Dendritic Cell Differentiation and Immune Tolerance to Insulin-Related Peptides in *Igf2*-Deficient Mice[1]

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There is some evidence that insulin-like growth factor 2 (IGF-2) may intervene in the control of T cell differentiation. To further study the immunoregulatory function of this growth factor, we analyzed the immune system of *Igf2*$^{-/-}$ mice. Phenotypically, some immunological parameters such as lymphoid organ morphology and cellularity were unaltered in *Igf2*$^{-/-}$ mice, but an increase of CD8$^+$ cells and a decrease of B220$^+$ cells were observed in spleen. In vitro, the development of bone marrow-derived dendritic cells was affected by the absence of *Igf2* expression. After maturation, a higher percentage of immature dendritic cells was observed in *Igf2*$^{-/-}$ population, together with a secondary decrease in allogenic T cell proliferation. Activation of T cells was also affected by the lack of expression of this growth factor. The profile of B cell response in mutant mice immunized with IGF-2 evidenced a T-dependent profile of anti-IGF-2 Abs that was absent in *Igf2*$^{+/+}$ mice. The influence of IGF-2 upon tolerance to insulin was also assessed in this model, and this showed that IGF-2 also intervenes in tolerance to insulin. The presence of a T-dependent response in *Igf2*-deficient mice should allow cloning of specific "forbidden" T CD4$^+$ lymphocytes directed against IGF-2, as well as further investigation of their possible pathogenic properties against insulin family. ***The Journal of Immunology,*** **2006, 176: 4651–4657.**

Insulin-like growth factors 1 and 2 (IGF-1/2)[3] are members of the insulin hormone family that exert a prominent role in fetal and postnatal development (1, 2). Null mutant mice for *Igf1* show a marked growth deficiency with 60% of normal body weight, a variable neonatal lethality frequency depending on genetic background, and they are infertile (3). The absence of *Igf2* induces similar growth failure, but *Igf2*$^{-/-}$ mice are viable and fertile (4, 5).

With regard to the implication of IGF in immune physiology and development, most of the studies have focused on the growth hormone/IGF-1 axis and type 1 IGF receptor (IGF-1R), a transmembrane tyrosine kinase homologous to the insulin receptor that mediates most of the biological effects of IGF-1 and IGF-2 (6, 7). Normal development and ex vivo activation of T and B cells are observed in chimeric *Rag2*-deficient C57BL/6 mice reconstituted with fetal liver cells from *Igf1r*$^{-/-}$ mice. However, this model revealed an unexpected decrease of the T-independent B cell response which is important in bacterial defense mechanisms (8).

So far, very few studies have investigated the function of IGF-2 in immune development and physiology. This growth factor is the dominant peptide of the insulin family expressed in the thymus epithelium of different species (9, 10). Thymic IGF-2 influences thymic development and T cell differentiation as evidenced by analysis of IGF-2 transgenic dwarf mice, which develop a thymic hyperplasia (11) with an increased number of thymocytes (and CD4$^+$ T lymphocytes in particular) (12, 13). This increase of T cells is also observed in the spleen compartment of IGF-2 transgenic mice, but there is no significant effect on B cell development. In vitro, T cell differentiation and proliferation are also impaired in fetal thymic organ culture treated with anti-IGF-2, anti-IGF-1R, or anti-IGF-2R. In these experimental conditions, T cell differentiation is inhibited at early CD4$^-$ CD8$^-$ stage, whereas anti-IGF-2 induces an increase in thymic CD8$^+$ T cells suggesting a role for IGF-2 in T cell final commitment to CD4 or CD8 lineage (14). Finally, a role of thymic IGF-2 in central immune self-tolerance of the insulin family has been suggested by the observation of a defect of *Igf2* transcription in the thymus of Bio-Breeding (BB) rats, an animal model of type 1 diabetes (15).

In this study, we further investigate the role played by IGF-2 in the immune function through the use of *Igf2*-deficient mice. Although *Igf2* deficiency does not interfere with the development of major lymphoid structures, bone marrow (BM)-derived dendritic cells (DC) from *Igf2*-deficient mice show a defect of maturation as evidenced by a higher persistence of immature DC (iDC) compared with wild-type (wt) mice. This is associated with a decrease of ability of *Igf2*$^{-/-}$ DC to activate allogenic T lymphocytes in MLR. T cell proliferation assessed by MLR is also severely impaired. The profile of the immune responses against IGF-2 and insulin in mutant mice was also evaluated and shows that tolerance to IGF-2 depends on *Igf2* expression, but that IGF-2 also contributes to tolerance to insulin.

Materials and Methods

Animals

Igf2$^{+/-}$ mice were provided by Dr. A. Efstratiadis and were bred at the Animal Department of Liege University under conventional conditions

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[3] Abbreviations used in this paper: IGF-1/2, insulin-like growth factor-1/2; IGF-1R, type 1 IGF receptor; IGF-2R, type 2 IGF receptor; DC, dendritic cell; iDC, immature DC; wt, wild type; mDC, mature DC; BM, bone marrow; TEC, thymic epithelial cell; rm, recombinant murine.

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with free access to food and water. DNA for typing was prepared from tail biopsies. BALB/c mice were also bred under the same conditions. Male and female mice were 5–8 wk old at the time of experiments, which were performed in agreement with the local ethic committee.

Phenotype of mice

Igf2$^{-/-}$ and wt mice were weighed and were sacrificed by cervical dislocation. The thymus and the spleen were removed and weighed. The number of living cells was estimated by trypan blue dye exclusion.

