The Journal of Experimental Biology 207, 55-65
Published by The Company of Biologists 2004
doi:10.1242/jeb.00710

Characterization of a novel set of resident intrathyroidal bone marrow-derived hematopoietic cells: potential for immune-endocrine interactions in thyroid homeostasis

John R. Klein* and Heuy-Ching Wang

Department of Basic Sciences, Dental Branch, University of Texas Health Science Center at Houston, Houston, TX 77030, USA

*Author for correspondence (e-mail: john.r.klein@uth.tmc.edu)

Accepted 9 September 2003

Summary

Immunofluorescent staining of thyroid tissues was done using monoclonal antibodies to dendritic cell (DC), lymphocyte, macrophage and granulocyte markers. Despite the presence of occasional CD11c$^+$ cells, CD11b$^+$ cells, morphologically characteristic of DCs, were abundant in thyroid of normal mice, at a density of ~2.0 cells per thyroid follicle, and were >tenfold more frequent than CD11c$^+$ cells. Thyroid tissues were non-reactive with antibodies to F4/80, CD8α, CD40, CD80, Gr-1, CD3, or CD19, indicating that the CD11b$^+$ cells were not macrophages, activated DCs, granulocytes, plasmacytoid DCs, T cells or B cells. Following systemic immune activation, DCs in secondary lymphoid tissues but not in the thyroid, upregulated CD80 expression. Using radiation chimeras made from bone marrow from enhanced green fluorescent protein (EGFP) transgenic mice, EGFP$^+$ DC-like cells were present in the thyroid from 1–20 weeks after bone marrow transfer, but were rare in the kidney and liver, although EGFP$^+$ cells were present in secondary lymphoid tissues. Additionally, DCs generated from EGFP$^+$ bone marrow cells localized in the thyroid of EGFP$^-$ mice following adoptive transfer. Double staining of thyroid tissue sections with antibodies to the thyroid stimulating hormone (TSH)-β molecule and to CD11b revealed co-expression of TSHβ and CD11b among intrathyroidal DCs. Moreover, RT-PCR analyses indicated expression of the TSHβ gene in thyroid tissues. These findings define a novel bone marrow-derived hematopoietic cell population that resides in the thyroid of normal mice, which may have a unique role in the microregulation of thyroid physiology and homeostasis.

Key words: immune–endocrine, bone marrow, homeostasis, thyroid.

Introduction

It is now increasingly evident that physiological systems once regarded to be functionally autonomous rely on a complex network of interdependent regulatory signals and events. Such biological intersections are particularly evident between many aspects of the immune system and the neuroendocrine system. Thus, a number of classical neuroendocrine hormones are produced by the cells of the human and murine immune systems (Smith and Blalock, 1981; Smith et al., 1983; Stephanou et al., 1990; Hiestand et al., 1986; Hattori et al., 1990; Wang et al., 2003), and receptors for those are widely distributed across many types of hematopoietic cells (Wang et al., 2003; Chabaud and Lissitzky, 1997; Kruger et al., 1989; Coutelier et al., 1990; Hiruma et al., 1990; Bagriacik and Klein, 2000; Wang et al., 1997). The functional significance of those immune–endocrine circuits, though not fully evident, is nonetheless gradually becoming clear. One area for which information has increased in recent years is the role of hormones of the hypothalamus–pituitary–thyroid (HPT) axis in the process of immune regulation. Conversely, a potential role for the immune system in the regulation of hormone activity also has been identified. In the case of the former, thyrotropin releasing hormone and thyroid stimulating hormone (TSH) have been shown to influence the development and immunobiological responses of T cells located in gut-associated lymphoid tissues, i.e., the intestinal intraepithelial lymphocytes (IELs) (Wang and Klein, 1994; Wang and Klein, 1995; Wang and Klein, 1996). Exogenous treatment of mice with either thyrotropin releasing hormone (TRH) or TSH resulted in an increase in the proportions of T cell receptor-$\alpha\beta^+$, CD8$\alpha\beta^+$ IELs (Wang et al., 1997; Wang and Klein, 1994; Wang and Klein, 1995). The activity of TSH appeared to be due to locally secreted TSH produced by small intestinal enterocytes (Wang et al., 1997). In a reciprocal manner, thyroxine was shown to suppress IEL development in normal mice (Wang and Klein, 1996), and to reduce autoimmune pathology in a model of experimental murine gastritis (Wang et al., 1998), thus pointing to bidirectional effects of HPT hormones on immunity.

Other studies of hypothalamus–pituitary–thyroid axis mediated immune–endocrine interactions have examined the

involvement of dendritic cells (DCs) in modulating thyroid cell function in normal non-autoimmune animals. *In vitro* co-culture of thyrocytes with DCs restricted thyrocyte growth and thyroid hormone synthesis (Simons et al., 1998). This may be mediated by interleukin-1β or interleukin-6 (Simons et al., 1998) produced from DCs, or possibly by secretion of those monokines directly from the thyrocytes themselves (Simons et al., 1998). Simons et al. (1998) showed that DCs were obtained from splenic tissues, however. Given the phenotypic and functional heterogeneity of splenic DCs, the relevance of those cells to intrathyroidal DCs is unclear. In other studies, thyroid-derived low density mononuclear cells isolated from rat thyroids expressed the rat monocyte ED1 marker, but lacked the macrophage markers ED2 and ED3 and had little or no expression of CD80 and CD86 (Simons et al., 2000). However, the inability to obtain sufficient numbers of cells for flow cytometric analyses required that analyses be done in cytospin preparation, and ~50% of the cells were contaminated with thyrocytes (Simons et al., 2000). In studies by Croizet et al. (2000, 2001) porcine thyroid was used as a source of both thyrocytes and DCs for *in vitro* analyses of DC–thyrocyte interactions. Thyroid-derived DCs were shown to proliferate in the presence of TSH and to retain the ability to endocytose labeled ligands (Croizet et al., 2001). Moreover, intrathyroidal DCs retained an immature phenotype until exposed to tumor necrosis factor-α (Croizet et al., 2000), a cytokine commonly produced by DCs. An important feature of that study pertains to its ability to compare DC-thyroid responses using thyroid-derived rather than peripheral DCs; however, studies using porcine tissues are limited by a paucity of immunological reagents for extensive phenotypic analyses.

In the present study, we have used multiple approaches to characterize intrathyroidal DCs in situ in mice. First, thyroid tissue sections from normal mice were examined using immunofluorescent staining and a panel of monoclonal antibodies (mAbs) to DC, macrophage (mϕ), lymphocyte and granulocyte markers (CD11b, CD11c, CD40, CD3, CD19, CD8α, F4/80, and Gr-1). This permitted us to evaluate the presence and distribution of DCs as they naturally occur in the thyroid, and to avoid problems associated with isolating DCs from thyroid tissue digests that would be compromised by difficulties in obtaining adequate numbers of DCs for *in vitro* phenotypic analyses. Second, the trafficking of DCs to the thyroid was examined to define the use in radiation chimeras of bone marrow cells from transgenic donor mice expressing enhanced green fluorescent protein $(EGFP)^+$ were injected into irradiated $EGFP^-$ host animals. Direct fluorescence analyses of thyroid, kidney and liver tissues were done to localize $EGFP^+$ cells in non-lymphoid tissues. Third, $EGFP^+$ bone marrow-derived DCs generated *in vitro* and transferred to $EGFP^-$ host mice were used to confirm that intrathyroidal $EGFP^+$ cells were in fact DCs. Fourth, thyroid tissue sections were stained with a mouse TSHβ-specific mAb to determine the spatial relationship between TSH-producing cells and $CD11b^+$ cells in the thyroid. Findings from these studies provide important new information by demonstrating: (i) that intrathyroidal DCs bear an uncommon 'myeloid-related' $CD11b^+$, $CD11c^-$, $F4/80^-$, $CD8\alpha^-$ non-macrophage cell phenotype; (ii) that they are seeded rapidly into the thyroid from the bone marrow within 1 week of cell transfer and are present in the thyroid up to 20 weeks post-bone marrow transfer; (iii) that within the thyroid they tend to cluster between and around thyroid follicles, and (iv) that they appear to express high levels of TSH. A hypothesis is proposed whereby intrathyroidal DCs in normal mice may be involved in the microregulation of thyroid hormone activity, thus establishing a novel immune-endocrine network used to maintain broad-spectrum physiological homeostasis.

