LPS and Freund's adjuvant initiate different inflammatory circuits in experimental autoimmune thyroiditis

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ABSTRACT. Although adjuvants are essential for the initiation of experimental autoimmune diseases, their precise contribution to the pathological manifestations is still poorly understood. Experimental autoimmune thyroiditis (EAT) is interesting in that respect because it can be initiated with the help of two different adjuvants — Freund's complete or LPS — which may initiate independent pathogenic pathways. In the present study, we have compared Freund's-induced *versus* LPS-induced EAT with respect to their dependence upon $CD8^+$ T cells, which are considered as major actors in the pathogenesis of thyroiditis. Our results reveal that whereas $CD8^+$ T cells are mandatory in the Freund's model, they can be bypassed in the LPS model. On the basis of this finding, we have examined the possibility that LPS may act directly upon *in vitro* cultured thyrocytes with no intermediate cell stages. Indeed, LPS triggers transcription and protein synthesis of several chemokines such as MIP-3α, RANTES, MCP-1 or TARC. Thus, beside enhancing the immunogenicity of autoantigens, probably *via* antigen trafficking and presentation, adjuvants such as LPS directly interact with the target organ through synthesis and release of powerful T cell attractants that facilitate its lymphocytic infiltration.

Keywords: Experimental autoimmune thyroiditis, LPS, Freund's adjuvant, CD8+ T cells, chemokines

INTRODUCTION

The experimental induction of autoimmune diseases in rodents is critically dependent upon the administration, together with the autoantigen, of an appropriate adjuvant. The role of the adjuvant is still not totally understood [1]. Adjuvants are believed to facilitate the response of autoreactive T cell precursors which would otherwise ignore self-antigens or be unresponsive to them, by providing a set of signals associated with antigen processing, trafficking and presentation. However, in some instances, the elicitation of a potent anti-self response is not sufficient to generate clinical and histological manifestations [2], yet conversely, in others, adjuvant alone can produce an autoimmune condition [3]. The severity of the disease seems to depend upon signals provided by the adjuvants, which initiate inflammatory circuits leading to lymphocytic infiltration and physiopathological alterations of the target organ.

Abbreviations: mTg: murine thyroglobulin / EAT: experimental autoimmune thyroiditis / NOD: non-obese diabetic / β2m: beta 2 microglobulin / FCA: Freund's complete adjuvant / FIA: Freund's incomplete adjuvant / MIP-3α: macrophage inflammatory protein-3 / MCP-1: monocyte chemoattractant protein-1 / RANTES: regulated on activation of normal T-cell-expressed and secreted / TARC: thymus and activation-regulated chemokine / IP-10: interferon-inducible protein-10

Experimental autoimmune thyroïditis (EAT), an animal model for Hashimoto's disease, can be achieved, in susceptible strains of mice, by active immunization with thyroid gland components — thyroglobulin (Tg) or thyroperoxidase — either emulsified in Freund's complete and incomplete adjuvant (FCA and FIA), or associated with LPS [4]. Although both adjuvants have a bacterial origin and stimulate components of the innate immune system, which in turn orchestrate the activation of T and B lymphocytes [5], it is not clear whether the circuits initiated by the two adjuvants are the same and subsequently whether the pathogenesis of the two EAT models is comparable. Recent experimental evidence suggests that the two models might not be identical, LPS initiating for instance, an inflammatory network more dependent on TNF than that induced by Freund's adjuvant [6].

The precise contribution of $CD8^+$ T lymphocytes in EAT is another debated question. $CD8^+$ T cells have been implicated as CTL effectors, on the basis of their lytic activity *in vitro* against thyrocytes or antigen-pulsed macrophages [7-9]. In other studies, $CD8^+$ T cells have also been shown to initiate lymphocytic invasion of the thyroid, with or without the help of $CD4^+$ T cells, in a way which is reminiscent of what has been demonstrated for insulitis in NOD type 1 diabetes [10]. However, $CD8^+$ T lymphocytes also appear to regulate the autoimmune process, as suggested by the fact that in some models, infiltration is more benign in the presence of

$CD8^+$ T cells than in their absence [11-13]. Whatever the contrasting functions they may fulfill, $CD8^+$ T cells appear to be neverthless, essential actors in association with $CD4^+$ T cells [14-16].

We have previously shown that wild type NOD and congenic NOD.$H2^k$ mice, following immunization with Tg in FCA and FIA, develop severe forms of EAT, which do not regress spontaneously as they do in most other permissive strains of mice [17]. This NOD chronic EAT model appears therefore to mimic more accurately the manifestations of Hashimoto's disease encountered in human patients. In view of the acute character of EAT in NOD mice, we thought that the model would be appropriate for examining the role of adujvants — Freund's or LPS — and the importance of $CD8^+$ T cells. In the first part of the present study, we have evaluated the susceptibility to disease induction of β2m knock-out (β2m KO) NOD mice, which lack MHC class I and $CD8^+$ T cell expression [18]. The finding that the two adjuvants initiate independent pathogenic pathways, one depending upon $CD8^+$ T cells, the other not, led us to consider the possibility that LPS might act directly upon thyrocytes. In the second part of this study, we show that the *in vitro* stimulation of cultured murine thyrocytes with LPS indeed triggers the synthesis of chemokines known for they capacity to attract T cells [19]. These original data highlight therefore the possible role for adjuvants in autoimmune disorder models, namely that of directly eliciting, in the target organ itself, the synthesis and secretion of pharmacological agents that will attract and facilitate T cell infiltration.

METHODS AND MATERIALS

Mice

NOD and NOD.β2m knock-out mice (β2m KO) were used throughout this study. The β2m *null* mutation originally generated in the 129 strain [18] was back-crossed onto NOD mice for 12 generations. The NOD.β2m knock-out mice used for this study were the progeny of homozygous breeders generated by intercrossing.

All mice were bred and maintained in our facility under strict pathogen-free conditions, in compliance with EEC recommendations.