Generation of BM-derived DC

BM-derived DC were isolated and differentiated according to the method developed by Lutz et al. (16). Briefly, *Igf2*$^{-/-}$ and wt mice were sacrificed by cervical dislocation and femur/tibiae were removed and freed of muscles and tendons. Both ends were cut with scissors and BM cells were flushed with DPBS without Ca^{2+} and Mg^{2+} (Cambrex) using a syringe with a needle 0.45 mm in diameter. Cell suspension was filtered through a 70-μm cell strainer (BD Biosciences) to remove remaining bone fragments. At day 0, the cells were seeded at 2×10^5 cells/ml in 100-mm petri dishes (Falcon no. 1029; VWR International) and 10 ml per dish. The medium used during differentiation (10 days) to obtain iDC was RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) (Cambrex), 2-ME (50 μM), 10% heat-inactivated FCS (Invitrogen Life Technologies) and recombinant murine (rm) GM-CSF (20 ng/ml, personal production). The vector pcDNA GM-CSF was a gift from J.-C. Renauld (Université Catholique de Louvain, Brussels, Belgium) and rmGM-CSF was produced by COS-7 transfection using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. At day 3, an additional 10 ml of complete medium containing rmGM-CSF (20 ng/ml) per dish were added, and at day 6 and 8, 10 ml were collected, centrifuged at $200 \times g$ for 7 min. The cell pellet was resuspended in fresh complete medium with rmGM-CSF (20 ng/ml) and was plated again. At day 10, the maturation to mDC was performed in 100 mm tissue culture petri dishes (Falcon no. 3003; VWR International) with the same medium containing rmGM-CSF (10 ng/ml) and LPS (2.5 μg/ml, *Escherichia coli* O26:B6; Sigma-Aldrich) for 24 or 48 h. For this step, the cells were seeded at 1.5×10^5 cells/ml and 10 ml per plate.

RNA isolation

Total RNA was extracted from BM cells, iDC, mDC, and 16-day-old fetal brains using RNeasy Mini kit (catalog no. 74104; Qiagen) according to the manufacturer's instructions. Pancreas and liver total RNA was isolated using Tripure isolation reagent (Roche). DNA contamination was removed by treatment of samples with RNase-free DNase (Roche). RNA was dosed with the RiboGreen RNA quantitation kit (Molecular Probes).

Actin, Insulin 1 (Ins1), Insulin 2 (Ins2), Igf1, Igf2, Igf1r, Igf2r RT-PCR and sequencing

Total RNA (250–500 ng) was reverse transcribed in a total of 20 μl by the 1st Strand cDNA synthesis kit for RT-PCR (AMV; Roche) using oligo(dT)primer. Reverse-transcription products (1:20) were used directly for PCR using FastStart *Taq*DNA polymerase (Roche) according to the manufacturer's instructions in a UNO II rapid thermocycler (Biometra). After a denaturation step at 94°C for 5 min, the samples were submitted to 35 cycles comprising 94°C for 45 s, variable temperature depending on primers for 45 s and 72°C for 45 s. To finish, an additional elongation step was performed at 72°C for 7 min. PCR products were analyzed in 2% agarose ethidium bromide-staining gels. The sequence of primers and the respective temperature are presented in Table I. The PCR products were sequenced (Genome Express) using their respective primers.

Flow cytometry

Immature DC, mDC, fresh thymocytes, BM cells, and splenic cells (5×10^5-10^6) were centrifuged at $200 \times g$ for 5 min and prepared in 100 μl of DPBS (Cambrex) for labeling. Cell preparations were stained for 20 min at 4°C and washed one time in DPBS.

Flow cytometric analyses were performed with FACStar^{Plus} (BD Biosciences). Abs (BD Biosciences) were CD86-PE clone GL1 (rat IgG2a), CD80-PE clone 16-10A1 (Armenian hamster IgG), Gr-1/Ly6G-PE clone RB6-8C5 (rat IgG2b), I-A/I-E-PE clone M5/114.15.2 (rat IgG2b), CD11c-FITC clone HL3 (Armenian hamster IgG), CD8α-FITC clone 53-6.7 (rat IgG2a), CD45R/B220-PE clone RA3-6B2 (rat IgG2a), and CD4-PE clone RH4-5 (rat IgG2a). The data analysis was performed using CellQuest software (BD Biosciences).

The MLR

The capacity of DC from wt and *Igf2*$^{-/-}$ mice on T cell activation was evaluated in a primary allogenic MLR. DC were harvested after 24 h of maturation with LPS and used as T cell stimulators after 3000 rad of gamma-irradiation. BALB/c spleen cell responders were prepared by mechanical disruption, erythrocyte lysis (Hybrid-Max; Sigma-Aldrich), and filtration through a 70-μm cell strainer (BD Biosciences). The MLR was performed with 10^6 responders $\times$ 10^5 irradiated stimulators/ml in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessential acid, 10 mM HEPES (Cambrex), 50 μM 2-ME (Invitrogen Life Technologies), and 10% heat-inactivated FCS (Invitrogen Life Technologies). The cells were incubated in 5% CO_2 at 37°C.

Igf2$^{-/-}$ and wt T cell proliferation was also assessed by MLR. BALB/c BM-derived DC were used as stimulators after 3000 rad of gamma-irradiation. *Igf2*$^{-/-}$ and wt T cell responders were prepared by mechanical disruption, erythrocyte lysis (Hybrid-Max; Sigma-Aldrich), and filtration through 70 μm cell strainer (BD Biosciences). The MLR was performed with 10^6 responders $\times$ 10^5 gamma-irradiated stimulators/ml in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessential acid, 10 mM HEPES (Cambrex), 50 μM 2-ME (Invitrogen Life Technologies), and 10% heat-inactivated FCS (Invitrogen Life Technologies). The cells were incubated in 5% CO_2 at 37°C.