Materials and methods

Mice

Adult female BALB/c mice were purchased from Harlan Sprague-Dawley (Houston, TX, USA). EGFP transgenic mice were made in the laboratory of Dr Masaru Okabe (Okabe et al., 1997), Research Institute for Microbial Diseases, Osaka, Japan, and were bred onto a BALB/c background in our laboratory (at the University of Texas HSC) by back-crossing onto BALB/c mice for more than six generations. STOCK TgN(TcrHEL3A9)Mmd mice, carrying the HEL3A9 T cell receptor transgene for the α and β chains of the T cell receptor for a hen egg lysozyme (HEL) peptide (Ho et al., 1994), were purchased from the Jackson Laboratories. 3A9 mice were bred at the UT vivarium and genotyped using the primer sequences recommended by Jackson Laboratories.

Antibodies and immunofluorescence

Antibodies used in this study were: phycoerythrin (PE)-anti-CD3 (2C11); PE-anti-CD8α (53-6.7); PE- and APC-anti-CD11b (M1/70); PE-anti-CD11c (HL3); PE-anti-CD19 (1D3); PE-anti-CD40 (3/23); PE-anti-CD80 (B7-1, 16-10A1); PE-anti-CD45 LCA (30-F11); biotinylated-anti-Ly-6G (RB6-8C5); streptavidin-PE; purified anti-CD16/32 (Fcγ III/II receptor) (2.4G2); PE-hamster and -rat Ig control mAbs (BD-PharMingen; San Diego, CA, USA, all reagents); PE-anti-F4/80 (CalTag; South San Francisco, CA, USA); biotin-anti-mouse TSHβ (1B11) (Zhou et al., 2002).

Mice were killed by CO_2 inhalation. Thyroid, mesenteric lymph nodes, kidney and liver tissues were recovered using a dissecting microscope. Tissues were frozen in liquid N_2, embedded in Tissue Freezing Medium (Triangle Biomedical Sciences; Durham, NC, USA), and 5 μm sections were prepared on a Model 2800 Frigocut N cryostat (Cambridge Scientific Products; Cambridge, MA, USA). Tissues were fixed in acetone, air dried, and washed three times with phosphate-buffered saline (PBS). Tissues were blocked for 15 min at room temperature with Avidin Block (DAKO Corp.; Carpinteria, CA, USA), washed, and blocked for 15 min at room temperature with Biotin Block (DAKO). Slides were washed and reacted with anti-Fc receptor mAb for 30 min at room temperature. Tissues were washed with PBS and reacted with a PE- or biotin-labeled mAb for 3 h at

4°C and then washed again. For direct PE-labeled staining, tissues were washed and observed for immunofluorescence. For staining using biotin-labeled mAb, tissues were reacted for an additional 30 min with 1 μg ml^{-1} of streptavidin–PE, washed and examined. For analyses of $EGFP^+$ cells in tissues, fresh-frozen tissue specimens were sectioned in the cryostat, fixed and examined directly without staining, or were reacted with PE-anti-CD45 LCA or PE-anti-CD11b for identification of hematopoietic cells and $CD11b^+$ cells, respectively, in thyroid tissue sections from chimeric mice. For experiments with fluorescently labeled beads, mice were injected intraperitoneally (i.p.) with a solution of 6 drops ml^{-1} of Fluoresbrite plain YG 1.0 μm microspheres (2.59% Solids-Latex) in PBS (Polysciences, Inc; Warrington, PA, USA). 18 h post-injection, mice were skilled and the spleen, mesenteric lymph nodes and thyroid were removed. Tissues were prepared by cryostat sectioning and observed directly for the presence of fluorescent beads. Fluorescence was analyzed using an Olympus BH-2 immunofluorescence microscope.

Bone marrow chimeras

12-week-old female $EGFP^-$ BALB/c mice were given 900 rad total body irradiation from a ^{60}Co source at the University of Texas M. D. Anderson Cancer Center, Houston, TX, USA. Whole bone marrow was recovered from $EGFP^+$ donor mice, washed with PBS and 17×10^6 cells were injected intravenously (i.v.) into the tail vein of irradiated female $EGFP^-$ recipient mice. At intervals after bone marrow reconstitution (1–20 weeks), mice were sacrificed, tissues were recovered and examined for immunofluorescence.

Generation of in vitro *bone marrow-derived DCs and flow cytometry*

In vitro generation of DCs from bone marrow was done according to published protocols (Inaba et al., 1992). Briefly, whole bone marrow cells were flushed from the femur canula of $EGFP^+$ mice. Erythrocytes were lysed by hypotonic shock with ammonium chloride. Cells were washed in PBS and seeded at a density of 10^6 cells ml^{-1} in 6-well tissue culture plates (Fisher Scientific; Houston, TX, USA) in RPMI-1640 supplemented with FBS (10% v/v), 100 U ml^{-1} penicillin–streptomycin, 2 mmol l^{-1} L-glutamine, and 5×10^{-5} mol l^{-1} 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA), containing mouse recombinant GM–CSF (10 ng ml^{-1}) (BD-PharMingen, San Diego, CA, USA). On days 2 and 4 of culture, the medium and floating cells containing many granulocytes were removed. DCs attached in clusters to the bottom of the plate were re-fed with fresh supplemented medium containing 10 ng ml^{-1} GM–CSF. By day 6 of culture, the majority of floating cells had the morphology of DCs. These cells were collected and some were stained for two-color flow cytometric analyses for expression of CD11b-PE or CD11c-PE and endogenous green fluorescence using a FACScalibur flow cytometer with CellQuest software (BD Biosciences). The remaining cells were washed extensively with PBS and 3.3×10^6 cells were injected i.v. into each of two non-irradiated $EGFP^-$ recipient mice. Host mice were sacrificed 25 days post-cell transfer, the thyroids were recovered, frozen sections were prepared, and tissues were examined for the presence of cells having endogenous green fluorescence.

Reverse transcription-polymerase chain reaction

Isolation of RNAs and reverse transcription-polymerase chain reactions (RT-PCRs) were done using protocols we described previously (Wang et al., 2002). Pituitary tissues were purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN, USA); thyroid tissues were dissected from healthy BALB/c mice. Primer sequences were: TSHβ forward 5′-GTGGGTGGAGAAGAGTGAGC-3′; TSHβ reverse 5′-TAGAAAGACTGCGGCTTGGT-3′; β-actin forward 5′-ATGGATGACGATATCGCTG-3′; β-actin reverse 5′-ATGA-GGTAGTCTGTCAGGT-3′. *Taq* polymerase (Promega; Madison WI) amplification consisted of 35 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min using a Biometra T-Gradient thermocycler (Whatman Biometra; Gottingen, Germany). Based on the published sequence of the murine TSHβ gene (Gordon et al., 1988), the amplified TSHβ product was expected to be 473 bp; PCR products were run on a 1.5% agarose gel.

Results

Phenotypic analysis of intrathyroidal cells

Thyroid tissue sections from normal mice were stained using mAbs reactive to CD11b, CD11c, CD40, CD3, CD19 and F4/80 antigens. Fig. 1 shows that within the same thyroid tissue, $CD11b^+$ cells were abundant and widely dispersed, whereas $CD11c^+$ wells were rare. Those findings were consistent in tissue sections from many animals. To quantify the number of $CD11b^+$ and $CD11c^+$ cells in the thyroid, cells of each type were counted, in tissue sections, relative to the number of thyroid follicles. These data, shown in Table 1, indicate that there are >tenfold more $CD11b^+$ than $CD11c^+$ cells, with each thyroid follicle surrounded by ~2 $CD11b^+$ cells.

The $CD11b^+$ cells may be either a myeloid-related DC population with high CD11b and low CD11c expression (Maraskovsky et al., 1999), or they may be mϕs, given that CD11b can be expressed on both DCs and mϕs

Table 1. *Relative numbers of intrathyroidal $CD11b^+$ and $CD11c^+$ cells*

Number of cells per follicle		
$CD11b^+$	$CD11c^+$	$CD11b^+/CD11c^+$
2.01±0.23	0.19±0.07*	10.58

*Statistically significant difference ($P<0.01$) as determined by Student's *t*-test for unpaired observations. $N=12$ tissue samples analyzed from six individual mice.

