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# Antigen-Presenting Dendritic Cells as Regulators of the Growth of Thyrocytes: A Role of Interleukin-1β and Interleukin-6*

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**ABSTRACT**

An accumulation of antigen-presenting dendritic cells (DC) in the thyroid gland, followed by thyroid autoimmune reactivity, occurs in normal Wistar rats during iodine deficiency, and spontaneously in diabetic-prone Biobreeding rats. This intrathyroidal DC accumulation coincides with an enhanced growth rate and metabolism of the thyrocytes, suggesting that both phenomena are related. Because DC are known to regulate the hormone synthesis and growth in other endocrine systems (*i.e.* the pituitary, the ovary, and the testis), we tested the hypothesis that DC, known for their superb accessory cell function in T cell stimulation, act as regulators of thyrocyte proliferation (and hormone secretion).

We investigated the effect of (Nycodenz density gradient) purified splenic DC from Wistar rats on the growth rate of and thyroid hormone secretion by Wistar thyroid follicles (collagenase dispersion) in culture. Various numbers of DC and follicles were cocultured during 24 h. The proliferative capacity of thyrocytes was measured by adding tritiated thymidine ($^3$H-TdR) and bromodeoxyuridine, the hormone secretion into the culture fluid was measured by using a conventional $T_3$ RIA. Furthermore, antibodies directed against interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) were added to these cocultures to determine the role of these cytokines in a possible DC regulation of thyrocyte growth. Cocultures were also carried out in the presence of antimajor histocompatibility complex-class I (MHC I), anti-MHC II, antiintercellular adhesion molecule-1 (ICAM-1), and antilymphocyte function-associated antigen-1α (LFA-1α) antibodies to possibly interfere with DC-thyrocyte interactions.

The addition of DC to thyroid follicles clearly inhibited their $^3$H-TdR uptake, particularly at a 10:1 ratio, in comparison to follicle cultures alone, both under basal conditions and after TSH stimulation (75 ± 7% and 49 ± 11% reduction, respectively, n = 4). The follicle $T_3$ secretion (after TSH stimulation) was also suppressed by DC in this system, but to a lesser extent (at best at an 1:1 ratio, 25 ± 7% reduction, n = 4). The DC-induced inhibition of thyroid follicle growth was totally abrogated after addition of anti-IL-1β antibodies; anti-IL-6 only had effect on the DC inhibition of non-TSH-stimulated thyrocytes, whereas anti-TNF-α demonstrated no effect at all. The antibodies to MHC and to adhesion molecules had also no effect on this DC-induced growth inhibition. The effect of the different anti-cytokine and anti-adhesion antibodies on the $T_3$ secretion from thyroid follicles was not investigated.

The clear inhibition of thyrocyte growth by splenic DC (classical antigen-presenting cells) again demonstrates the regulatory role of DC in endocrine systems. Proinflammatory cytokines such as IL-1β and IL-6 are important mediators in this regulation. The here shown dual role of DC represents a link between the immune and endocrine system, which may form the gateway to the understanding of the initiation of thyroid autoimmune reactions and the thyroid autoimmune phenomena seen in iodine deficiency. (*Endocrinology* **139:** 3148–3156, 1998)

DESPITE RELATIVELY SIMPLE treatment, 1.6 billion people are still at risk for iodine deficiency. The effects of iodine deficiency on individuals are (severely) impaired mental and physical development, hypothyroidism, and endemic goitre development. Although the latter phenomenon is well established, it is still unknown which pathobiological mechanisms play a role in the trophic and metabolic stimulation of iodine-deprived thyrocytes. A raised serum TSH (1), a deficiency of thyroid-derived antitrophic iodinated lipids (2, 3) and a specific trophic action of thyroid reactive antibodies (4, 5) have all been proposed and disputed (6, 7) as driving forces behind iodine-deficient goitrogenesis.

With regard to the involvement of the immune system in iodine-deficient goitrogenesis, we (8–10) previously described an accumulation of monocyte-derived cells such as macrophages (MΦ) and dendritic cells (DC) in human endemic goitre and in the thyroid glands of rats and mice a few weeks after starting an iodine-deficient diet. Because DC are antigen-presenting cells (APC) par excellence and the only stimulators of naive T lymphocytes, these early DC/MΦ accumulations were proposed as inducers of the relatively mild thyroid autoimmune reactions, which often later develop in endemic goitre patients (11, 12) and iodine-deficient rats (8). Indeed, the first local sign of an autoimmune thyroiditis process is, in all animal models of spontaneous disease studied so far, the accumulation and cluster formation of DC and MΦ in the thyroid gland (10, 13, 14). This accumulation occurs prior to thyroid autoantibody formation and prior to the influx of large numbers of T and B cells. Relevant to this report is that the diabetic-prone Biobreeding (BB-DP) rat, one of the animal models of spontaneous thyroid autoimmune disease, shows the formation of a small goitre at the time of the early intrathyroid accumulation of DC (15). The question thus arises whether there is a relationship between goitre formation and DC accumulation.

There is a growing body of evidence that locally accumulated DC and MΦ are not only acting as APC and effector cells in host defence, but also as cells involved in morphogenesis (woundhealing and matrix repair; 16) and, more

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relevant to this report, in the regulation of growth and function of various endocrine cells (17), *i.e.* of anterior pituitary cells, ovarian granulosa cells, theca cells, luteal cells, and testicular Leydig cells. MΦ- and DC-derived cytokines, interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF), are thought to play a role in such immuno-endocrine regulatory interactions (17).

This notion has led to the aim of this study, *i.e.* the investigation of the regulatory effects of rat splenic DC (excellent APC) on particularly the growth, but also the function (thyroid hormone secretion) of rat thyrocytes in a thyroid follicle culture system. Splenic DC and thyroid follicles were isolated and enriched from Wistar rats, and subsequently cocultured and their interactions studied by time lapse cinematography. The tritiated thymidine ($^3$H-TdR) and bromodeoxyuridine (BrdU) incorporation as well as the $T_3$ release of thyrocytes exposed to DC under basal conditions and under TSH stimulation was measured. Furthermore, the effect of neutralizing antibodies to IL-1$\beta$, IL-6, TNF-$\alpha$, and to major histocompatibility complex-class I (MHC I), MHC II, intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1$\alpha$ (LFA-1$\alpha$) was studied in this DC-thyroid follicle coculture system.

## Materials and Methods

### *Animals*

Wistar rats were purchased from Harlan (Zeist, The Netherlands) and were kept under controlled light conditions (12-h light, 12-h dark cycle) throughout this study. A standard pelleted diet (0.35 mg iodine/kg; AM-II, Hope Farms BV, Woerden, The Netherlands) and tap water were provided *ad libitum*.

