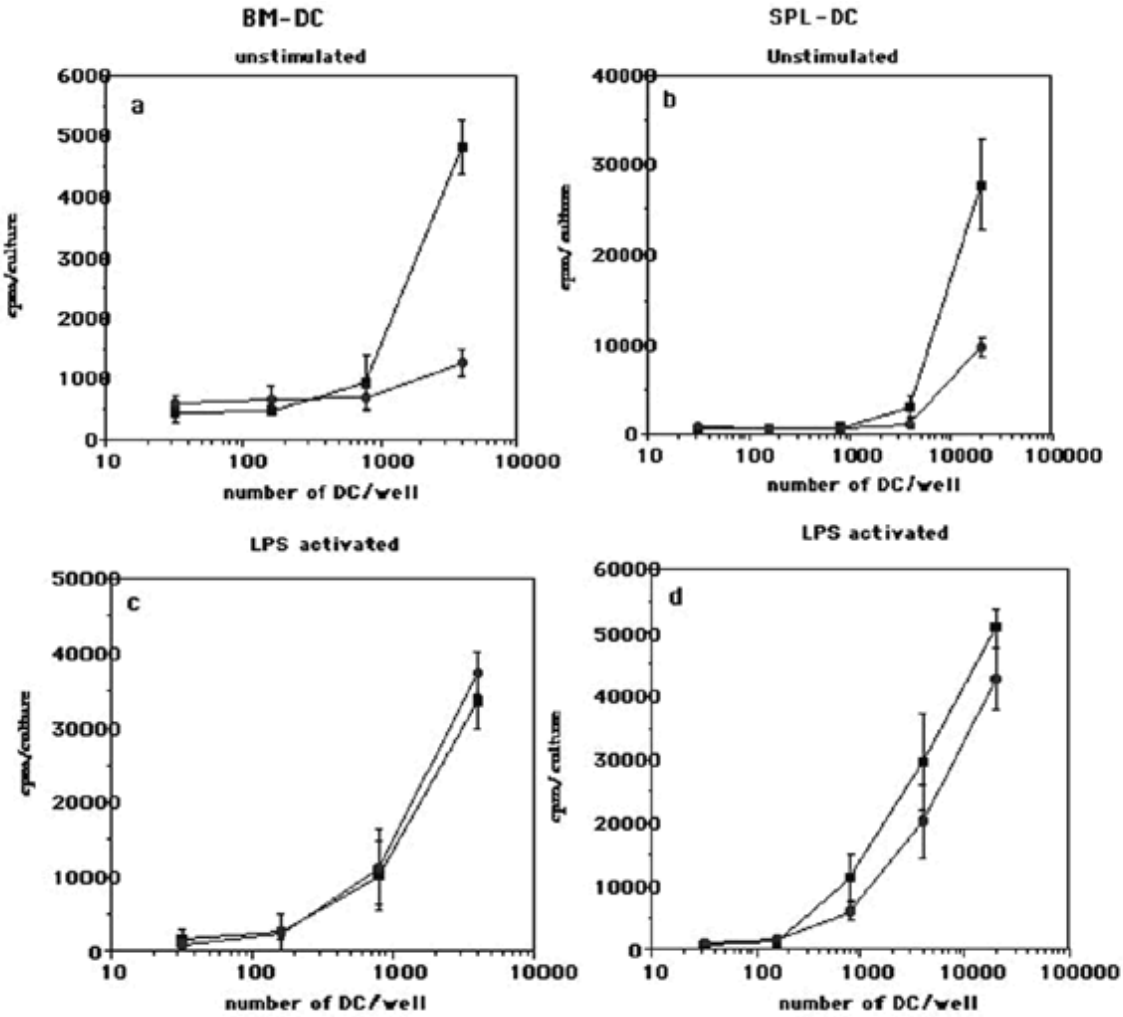


Figure 3

Functional analysis of NOD DC in MLR. BM-DC and SPL-DC from DBA/2 (square) and NOD (circle) were stimulated for 48 hours with or without LPS. Cells recovered were used to stimulate B6 splenocytes (1×10^5 cells/well). T cell proliferation was measured by ^{3}H-Tdr incorporation (0.1 muCi/well) after 3 days of culture. Data represent mean values ± SD of triplicates. The level of incorporation in syngeneic MLR was < to 1000 cpm/culture.