After 0, 48, and 96 h, 800 μl of culture were collected and distributed in triplicate on 96-well flat-bottom (200 μl/well). Then, 0.5 μCi of [^{3}H]thymidine (Amersham Biosciences) was added to each well for an additional 18 h of culture. Cellular DNA was harvested using a Titertek cell harvester (Flow Laboratories), and scintillation counting was performed in a Beckman liquid scintillation counter (BD Biosciences). wst-1 tests were performed with 10 μl of wst-1 Cell Proliferation Reagent (Roche) for an additional 4 h of culture. The absorbance was measured at 450–620 nm.

Table I. *Synthetic primers selected for RT-PCR and sequencing*

Genes	Strand	Primer Sequence	Annealing T°	PCR Products Size (bp)	Reference
Ins1	Sense	AGACCATCAGCAAGCAGGTC	65°C	324	17
	Antisense	CTGGTGCAGCACTGATCCAC			
Ins2	Sense	GTGGAGGACCCACAAGTGG	65°C	198	17
	Antisense	ATTCATTGCAGAGGGGTAGGCT			
Igf1	Sense	GCTGAGCTGGTGGAATGCTCTTCAGTTC	55°C	215	18
	Antisense	CTTCTGAGTCTTGGGCATGTCAGTGTG			
Igf2	Sense	GAGCTTGTTGACACGCTTCAGTTTGTC	55°C	356	18
	Antisense	ACGTTTGGCCTCTCTGAACTCTTTGAG			
Igf1r	Sense	GACATCCGCAACGACTATCAG	50°C	395	19
	Antisense	GTAGTTATTGGACACCGCATC			
Igf2r	Sense	CTGGAGGTGATGAGTGTAGCTTGGC	55°C	235	20
	Antisense	GAGTGACGAGCCAACACAGACAGGTC			
Actin	Sense	TAAAGACCTCTATGCCAACACAGT	54°C	250	21
	Antisense	CACGATGGAGGGGCCGGACTCATC			

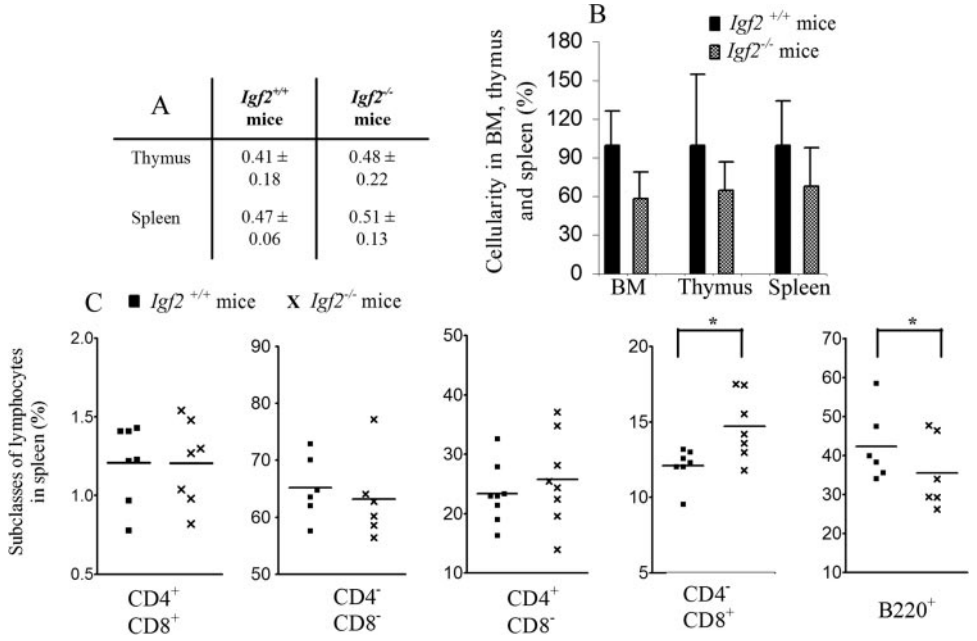

FIGURE 1. Analysis of $Igf2^{+/+}$ and $Igf2^{-/-}$ phenotype. *A*, Thymic and splenic weights of $Igf2^{+/+}$ and $Igf2^{-/-}$ mice ($n = 4$ of each genotype). Tissue weights were calculated in percentage of total body weight. *B*, Cellularity of $Igf2^{+/+}$ (filled histogram) and $Igf2^{-/-}$ (hatched histogram) in BM, thymus, and spleen. The number of $Igf2^{-/-}$ cells was expressed in percentage of $Igf2^{+/+}$ cells (100%) ($n = 8$ of each genotype). *C*, Lymphoid cell subclasses (%) in spleen of the two kinds of mice. These subclasses were analyzed by flux cytometry ($n = 8$ of each genotype). Mean ± SD; *, $p < 0.05$.

Mice immunization

$Igf2^{-/-}$ and wt mice were injected i.p. with 5 μg of rmIGF-2 (R&D Systems) or with 5 μg of human insulin (Roche) to study the humoral response and with 50 μg of murine IGF-2-derived B11-25 (NEOSYSTEM) to analyze the cellular response. CFA (Sigma-Aldrich) (v/v) is used for the first injection and IFA (Sigma-Aldrich) (v/v) for the other injections. For humoral response, three injections separated by 20 days were performed. Blood test was performed 15 days after each injection in agreement with the Liege University ethic committee. For cellular response, two injections separated by 15 days were performed.