Induction of EAT with Freund's adjuvant or LPS

EAT was induced in 6-8 week-old female mice by immunization with mouse Tg prepared from a pool of thyroid lobes from normal mice, as previously described [24]. In the protocol using Freund's adjuvant, the mice received 2 injections, at two week intervals, of 100 µg of mouse Tg emulsified in an equal volume of complete adjuvant for the first injection and incomplete for the second. Complete adjuvant contained 1 µg/ml of *Mycobacterium tuberculosis*, strain H37 Ra (Difco Lab, Detroit, MI, USA) [17]. The emulsion was injected sub-cutaneously at 4 different sites. In the LPS protocol, mice received a 100 µg i.v. injection of autologous Tg followed 2 hours later by 20 mg of LPS (*E. Coli*, 0111: B4; Sigma) inoculated by the same route. This was repeated 2 weeks later. The mice were sacrificed for thyroid scoring 3 weeks after the second antigen challenge.

Adoptive transfer of EAT

Donors were NOD wild type or NOD.β2m KO female mice immunized with mouse Tg in CFA as described above. The spleens were collected 2 weeks after the second challenge and processed into cell suspensions in PBS plus 2% FCS. Red cells were lysed by osmotic shock in lysis buffer (Sigma). Twenty five $\times 10^6$ viable splenocytes were transferred i.v. into NOD.SCID female recipients, together with 100 µg of mouse Tg. Thyroids were collected for histopathological scoring 17 days later.

Histopathology

Tissues were fixed in 4% formalin solution and processed according to standard procedures. Thyroid specimens were examined in 4 non-contiguous, 5 µm thick, sections stained with hematoxylin-eosin-safran. Histological thyroiditis was scored from 0 to 4 as previously described: 0 for an absence of infiltrate, 1 for interstitial accumulation of inflammatory cells limited to one or two follicles, 2 for the presence of 1-2 foci of inflammatory cells reaching the size of a follicle, 3 for the replacement of 10 to 40% of the thyroid tissue by inflammatory cells and 4 for lesions exceeding 40% of the total surface. The final score is the arithmetic mean of 4 sections.

T cell proliferation assay

Draining lymph nodes (inguinal, lombo-aortic, axillary and brachial), collected 10 days after 1 injection of 100 µg mouse Tg in CFA, were teased into a cell suspension, washed twice in PBS plus 2% FCS and resuspended in culture medium composed of RPMI-1640 supplemented with glutamax, sodium pyruvate, non-essential amino-acids, HEPES buffer (all reagents from Life Technologies LTD, Paisley, Scotland), 50 mM 2-mercaptoethanol (Sigma) and 10% FCS (Techgen, France). LN lymphocytes were distributed at a concentration of 5×10^5 cells/well in 96 flat-bottomed microtiter plates (Becton Dickinson, Lincoln Parle, NJ, USA). Irradiated (1 800 rad) spleen cells from naive syngenic mice were added as antigen-presenting cells (APC) at a standard concentration of 5×10^5 cells/well, together with Tg at 50 µg/ml. The plates were incubated for 5 days at 37 °C in 5% CO_2 humid atmosphere, and pulsed with 1 mCi/well of [^{3}H] thymidine (CEA-ORIS, France) for the last 18 hrs. Culture contents were harvested automatically on filters and counted in a β-plate scanner (Pharmacia, France).

Cytokine detection by intracytoplasmic staining

Cytokine secreting lymphocytes were detected by a single cell assay as described by Openshaw *et al.* [33]. LN cells from Tg-primed mice were collected at day 10, restimulated *in vitro* for 3 days in the presence of APC and Tg as described above and boosted for an additional 4 hours in PMA (10 ng/ml) and Ionomycin A23187 (500 ng/ml) (Calbiochem, La Jolla, CA, USA). Brefeldin A (Sigma) at 10 µg/ml was added during the last 2 hours of culture to favor cytokine accumulation. The cells were harvested and centrifuged at 800 g through 4 ml of Ficoll (Lympholite-M, Cedarlane, Hornby, Canada). They were incubated first for cell surface staining with anti-CD4 mAb (Pharmingen) coupled to PE, then fixed in 2% paraformaldehyde in PBS for 20 min, permeabilized for

10 min in 0.5% saponin (Sigma) in PBS-2%FCS plus 2 mM sodium azide and then incubated in an appropriate antibody dilution of clone AN18 against IFN-γ coupled to FITC (Pharmingen). Cell aliquots processed under the same conditions were incubated with an isotype matched rat IgG control coupled to FITC (Southern Biotechnology, Birmingham, AL, USA), for delimiting the threshold between negative and positive cells. The cell samples were analyzed on a FACScan (Becton Dickinson) with Cellquest software. A minimum of 1×10^5 FSC-SSC gated events were stored for fluorescence analysis.

Stimulation of mouse thyroid epithelial cell cultures

Murine thyroid epithelial cell cultures (TEC) were prepared as previously described [4]. Thyroid glands were carefully dissected and cut into small pieces with scissors in chilled, complete RPMI plus 10% FCS. After several washes to remove debris and blood cells, the preparations were incubated with 1.5 mg/ml of collagenase (Boehringer-Mannheim, Mannheim, Germany) in a shaking water bath at 37 °C for 30 min. After washing and collection of the cells, a second treatment with a new batch of collagenase was performed as described above. The cells of the two batches were pooled, centrifuged and resuspended in complete RPMI plus FCS. About 2×10^4 cells in 0.2 ml (mainly viable single thyroid cells as well as complete follicles) were left in flat-bottomed 96-well plates at 37 °C, in a 5% CO_2 atmosphere. The cultures were used between day 10 and 13, when confluence was reached. LPS at 10 µg/ml, IFN-γ and TNF-α (both from R&D Systems, Abingdon, UK) at 0.1 µg/ml were introduced into culture wells for 12 h to 48 h. Supernatants were collected for measurement of chemokines. Cell cultures were processed for RNA extraction.