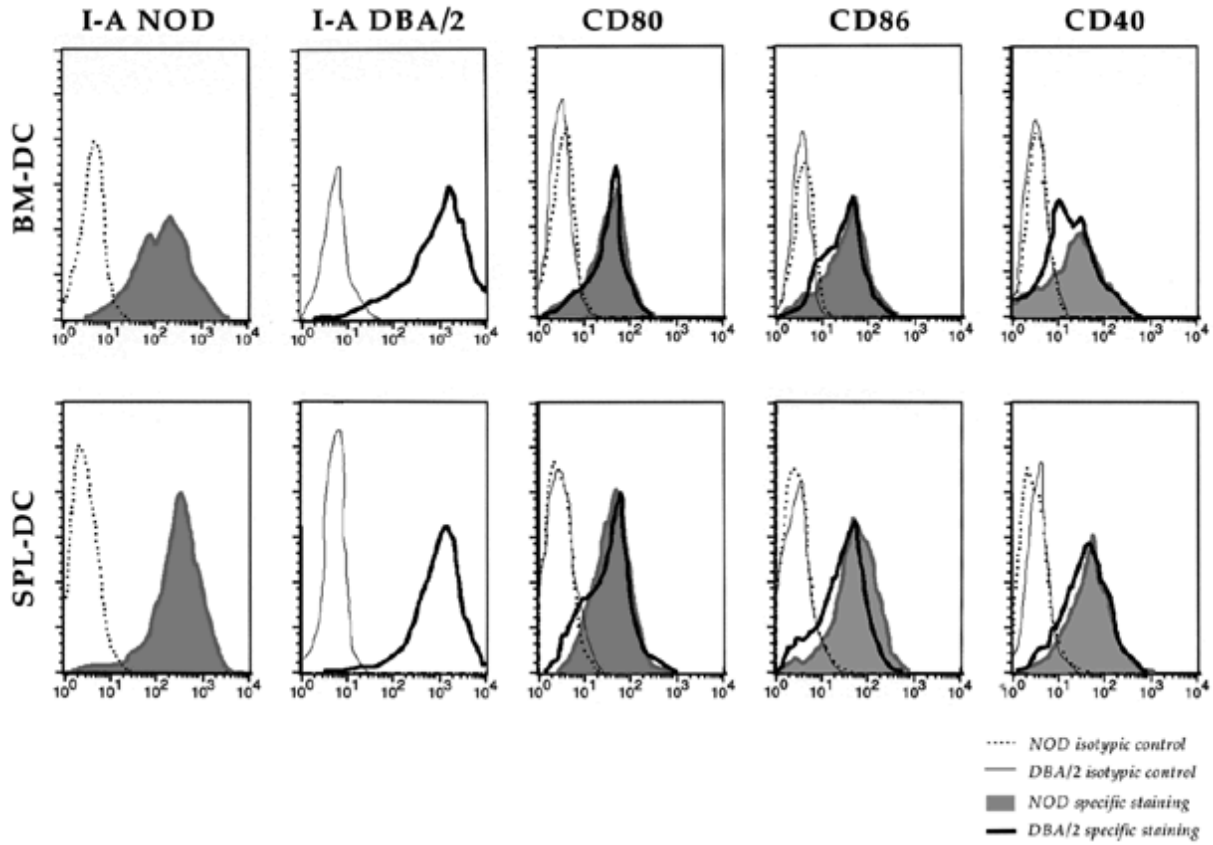


Figure 4 Phenotype of LPS activated NOD DC. BM-DC and SPL-DC were stimulated for 48 hours in the presence of LPS. The recovered cells were double stained with anti-CD11c and the indicated Ab and were analyzed by flow cytometry. Histograms represent I-A, CD80, CD86 and CD40 staining on the CD11c positive cells and are representative of 3 independent experiments, each consisting of 3 cultures of BM-DC and SPL-DC or 3 suspensions of *ex vivo* DC from individual mice.