### *Isolation and 24 h reconstitution culture of thyroid follicles*

Rats (females and males, 8–22 weeks of age) were asphyxiated with carbon dioxide. Thyroid follicles were isolated as previously described (15). Thyroid glands were excised and carefully trimmed of fat and connective tissue. Subsequently, thyroid lobes were washed (4 C) in calcium- and magnesium-free HBSS (Gibco BRL Life Technologies, Breda, The Netherlands). The HBSS was removed and replaced with dispersion solution, containing the following substances: HBSS supplemented with 475 U/ml collagenase (type II; Sigma Chemical Co., Axel, The Netherlands) and 0.1 mg/ml DNase (type I, grade II; Boehringer Mannheim BV, Almere, The Netherlands). Thyroid lobes were minced and incubated for 30 min (37 C, 5% $CO_2$, water-saturated atmosphere). The tissue was then further mechanically disrupted by gentle pipetting. Thyroid fragments were left to sediment during 1 min at $1 \times g$ and released follicles present in the supernatant were harvested and kept at 4 C. Fresh dispersion solution was added to the remaining undigested tissue for 10 min and the above described procedure was repeated. After several reincubation steps the dissociation of thyroid tissue was complete.

The obtained suspension of thyroid follicles was washed twice with ice-cold HBSS containing 0.1 mg/ml DNase and centrifuged for 2 min at $50 \times g$. The pellet consisted of intact and partly fragmented follicles. Single follicular and endothelial cells, fibroblasts, and cell debris remaining in the supernatant were discarded. The isolated follicles were resuspended and cultured in Ham's F-12 medium (Gibco), supplemented with a 5-hormone and peptide mixture containing 10 $\mu$g/ml insulin (Sigma), 10 nM hydrocortisone (Sigma), 5 $\mu$g/ml transferrin (Sigma), 10 ng/ml glycyl-L-histidyl-L-lysine-acetate (Sigma), 10 ng/ml somatostatin (Sigma) and 1% (vol/vol) heat-inactivated (30 min, 56 C) FCS (Integro BV, Zaandam, The Netherlands). The following antibiotics were also added: 100 U/ml penicillin (Seromed, Biochrom, Berlin, Germany), 0.1 mg/ml streptomycin (Seromed), and 2.5 $\mu$g/ml amphotericin (Boehringer).

At 24 h after initial isolation (37 C, 5% $CO_2$, water-saturated atmosphere), the partly fragmented follicles became spheric-like structures again and contained 30–50 thyrocytes per follicle as judged by using an inverted microscope and hematoxylin-eosin staining, respectively.

### *Determination of thyrocyte proliferation in reconstituted thyroid follicles stimulated for 24 h*

The reconstituted follicles were washed and seeded in 24-wells plates (Costar Europe Ltd., Badhoevedorp, The Netherlands) at an averaged density of 1000 follicles/well (proliferation in thyroid follicles, see below) or 2000 follicles/well (thyroid hormone secretion by thyroid follicles, see below). Bovine-TSH (Sigma; concentrations ranging from $1 \cdot 10^{-1}$ to $5 \cdot 10^2$ mU/ml) and 0.1 $\mu$M KI (Merck, Darmstadt, Germany) were added to the follicles. At this stage, splenic DC were also added at various ratios to the thyroid follicles (see below, *Coculture of splenic dendritic cells and thyroid follicles*). The follicles were cultured for another 24 h. Basal controls consisted of cultures in the absence of TSH.

The proliferation assays were designed to examine the entry of cells into the S-phase of the cell cycle. Two methods were used.

*Tritiated thymidine uptake.* Thyroid follicles were $^3$H-TdR labeled during the last 5 h of the 24 h exposure to TSH.

In brief, the follicles were washed and put on fresh Ham's F12 medium (plus supplements and different concentrations TSH) in 96-wells plates (Costar). Subsequently, 1 $\mu$Ci/well methyl-$^3$H-TdR (Amersham International, Buckinghamshire, UK) was added. After the mentioned labeling period at 37 C in a 5% $CO_2$ water-saturated incubator, the follicles were harvested on filter paper and radioactivity was counted in a liquid scintillation analyzer (LKB Betaplate, Wallac, Turku, Finland).

In some experiments, follicles were (a) cocultured with 5 $\mu$g/ml aphidicolin (inhibitor DNA polymerase, 18; Sigma) during or (b) 2,000 rad X-irradiated before TSH exposure. These treatments were applied to prevent replication of DNA, and thus to ascertain that measured radioactivity in the samples without these treatments reflect the S-phase of the cell cycle. In other words; exclusion of erroneous positive measurements, as discussed by Maurer (19). Each individual experiment was done in duplicate.

*5-Bromo-2′-deoxyuridine labeling.* The follicles were incubated at 37 C in a 5% $CO_2$ water-saturated incubator in the presence of 10 $\mu$M BrdU (BrdU-kit I; Boehringer) during the last 5 h before the 24 h of TSH stimulation ended. Thereafter, the samples were thoroughly washed in 0.01 M PBS (Merck), cytocentrifuged onto glass slides, air-dried, and fixed in precooled 70% (vol/vol) ethanol (Merck) during exactly 30 min at −20 C.

The BrdU incorporation was immunocytochemically visualized according to the instructions of the manufacturer (see above) with slight modifications: 1) incubation with anti-BrdU antibody, diluted 1:10 in the manufacturer's incubation buffer (containing nucleases that allow access to BrdU without denaturation of DNA), during 40 min at 37 C; 2) washing steps (3×) in PBS; 3) incubation with antimouse-immunoglobulins antibody, conjugated with fluorescein isothio-cyanate, diluted 1:10 in PBS containing 0.1% (wt/vol) BSA (Sigma), during 45 min at 37 C; 4) washing steps (3×) in PBS. The cytospin preparations were mounted in a solution containing 90% (vol/vol) glycerol (Sigma) and 2.3% (wt/vol) 1,4-diazobicyclo-(2,2,2)-octane (Sigma) to delay fading of the fluorescent dye during examination using an ordinary fluorescence microscope (Zeiss, Weesp, The Netherlands). The percentage BrdU-positive ($BrdU^+$) follicles was evaluated by counting at least 500 follicles/sample. For negative controls, the primary antibody incubation step consisted of buffer solution without anti-BrdU antibody.

This thyroid follicle system also enabled us to determine the thyroid hormone secretion into the culture fluid. After 24 h of exposure to TSH, the culture supernatants were centrifuged ($500 \times g$, 5 min), carefully aspirated (devoid of thyrocytes) and stored at −20 C until $T_3$ levels were determined. The $T_3$ contents in these supernatants were measured by a conventional RIA (Dr. T. J. Visser, Dr. R. Docter, Mr. H. van Toor, Department of Internal Medicine, University Hospital Dijkzigt, Rotterdam, The Netherlands). $T_3$ standards were diluted in Ham's F12 medium plus supplements. Each individual supernatant sample was assayed in duplicate.