RT-PCR

Total cellular RNA was extracted from pools of 4 wells of confluent TEC cultures using the guanidium isothiocyanate-phenol procedure and RT-PCR was achieved using a standart two-step procedure. Chemokine cDNA amplifications were achieved using the following nucelotides: RANTES 5'-GTACCATGAAGATCTCTGCA-3', 5'-TCTATCCTAGCTTCATCTCCA-3'; hybridation t°: 53 °C and amplicon size 297 bp. MCP-1: 5'-ATGCAGGTCCCTGTCATG-3', 5'-GTTCACTGTCACACTGGT-3'; hybridation t°: 55 °C and amplicon size 443 bp. MIP-3a: 5'-GGCAGAAGCAAGCAACTACG-3', 5'-ACAGCCCTTTTCACCCAGTT-3'; hybridation t°: 63 °C and amplicon size 232 bp. TARC: 5'-CAGGAAGTTGGTGAGCTGGTATA-3', 5'-TTGTGTTCGCCTGTAGTGCATA-3': hybridation t°: 60 °C and amplicon size 300 bp. IP-10: 5'-ACCATGAACCCAAGTGCTGCCGTC-3', 5'-GCTTCACTCCAGTTAAGGAGCCCT-3': hybridation t°: 63 °C and amplicon size 286 bp. β-actine: 5'-TGGAATCCTGTGGCATCCATGAAAC-3', 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'; hybridation t°: 60 °C and amplicon size 348 bp. The PCR products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide. Each amplicon was quantified for both β-actin and speific chemokine using a scanner densitometer (Vilber Lourmat, Marnes-la-Vallée, France). Results are expressed as ratios of arbitrary units of the chemokine to β-actin.

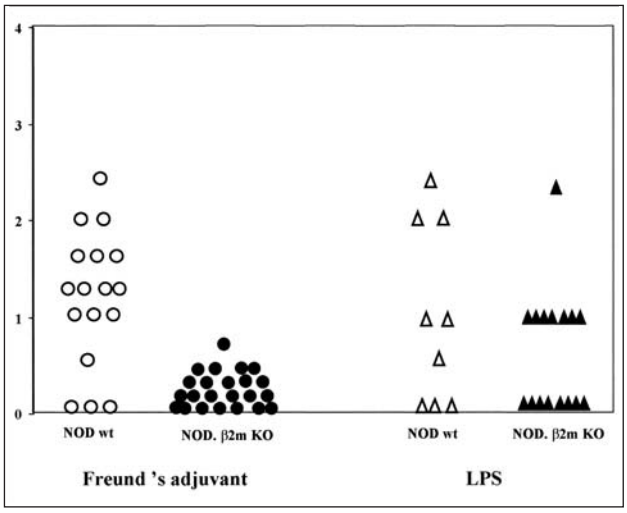

Figure 1
Susceptibility of NOD β2m KO versus NOD wild type mice to EAT, induced with either Tg and Freund's adjuvant or with Tg and LPS. Thyroiditis scores (as described in Methods and materials) are from individual NOD and NOD β2m KO mice immunized with porcine or mouse Tg.

ELISA for chemokine titration

Production of RANTES and MCP-1 was assayed using standard ELISA. Recombinant chemokines (R&D systems Minneapolis, MN, USA) or 100 µl thyroid cell culture supernatants, in coating buffer pH = 9.5, obtained 12 hrs after addition of LPS, TNF-α or IFN-γ at concentrations specified above were left at 4 °C overnight. After blocking with PBS/Tween/BSA, the developing monoclonal Abs anti-RANTES and anti-MCP-1 (R&D) conjugated to alkaline phosphatase were added. After washings, the substrate, p-nitrophenyl phosphatase (1 mg/ml, Sigma) was added at 100 µg/well. The reaction was stopped with 25 µg/ml 4M NaOH after which the OD was read at 410 nm. Results were expressed as ng of chemokine/ml.

RESULTS

The requirement for $CD8^+$ T cells in EAT induction depends upon the adjuvant used for immunization

In order to assess the contribution of $CD8^+$ T cells to the elicitation of EAT, normal and β2m KO NOD mice, which lack MHC class I and $CD8^+$ T cell expression, were challenged at 2 week intervals with autologous Tg, together with either CFA/IFA, or LPS. Thyroids were collected 3 weeks later and examined histologically for the presence of lymphocytic infiltrates. As shown in Figures 1 and 2, the response of the β2m KO mice was totally different depending upon whether they had been immunized with Freund's or LPS. Those injected with Freund's adjuvant were free of lesions, indicating that $CD8^+$ T cells are mandatory in this model, whereas those injected with LPS were as permissive as their wild type controls. Compared to Freund's adjuvant, the lymphocytic infiltration of diseased thyroid glands was slightly less in the LPS model, but the absence or presence of $CD8^+$T cells did not introduce any significant difference.