Table 1
Low number of DC in NOD mice

	Number of CD11c+/MHC class II+ cells		
	(x 10^{-6}/10 x 10^{6} initial cells)		(x 10^{-3}/10^{8} splenocytes)
strain (n = 6)	BM-DC	SPL-DC	*ex vivo* DC
NOD	1.91 ± 1.08*	3.37 ± 2.47*	2.44 ± 1.39*
DBA/2	9.17 ± 2.87	139.30 ± 36.50	7.6 ± 2.18
C57BL/6	9.67 ± 3.51		
CBA	4.30 ± 0.26		6.65 ± 1.20
AB/HBiozzi	5.20 ± 2.26		5.35 ± 0.2

DC were derived from BM or SPL progenitors (3 week culture) with GM-CSF + IL-4 and GM-CSF + FL (+IL-6) respectively. *Ex-vivo* DC were purified from the spleen as described in material and methods. The number of cells was indicated as mean ± SD. Statistical analysis using the non parametric Mann-Whitney test reveals significant difference between NOD and control strains ($p < 0.05$)*. No significant difference is observed between control strains ($p > 0.05$).

Table 2
IL-12p70 production by NOD DC

	IL-12p70 (pg/ml)			
	BM-DC		SPL-DC	
	DBA/2	NOD	DBA/2	NOD
Stimulation				
–	< 30	< 30	< 30	< 30
LPS + anti-CD40	753 (± 204)	2,642* (± 1,135)	2,643 (± 1,096)	2,564 (± 1,051)

Day 6 BM-DC and day 12 SPL-DC (2 x 10^{5} C/ml) were stimulated for 48 hours with 1 µg/ml LPS and 1 µg/ml anti-CD40 agonist Ab. Amounts of IL-12p70 was measured in culture supernatant. Data are indicated as mean ± SD. Statistical analysis using the non parametric Mann-Whitney test reveals significant difference between NOD and DBA/2 values for BM-DC ($p = 0.035$)* and no significant difference between NOD and DBA/2 values for SPL-DC ($p = 0.280$).

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In this issue

Viral and cellular interleukin-10 (IL-10)-related cytokines: from structures to functions

Laure Dumoutier, Jean-Christophe Renauld
IL-10, IL-19, IL-20, IL-22, mda-7, AK155.

Dendritic cells (DC) promote natural killer (NK) cell functions: dynamics of the human DC/NK cell cross talk

Nadine C. Fernandez, Caroline Flament, Florent Crépineau, Eric Angevin, Eric Vivier, Laurence Zitvogel
NK cells, dendritic cells, cell-to-cell interaction, activation, proliferation.

Altered dendritic cells (DC) might be responsible for regulatory T cell imbalance and autoimmunity in nonobese diabetic (NOD) mice

Sarah Boudaly, Joëlle Morin, Rolande Berthier, Patrice Marche, Christian Boitard
dendritic cell, nonobese diabetic mice, autoimmunity, insulin-dependent diabetes mellitus.

Estradiol and raloxifene decrease the formation of multinucleate cells in human bone marrow cultures

Ana Claudia Ramalho, Philippe Couttet, Claude Baudoin, Caroline Morieux, Anne-Marie Graulet, Marie-Christine de Vernejoul, Martine E. Cohen-Solal
osteoclast, estrogen receptor, SERM, cytokines, apoptosis.

Interleukin-10 and cAMP-elevating agents cooperate to induce suppressor of cytokine signaling-3 via a protein kinase A-independent signal

Sara Gasperini, Luca Crepaldi, Federica Calzetti, Luana Gatto, Chiara Berlato, Flavia Bazzoni, Akihiko Yoshimura, Marco A. Cassatella
SOCS-3, PGE, IL-10, PBMC, neutrophils, PKA.

Different patterns of interleukin-1alpha and interleukin-1beta expression in organs of normal young and old mice

Moshe Hacham, Shmuel Argov, Rosalyn M. White, Shraga Segal, Ron N. Apte
IL-1alpha, IL-1beta, organ cytokine expression, young/old age, homeostasis.