### *Isolation of splenic dendritic cells and mixed leucocyte reaction*

Splenic DC from Wistar rats were enriched according to the method of Knight *et al.* (20), with slight modifications. Briefly, the spleen was minced and digested for 1 h at 37 C in RPMI 1640 (Gibco) containing: 125 U/ml collagenase (type III; Gibco) and 0.1 mg/ml DNase. The remaining tissue was teased through a (105 $\mu$m) filter and the erythrocytes were removed by lysis. Finally, the cells were washed and cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. After an overnight culture period in culture flasks (Costar; 37 C, 5% $CO_2$ incubator), the DC were isolated from the nonadherent cells by using a 14.5% (wt/vol) Nycodenz (Nycomed Pharma As, Oslo, Norway) density gradient (800 $\times$ *g* for 20 min). Low density cells were collected from the interphase, thoroughly washed in PBS/0.5% BSA and put on RPMI 1640 with 10% FCS and antibiotics. This cell fraction demonstrated in 70–95% of cells a dendritic morphology, a strong MHC II expression, and absence of acid phosphatase activity.

For the mixed leucocyte reaction (MLR), splenic T lymphocytes were isolated from the (high density cells) pellet fraction after 14.5% Nycodenz density gradient centrifugation. T lymphocytes were separated from B lymphocytes and some residual MΦ by using a nylon wool column (Polyscience Ltd., Eppelheim, Germany) during 1 h in 5% $CO_2$ incubator. These responder T (80–90% $CD3^+$) cells were used in the MLR by adding stimulator (2,000 rad irradiated) DC at various ratios (fixed number of 150,000 T cells/well) in a 96-wells plate, and were subsequently cultured for 5 days in RPMI 1640 containing 50 mM HEPES buffer (Gibco), 110 $\mu$g/ml Na-pyruvate (Merck), 0.5‰ (vol/vol) $\beta$-mercapto-ethanol (Merck) and antibiotics. T cell proliferation was measured via $^3$H-TdR incorporation (0.5 $\mu$Ci/well during the last 16 h of total culture period).

To study the role of MHC and adhesion molecules in this MLR, neutralizing antibodies against MHC I (rat RT1A; 1:1000; Serotec Ltd., Oxford, UK) and MHC II (rat RT1B; 1:400; Serotec), and against LFA-1$\alpha$ (rat CD11a; 1:50; Serotec) and ICAM-1 (rat CD54; 1:50; Serotec) were added on the first and second day of a total culture period of 3 days. Each individual experiment was done in triplicate.

### *Coculture of splenic dendritic cells and thyroid follicles*

Splenic DC (in hormone supplemented Ham's F-12 medium) were 2,000 rad X-irradiated to prevent proliferation and cocultured with thyroid follicles at various ratios (10:1, 1:1 and 1:10) for 24 h (with and without addition of TSH 100 mU/ml). Before coculturing, the thyroid follicle suspensions (24 h after initial isolation) were checked for possible presence of residual DC. A cytochemical staining method (against MHC II) was used for this purpose and demonstrated that these suspension contained no DC.

After the period of coculture, the effect of splenic DC on thyroid follicle $^3$H-TdR (and BrdU) uptake and $T_3$ release was measured (see above, *Determination of thyrocyte proliferation in reconstituted thyroid follicles stimulated for 24 h*). The viability of the thyroid follicles and DC remained unchanged when cultured alone or cocultured (data not shown). Control cultures consisted of thyroid follicles and splenic DC alone (with and without TSH 100 mU/ml).

In some experiments (n = 3), the specificity of the DC effect on thyrocyte proliferation and $T_3$ release was tested by fixing DC in a 4% (vol/vol) formaldehyde-PBS solution (Merck) for 20 min at room temperature before adding these DC to the follicles. This procedure prevented the motility of (as verified by time lapse video recording, see below) and production of cytokine(s) by DC.

To establish whether cytokines (IL-1$\beta$, IL-6 and TNF-$\alpha$) or adhesive interactions (MHC I and II, and LFA-1$\alpha$/ICAM-1) might contribute to the effect of DC on the examined thyrocyte growth, the following antibodies were added to our coculture system (DC:follicle ratio of 1:1) for 24 h: antihuman II-1$\beta$ (1:5000; Glaxo, Geneva, Switzerland), antirat IL-6 (1:100; kindly provided by Dr. J. Gauldie, Department of Pathology, McMaster University, Hamilton, Canada), antihuman TNF-$\alpha$ (1:1000; a kind gift from Dr. W. A. Buurman, Department of Surgery, University of Limburg, Maastricht, The Netherlands), antirat MHC I and II (see above, *Isolation of splenic dendritic cells and mixed leucocyte reaction*), antirat LFA-1$\alpha$ and ICAM-1 (see above). Each individual experiment was done in duplicate.