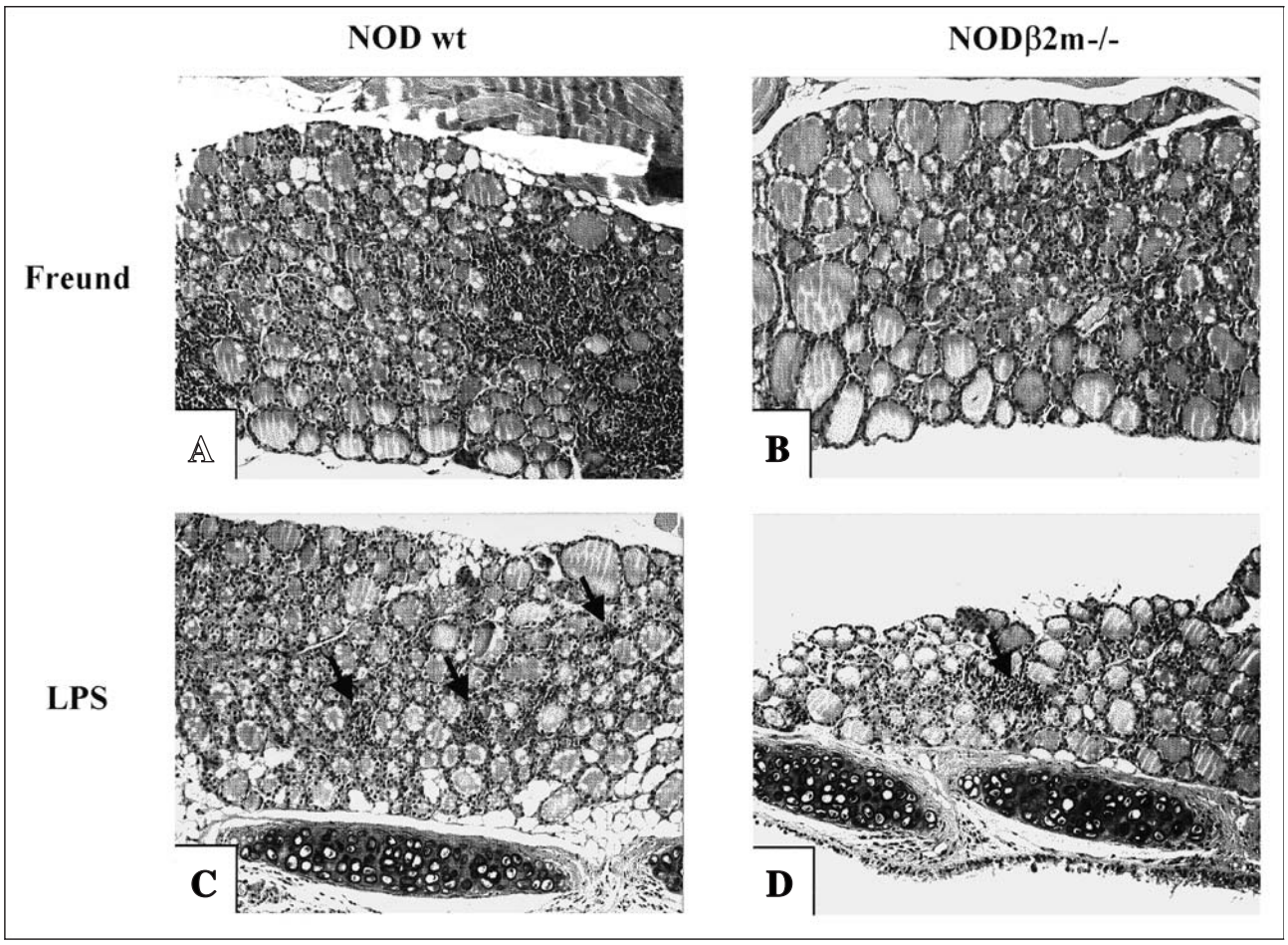

Figure 2
Thyroids of mice, 4 weeks after immunization (magnification × 200). Histological aspect of thyroiditis in CFA and Tg-immunized NOD wild type mice (Figure 2A). NOD β2m KO mice immunized with the same adjuvant do not display any inflammation (Figure 2B). In NOD wild type and NOD β2m KO mice (Figure 2C and 2D respectively), immunization with thyroglobulin and LPS induce mild thyroiditis in both strains.

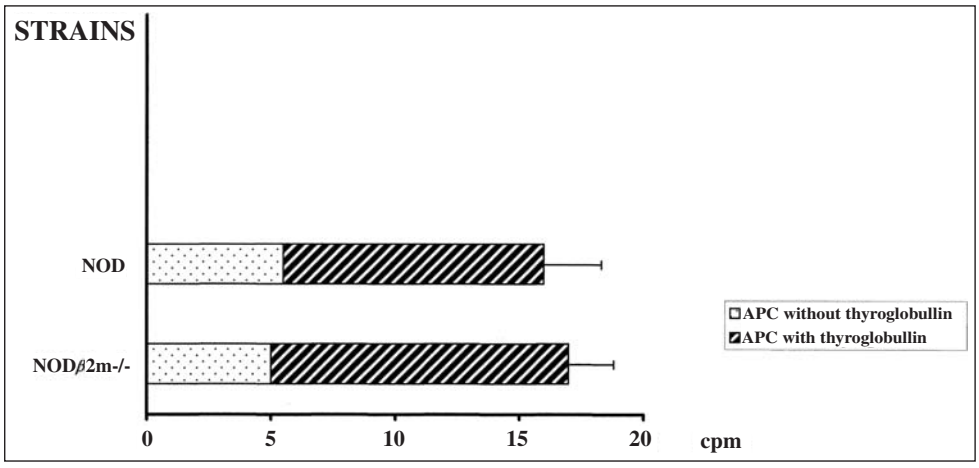

Figure 3A
Proliferation assay of lymph node lymphocytes from mTg-primed donors stimulated *in vitro* with mTg. 5×10^5 cells from regional lymph node were collected 10 days after priming with 100 µg of porcine Tg in CFA.

Several alternatives that could account for the requirement of $CD8^+$ T cells in the Freund's model were then tested. The first one was the possibility that the absence of $CD8^+$ T cells might jeopardize the $CD4^+$ T cell response to Tg. As shown in Figure 3A, this is not the case, LN lymphocytes from β2m KO mice proliferate as vigourously as LN cells from wild type mice in the presence of APC pulsed with Tg. They also release as much IFN-γ in the culture supernatants (Figure 3B), but did not produce detectable amounts of IL-4 (not shown). Thus the absence