(Maraskovsky et al., 1999; Ho and Springer, 1982). To distinguish between those possibilities, thyroid tissue sections from normal mice were stained with mAb F4/80, a reagent that recognizes a determinant on nearly all mouse mϕs (Hirsch et al., 1981; Austyn and Gordon, 1981). As seen in Fig. 2, thyroid tissue sections displayed a pattern of CD11b staining similar to that described in Fig. 1, yet they were devoid of F4/80$^+$ cells. Additionally, immunofluorescent analyses indicated a lack of staining with anti-CD40, anti-CD80, anti-CD19 and anti-CD3 mAbs. Because CD40 and CD80 are acquired on DCs with activation (Quadbeck et al., 2002), the lack of expression of those markers is reflective of the non-activated status of the intrathyroidal DCs. Similarly, the lack of anti-CD19 and anti-CD3 staining further indicates the non-inflamed status of the thyroid given that antigen-reactive lymphocytes, in particular T cells, would be expected to accompany activated DCs, if present. The absence of CD8α staining suggests that the intrathyroidal DCs are myeloid-related (Maraskovsky et al., 1996; Pulendran et al., 1997; Vrmec and Shortman, 1997) and not lymphoid-related DCs (Ardavin et al., 1993; Kronin et al., 1997; Saunders et al., 1996; Wu et al., 1995); the lack of expression of the Ly-6G antigen (Gr-1) suggests that they are not related to plasmacytoid DCs (Nakano et al., 2001).

CD80 is expressed in the lymph nodes but not in the thyroid

To better address the issue of the activation characteristics of intrathyroidal DCs, studies were done in which mice with a transgenic T cell receptor for a HEL peptide (3A9 mice) were injected i.p. with 100 µl of 10 mg ml^{-1} HEL. 48 h later mice were killed and the spleen, lymph nodes and the thyroid were removed and stained for expression of CD11b and CD80. In the spleen there was an abundance of both CD11b$^+$ (Fig. 3A) and CD80$^+$ (Fig. 3B) cells. Note the presence of these in the marginal zones around the germinal centers (Fig. 3A,B), suggesting that DC in 3A9 mice had been activated as a consequence of exposure to HEL. Similar findings also were observed for lymph node tissues (data not shown). Unlike the spleen and lymph nodes, however, no CD80$^+$ cells were present in the thyroid of 3A9 mice injected with HEL (Fig. 3C), despite an abundance of CD11b$^+$ cells in that tissue (Fig. 3D). Of interest was the finding that in HEL-primed mice, both CD11b$^+$ (Fig. 3E) and CD80$^+$ (Fig. 3F) cells were present in the pericapsular lymph nodes associated with the thyroid, even though no CD80$^+$ cells were not present in the thyroid itself (Fig. 3C). This suggests either that CD80$^+$ cells do not enter the thyroid, or that DC cells of the thyroid are limited in their capacity to become activated in a conventional manner.

Bone marrow origin of intrathyroidal cells

The findings described above indicate that intrathyroidal CD11b$^+$ cells are present in the thyroid of normal mice. In an

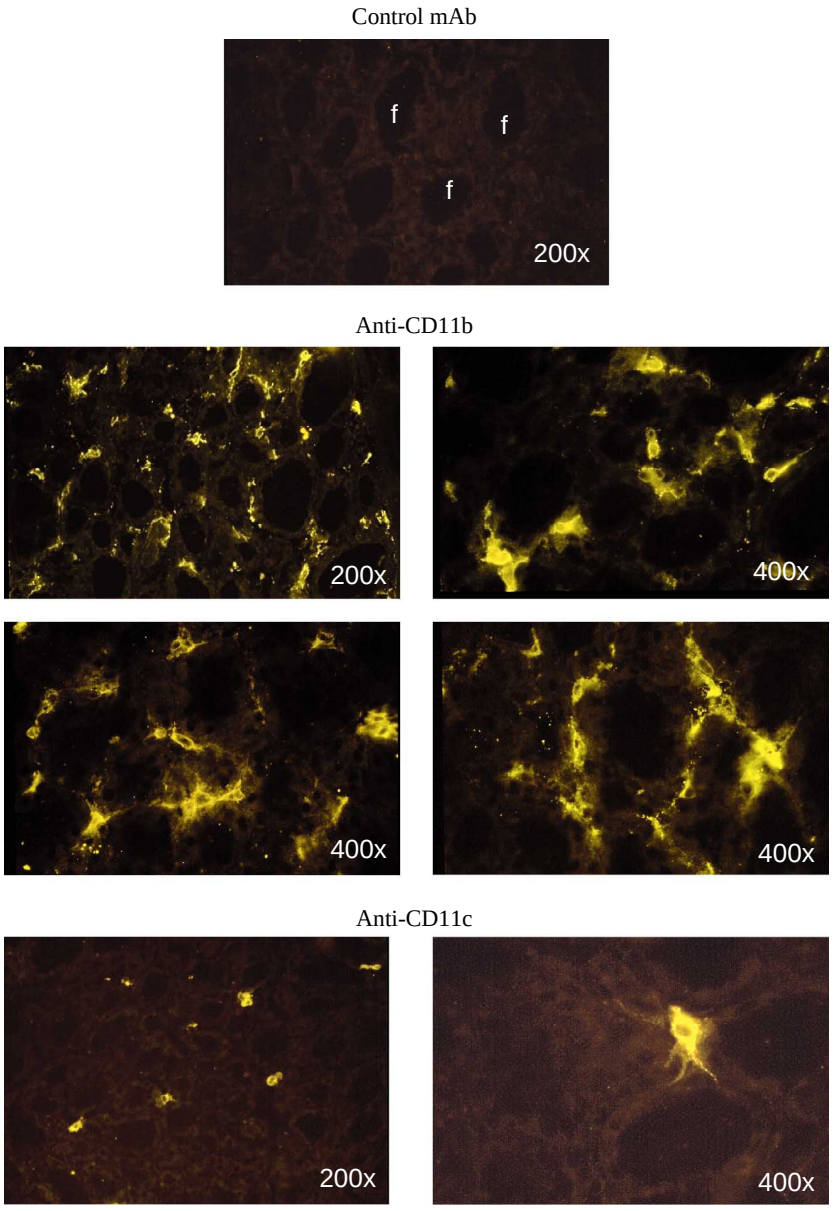


Fig. 1. Expression of CD11b and CD11c in normal mouse thyroid. Thyroid tissues from normal healthy mice have significantly more CD11b$^+$ than CD11c$^+$ cells as determined by the reactivity of PE-anti-CD11b and PE-anti-CD11c mAbs. Reactivity of PE-labeled control antibody to mouse thyroid tissue sections is shown, and the location of some thyroid follicles are indicated (f). Note the high density of CD11b$^+$ cells with typical dendritic cell morphology in four sections from the same thyroid, and the sparse number of CD11c$^+$ cells in that tissue. Sections are from the thyroid from one BALB/c mouse, but were typical of four mice examined.

effort to understand the origins of those cells, hematopoietic radiation chimeras were constructed by injecting bone marrow cells from EGFP$^+$ transgenic mice into lethally irradiated EGFP$^-$ recipient mice. At intervals post-cell transfer, mice were killed, cryostat sections were made of the thyroid, and sections were examined for green fluorescence *without* staining to identify donor cells based on endogenous green fluorescence. As seen in Fig. 4, donor EGFP$^+$ cells were readily detected in the thyroid as early as 1 week post-bone marrow transfer, and were present at all points examined up to 20 weeks post-transfer. Typically, EGFP$^+$ cells clustered near or around thyroid follicles (Fig. 4) similar to that observed for CD11b$^+$ cells (Figs 1 and 2). Note that the differences in staining pattern seen in Figs 1 and 2 are because CD11b and CD11c are membrane bound but EGFP fluorescence is cytoplasmic in EGFP$^+$ cells. These findings are of particular interest for several reasons. First, the rapid appearance of EGFP$^+$ cells in the thyroid after bone marrow injection suggests that some bone marrow cells traffic quickly to the thyroid. Second, the fact that EGFP$^+$ cells were present as late as 20 weeks post-bone marrow injection suggests either that bone marrow-derived EGFP$^+$ cells in the thyroid are long-lived cells, or that they are continually replenished from the bone marrow.

To determine whether bone marrow-derived EGFP$^+$ cells randomly trafficked into the thyroid and other non-lymphoid tissues, or whether their presence in the thyroid reflects a selective process of cell trafficking, tissue sections were made from the thyroid, mesenteric lymph nodes, the kidney and the liver of EGFP$^+$ $\rightarrow$ EGFP$^-$ mice 2 weeks after bone marrow transfer. As seen in Fig. 5, EGFP$^+$ cells similar to those in Fig. 4 were present in the thyroid. Additionally, there was a high density of EGFP$^+$ cells in the lymph nodes, particularly in or near lymphocyte-enriched follicular regions. In contrast, few if any EGFP$^+$ cells were found in the kidney or liver of EGFP$^-$ recipient mice. These finding strongly imply that at least some bone marrow CD11b$^+$ cells traffic directly to the thyroid, and that the distribution of those cells reflects a specific rather than a stochastic process.