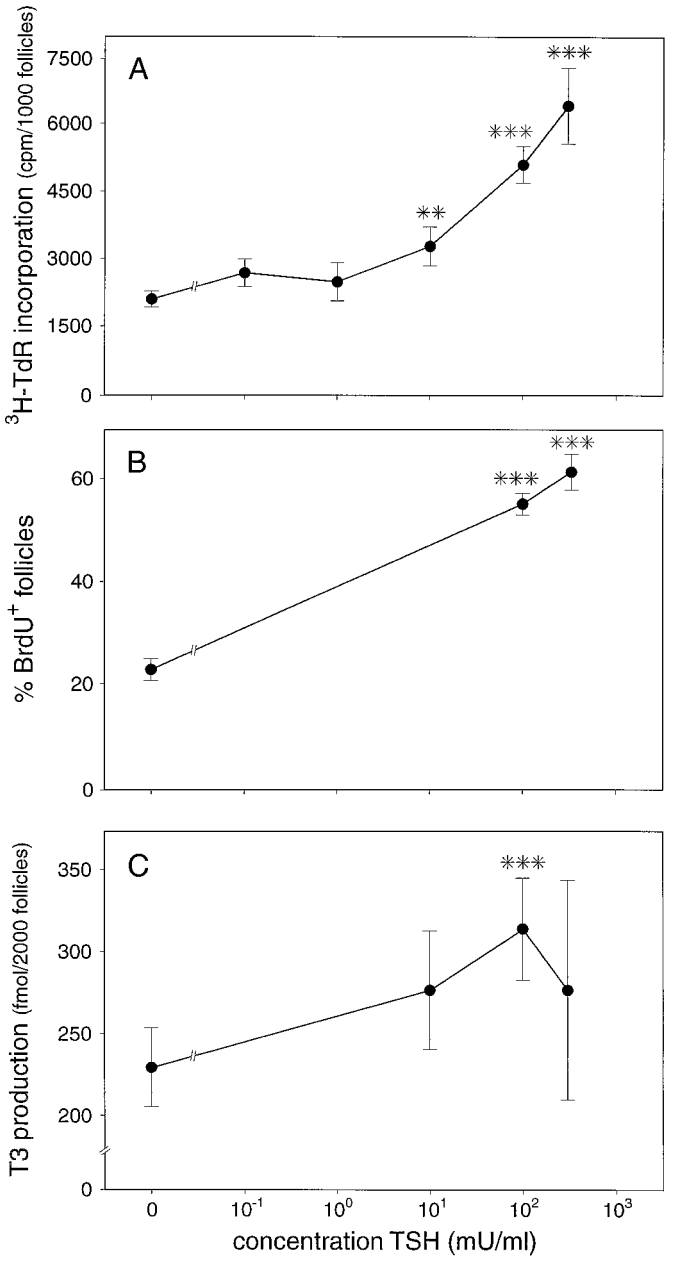
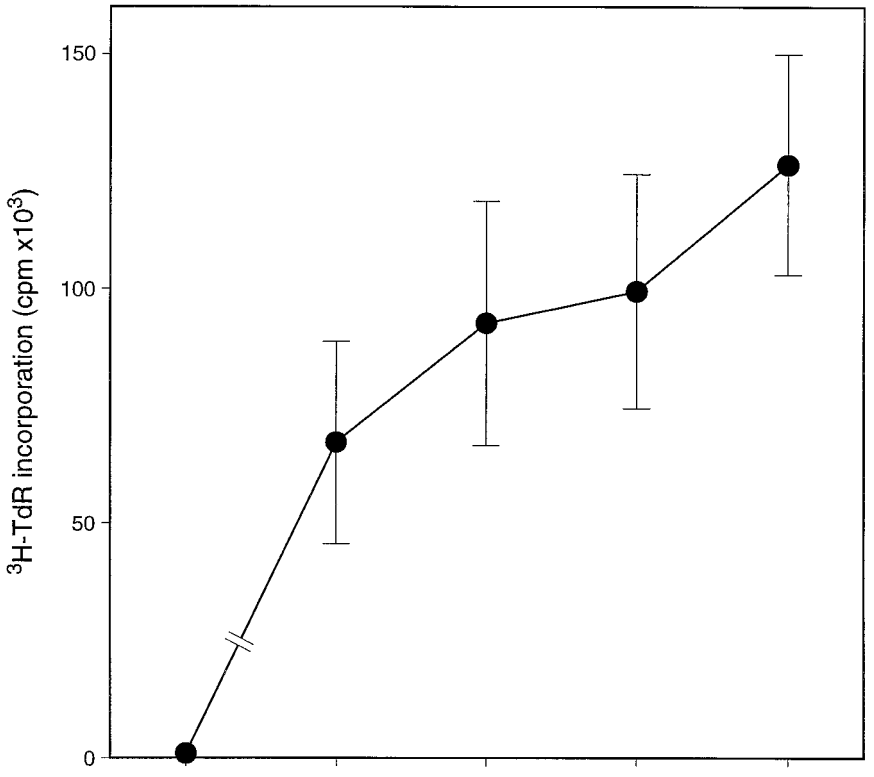



FIG. 1. The $^3$H-TdR incorporation in (A, n = 4–16), the percentage of positive BrdU labeled (B, n = 7–15) and the $T_3$ secretion by (C, n = 7–14) Wistar thyroid follicles kept in suspension culture under basal conditions and stimulated by various concentrations TSH. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ stimulated *vs.* basal conditions.

### *Time lapse cinematography*

To investigate DC motility and cell-cell interactions between DC and thyroid follicles under the various above described coculture conditions, time lapse cinematographic studies were performed. Again, DC and thyroid follicles (ratio 1:10), with or without the various anticytokines or antiadhesions, were cocultured and recorded during 45 min in a microincubator (37 C, 5% $CO_2$) on an inverted microscope (Axiovert, Zeiss) attached to a video camera (Sony Co., Japan) and a time lapse video recorder (Panasonic, Matsushita Electric Industrial Co., Osaka, Japan). The video recording interval was set at 0.18 sec and the tape running speed at 2.599 mm/sec. After recording, the images were analyzed using a computer (Acorn Computers Ltd., Cambridge, UK).




| DC/lymphocyte ratio | | no DC | 1:40 | 1:20 | 1:10 | 1:5 |
|---|---|---|---|---|---|---|
| percentage blockade by ab | α-LFA1α | ND | 56±18 | ND | 43±15 | 54±16 |
| | α-ICAM1 | ND | 68±18 | ND | 92± 1 | 80± 3 |
| | α-MHC I | ND | 100± 0 | ND | 100± 0 | 100± 0 |
| | α-MHC II | ND | 89±10 | ND | 79±12 | 81± 8 |

FIG. 2. The $^3$H-TdR incorporation into Wistar T lymphocytes stimulated by syngeneic splenic DC in a mixed leucocyte reaction (n = 7–8). In the added table the blocking effect (n = 3) of the listed antibody is given at the indicated DC:T cell ratio. Effective antibody dilutions interfering with DC clustering were 1:50 for anti-LFA-1α and anti-ICAM-1, 1:1000 for anti-MHC I and 1:400 for anti-MHC II; these effective dilutions have been used in this set of experiments and Fig. 5. (ND, Not determined).

*Statistical analysis*

The results are presented as means ± SEM and statistical analysis was performed using the two-tailed and paired Students' *t* test.

## Results

*Effects of the coculture of splenic dendritic cells with thyroid follicles on thyrocyte proliferation and $T_3$ secretion*

In a first set of experiments, we established the time- and dose-dependent effects of the spontaneous and TSH-induced $^3$H-TdR uptake in and $T_3$ release from Wistar thyroid follicles in suspension culture. During the follicle reconstitution time (first 24 h after isolation), there was hardly any $^3$H-TdR uptake (<100 cpm). Maximal $^3$H-TdR uptake was obtained in follicle cultures of 24 h after the reconstitution time. During this time, detectable amounts of $T_3$ were also released in the culture fluid. Exposure to TSH during these 24 h increased both the $^3$H-TdR uptake and the $T_3$ release in a dose-dependent manner (Fig. 1, A and C), 100 mU/ml being the most optimal dosage for measuring both $T_3$ release and $^3$H-TdR incorporation in our assay system. The specificity of the $^3$H-TdR uptake with regard to true DNA proliferation was demonstrated with aphidicolin and X-irradiation treatment. Both treatments reduced >80% (n = 5) of the total measured incorporated radioactivity (spontaneous and TSH-stimulated). This reduction could not be ascribed to cytotoxicity of aphidicolin or X-irradiation: none of the follicles showed a change in viability (trypan blue exclusion test) or an altered morphology, and moreover the $T_3$ release remained unchanged (data not shown). In accordance with the $^3$H-TdR uptake, our assay system showed a clearly measurable BrdU labeling of the thyroid follicles with TSH at 100 and 500 mU/ml after 24 h of culture (Fig. 1B). Under basal (non-TSH-stimulated conditions) < 5 BrdU$^+$ nuclei per follicle were always found, whereas exposure to TSH often resulted in 10–20 BrdU$^+$ nuclei per follicle.