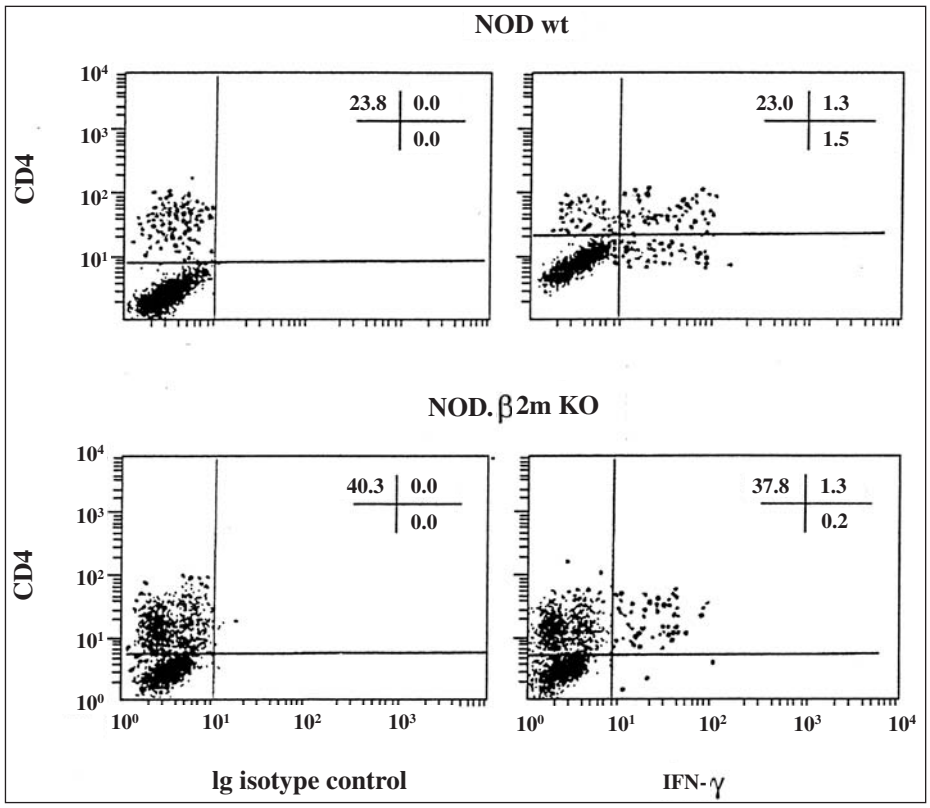

Figure 3B
Intracytoplasmic staining of IFN-γ in activated LN cells from NOD and NOD β2m KO mice 10 days after *in vivo* priming with porcine Tg and CFA. Numbers in quadrants represent percentage of positive cells.

of lymphocytic infiltrates in the thyroids of mice devoid of $CD8^+$ T cells, is not the consequence of an aborted $CD4^+$ T cell response to Tg.

We next asked whether $CD4^+$ T cells from immune β2m KO donors might adoptively transfer thyroiditis into NOD SCID mice. As shown in Table 1, recipients of spleen cells from β2m KO donors did not develop thyroiditis, whereas all the recipients of spleen cells from wild type mice developed florid infiltrates. Thus, $CD4^+$ T cells even injected in massive doses are not capable, by themselves, of transferring disease. They need the presence of $CD8^+$ T cells, which seem to help them home into the thyroid.

Table 1
Tg-activated T cells from NOD. β2m KO mice cannot transfer EAT into NOD.SCID recipients

Donor cells mouse strains	*EAT incidence in NOD.SCID mice	EAT mean score
NOD	7/7 (100%)	2.2
NOD. β2m knock-out	0/7 (0%)	0.0

Spleens cells from NOD wild type or from NOD. β2m knock-out mice primed with mTg in Freund's adjuvant were collected 2 weeks after priming and transferred i.v. into NOD.SCID mice together with 100 µg of mTg.
*Thyroids were collected for histopathological scoring 17 days later.

LPS triggers chemokine synthesis in vitro cultured thyrocytes

In view of the fact that the mediation by $CD8^+$ T cells is not needed in the LPS model, and the role played by chemokines in the attraction of T cells [19], we investigated the possibility that LPS might directly trigger chemokine transcription and synthesis in thyrocytes. Ten to 12 day-old cultures of thyroid glands from unprimed CBA/J mice, and therefore totally free of infiltrating lymphocytes, were challenged with 10 µg/ml LPS for 12, 24 or 48 hrs. Parallel cultures were incubated with 0.1 mg/ml IFN-γ or TNF-α, two potent inducers of chemokines. Results in Figure 4, from cultures at 12hrs, show that LPS is as strong inducer as the two pro-inflammatory cytokines, although with some differences. LPS initiates transcription of MIP-3α, MCP-1, RANTES and TARC, sometimes more efficiently than IFN-γ or TNF-α, but does not trigger IP-10 which, as expected, is mainly elicited by IFN-γ [20]. MCP-1 and RANTES were also measured as proteins in the supernatants of LPS-stimulated thyrocyte cultures. Their concentrations at 12 hrs were respectively 35 ng/ml and 13 ng/ml as compared with 4 ng/ml and 0 ng/ml in supernatants of non-activated cultures.

DISCUSSION

Adjuvants are crucial in most experimentally-induced models of autoimmune disease. Yet, each model has its

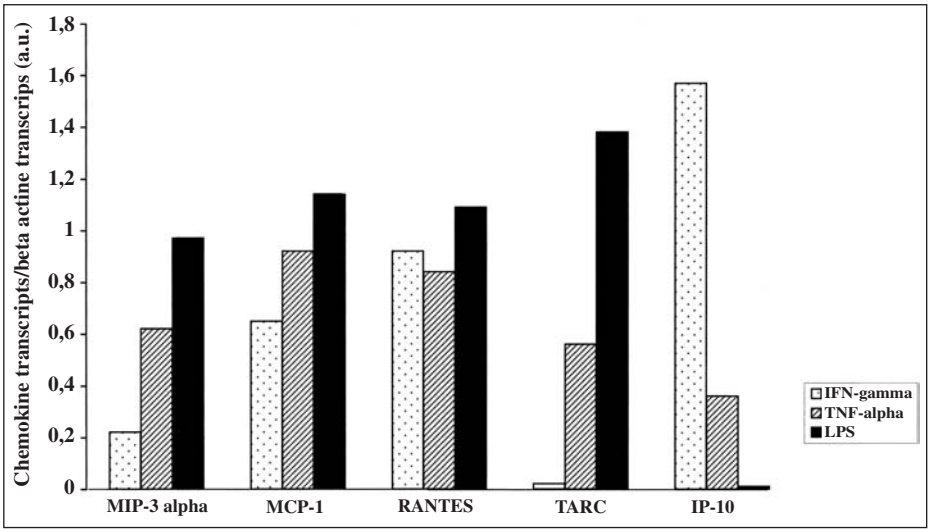

Figure 4
Chemokine transcripts induced by IFN-γ, TNF-α and LPS added in murine thyrocyte culture. Results represent arbitrary units of the ratio of chemokine transcripts to β actin transcripts.