Dosage of anti-IGF-2 and anti-insulin Abs

Abs against IGF-2 and insulin were detected and quantified using a specific ELISA procedure. Microplates (Nunc Maxisorp 468667; VWR International) were incubated overnight at 4°C with 2 μg/ml rmIGF-2 (R&D Systems) or 5 μg/ml human insulin (Roche) in 100 μl of 0.1 M $NaHCO_3$ (pH 8.4). From this step, the procedure was identical between the two kinds of ELISA. SL buffer (0.01 M phosphate buffer, pH 7.4) was used as the basic buffer, and all reactions were incubated at 37°C under agitation. To block nonspecific binding, 200 μl of SL buffer containing 1% of BSA (Sigma-Aldrich) was added per well for 2 h. Four washes with 0.01 M phosphate buffer with 0.5‰ of Tween 20 (washing buffer) were performed, and the sera were diluted in SL buffer containing 0.5% BSA and 0.1% Tween 20 (VWR International) and incubated to 100 μl/well for 2 h. After four washes, the samples were incubated with peroxidase-labeled anti-mouse Abs diluted in the same buffer at a different concentration: 1/4000 for anti-IgG (Prosan), 1/2000 for anti-IgG1, 1/2000 for anti-IgG2a, 1/2000 for anti-IgG2b, 1/2000 for anti-IgG3, and 1/3000 for anti-IgM (Serotec). The incubation was performed in 100 μl for 1 h. The microplates were washed 4 times and 200 μl/well of substrate buffer (TMB; Biosource Europe) were added for 20 min at room temperature under agitation. The reaction was stopped with 50 μl of 0.4 N HCl, and the absorbance was measured at 450–620 nm with a spectrophotometer.

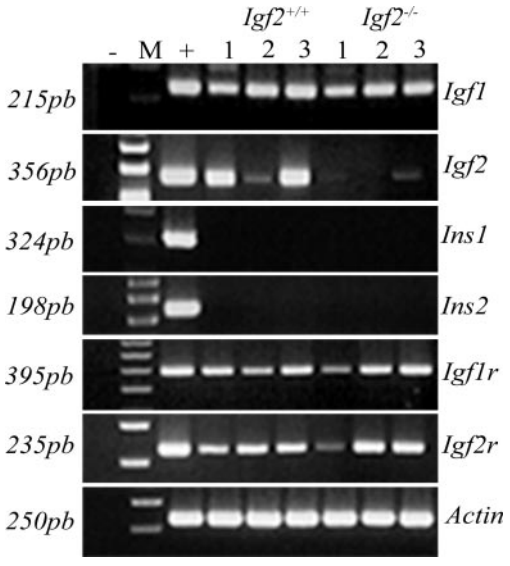

FIGURE 2. Expression of insulin family genes and their receptors in BM (*lane 1*), iDC (*lane 2*), and mDC (*lane 3*) by RT-PCR. H_2O served as negative control (*lane −*). Liver and pancreas served as positive control (*lane +*) for *Igf1* and *Igf2*, and *Ins1* and *Ins2*, respectively. Fetal brain was the positive control (*lane +*) for *Igf1r* and *Igf2r*. M represented the m.w. marker (*lane M*). One experiment representative of three individual experiments is shown.

Specific T cell culture

After immunization with murine IGF-2-derived B11-25, spleen cells were prepared by mechanical disruption, erythrocyte lysis (Hybrid-Max; Sigma-Aldrich), and filtration through a 70-μm cell strainer (BD Biosciences). The culture was performed with 3×10^6 spleen cells without or with murine IGF-2-derived B11-25 (20 μg/ml) in Ex vivo-15 medium (Cambrex) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessential acid, 10

Table II. *Comparison of surface phenotype on $Igf2^{+/+}$ and $Igf2^{-/-}$ mDC ($n = 5$ of each genotype)*

	$Igf2^{+/+}$	$Igf2^{-/-}$
A		
$CD11c^+$ $Ly6G^-$	76.79 ± 9.191	67.76 ± 6.224*
$CD11c^+$ $Ly6G^+$	9.10 ± 3.660	19.18 ± 6.116*
B		
$CD80^{low/int}$	21.45 ± 12.85	37.22 ± 13.94*
$CD80^{high}$	78.82 ± 13.10	63.17 ± 13.97*
$CD86^{low/int}$	19.60 ± 12.01	39.21 ± 16.62*
$CD86^{high}$	80.60 ± 12.22	61.19 ± 16.58*
$MHC\text{-}II^{low/int}$	21.34 ± 17.56	38.94 ± 15.45*
$MHC\text{-}II^{high}$	78.68 ± 17.53	61.16 ± 15.34*

*, $p < 0.05$; mean (%) ± SD.