Addition of 6 propyl-2-thiouracil (Sigma; 2 mM), during the 24-h culture period after follicle reconstitution, clearly inhibited basal and TSH-stimulated $T_3$ levels in the culture fluid (data not shown) indicating that the hormone measured in the culture supernatants was mainly due to secretion rather than from aspecific release.

On the basis of the above-described findings, we decided to use a culture period of 24 h and a dosage of 100 mU/ml TSH for stimulation, and to consider the $^3$H-TdR incorporation as a reliable parameter of thyrocyte proliferation.

In a second set of experiments, we verified the accessory capability of the splenic DC population. As expected, the Wistar rat splenic DC (Nycodenz-enriched low density splenic cell fraction) were potent stimulators of syngeneic T lymphocytes in MLR (Fig. 2). Already at a ratio of 1:40 (DC:T

cell) high incorporation values of around 70,000 cpm were found. In contrast, resident peritoneal MΦ or unfractionated splenic cells were hardly capable of stimulating syngeneic T cells (values of at best 2,000–3,000 cpm were reached at a ratio 1:1), illustrating the superb stimulatory capability of splenic DC.

The addition of the splenic dendritic APC to syngeneic thyroid follicles resulted in a much lower $^3$H-TdR and BrdU uptake by the thyrocytes both under basal and TSH-stimulated conditions (Fig. 3). In the case of $^3$H-TdR uptake values between 25–75% DC-induced inhibition were found depending on the ratio DC:follicle and the conditions; the strongest inhibition was found under basal conditions and at a ratio of 1:10 (follicle:DC), viz. 25 ± 7% of control (= thyroid follicle culture in the absence of DCs, Fig. 3). The decreased BrdU labeling largely confirmed the $^3$H-TdR uptake data, however, values only (probably because of the semiquantitative nature of this method) reached statistical significance under non-TSH-stimulated conditions and at a ratio of 1:10 (follicle:DC, 71 ± 6% of control, Fig. 3). With regard to the $T_3$ secretion, splenic DC induced a subtle but consistent inhibition (*i.e.* 75 ± 7% of control) on TSH-stimulated thyroid follicles at a ratio of 1:1 (Fig. 3). The $T_3$ release in cocultures not stimulated with TSH was not affected by the presence of splenic DC.

Time lapse phase-contrast cinematographic studies of the DC-thyroid follicle cocultures showed that DC were very active in the system (Fig. 4). They had more or less rounded or elongated shapes and the vast majority were constantly extending and withdrawing long cytoplasmic veils approaching and touching the thyroid follicles. The dendritic (or veiled) cells often formed small cell clusters with each other (containing 4–8 cells, so-called homotypic clustering), and some of these DC clusters also made contact with the thyroid follicles. The contacts of DC with the thyroid follicles varied from short (mere touching at several spots of the thyroid follicle) to longlasting and firm. During these firm contacts, the actively moving DC were able to tilt a whole thyroid follicle. When formaldehyde-fixed DC were used in this coculture system, their moving and active behavior were completely lost as well as their homotypic clustering and contacting with the thyroid follicles. This treatment also resulted in a complete abrogation (data not shown; n = 3) of the inhibitory effects of splenic DC on both the $^3$H-TdR uptake (basal and TSH stimulated) and the $T_3$ release (TSH stimulated).

### *Effects of neutralizing antiadhesion antibodies on DC-thyroid follicle interactions*

Because the intensive physical interactions of the splenic DC with the thyroid follicles suggest the involvement of adhesion molecules and because the DC constitutively expressed MHC I, MHC II, LFA-1$\alpha$, and ICAM-1, whereas thyroid follicles were positive for ICAM-1 and LFA-1$\alpha$ (data not shown), we added anti-MHC I, anti-MHC II, anti-LFA-1$\alpha$, and anti-ICAM-1 antibodies to the coculture system.

Before these experiments, the effect of these antibodies and their effective dilutions were studied on homotypic clustering of splenic DC and APC capability in syngeneic MLR. Effective dilutions for interfering with DC clustering ap-