The combination of the interleukin-1alpha (IL-1alpha-889) genotype and the interleukin-10 (IL-10 ATA) haplotype is associated with increased interleukin-10 (IL-10) plasma levels in healthy individuals

Sanna Kilpinen, Heini Huhtala, Mikko Hurme
interleukin-1, interleukin-10, gene polymorphism, plasma.

The interleukin-6 G(-174)C polymorphism and the ex vivo interleukin-6 response to endotoxin in severely injured blunt trauma patients

Michael Heesen, Udo Obertacke, F. Ulrich Schade, Brunhilde Bloemeke, Matthias Majetschak
interleukin-6, endotoxin, genetic polymorphism, polymerase chain reaction, whole blood, critical illness.

Comparison of leptin- and interleukin-6-regulated expression of the rPAP gene family: evidence for differential co-regulatory signals

Daniël Broekaert, Sven Eyckerman, Delphine Lavens, Annick Verhee, Wim Waelput, Joël Vandekerckhove, Jan Tavernier
forskolin, IL-6, leptin, rPAP/Reg gene family, signal transduction.

Mice lacking alpha2-macroglobulin show an increased host defense against Gram-negative bacterial sepsis, but are more susceptible to endotoxic shock

Tino Hochepied, Fred Van Leuven, Claude Libert
TNF, shock, acute phase, inflammation, alpha2-macroglobulin.

Tumor necrosis factor-alpha mRNA stability in human peripheral blood cells after lipopolysaccharide stimulation

Lucile Baseggio, Carole Charlot, Jacques Bienvenu, Pascale Felman, Gilles Salles
tumor necrosis factor, mRNA stability, lipopolysaccharide, tristetraprolin.

Caspase-1 levels in biological fluids from patients with multiple sclerosis and from patients with other neurological and non-neurological diseases

Diego Franciotta, Gianvito Martino, Elisabetta Zardini, Roberto Furlan, Roberto Bergamaschi, Maira Gironi, Alessandra Bergami, Giovanna Angelini, Fabrizio De Benedetti, Patrizia Pignatti, Gianna Moscato, Vittorio Cosi
asthma, cerebrospinal fluid, juvenile idiopathic arthritis, sputum, synovial fluid, THP-1 cells.

Divergent effects of tumor necrosis factor-alpha and lymphotoxin-alpha on lethal endotoxemia and infection with live Salmonella typhimurium in mice

Edi Dharmana, Monique Keuter, Mihai G. Netea, Ineke C.M.M. Verschueren, Bart Jan Kullberg
tumor necrosis factor, lymphotoxin, salmonella typhimunium, knock-out mice.

Th1/Th2 cytokine balance and nitric oxide in cerebrospinal fluid and serum from patients with multiple sclerosis

María del Carmen Rodríguez-Sáinz, Silvia Sánchez-Ramón, Clara de Andrés, Margarita Rodríguez-Mahou, M Angeles Muñoz-Fernández
cytokine, nitric oxide, multiple sclerosis, cerebrospinal fluid, oligoclonal bands, Igs.

Retinoids, cisplatin and interferon-alpha in recurrent or metastatic cervical squamous cell carcinoma: clinical results of 2 phase II trials

Anne-Chantal Braud, Laurence Gonzague, François Bertucci, Dominique Genre, Jacques Camerlo, Gwenaelle Gravis, Anthony Goncalves, Vincent Moutardier, Frédéric Viret, Dominique Maraninchi, Patrice Viens
all-trans-retinoic acid, 13 Cis retinoic acid, interferon-alpha, cervical squamous cell carcinoma.

Circulating macrophage colony stimulating factor as a marker of tumour progression

Raymond S. McDermott, Laurent Deneux, Véronique Mosseri, Jacques Védrenne, Krishna Clough, Alain Fourquet, José Rodriguez, Jean-Marc Cosset, Xavier Sastre, Philippe Beuzeboc, Pierre Pouillart, Susy M. Scholl
CSF-1, tumour progression, metastasis, de-differentiation.

The effect of interleukin-1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) on granulocyte macrophage-colony stimulating factor (GM-CSF) production by neuronal precursor cells

J. Benjamin Dame, Nasser Chegini, Robert D. Christensen, Sandra E. Juul

IL-1beta, TNF-alpha, GM-CSF, neuron, mRNA, protein.

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