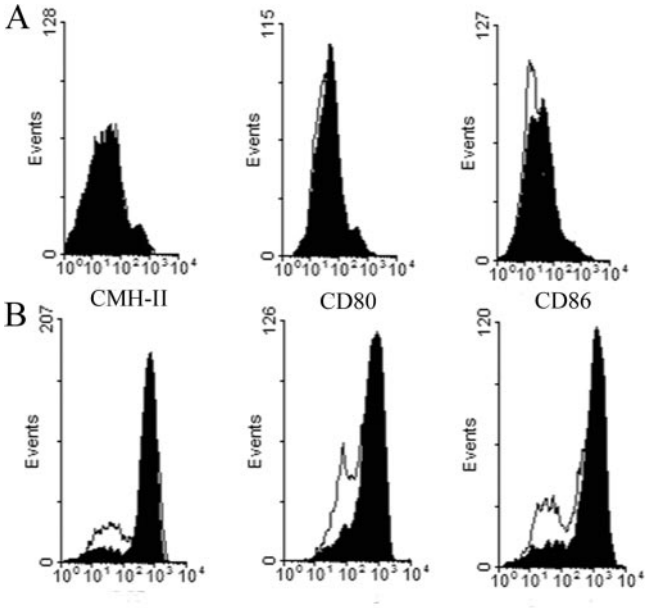

FIGURE 3. Comparison of surface expression of MHC-II, CD80, and CD86 by iDC (*A*) and mDC (*B*) from wt (filled histograms) and $Igf2^{-/-}$ mice (open histograms). Flow cytometric analysis of gated $CD11c^{+}$ cells represent the three markers implicated in Ag presentation and T cell activation. After 10 days with rmGM-CSF, BM cells were differentiated to iDC. Maturation was performed with rmGM-CSF and LPS for 24 h. One experiment representative of five individual experiments is shown.

mM HEPES (Cambrex), and 50 μM 2-ME (Invitrogen Life Technologies). The cells were incubated in 5% CO_2 at 37°C.

IL-2 production

After 48, 72, and 96 h of MLR or after 24 and 48 h of specific T cell culture, 800 μl were removed of each condition and IL-2 production released by $Igf2^{-/-}$ and wt T cells in MLR was assessed by specific ELISA (Biosource Europe).

Statistical analyses

One way ANOVA was used for MLR experiments (Newman-Keuls post-test) and *t* test was used to compare $Igf2^{-/-}$ and wt mice in cytometric experiments, IL-2 production, and Ab production after immunization. The level of significance was $p < 0.05$.

Results

Normal phenotype of lymphoid organs in Igf2-deficient mice

$Igf2^{-/-}$ mice presented a significant reduced body weight (61.6% of normal body weight) when compared with wt mice. Thymus and spleen from mutant mice were not significantly affected by the deletion of this gene because they presented a similar size and morphology compared with wt organs (Fig. 1*A*). The cellularity of different lymphoid organs such as thymus, spleen, and BM, was also analyzed and did not show any significant difference between wt and $Igf2^{-/-}$ mice. The number of $Igf2^{-/-}$ cells in the three studied organs was reduced in the same proportion than the size reduction (60%) (Fig. 1*B*). In vivo analyses of lymphoid compartment (T and B lymphocytes) showed no significant difference in thymocyte subpopulations (data not shown). In spleen, T cell analysis revealed no difference for $CD4^{+}CD8^{+}$ cells, $CD4^{-}CD8^{-}$ cells and $CD4^{+}CD8^{-}$ cells but the $CD4^{-}CD8^{+}$ population was significantly higher in mutant mice compared with wt mice. $Igf2^{-/-}$ mice also presented a significant decrease of B cell population ($B220^{+}$ cells) (Fig. 1*C*).

Influence of IGF-2 on DC differentiation and maturation

Insulin-related genes and their receptors expressed by BM, iDC, and mDC. RNA expression of *Igf1*, *Igf2*, *Ins1*, *Ins2*, and their receptors *Igf1r* and *Igf2r* was assessed by RT-PCR with specific primers. The positive controls were liver for *Igf1* and *Igf2*, fetal brain for *Igf1r* and *Igf2r,* and pancreas for *Ins1* and *Ins2*. Negative control was H_2O. *Igf1*, *Igf2*, *Igf1r*, and *Igf2r* transcripts (215, 356, 395, and 235 bp, respectively) were detected in wt BM cells, wt iDC, and wt mDC extracts but, on the other hand, neither *Ins1*, nor *Ins2* transcripts (324 and 198 bp, respectively) were found in wt DC samples contrary to positive controls (Fig. 2). The semiquantitative PCR pattern suggested that *Igf2* expression is up-regulated between iDC and mDC. Sequencing of *Igf1*, *Igf2*, *Ins1*, *Ins2, Igf1r*, and *Igf2r* PCR products confirmed the specificity and the selectivity of different primers. Each product presented 97–100% homology compared with their respective GenBank sequence. All genes detected in wt samples were found in $Igf2^{-/-}$ BM cells, $Igf2^{-/-}$ iDC and $Igf2^{-/-}$ mDC except *Igf2* for which the expression was severely decreased.

Surface phenotype of iDC and mDC is affected by deficiency of Igf2 expression. DC population was determined by analysis of CD11c expression in flow cytometry. From day 0 to day 11, wt