Intrathyroidal CD11b$^+$ cells are not bone marrow stromal cells

To better define the origins of EGFP$^+$ cells in the thyroid of EGFP$^+$ $\rightarrow$ EGFP$^-$ chimeras, thyroid tissue sections were made from mice 3 weeks post-bone marrow transfer. Unstained tissues contained cells with endogenous green fluorescence (Fig. 6) similar to that of other chimeric mice (Fig. 5). Additionally, however, thyroid tissue sections stained with PE-anti-CD11b mAb or PE-anti-CD45 LCA, and examined for yellow fluorescence, had many CD11b$^+$ and CD45 LCA$^+$ cells (Fig. 6). Because LCA is expressed on all hematopoietic cells except erythrocytes (van Ewijk et al., 1981), and is not expressed on bone marrow stromal cells, this indicates that the intrathyroidal EGFP$^+$ cells in EGFP$^+$ $\rightarrow$ EGFP$^-$ chimeras were hematopoietic cells and were not derived from bone marrow stromal cells.

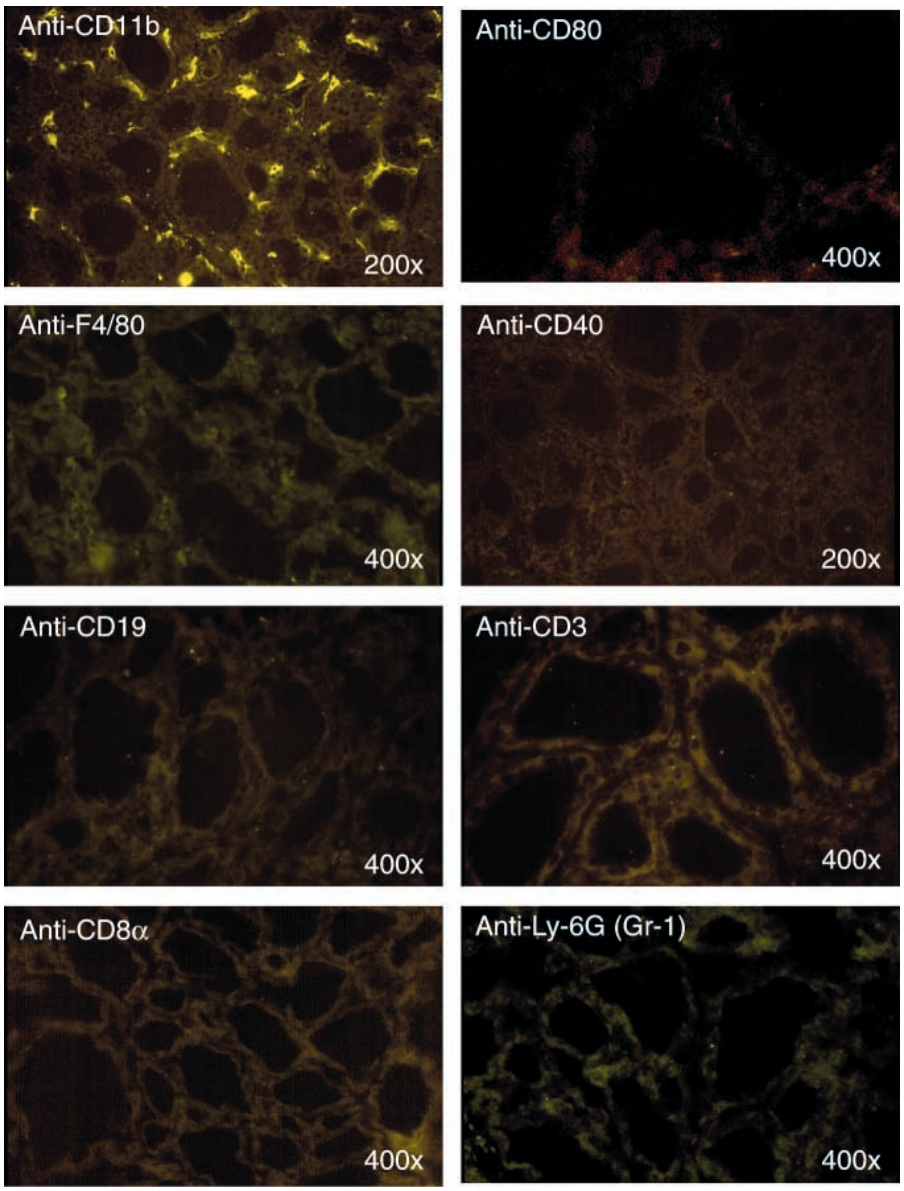


Fig. 2. Expression of dendritic cell markers within the thyroid. Thyroid tissues from normal healthy mice have many widely dispersed CD11b$^+$ cells in areas near thyroid epithelial cells and follicles, but lack cells expressing F4/80, CD40, CD80, CD19, CD3, CD8α and Ly-6G. Tissues are from the same animal, but are typical of the results from two mice.

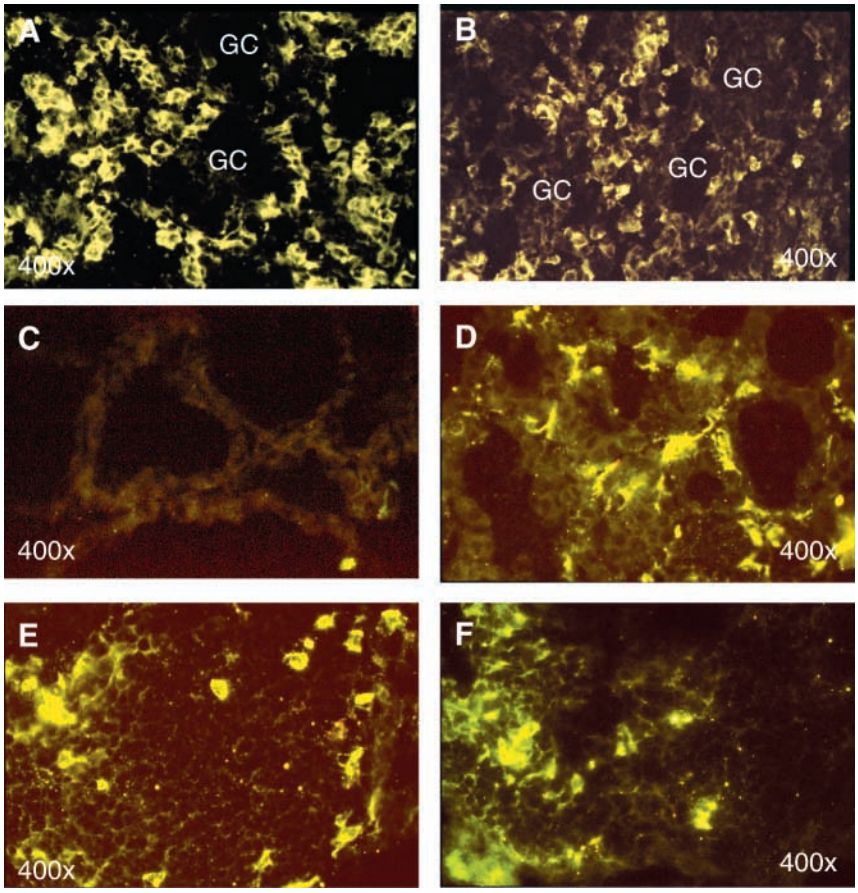


Fig. 3. Both (A) CD11b$^+$ and (B) CD80$^+$ cells are present in the spleen of 3A9 transgenic mice 48 h after i.p. injection with hen egg lysozyme (HEL) (GC, germinal centers). (C) CD80$^+$ cells are not present in the thyroid of HEL-injected 3A9 mice despite the presence of (D) CD11b$^+$ cells in that tissue. Both (E) CD11b$^+$ and (F) CD80$^+$ cells are present in the thyroid pericapsular lymph nodes of HEL-injected mice.