own requirements, suggesting that the role of the adjuvant is not only to enhance the immune response against the autoantigen, but also to initiate inflammatory circuits specific to a given tissue or a given organ [21]. EAT presents the interesting particularity that it can be induced by two independent adjuvants – CFA/IFA or LPS. From the knowledge of what is common and different in the two models, it is thus possible to delineate more precisely the contribution of the adjuvants in the pathogenic process. As already reported by Zaccone *et al.* [22], who compared the two EAT models in terms of IL-12 involvement, Freund's adjuvant induces a more agressive disease than LPS. Our own data confirm that the lesions are relatively mild in the LPS cohort, although the mice are of NOD origin (see Figure 2). Yet, the important point is that there is no difference in this cohort, between the wild type mice and the β2m KO mice, which lack MHC class I antigens and $CD8^+$ T cells. Thus, in the LPS-induced EAT model, $CD8^+$ T cells are not mandatory. Using a different strain of mice, Lomo *et al.* [23] came to the same conclusion, which was rather unexpected in view of the abundant literature showing an involvement of $CD8^+$ T cells in EAT [7-9, 14-16, 24]. This apparent contradiction can be now resolved if we consider that most of the data from the literature, demonstrating a $CD8^+$ T cell contribution were obtained in the Freund's model [4] and that in this model, $CD8^+$ T cells are indeed mandatory, as they are in the iodine-accelerated model of thyroiditis in NOD and NOD.$H2^{h4}$ mice [15, 25].

As suggested by Zaccone *et al.* [6], there are significant differences between the pathogenic processes involved in Freund's — and LPS — induced models of EAT. These authors have recently shown that the inhibition of the common signalling pathway used by TNFα and LT through TNFR1 receptors, decreases the severity of the thyroid lesions when EAT is induced by administration of LPS, but not when it is induced by Freund's adjuvant. This result stresses the importance of considering the adjuvant not as a mere experimental tool, but as a full element of pathogenicity, triggering its own specific inflammatory circuits.

$CD8^+$ T cells appear to perform some specific task that is essential for the initiation of lymphocytic infiltration in the thyroid when CFA is used, but not when LPS is used. The analysis of the cellular response to Tg at the level of the regional LN indicates that the absence of $CD8^+$T cells has practically no effect upon the $CD4^+$ T cell response, both in terms of proliferation and of INF-γ release. $CD8^+$ T cells do probably not contribute to the strength of the $CD4^+$ T cell response, nor do they seem to skew the response in a particular direction, because we systematically detected INF-γ, but not IL-4, both in β2m KO and in wild type mice. On the basis of adoptive transfer experiments, it seems that the contribution of $CD8^+$ T cells to the initiation of thyroid lesions is that of directing $CD4^+$ T cells toward the thyroid and facilitating their penetration into the tissue. Several years ago, we proposed a similar scenario, also based on adoptive transfer experiments, for explaining the cooperation between $CD4^+$ and $CD8^+$ T lymphocytes in NOD insulitis [26]. What $CD8^+$ T cells do precisely in this process is still not clear. They might directly release proinflammatory cytokines or chemokines attracting $CD4^+$ T cells. Alternatively, they might cause some minimal lesions which would mobilize phagocytes or antigen presenting cells in the gland and in turn, those cells would attract $CD4^+$ T lymphocytes. In any event, Freund's adjuvant does not have a direct impact on the target organ. This is not the case for LPS which circulates freely in the body and, as suggested by our results, is able to trigger chemokine synthesis in thyrocytes. The chemokines produced *in vitro* by LPS-stimulated thyrocytes cultures: MCP-1, RANTES, MIP-3α and TARC, are powerful T cell attractors [27-29] known for stimulating the migration of T cells, and consequently their proliferation, when *in situ* in the presence of thyroid auto-Ags. Furthermore, we have recently shown [30] that MCP-1 and RANTES are synthetized *in vivo* in diseased thyroid glands from CBA/J mice immunized by murine Tg (mTg) and CFA/IFA. However, the kinetics of production and their functions differ: we observed that the MCP-1 that is produced in higher amounts in the first 7 days after immunization attract mTg-specific proliferating T cells,

whereas the RANTES that is produced later (21-27 days after immunization) attract regulatory $CD4^+$, $CD25^+$T cells that secrete IL-10. In line with these *in vitro* and *in vivo* results obtained in murine EAT, Garcia-Lopez *et al.* have shown that IP-10 and monocytes induced by IFN-γ were expressed in thyroid follicular cells of patients with autoimmune thyroid disorders and were involved in the recruitment of specific subsets of activated lymphocytes [20]. Thus, LPS acting upon thyrocytes could provide the appropriate conditions for attracting $CD4^+$ T cells into the gland and therefore circumvent the otherwise necessary requirement for $CD8^+$ T cells. However, it must also be borne in mind that LPS exerts a large array of pleiotropic effects, among which are the massive release of TNF-α-enhancing antigen presentation [31] and the up-regulation of ICAM-1 [32], which could also attract committed $CD4^+$ T cells into the thyroid.

In conclusion, our results underscore, like those of Zaccone *et al.* [6] the importance of considering the adjuvant as a component of pathogenicity in itself. Furthermore, they focus on a new mode of action of LPS that might throw some light on the pathogenesis of experimental autoimmune conditions. The challenge is now to find out whether adjuvants have an equivalent in clinical situations.