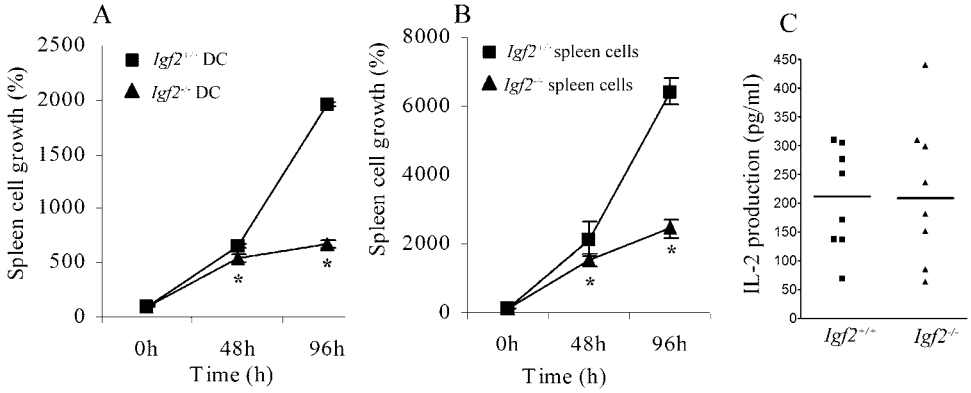

FIGURE 4. Implication of IGF-2 in DC activation capacity and in T cell proliferation. *A*, $Igf2^{+/+}$ and $Igf2^{-/-}$ DC capacity to stimulate BALB/c spleen cells in MLR. *B*, wt and $Igf2^{-/-}$ spleen cell proliferation in MLR with BALB/c DC stimulators. Proliferations was assessed by [^{3}H]thymidine uptake and measured after 48 and 96 h. Data are expressed as growth percentage vs time (h). Mean ± SD; *, $p < 0.01$, one experiment representative of three individual experiments is shown, each performed in triplicate ($n = 3$ of each genotype). *C*, IL-2 production of $Igf2^{+/+}$ and $Igf2^{-/-}$ spleen cells after 48 h of culture ($n = 8$ of each kind of mice).

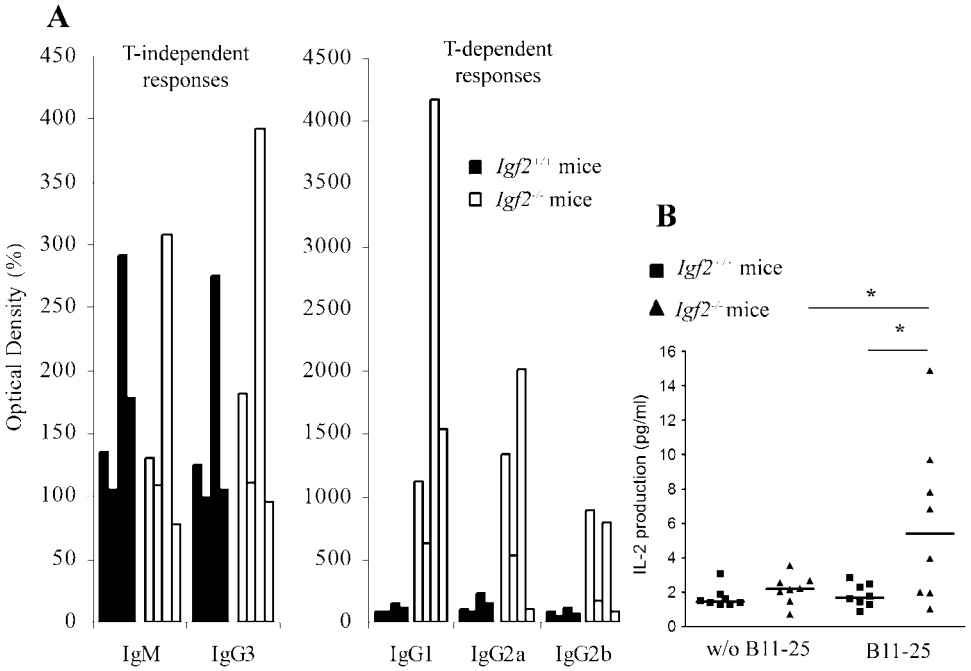

FIGURE 5. Production of IGF-2-specific Abs and T cells by wt and $Igf2^{-/-}$ mice after i.p. immunization. *A*, Analysis of different isotypes: IgM and IgG3 present the T-independent responses and IgG1, IgG2a, and IgG2b the T-dependent responses. Serial dilutions were performed, and data represent the percentage of the OD in preimmune serum at the dilution 1/200 for IgM and IgG3, 1/500 for IgG2a and IgG2b, and 1/10,000 for IgG1. These histograms represent four representative wt mice and four representative $Igf2^{-/-}$ mice ($n = 8$ of each kind of mice). *B*, IL-2 production by IGF-2-specific T cells from wt and $Igf2^{-/-}$ mice after two i.p. injections of IGF-2-derived B11-25 and one in vitro stimulation; *, $p < 0.05$ ($n = 8$ of each kind of mice).

$CD11c^+$ cells increased in the same way than $Igf2^{-/-}$ $CD11c^+$ cells to reach 84.75 ± 10.75% and 86.94 ± 10.46%, respectively, at mature stage. The percentage of $CD11c^+$ cell population from *Igf2*-deficient mice was identical than the $CD11c^+$ population from wt mice. These $CD11c^+$ cells showed no CD8α expression during differentiation and maturation. Among mature $CD11c^+$ cells, two populations were analyzed: $CD11c^+Ly6G^-$ cells and $CD11c^+Ly6G^+$ cells. The distribution of these classes was modulated according to the origin of cells: the wt $CD11c^+Ly6G^-$ population was significantly higher than $Igf2^{-/-}$ $CD11c^+Ly6G^-$ population, and this was the opposite for $CD11c^+Ly6G^+$ cells (Table II, part A). This difference was not observed at day 10 but only after maturation. $CD11c^-Ly6G^+$ population was also analyzed and showed no difference between wt and mutant mice after differentiation and maturation (data not shown). Expression of different molecules involved in T cell activation such as class II MHC (MHC-II), CD80, and CD86 was assessed by flow cytometry on $CD11c^+$ cells. After differentiation (Fig. 3*A*), wt iDC expressed a low or intermediate level of the three markers, with no significant difference with $Igf2^{-/-}$ iDC. In wt mice, LPS treatment promoted overexpression of these molecules during DC maturation (Fig. 3*B*). The persistence of iDC population with a lower expression of MHC-II, CD80, and CD86 was higher in $Igf2^{-/-}$ culture compared with wt culture (Table II, B). This immature population in $Igf2^{-/-}$ culture was observed after 24 and 48 h of maturation.