Adoptively transferred DCs localize in the thyroid

To further confirm that intrathyroidal DCs originated from bone marrow-derived DCs, bone marrow cells from EGFP$^+$ mice were cultured for 6 days with GM-CSF according to published protocols (Inaba et al., 1992) as described in the Materials and methods. Floating cells with the morphology of DCs were collected and stained for two-color PE-anti-CD11b expression plus endogenous green fluorescence, and PE-anti-CD11c expression plus endogenous green fluorescence. As seen in Fig. 7A, 96.5% of the cells showed endogenous green fluorescence and also expressed high levels of CD11b. In contrast, only 38.9% of the cells expressed CD11c and showed green fluorescence, whereas 58.7% of the cells did not express CD11c but showed green fluorescence (Fig. 7B). Hence, the day-6 DCs consisted of a population of CD11b$^+$ CD11c$^+$ cells, and a population of CD11b$^+$ CD11c$^-$ cells. Cells were collected, washed extensively in PBS, and 3×10^6 cells were injected into each of two non-irradiated EGFP$^-$ recipient mice. 25 days later, thyroid tissue sections were prepared and examined for the presence of green fluorescent cells. As seen in Fig. 7C-F, intrathyroidal EGFP$^+$ cells were present adjacent to and surrounding thyroid follicles as seen in EGFP$^+$ $\rightarrow$ EGFP$^-$ radiation chimeras (Fig. 4). It is important to note that because the host mice in this experiment were not irradiated, the homing of bone marrow-generated DCs into the thyroid cannot be attributed to tissue damage imparted by ionizing radiation. Additionally, and perhaps most important, these findings indicate that DCs grown using conventional *in vitro* methods migrate to the thyroid after cell transfer, thus reinforcing the notion that the EGFP$^+$ intrathyroidal cells present in EGFP$^+$ $\rightarrow$ EGFP$^-$ chimeras were derived from a bone marrow DC precursor population.

CD11b$^+$ thyroid cells produce TSHβ

The finding that there is an abundance of CD11b$^+$ intrathyroidal cells, and the homing of bone marrow cells to the thyroid of normal mice, were unexpected and surprising. Although DCs can be found in nearly all tissues of the body (in part because of their natural ability to sample and transport antigens to secondary lymphoid tissues during the early phase of an adaptive immune response) the presence of large numbers of DCs in internal organs of normal animals with no evidence of pathology has, to our knowledge, not been described. The rare but not totally atypical phenotype of the intrathyroidal DCs described here therefore prompted us to explore the possibility that they may function in some capacity other than in antigen handling and processing, given that the thyroid is not a tissue known to have excessive amounts of foreign antigens. Similarly, we have recently demonstrated that bone marrow cells, in particular a CD11b$^+$ population, produce high levels of TSH (Wang et al., 2003). To further explore the possibility that intrathyroidal CD11b$^+$ cells might be a source of TSH production within the thyroid, thyroid sections were stained with APC-labeled anti-CD11b mAb to identify CD11b$^+$ cells, and with biotin-labeled anti-mouse TSHβ-specific mAb (Zhou et al., 2002) plus streptavidin-FITC to identify TSH-producing cells in the thyroid. Of many sections examined, the general pattern was one in which CD11b$^+$ cells (Fig. 8A) also expressed TSHβ (Fig. 8B), strongly suggesting that CD11b$^+$ cells routinely produce TSHβ. Occasionally, however, some CD11b$^+$ cells did not express intracellular TSHβ (Fig. 8A, circled area), though overall these were rare. It is possible either that those cells constitute a population of CD11b$^+$ cells that do not normally secrete TSH, or that they were not producing TSH at the time of analyses.

Owing to the high phagocytic potential of most DCs, the possibility was considered that the presence of intracellular

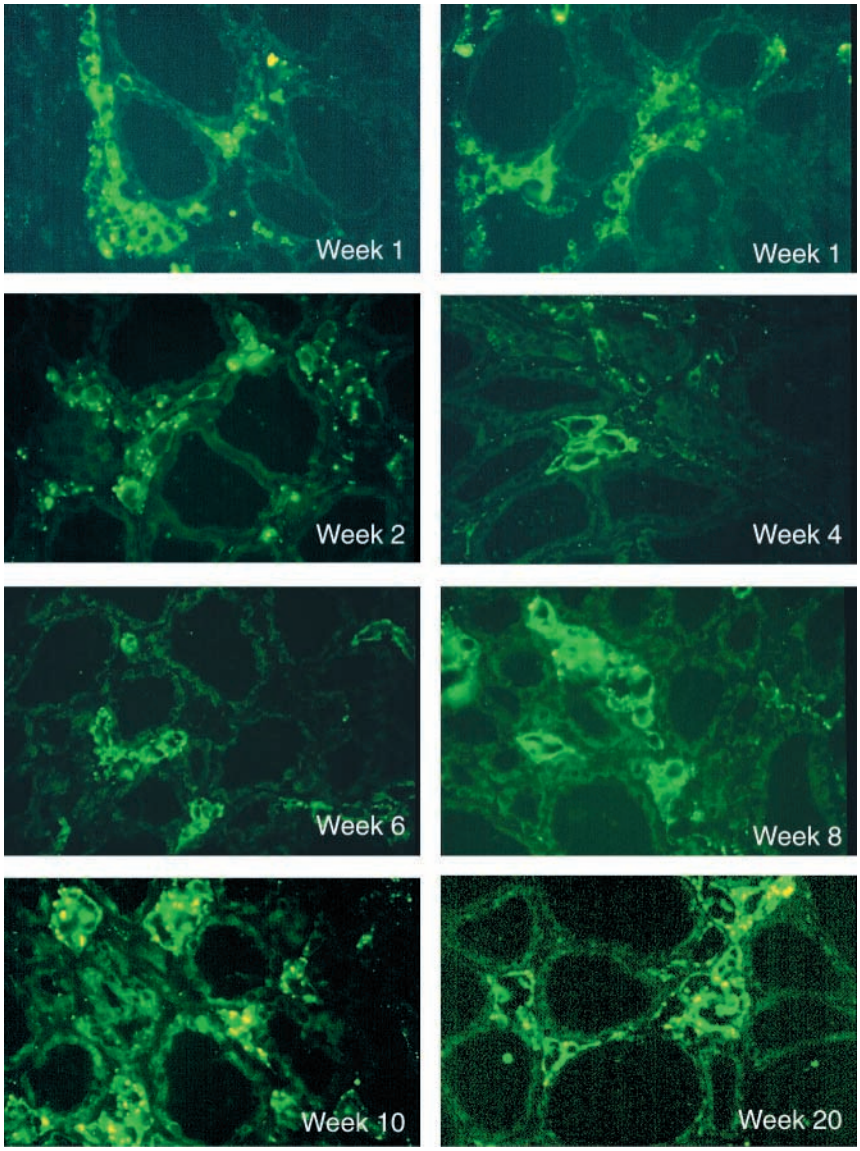


Fig. 4. Presence of EGFP$^+$ cells in the thyroid of EGFP$^+$ → EGFP$^-$ bone marrow radiation chimeras. Unstained thyroid tissue sections from EGFP$^+$ → EGFP$^-$ bone marrow radiation chimeras have intrathyroidal cells with endogenous green fluorescence. Note the presence of EGFP$^+$ cells surrounding thyroid follicles from 1–20 weeks post-bone marrow transfer. Magnification 400×.

TSH could reflect a process of TSH engulfment rather than one in which TSH is manufactured by intrathyroidal DCs. Two experiments were done to test this. First, normal BALB/c mice were injected with green fluorescent microspheres as described in the Materials and methods. 18 h later, the mice were killed and the thyroid, mesenteric lymph nodes and spleen were examined directly in cryostat sections for the presence of green fluorescent particles. As seen in Fig. 8C, there was a distinct rim of green fluorescence in the marginal zones around follicular regions in the spleen, as would be indicative of a phagocytic process of microspheres by DCs and mϕs that are present at high density in those areas. A similar pattern was also noted for the lymph node tissues surrounding germinal centers and follicles (data not shown). Notably, however, fluorescent spheres were not present in the thyroid (Fig. 8D).

In the second experiment, RT-PCR analyses was done using thyroid tissues from normal mice to determine whether the TSHβ gene was expressed locally. Control material consisted of tissue from the anterior pituitary, which actively expresses the TSHβ gene. As seen in Fig. 9, a clearly detectable TSHβ band of the correct size (473 bp) was amplified from thyroid tissue and the pituitary tissue. Collectively, these findings make a strong case for TSH in intrathyroidal DCs not being the consequence of phagocytosis but, rather, the result of local synthesis of TSH within the thyroid itself.