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REFERENCES

1. Billiau A, Matthys P. 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. (Review). *J. Leukocyte Biol*. 70: 849.
2. Theofilopoulos A. 2002. Molecular pathology of autoimmune diseases: an overview. In Theofilopoulos A and Bona C, (Eds). The molecular pathology of autoimmune diseases. Taylor & Francis, N.Y. pp 1-16.
3. Joe B, Wilder RL. 1999. Animal models of rheumatoid arthritis (Review). *Mol. Med. Today*. 5: 367.
4. Charreire J. 2002. Experimental models of autoimmune thyroiditis. In Theofilopoulos, A. and Bona, C., (Eds). *The molecular pathology of autoimmune diseases.*Taylor & Francis, N.Y. pp 685.
5. Janeway C. Jr., Medzhitou R. 2002. Innate immune recognition. (Review). *Ann. Rev. Immunol.* 20: 197.
6. Zaccone P, Fehérvari Z, Blanchard L, Nicoletti F, Edwards CK 3rd, Cooke A. 2002. Autoimmune thyroid disease induced by thyroglobulin and lipopolysaccharide is inhibited by soluble TNF receptor type I. *Eur. J. Immunol*. 32: 1021.
7. Creemers P, Rose NR, Kong YM. 1983. Experimental autoimmune thyroiditis. *In vitro* cytotoxic effects of T lymphocytes on thyroid monolayers. *J. Exp. Med.* 157: 559.
8. Salamero J, Charreire J. 1985. Syngeneic sensitization of mouse lymphocytes on monolayers of thyroid epithelial cells. VII. Generation of thyroid-specific cytotoxic effector cells. *Cell. Immunol*. 91: 111.
9. Remy JJ, Texier B, Chiocchia G, Charreire J. 1989. Characteristics of cytotoxic thyroglobulin-specific T cell hybridomas. *J. Immunol*. 142: 1129.
10. Bendelac A, Carnaud C, Boitard C, Bach JF. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4 + and Lyt-2 + T cells. *J. Exp. Med.* 166: 823.
11. Jiang H, Zhang SI, Pernis B. 1992. Role of CD8+ T cells in murine experimental allergic encephalomyelitis. *Science.* 256: 1213.
12. Tada Y, Ho A, Koh DR, Mak TW. 1996. Collagen-induced arthritis in CD4- or CD8-deficient mice: CD8+ T cells play a role in initiation and regulate recovery phase of collagen-induced arthritis. *J. Immunol*. 156: 4520.
13. Zhang GX, Xiao BG, Bakhiet M, van der Meide P, Wigzell H, Link H, Olsson T. 1996. Both CD4+ and CD8+ T cells are essential to induce experimental autoimmune myasthenia gravis. *J. Exp. Med.* 184: 349.
14. Sugihara S, Fujiwara H, Niimi H, Shearer GM. 1995. Self-thyroid epithelial cell (TEC)-reactive CD8+ T cell lines/clones derived from autoimmune thyroiditis lesions. They recognize self-thyroid antigens directly on TEC to exhibit T helper cell 1-type lymphokine production and cytotoxicity against TEC. *J. Immunol*. 155: 1619.
15. Verma S, Hutchings P, Guo J, McLachlan S, Rapoport B, Cooke A. 2000. Role of MHC class I expression and CD8(+) T cells in the evolution of iodine-induced thyroiditis in NOD-H2(h4) and NOD mice. *Eur. J. Immunol*. 30: 1191.
16. Braley-Mullen H, McMurray RW, Sharp GC, Kyriakos M. 1994. Regulation of the induction and resolution of granulomatous experimental autoimmune thyroiditis in mice by CD8+ T cells. *Cell. Immunol*. 153: 492.
17. Damotte D, Colomb E, Cailleau C, Brousse N, Charreire J, Carnaud C. 1997. Analysis of susceptibility of NOD mice to spontaneous and experimentally induced thyroiditis. *Eur. J. Immunol*. 27: 2854.
18. Zijlstra M, Li E, Sajjadi F, Subramani S, Jaenisch R. 1989. Germ-line transmission of a disrupted beta 2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature*. 342: 435.
19. Baggiolini M. 1998. Chemokines and leukocyte trafic. (Review). *Nature.* 392: 565.
20. Garcia-Lopez MA, Sancho D, Sanchez-Madrid F, Marazuela M. 2001. Thyrocytes from autoimmune thyroid disorders produce the chemokine IP-10 and Mig and attract CXCR3 + lymphocytes. *J. Clin. Endocrinol. Metab*. 86: 5008.
21. White RG. 1967. Role of adjuvants in the production of delayed hypersensitivity. (Review). *Br. Med. Bull.* 23: 39.
22. Zaccone P, Hutchings P, Nicoletti F, Penna G, Adorini L, Cooke A. 1999. The involvement of IL-12 in murine experimentally induced autoimmune thyroid disease. *Eur. J. Immunol*. 29: 1933.
23. Lomo LC, Zhang F, McCormick DJ, Giraldo AA, David CS, Kong YC. 1998. Flexibility of the thyroiditogenic T cell repertoire for murine autoimmune thyroiditis in CD8-deficient (beta2m – / –) and T cell receptor Vbeta(c) congenic mice. *Autoimmunity*. 27: 127.
24. Mignon-Godefroy K, Rott O, Brazillet MP, Charreire J. 1995. Curative and protective effects of IL-10 in experimental autoimmune thyroiditis (EAT). Evidence for IL-10-enhanced cell death in EAT. *J. Immunol*. 154: 6634.
25. Hutchings PR, Verma S, Phillips JM, Harach SZ, Howlett S, Cooke A. 1999. Both CD4(+) T cells and CD8(+) T cells are required for iodine accelerated thyroiditis in NOD mice. *Cell. Immunol*. 192: 113.
26. Thivolet C, Bendelac A, Bedossa P, Bach JF, Carnaud C. 1991. CD8+ T cell homing to the pancreas in the nonobese diabetic mouse is CD4+ T cell-dependent. *J. Immunol*. 146: 85.

27. Mantovani A. 1999. The chemokine system: redundancy for robusts outputs. (Review). *Immunol. Today.* 20: 254.

28. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. 1994. Monocyte chemoattractant protein-1 acts as a T-lymphocyte chemoattractant. *Proc. Natl. Acad. Sc. USA* 91: 3652.