Higher iDC population influences T cell activation. The influence of a higher iDC population in $Igf2^{-/-}$ mice on T cell activation was studied using [^{3}H]thymidine incorporation assays (Fig. 4*A*). After 48 h of MLR, a significant decrease in T cell proliferation with $Igf2^{-/-}$ DC was observed when compared with wt DC. The decreased proliferation with $Igf2^{-/-}$ DC was time persistent and still observed after 96 h of MLR.

IGF-2 influence on T cell proliferation and activation

The absence of *Igf2* expression in T lymphocytes caused a significant decrease of proliferation of spleen cells as shown by [^{3}H]thymidine incorporation assays (Fig. 4*B*). A similar decrease was observed with wst-1 tests but did not reach the level of significance (data not shown). This reduction of proliferation was persistent until 120 h of MLR. However, no significant difference in IL-2 production was observed between wt and $Igf2^{-/-}$ culture along the time of culture (Fig. 4*C*).

Characterization of anti-IGF-2-specific immune responses

$Igf2^{-/-}$ and wt mice were immunized with IGF-2 to characterize anti-IGF-2-specific immune responses. The analysis of humoral response (Fig. 5*A*) showed that immunization of *Igf2*-deficient mice rapidly induced anti-IGF-2 Abs. This response was characterized by a T-independent response (both IgM and IgG3) and after the second injection, a T-dependent response with IgG1, IgG2a, and IgG2b production. Contrary to $Igf2^{-/-}$ mice, wt mice presented only IgM and IgG3 production with a similar titer, but no T-dependent response was observed.

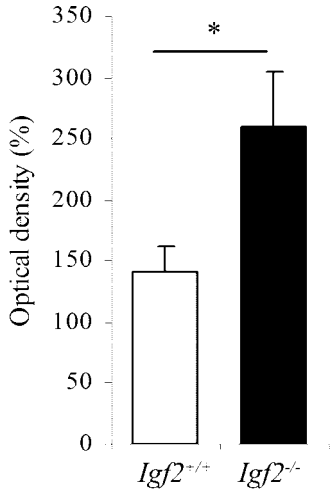

FIGURE 6. Insulin-specific humoral response in wt and $Igf2^{-/-}$ mice after i.p. immunization. The analysis of Ig production following injection was performed in mice with Ig production at day 0 lower than 1 in OD. Serial dilutions were performed, and data represent the percentage of the OD in preimmune serum at the dilution 1/100. Mean ± SEM; *, $p < 0.05$ ($n = 6/8$ of each kind of mice).

The proliferation of IGF-2-derived B11-25-specific T cells activated after immunization was characterized by IL-2 production with or without in vitro stimulation by IGF-2-derived B11-25 (Fig. 5*B*). In culture with murine IGF-2-derived B11-25, spleen cells from $Igf2^{-/-}$ mice present a significant increase of IL-2 production when compared with wt mice. This IL-2 production was specific of B11-25 because cultures without B11-25 did not show any IL-2 secretion.

Characterization of anti-insulin specific humoral response

$Igf2^{-/-}$ and wt mice were immunized with insulin to evaluate anti-insulin-specific humoral response. We observed that Ig response following immunization was dependent of anti-insulin Ig level in preimmune serum. Mice with high insulin-specific Ig production before immunization did not display any response to injections (data not shown). We decided to consider mice with anti-insulin Ab level at day 0 lower than 1 in OD. $Igf2^{-/-}$ mice presented a higher anti-insulin Ig-specific humoral response following the three injections compared with $Igf2^{+/+}$ mice (Fig. 6).

Discussion

The main objective of this study was to evaluate the importance of IGF-2 in immune development and physiology. The absence of *Igf2* expression does not influence lymphoid organ development as shown by a similar growth of thymus and spleen, as well as a cellularity in both organs and BM, which is identical in wt and $Igf2^{-/-}$ mice. In *Igf2*-deficient mice, thymocyte development is not affected but, in the spleen, a significant increase of $CD4^{-}CD8^{+}$ cells and a lower $B220^{+}$ population is observed.