Discussion

Although DCs are known to infiltrate the thyroid in disease conditions such as Graves' disease, Hashimoto's thyroiditis, and autoimmune thyroiditis (Quadbeck et al., 2002; Kabel et al., 1988; Wilders-Trushnig et al., 1989; Imal and Yamakawa, 1996; Jansson et al., 1984; Aichinger et al., 1985), considerably less is known about the extent to which DCs are present in the thyroid of healthy mice. Of the studies done to date, phenotypic analyses have been limited or cells have been characterized using only CD11c as a marker of DCs (Quadbeck et al., 2002; Kabel et al., 1988; Bagriacik et al., 2001). However, DCs are an extremely heterogeneous population of cells, consisting of phenotypic subsets that can be differentiated in part according to lineage variations, distribution within secondary lymphoid tissues, and changes that occur as cells proceed from an immature to mature or activated form (Maraskovsky et al., 1996; Pulendran et al., 1997; Vrmec and Shortman, 1997; Ardavin et al., 1993; Kronin et al., 1997; Saunders et al., 1996; Wu et al., 1995). The present study demonstrates that the thyroid of normal mice contains an abundance of CD11b$^+$ cells and at least tenfold less CD11c$^+$ than CD11b$^+$ cells (Table 1). Interestingly, in one study of DCs in human thyroid tissues from normal individuals and patients with Graves' disease or sporadic nontoxic goiter (autoimmune thyroid disease), mAb FK24, a reagent that recognizes a human equivalent of murine CD11b, was found to be the most reliable indicator of intrathyroidal DCs (Kabel et al., 1988). Although CD11b is expressed on mϕs (Ho and Springer, 1982), most murine mϕs also express the antigen recognized by mAb F4/80 (Hirsch et al., 1981; Austyn and

Gordon, 1981). The lack of expression of F4/80 on intrathyroidal DCs, plus the basic DC-like appearance of the $CD11b^+$ cells (Figs 1 and 2), favor the possibility that they are not mϕs but are similar to the myeloid-related DC population (Maraskovsky et al., 1996; Pulendran et al., 1997; Vrmec and Shortman, 1997). Moreover, although some myeloid DCs express both CD11c and CD11b, a subset of those cells are $CD11b^+$ $CD11c^{low}$, $F4/80^-$ cells (Maraskovsky et al., 1999), which is essentially the phenotype of the cells described here. The lack of CD8α expression on intrathyroidal DCs (Fig. 2) further suggests that they are a myeloid-related (Maraskovsky et al., 1996; Pulendran et al., 1997; Vrmec and Shortman, 1997) rather than a lymphoid-related (Ardavin et al., 1993; Kronin et al., 1997; Saunders et al., 1996; Wu et al., 1995) DC population, and the lack of Gr-1 (Ly-6G) expression rules out that they are granulocytes (Fleming et al., 1993) or plasmacytoid DCs (Nakano et al., 2001). The absence of CD40 and CD80 expression indicates that the intrathyroidal cells are not activated DCs, given that expression of those markers is negligible on non-activated DCs, but increases markedly following antigen capture leading to DC maturation and activation (Quadbeck et al., 2002). It should be noted that staining of thyroid tissues with anti-MHC-II antibody as an indicator of DCs was not done in our study because of the wide-spread expression of that marker on thyroid endothelial cells.

Studies from the adoptive transfer experiments using *in vitro*-derived DCs indicate strongly that some cells with DC characteristics traffic to the thyroid. It will be of interest to determine whether this occurs from the $CD11b^+$ $CD11c^+$ or the $CD11b^+$ $CD11c^-$ population of bone marrow cells. Experiments are underway to differentiate between those possibilities using cell-sorted populations of *in vitro*-generated DCs. Also of interest is the identification of thyroid-specific homing markers that might be involved in regulating the trafficking of DCs to the thyroid, possibly involving integrins similar but different from those used to direct hematopoietic cells to mucosal and other tissue sites (Kilshaw and Murant, 1991; Streeter et al., 1988).

An interesting finding from this study was the extensive TSHβ staining of cells with features very similar to the $CD11b^+$ intrathyroidal cells, and the location of those cells near thyroid follicles as occurred with $CD11b^+$ cells (Figs 1 and 2) and bone marrow-derived $EGFP^+$ cells (Figs 3–5). The implication of this is that $TSH\beta^+$ intrathyroidal cells are a potential source of non-endocrine TSH that is directly available to the thyroid itself.

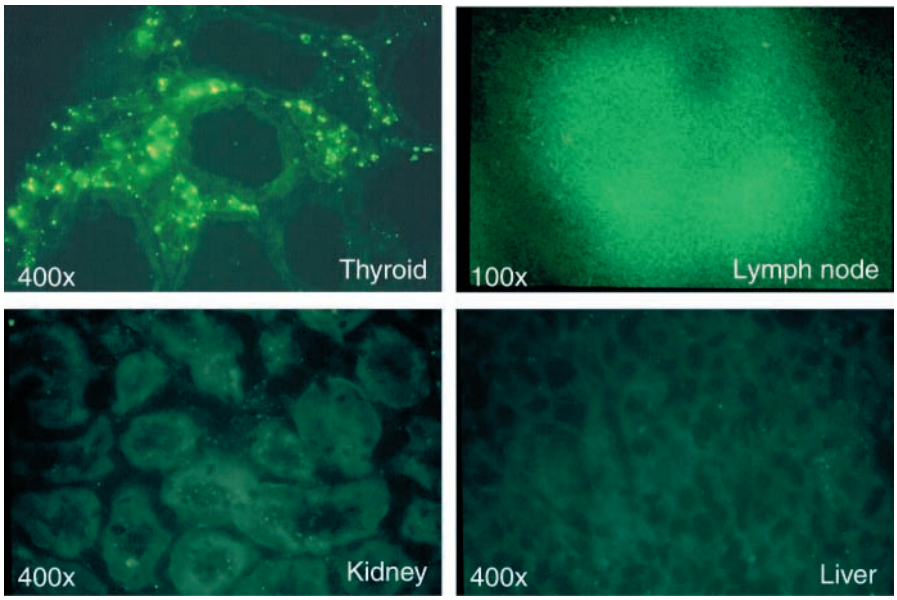


Fig. 5. $EGFP^+$ cells are abundant in the thyroid and the mesenteric lymph node, but not in the kidney or the liver, of $EGFP^+ \rightarrow EGFP^-$ bone marrow radiation chimeras 2 weeks post-bone marrow transfer.

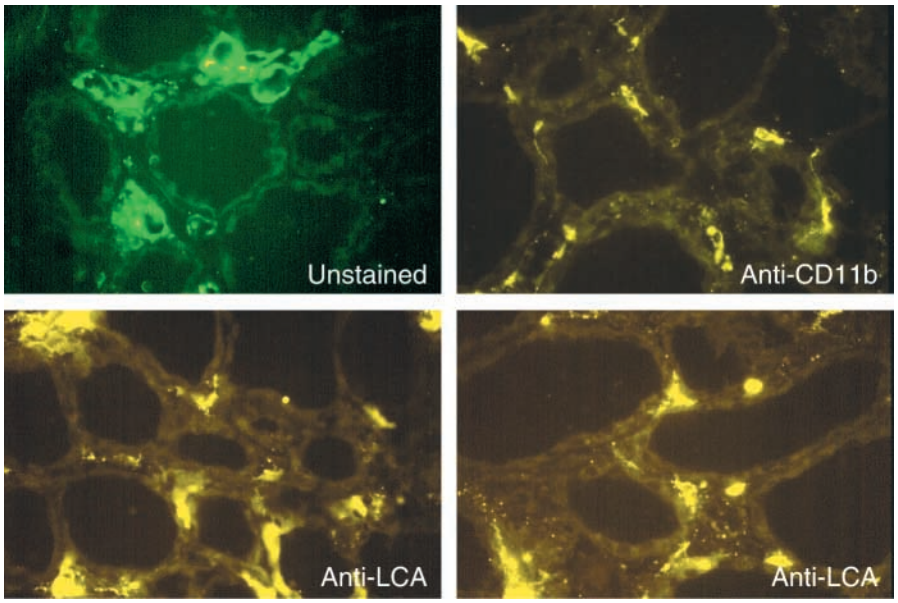
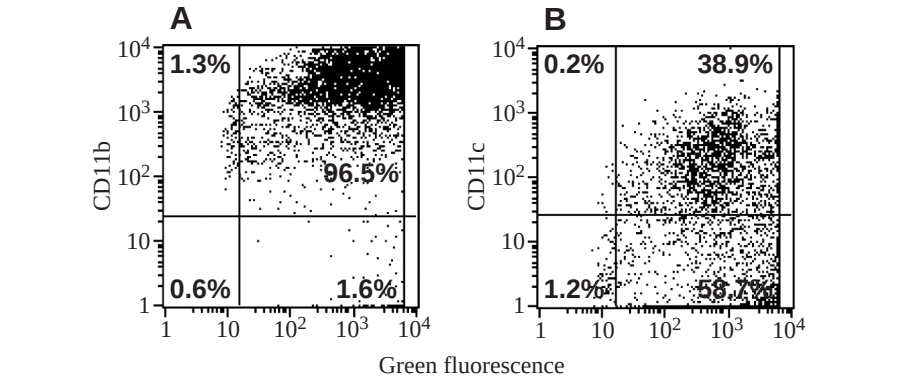


Fig. 6. Presence of $EGFP^+$ cells, $CD11b^+$ cells and LCA^+ cells in the thyroid of $EGFP^+ \rightarrow EGFP^-$ bone marrow radiation chimeras (week 3). Thyroid tissue sections from $EGFP^+ \rightarrow EGFP^-$ bone marrow radiation chimeras have $EGFP^+$ cells (unstained section), and cells reactive with anti-CD11b and anti-CD45 LCA, suggesting that $EGFP^+$ cells originate from bone marrow hematopoietic cells and not from bone marrow stromal cells. Magnification 400×.