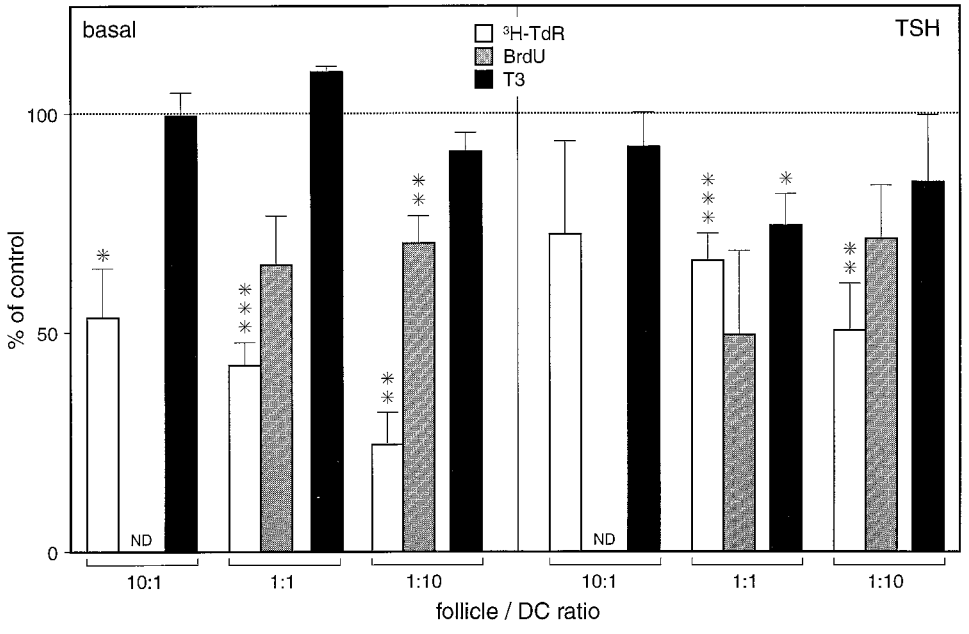



FIG. 3. The effect of Wistar splenic DC on the growth of and $T_3$ secretion (*black bars*, n = 4) by Wistar thyroid follicles kept in suspension culture under basal (*left panel*) and TSH-stimulated (*right panel*) conditions. Follicle growth was measured by $^3$H-TdR incorporation (*open bars*, n = 4–17) and by BrdU labeling (*shaded bars*, n = 4–5). Basal or TSH-stimulated (100 mU/ml) outcomes in the absence of DC were set at 100% (*dotted line*) in each separate experiment because these outcomes showed considerable interassay variation in this set of experiments ($^3$H-TdR incorporation: basal 1673 ± 227 cpm/1000 follicles, range 247–3777 cpm and TSH-stimulated 4438 ± 483 cpm/1000 follicles, range 1070–8888 cpm; BrdU labeling: basal 18 ± 4% BrdU$^+$ follicles, range 12–28% and TSH-stimulated 52 ± 1% BrdU$^+$ follicles, range 50–55%; $T_3$ secretion: basal 194 ± 14 fmol/2000 follicles, range 155–220 fmol and TSH-stimulated 309 ± 37 fmol/2000 follicles, range 250–320 fmol). Despite this variation, coculture with DC invariably gave a clear inhibition of $^3$H-TdR incorporation or BrdU labeling, and of $T_3$ secretion. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ presence *vs.* absence of DC in thyroid follicle cultures. (ND, Not determined).

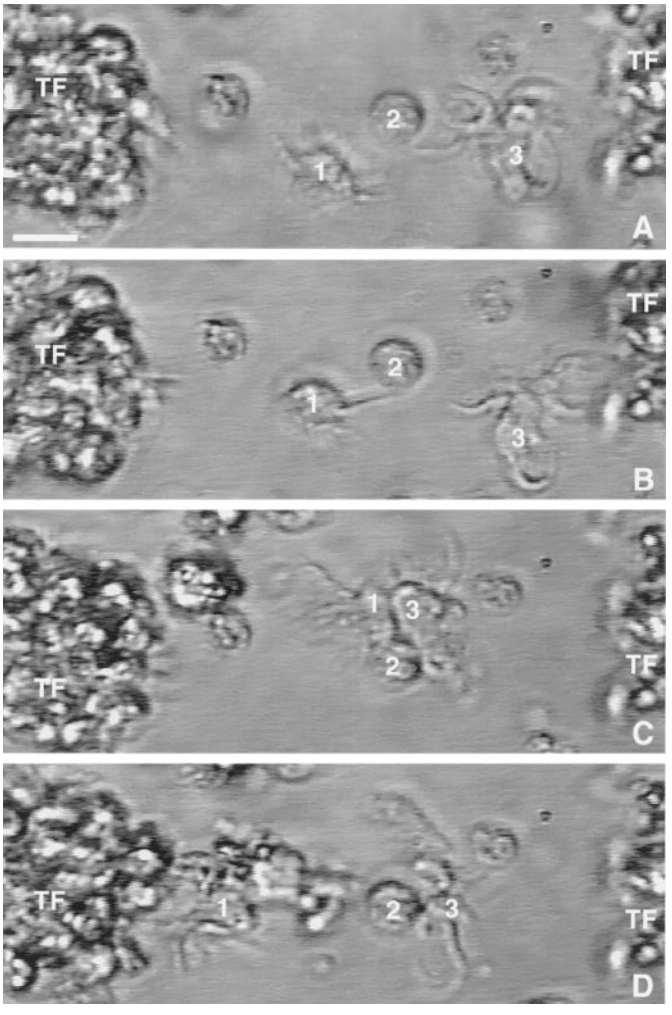



FIG. 4. Photographs of time lapse phase-contrast cinematography demonstrating the interactions of two active veiled splenic DC (1, 3) with each other, with nonactive rounded DC (2) and with two thyroid follicles (TF) in suspension culture. Four sequential pictures (A–D) are given 2 min apart. Note the moving and active behavior of the veiled DC and the short contacts established with each other and the thyroid follicles. Magnification, ×1000; *bar*, 10 μm.

peared to be 1:50 for anti-LFA-1α and anti-ICAM-1, 1:1000 for anti-MHC I, and 1:400 for anti-MHC II. The homotypic clustering was delayed for several hours (2–3 h) by the addition of anti-LFA-1α and anti-ICAM-1; normally homotypic clustering starts at 30 min to 1 h. The anti-MHC I and anti-MHC II intensified the homotypic clustering of DC and very large clusters of over 50 cells ($1.5 \times 10^5$ DC/well) were detected after 30 min in culture. With regard to the accessory capability of the splenic DC, the antiadhesion and the anti-MHC antibodies clearly reduced this function of these cells in syngeneic T cell MLR (Fig. 2).

Despite their clear effects on the immune functions of the splenic DC, the antibodies were not able, using the above indicated dilutions, to interfere with the DC-induced inhibitory effects on $^3$H-TdR uptake of thyroid follicles (Fig. 5, both basal and TSH-stimulated conditions). In the absence of the splenic DC these antibodies had also no effect on the $^3$H-TdR uptake of thyrocytes (Fig. 5). By using time lapse cinematography it appeared that the antibodies were effective in our follicle-DC coculture system because homotypic DC clustering was affected in the system; the interactions of splenic DC with thyroid follicles were, however, similar in the presence or absence of anti-MHC I, anti-MHC II, anti-LFA-1α, and anti-ICAM-1.

*Effects of neutralizing anticytokine antibodies on DC-induced inhibition of thyrocyte $^3$H-TdR uptake*

To determine whether IL-1β, IL-6, and TNF-α were involved in the antiproliferative effect of splenic DC on thyroid follicles, experiments with neutralizing anticytokine antibodies were performed. In earlier published experiments, we had established the effective dilutions of these antibodies to neutralize the bioactivity of their target cytokines in the appropriate bioassays (21, 22).

Anti-IL-1β antibodies (1:5000) were able to abrogate the DC-induced inhibition of $^3$H-TdR uptake in thyroid follicles, both under basal and TSH-stimulated conditions (Fig. 6). This shows a key role of IL-1β in the thyrocyte antiproliferative effect of splenic DC. The $^3$H-TdR uptake of thyroid follicles in the absence of DC was not affected by the anti-IL-1β antibodies, demonstrating that the cytokine was not involved in thyrocyte proliferation under these conditions of the assay system.