We decided in a second step to investigate the effect of *Igf2* deficiency on the maturation and function of DC, the most potent APC (22) implicated in polarization of the immune response, i.e., immunity vs tolerance (23, 24). It has been previously reported that IGF-1 promotes maturation and inhibits apoptosis of immature cord blood monocyte-derived DC (25). The switch from iDC to mDC stage reduces the IGF-1 mRNA level as shown by DNA array (26). In normal mice, BM-derived DC express IGF and IGF-R genes at all differentiation stages (BM cells, iDC, and mDC), but our RT-PCR conditions failed to detect any *Ins1* and *Ins2* transcripts in all DC subtypes. All genes detected in wt samples were found in $Igf2^{-/-}$ BM cells, $Igf2^{-/-}$ iDC, and $Igf2^{-/-}$ mDC except *Igf2* for which the expression was severely decreased. Sequencing of PCR products confirmed the specificity of these $Igf2^{-/-}$ transcripts. With a high number of PCR cycles, some products can be detected from the turnover of read-through primary transcript. However, these transcripts cannot generate a functional protein because the deleted exon 4 encodes the signal peptide and 28 residues of mature IGF-2 (4, 27). After having checked that DC express the genes of different factors implicated in a potential action of insulin-related factors, we analyzed the implication of *Igf2* deficiency in DC development and acquirement of molecules implicated in T-DC interactions. In wt mice, BM-derived DC express CD11c and the majority of these cells are $CD11c^{+}Ly6G^{-}CD8\alpha^{-}$ myeloid DC as described by other groups with this method using GM-CSF only for DC differentiation (16, 28–30). However, a $CD11c^{+}Ly6G^{+}CD8\alpha^{-}$ population was also identified in our culture conditions. This population could be due to the source of GM-CSF generated by COS-7 transfection because the conditioned medium is used in BM cell culture. Other cytokines present in this medium could also influence DC differentiation. This second DC subset in culture exhibits the characteristics of plasmacytoid DC (pDC) expressing CD11c and Ly6C/Ly6G (clone RB6-8C5 recognized Ly6G/Gr1 and Ly6C Ag) (31, 32), and this population is higher in deficient mice compared with wt mice. In $Igf2^{-/-}$ mice, we also observed a significantly higher iDC population, which persisted 24 to 48 h after LPS maturation suggesting that this effect was not due to a delay of maturation but to its absence. The increased immature population as well immature myeloid DC as pDC in $Igf2^{-/-}$ mice is considered to be tolerogenic DC (33, 34), which are not effective in T cell activation. Our results concur with this functional status because we observed a decreased allogenic T cell proliferation in $Igf2^{-/-}$ MLR. The implication of IGF-2 is only observed in DC maturation (no significant difference is observed after 10 days of differentiation); this factor is not implicated in DC development to obtain cell population with DC characteristics because the percentage of $CD11c^{+}$ population after culture is identical between the two kinds of mice. The role of IGF-2 in maturation is further confirmed by semiquantitative PCR showing a higher *Igf2* transcription in wt mDC than in wt iDC. The mechanisms by which IGF-2 intervenes in maturation is not determined, but in our culture conditions, BM-derived DC differentiated and maturated in medium supplemented with 10% FCS (which contains a significant amount of IGF-2). $Igf2^{-/-}$ DC could easily use external IGF-2 to promote their maturation if only the absence of IGF-2 production was implicated. *Igf2* expression deficiency seems to be responsible for the defect observed in DC maturation, and a deficiency in an IGF-2-mediated intracrine signaling could be speculated in our experimental model.

With regard to the role of IGF-2 in T cell development, a thymic hyperplasia was observed in *Igf2* overexpression transgenic mice, with an increased number of thymocytes and $CD4^{+}$ cells (11, 12). In our experiments, we observed a significant defect of proliferation as shown by a decrease in [^{3}H]thymidine uptake, but an early event in T cell activation such as IL-2 production was not affected. A recent study based on the inhibitory implication of NK cells on T cell proliferation suggests that this effect could be due to a direct impact on p21 and on the cellular cycle without affecting an early event in T cell activation including IL-2 secretion and IL-2R upregulation (35). IGF-2 could also influence the apoptotic phenomenon to promote T cell survival in the same way as IGF-1, which influences T cell survival via Akt and JNK pathway (36). The role played by IGF-2 could be mediated through IGF-1R, which binds both IGF-1 and IGF-2, and is regulated during T-lymphocyte activation (37). These different ways are under current investigation.

Central self-tolerance of the immune system is established in the thymus through the MHC presentation of self-antigens transcribed by thymic stromal cells to immature T cells and through the generation of self-antigen-specific regulatory T cells (for review, see Refs. 38 and 39). With regard to the insulin gene family, all members of this family are transcribed in the murine thymus according to a precise hierarchy and topography of their expression profile: *Igf2* (cortical (c) and medullary (m) thymic epithelial cells (TEC)) > *Igf1* (macrophages) ≫ *Ins2* (mTEC) > *Ins1* (mTEC) (9, 40). A thymus-specific defect of *Igf2* transcription has been observed in BB rats, an animal model of type 1 diabetes, strongly suggesting some impaired tolerance to IGF-2 in this disease (15). As expected, $Igf2^{-/-}$ mice are completely intolerant of IGF-2 as confirmed by our immunization experiments. Indeed, only *Igf2*-deficient mice immunized with IGF-2 develop IgG1, IgG2a, and IgG2b following an isotypic switch that requires the help of IGF-2-specific $CD4^{+}$ T cells. These T cells were detected by IL-2 production in culture stimulated by IGF-2-derived B11-25. In wt mice immunized with IGF-2, these switch and cellular responses were not observed confirming a complete tolerance to IGF-2. In the same mouse model, influence of IGF-2 upon tolerance to insulin was also assessed. This study showed that $Igf2^{-/-}$ mice present a lower tolerance to insulin than $Igf2^{+/+}$ mice. The insulin-specific

Ab level in preimmune serum is correlated with the immune response suggesting the capture of insulin by these specific Abs. This phenomenon would influence the Ag availability for immune response. We can conclude that IGF-2 is able to mediate its own tolerance and also contributes to insulin tolerance. Insulin is the only β-cell-specific autoantigen in this disease and was recently demonstrated to be the primary target recognized by the immune system and initiator of the autoimmune response (41, 42). Theoretically, immunization of *Igf2*$^{-/-}$ mice with IGF-2 should allow cloning of IGF-2-specific CD4^{+} lymphocytes and to investigate the pathogenic properties of these "forbidden" self-reactive clones. This will further provide significant help in understanding the consequences of a thymic *Igf2* defect in the establishment of immune self-tolerance to the whole insulin family and in type 1 diabetes pathogenesis.

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Disclosures

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