29. Schall TJ, Bacon K, Toy KJ, Goeddel DV. 1990. Selective attraction of lymphocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 347: 669.

30. Goulvestre C, Batteux F, Charreire J. 2002. Chemokines modulate experimental autoimmune thyroiditis through attraction of autoreactive or regulatory T cells. *Eur. J. Immunol.* 32: 3435.

31. Green EA, Wong FS, Eshima K, Mora C, Flavell R. 2000. A. Neonatal tumor necrosis factor alpha promotes diabetes in non-obese diabetic mice by CD154-independent antigen presentation to CD8(+) T cells. *J. Exp. Med*. 191: 225.

32. Xu H, Gonzalo JA, St Pierre Y, Williams IR, Kupper TS, Cotran RS, Springer TA, Gutierrez-Ramos JC. 1994. Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. *J. Exp. Med*. 180: 95.

33. Openshaw P, Murphy EE, Hosken NA, Maino V, Davis K, Murphy K, O'Garra A. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med*. 182: 1357.

Erratum 2

In the article entitled *Prolactin-cytokine network in defense against* Acanthamoeba castellani *in murine microglia,* published in *European Cytokine Network* Vol 13 Nr 4, December 2002, pp. 447-455, there were several mistakes:
– The word *castellani* should be replaced everywhere with *castellanii*
– p. 450, the same figure appears twice. Figure 3 of page 451 should replace Figure 2 on page 450 as follows:

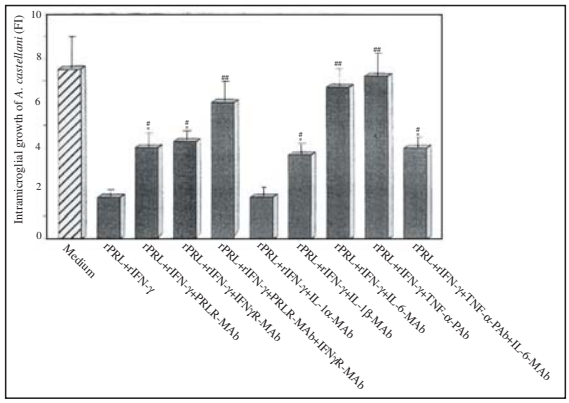

Figure 2
Effect of antibodies against PRLR, IFN-γR, IL-α, Il-1β, Il-6 or TBF-α on the amebastatic activity in MGC.
MGC cultures (5×10^4 cells/well) were incubated with medium alone or containing PRL (25 ng/ml) and rIFN-γ (100 U/ml) for 24 hours. At 3 hours of incubation, monoclonal antibodies (10 µg/ml) specific for either PRLR or IFN-γR or neutralizing antibodies (5 µg/ml) specific for IL-1α, Il-1β, Il-6 or TNF-α were added under normal condition cultures. The cells were then challenged with trophozoites. Intramicroglial growth of *A. castellanii* was determined after 20 hours of infection as above. Data are the means ± SD of triplicate counts of four experiments.
*: significant difference *versus* control; #: significant difference *versus* rPRL + rIFN-γ cells (*, # $p < 0.05$; **,** $p < 0.01$).

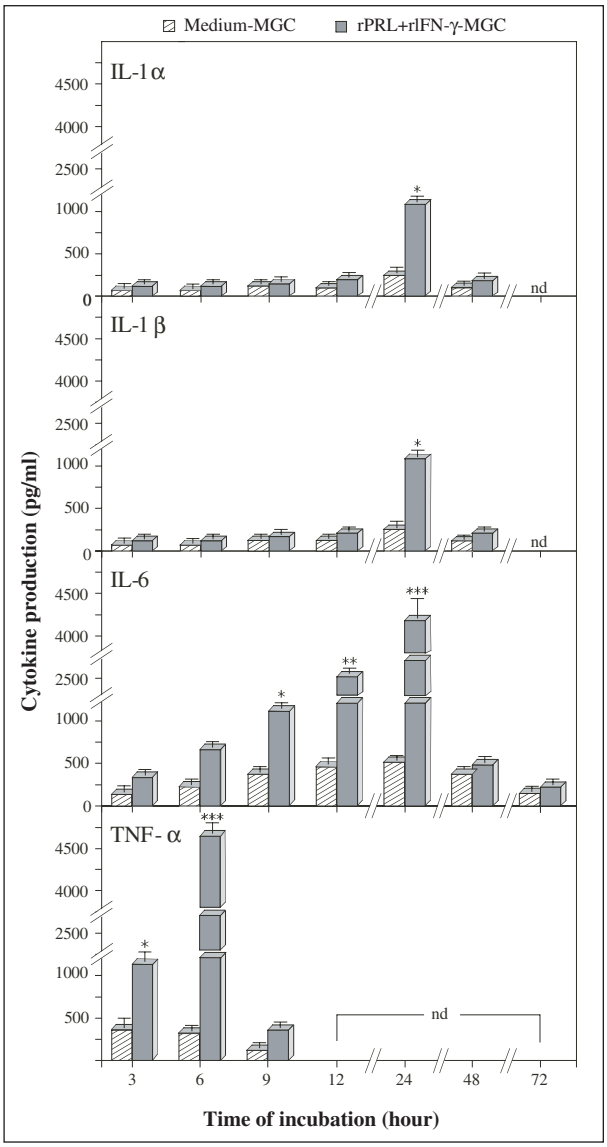

Figure 3
Course of the release of IL-α, Il-1β, Il-6 and TNF-α by MGC during incubation with rPRL and rIFN-γ.
Cell cultures (5×10^4 cells/well) were incubated with medium alone or containing rPRL (25 ng/ml) and rIFN-γ (100 U/ml). At various time points during the priming process, the culture supernatant was collected and the levels of IL-α, Il-1β, Il-6 or TNF-α were determined by ELISA methods. At 72 hours no TNF-α, IL-1α or IL-1β could be detected; nd: not detected. Values are the means ± SD of three determinations of four performed experiments. Significant difference *versus* control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).