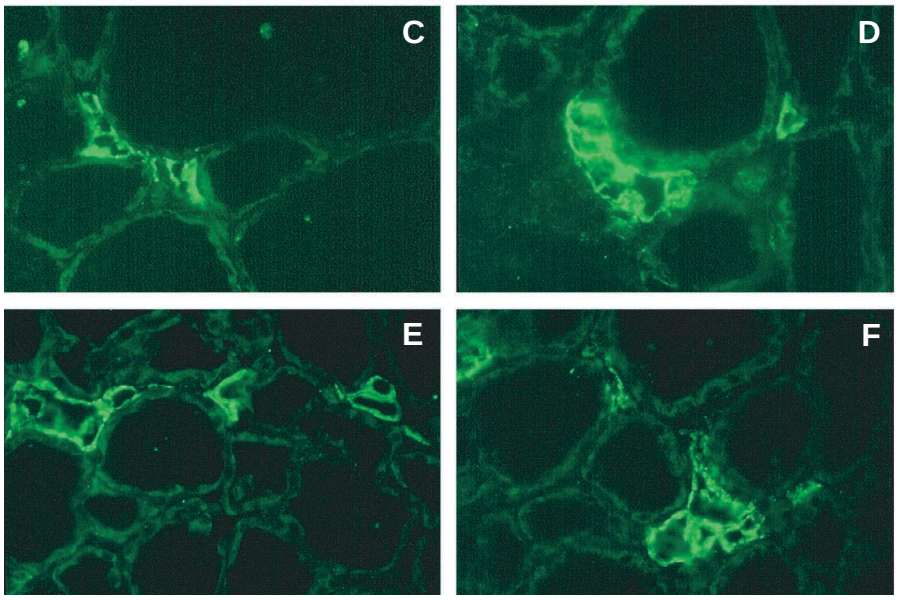


Fig. 7. Bone marrow-derived dendritic cells (DCs) generated *in vitro* migrate to the thyroid. Bone marrow cells from EGFP$^+$ mice grown with 10 ng ml^{-1} GM-CSF express high levels of (A) CD11b, of which (B) ~40% expressed CD11c. (C–F) Unstained thyroid tissue sections from non-irradiated EGFP$^-$ recipient mice 25 days post-DC transfer indicate the presence of EGFP$^+$ intrathyroidal cells. Magnification 400×.

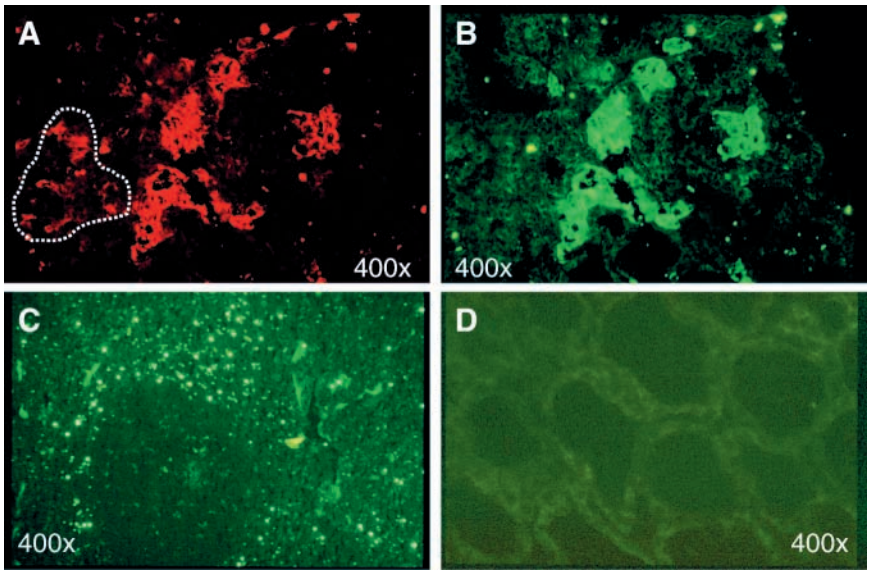


Fig. 8. Double staining of thyroid tissue from normal BALB/c mice, demonstrating the co-expression of (A) CD11b and (B) TSHβ; encircled area demarcates a region of CD11b$^+$ cells not containing intracellular TSHβ. 18 h post-i.p. injection of green fluorescent microspheres, there is an accumulation of microspheres in regions of high DC/mϕ density in the (C) spleen, despite the absence of microspheres in the (D) thyroid of the same animals.

Consistent with this, studies from our laboratory recently demonstrated that some bone marrow cells are a potent source of TSH as seen from intracellular TSHβ staining and by the presence of secreted TSH in the supernatants of cells cultured *in vitro* (Wang et al., 2003). Moreover, the primary TSH-producing cells were CD11b$^+$ cells, although CD11b$^-$ cells also produced TSH albeit at significantly lower levels. Bone marrow cells expressing TCR, B220 or Thy-1 did not synthesize TSH (Wang et al., 2003). In an opposite manner, the TSH receptor was heavily expressed on a population of CD11b$^-$ bone marrow cells distributed among monocyte precursors, as well as lymphocyte and granulocyte precursors (Wang et al., 2003). Stimulation of CD11b$^-$ (TSH receptor positive) bone marrow cells with TSH resulted in a dose-dependent release of TNFα, whereas CD11b$^+$ cells produced TNFα independent of TSH stimulation (Wang et al., 2003). Those observations are particularly curious in light of the findings by Croizet et al. (2001), demonstrating an effect of TNF on the phenotype of intrathyroidal DCs. Finally, the possibility that intrathyroidal DCs have phagocytosed or pinocytosed TSH within the thyroid seems unlikely in light of the finding that those cells failed to ingest fluorescent microspheres in a manner comparable to DCs in lymphoid tissues, and because of evidence from RT-PCR analyses suggesting that TSHβ may be produced locally within the thyroid.

In summary, this study has provided detailed phenotypic analyses of intrathyroidal DCs in normal mice, and has traced the origins of those cells to populations of bone marrow hematopoietic cells. The unconventional phenotype of

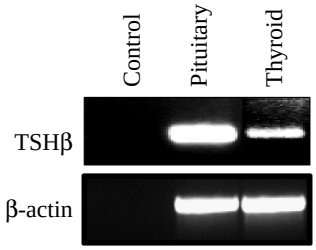


Fig. 9. RT-PCR amplification of a 473 bp TSHβ product from pituitary and thyroid tissues. The control lane consisted of PCR amplification using reagents and primers in the absence of added nucleic acids.

this DC population, plus the observation of TSH production by intrathyroidal DC-like cells, suggest a role for the immune system in the maintenance of thyroid homeostasis in ways not appreciated before.

The authors thank Dr Cindy Zhou for technical assistance. This research was supported by NIH grant DK35566.

References

Aichinger, G., Fill, H. and Wick, G. (1985). In situ immune complexes, lymphocyte subpopulations, and HLA-DR-positive epithelial cells in Hashimoto's thyroiditis. *Lab. Invest.* **52**, 132-140.

Ardavin, C., Wu, L., Li, C. L. and Shortman, K. (1993). Thymic dendritic cells and T cells develop simultaneously in the thymus from common precursor populations. *Nature* **362**, 761-763.

Austyn, J. M. and Gordon, S. (1981). F4/80, a monoclonal antibody directed against the mouse macrophage. *Eur. J. Immunol.* **11**, 805-815.

Bagriacik, E. U. and Klein, J. R. (2000). The thyrotropin (thyroid-stimulating hormone) receptor is expressed on murine dendritic cells and on a subset of $CD45RB^{high}$ lymph node T cells: Functional role for thyroid-stimulating hormone during immune activation. *J. Immunol.* **164**, 6158-6165.

Bagriacik, E. U., Zhou, Q., Wang, H. C. and Klein, J. R. (2001). Rapid and transient reduction in circulating thyroid hormones following systemic antigen priming: Implications for functional collaboration between dendritic cells and thyroid. *Cell. Immunol.* **212**, 92-100.