Anti-IL-6 antibodies (1:100) showed a strong stimulating effect on thyrocyte proliferation in the absence of splenic DC ($P < 0.01$) but only under basal (non-TSH-stimulated) conditions (Fig. 6). This suggests an antiproliferative effect of thyrocyte-derived IL-6 under basal conditions (note: DC are absent from these reconstituted follicles). The anti-IL-6 antibodies were also able to completely abrogate the DC-induced inhibition of thyrocyte $^3$H-TdR uptake under non-TSH-stimulated conditions (Fig. 6), hence an involvement of this cytokine in DC-thyrocyte interactions is most likely.

Anti-TNF-α antibodies (1:1000) had a dampening effect ($P < 0.05$) on TSH-stimulated $^3$H-TdR uptake of thyroid follicles (Fig. 6), indicating that this cytokine (thyrocyte-derived?) stimulates thyrocyte proliferation. No neutralizing effect of anti-TNF-α could be found on the thyrocyte growth inhibiting effect of added splenic DC (Fig. 6).

## Discussion

This study shows that rat splenic DC, apart from being excellent APC in MLR, also regulate the proliferation (and to some extent the thyroid hormone release) of thyrocytes in a rat thyroid follicle *in vitro* coculture system. Also *in vivo*, albeit in other endocrine systems, similar endocrine down-regulating effects of monocyte-derived cells have been described: Gu *et al.* (23) detected, after preventing the influx of DC/MΦ in the pancreas, a slight but consistent (25%) increase of islet and duct cell growth in interferon-γ transgenic mice, and Gaytan *et al.* (24) observed raised testosterone levels in rats after DC/MΦ depletion of the testis. Whether an *in vivo* blockade of the intrathyroid accumulation of DC (*e.g.* via the usage of the antibodies ED 7 and ED 8; Ref. 25) leads to an even more enhanced proliferation of thyrocytes in iodine-deficient Wistar rat goitre or in BB-DP rat goitre needs to be investigated.

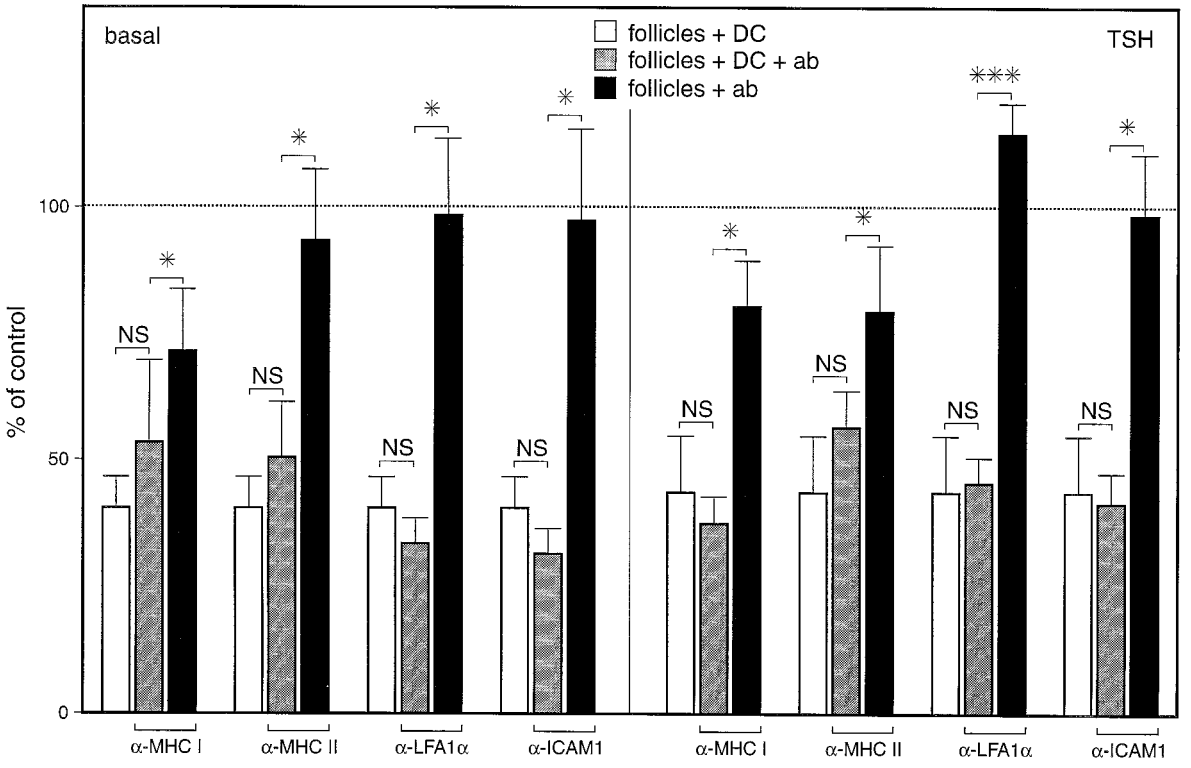



FIG. 5. The effects of anti-MHC I, anti-MHC II, anti-LFA-1α, and anti-ICAM-1 antibodies (ab) on the $^3$H-TdR incorporation of thyroid follicles in suspension culture under basal (*left panel*) and TSH-stimulated (*right panel*) conditions (n = 3–5). Coculture conditions (presence/absence of DC and/or ab) are given at the top of the figure. See Fig. 3 for explanation of *dotted line* (basal 1977 ± 470 cpm/1000 follicles, range 1268–3777 cpm; TSH-stimulated 4599 ± 1176 cpm/1000 follicles, range 2061–8888 cpm) and *P* values.

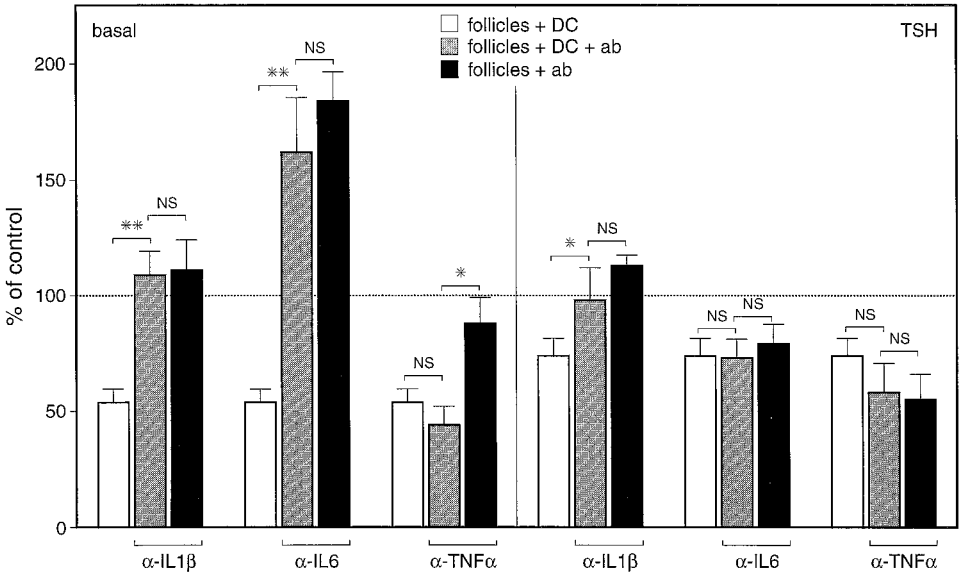



FIG. 6. The effects of anti-IL-1β (1:5000), anti-IL-6 (1:100), and anti-TNF-α (1:1000) antibodies on the $^3$H-TdR incorporation of thyroid follicles in suspension culture under basal conditions (*left panel*) and TSH-stimulated (*right panel*) conditions (n = 7–9). See Fig. 3 for explanation of *dotted line* (basal 1183 ± 219 cpm/1000 follicles, range 247–2556 cpm; TSH-stimulated 3666 ± 595 cpm/1000 follicles, range 1070–7328 cpm) and *P* values.

Our study also shows that the cytokine IL-1β is a key controlling signal in the *in vitro* DC-down-regulated thyrocyte growth. IL-6 also contributed to the immuno-endocrine interaction, but only under non-TSH-stimulated conditions. TNF-α, if any, had a growth promoting effect in our culture system. Though epidermal DC (the Langerhans cells) have been described as major sources of IL-1β and IL-6 (26), it is the general concept that cells belonging to the DC series are poor producers of these cytokines. In our experience (lung DC; 27) and that of others (28, 29), DC do not or hardly produce IL-1β and IL-6. Nevertheless, DC-derived IL-1β signals play a major role in DC-induced T cell proliferation as shown in cytokine neutralization experiments (27) and apparently also in DC-thyrocyte regulation (see this report).

Effects of IL-1β and IL-6 on thyrocyte growth and function have amply been described. In the majority of the reported

experiments the cytokines inhibit differentiated functions such as thyroid peroxidase expression, thyroglobulin release, iodide uptake, and $T_3$ secretion; reports on stimulation, however, also exist (reviewed in Refs. 30 and 31). The effect of these cytokines on thyrocyte proliferation is also conjectural. Upon injection, IL-1$\alpha$ and IL-1$\beta$ stimulate thyroid growth in rats (32), and IL-1$\alpha$ promotes the growth of a thyroid carcinoma cell line via $Ca^{2+}$/calmodulin pathways (33). In contrast, the group of Gärtner found no effect of IL-1$\beta$ on thyrocyte proliferation using a porcine thyroid follicle culture system (34). IL-6 was described as not affecting basal cell proliferation of FRTL-5 cells, it stimulated DNA-synthesis in a dose-dependent manner in the presence of TSH (35). This growth promoting effect of IL-6 is, however, controversial and Zeki *et al.* (36) have reported very little effect on FRTL-5 cell proliferation. Our data give evidence for a growth inhibiting effect of DC-derived IL-1$\beta$ and IL-6. Obviously, culture conditions (the state of the thyrocytes and the DC), local cytokine concentrations (31), and possibly other cofactors provided in the close DC-thyrocyte interactions may play a role as well. With regard to the growth-inhibiting effect of DC-derived IL-1$\beta$, it is also worthy to note that recent reports show that this cytokine induces Fas expression on thyrocytes, subsequently leading to the apoptosis of the thyrocytes (37). Whether DC are able to induce Fas expression on rat thyrocytes in our culture system needs further investigation.

Our cinematographic study showed that the DC were very active, moving around and touching the thyroid follicles, while constantly extending and withdrawing their veils. In some cases the DC made firm contacts with the follicles and were able to tilt the follicles. It is possible that these close contacts allow an efficient and special local cytokine signalling between the DC and the thyrocytes, explaining the above-discussed IL-1$\beta$ (and IL-6) induced inhibiting action on thyrocyte proliferation. A specific triggering of the adhesion molecules (receptors in itself) may also play a role in the DC-induced thyrocyte growth inhibitory action; indeed, DC constitutively express many adhesion molecules (38), whereas thyrocytes acquire such molecules on their surface after cytokine/TSH stimulation (39). However, antibodies to MHC I and II and to ICAM-1 and LFA-1$\alpha$ had no effect on the contacting behavior and the growth controlling action of DC on thyrocytes in coculture. These antibodies did show an effect on the physical and functional interactions between DC and T lymphocytes. This argues against at least a role of MHC molecules and LFA-1$\alpha$/ICAM-1 in the functional contacts between DC and thyrocytes. It is obvious that other adhesion molecules need to be tested in future experiments.

What could be the molecular signals attracting the DC to the thyroid? In the nonobese diabetic mouse, DC infiltration can be triggered by inducing thyrocyte necrosis via iodine-intoxication (10). In the thyroid of the BB-DP rat, the infiltration of DC occurs spontaneously at around 10 weeks of age, in the absence of early microscopic thyrocyte necrosis (13, 40, 41). At that time, isolated BB-DP thyrocytes produce *in vitro* more $T_4$ and IL-6 as compared to age-matched Wistar thyrocytes (15), and these molecules may be instrumental in the local accumulation of DC because thyroid hormones and IL-6 are *in vitro* active in the monocyte to DC transition (42). Monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are known regulators for the attraction (43) and maturation (42) of monocyte-derived DC. However, MCP-1 is not expressed in the thyroid of 10-week-old BB-DP rats but becomes expressed later when lymphocytic infiltrates are present (15). IL-1 has been described as inducing MCP-1 and GM-CSF expression in cultured thyrocytes (44, 45). Hence, a scenario can be envisioned where DC accumulated in the thyroid by yet unknown signals (IL-6?) provide IL-1 stimuli to the neighboring thyrocytes, upon which these endocrine cells are not only regulated in their proliferation but also start to secrete cytokines (MCP-1, GM-CSF) that further stimulate the accumulation of DC.

In conclusion, the present study shows that classical APC, such as the DC, inhibit the *in vitro* growth (and hormonal output) of thyrocytes and that IL-1$\beta$ and to a lesser extent IL-6 are involved. Further studies are needed to establish the *in vivo* effects of DC on thyroid growth itself and a possible involvement of Fas molecules. Unveiling the effect of intrathyroidal DC on the growth of neighboring thyrocytes will probably lead to a better understanding of the initial interactions between thyrocytes and immune cells during the development of iodine-deficient goitre and thyroid autoimmune disease.

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