Chabaud, O. and Lissitzky, S. (1977). Thyrotropin-specific binding to human peripheral blood monocytes and polymorphonuclear leukocytes. *Mol. Cell. Endocrinol.* **7**, 79-87.

Coutelier, J. P., Kehrl, J. H., Bellur, S. S., Kohn, L. D., Notkins, A. L. and Prabhakar, B. S. (1990). Binding and functional effects of thyroid stimulating hormone on human immune cells. *J. Clin. Immunol.* **10**, 204-210.

Croizet, K., Rabilloud, R., Kostrouch, S., Nicolas, J. F. and Rousset, B. (2000). Culture of dendritic cells from nonlymphoid organ, the thyroid gland: Evidence for TNFα-dependent phenotypic changes of thyroid-derived dendritic cells. *Lab. Invest.* **80**, 1215-1225.

Croizet, K., Masson, S. T., Rabilloud, R., Nicolas, J. F., Bernier-Valentin, F. and Rousset, B. (2001). Signaling from epithelial to dendritic cells of the thyroid gland: Evidence for thyrocyte-derived factors controlling the survival, multiplication, and endocytic activity of dendritic cells. *Lab. Invest.* **81**, 1601-1613.

Fleming, T. J., Fleming, M. I. and Malek, T. R. (1993). Selective expression of Ly-6G on myeloid cells in mouse bone marrow. RB6-8C5 monoclonal antibody to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J. Immunol.* **151**, 2399-2408.

Gordon, D. F., Wood, W. M. and Ridgway, E. C. (1988). Organization and nucleotide sequence of the gene encoding the β-subunit of murine thyrotropin. *DNA* **7**, 17-26.

Hattori, N., Shimatsu, A., Sugita, M., Kumagai, S. and Imura, H. (1990). Immunoreactive growth hormone (GH) secretion by human lymphocytes augmented exogenous GH. *Biochem. Biophys. Res. Comm.* **168**, 396-401.

Hiestand, P. C., Meklar, P., Nordmann, R., Grieder, A. and Permmongko, I. C. (1986). Prolactin as a modulator of lymphocyte responsiveness provides a possible mechanism of action for cyclosporine. *Proc. Natl. Acad. Sci. USA* **83**, 2599-2603.

Hirsch, S., Austyn, J. M. and Gordon, S. (1981). Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. *J. Exp. Med.* **154**, 713-725.

Hiruma, K., Nakamura, K. H., Sumida, T., Maeda, T., Tomioka, H., Yoshida, S. and Fujita, T. (1990). Somatostatin receptors on human lymphocytes and leukemia cells. *J. Immunol.* **71**, 480-485.

Ho, M. K. and Springer, T. A. (1982). Mac-1 antigen: quantitative expression in macrophage populations and tissues, and immunofluorescent localization in spleen. *J. Immunol.* **128**, 2281-2286.

Ho, W. Y., Cooke, M. P., Goodnow, C. C. and Davis, M. M. (1994). Resting and anergic B cells are defective in CD28-dependent costimulation of naïve $CD4^+$ T cells. *J. Exp. Med.* **179**, 1539-1549.

Imal, Y. and Yamakawa, M. (1996). Morphology, function and pathology of follicular dendritic cells. *Pathol. Int.* **46**, 807-833.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S. and Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176**, 1693-1702.

Jansson, R., Karlsson, A. and Forsum, U. (1984). Intrathyroidal HLA-DR expression and T lymphocyte phenotypes in Graves' toxicosis, Hashimoto's thyroiditis and nodular colloid goiter. *Clin. Exp. Immunol.* **58**, 264-272.

Kabel, P. J., Voorbij, H. A., De Haan, M., van der Gaag, R. D. and Drexhage, H. A. (1988). Intrathyroidal dendritic cells. *J. Clin. Endocrinol. Metab.* **66**, 199-207.

Kilshaw, P. J. and Murant, S. J. (1991). Expression and regulation of $\beta_7(\beta p)$ integrins on mouse lymphocytes: relevance to the mucosal immune system. *Eur. J. Immunol.* **21**, 2591-2597.

Kronin, V., Vremec, D., Winkel, K., Classon, B. J., Miller, R. G., Mak, T. W., Shortman, K. and Suss, G. (1997). Are $CD8^+$ dendritic cells (DC) veto cells? The role of CD8 on DC in DC development and in the regulation of CD4 and CD8 T cell responses. *Int. Immunol.* **9**, 1061-1064.

Kruger, T. E., Smith, L. R., Harbour, D. V. and Blalock, J. E. (1989). Thyrotropin: an endogenous regulator of the in vitro immune response. *J. Immunol.* **142**, 744-747.

Maraskovsky, E., Brasel, K., Teepe, M., Roux, E. R., Lyman, S. D., Shortman, K. and McKenna, H. J. (1996). Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell populations identified. *J. Exp. Med.* **184**, 1953-1962.

Maraskovsky, E., Pulendran, B. and Shortman, K. (1999). Lymphoid-related dendritic cells. In *Dendritic Cells* (ed. M. T. Lotze and A. W. Thomson), pp. 93-107. New York: Academic Press.

Nakano, H., Yanagita, M. and Gunn, M. D. (2001). $CD11c^+$ $B220^+$ $Gr\text{-}1^+$ cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J. Exp. Med.* **194**, 1171-1178.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313-319.

Pulendran, B., Lingappa, J., Kennedy, M. K., Smith, J., Teepe, M., Rudensky, A., Maliszewski, C. R. and Maraskovsky, E. (1997). Developmental pathways of dendritic cells in vivo. Distinct function, phenotype and location of dendritic cell subsets in FLT3 ligand-treated mice. *J. Immunol.* **159**, 2222-2231.

Quadbeck, B., Eckstein, A. K., Tews, S., Walz, M., Hoermann, R., Mann, K. and Gieseler, R. (2002). Maturation of thyroidal dendritic cells in Graves disease. *Scand. J. Immunol.* **55**, 612-620.

Saunders, D., Lucas, K., Ismaili, J., Wu, J., Maraskovsky, E., Dunn, E. and Shortman, K. (1996). Dendritic cell development in culture from thymic precursors cells in the absence of granulocyte/macrophage colony stimulating factor. *J. Exp. Med.* **184**, 2185-2196.

Simons, P. J., Delemarre, F. G. A. and Drexhage, H. A. (1998). Antigen-presenting dendritic cells as regulators of the growth of thyrocytes: A role of interleukin-1β and interleukin-6. *Endocrinology* **139**, 3148-3156.

Simons, P. J., Delemarre, F. G. A. and Drexhage, H. A. (2000). A functional and phenotypic study on immune accessory cells from the thyroid of Wistar rats and autoimmune-prone BB-DP rats. *J. Autoimmun.* **15**, 417-424.

Smith, E. M. and Blalock, J. E. (1981). Human lymphocyte production of ACTH and endorphin-like substances: Association with leukocyte interferon. *Proc. Natl. Acad. Sci. USA* **78**, 7530-7534.

Smith, E. M., Phan, M., Copenhagen, D., Kruger, T. E. and Blalock, J. E. (1983). Human lymphocyte production of immunoreactive thyrotropin. *Proc. Natl. Acad. Sci. USA* **80**, 6010-6013.

Stephanou, A., Jessop, D. S., Knight, R. A. and Lightman, S. L. (1990). Corticotropin-releasing factor-like immunoreactivity and mRNA in human leukocytes. *Brain. Behav. Immun.* **4**, 67-73.

Streeter, P. R., Berg, E. L., Rouse, B. T. N., Bargatze, R. F. and Butcher,

E. C. (1988). A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature* **331**, 41-46.

van Ewijk, W., van Soest, P. L. and van den Engh, G. J. (1981). Fluorescence analyses and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2, and T-200. *J. Immunol.* **127**, 2594-2604.

Vremec, D. and Shortman, K. (1997). Dendritic cell subtypes in mouse lymphoid organs. Cross-correlation of surface markers, changes in incubation, and differences among thymus, spleen, and lymph nodes. *J. Immunol*. **159**, 565-573.

Wang, J. and Klein, J. R. (1994). Thymus-neuroendocrine interactions in extrathymic T cell development. *Science* **265**, 1860-1862.

Wang, J. and Klein, J. R. (1995). Hormone regulation of extrathymic gut T cell development: Involvement of thyroid stimulating hormone. *Cell. Immunol.* **161**, 299-302.

Wang, J. and Klein, J. R. (1996). Hormone regulation of murine T cells: potent tissue-specific immunosuppressive effects of thyroxine targeted to gut T cells. *Int. Immunol.* **8**, 223-235.

Wang, J., Whetsell, M. and Klein, J. R. (1997). Local hormone networks and intestinal T cell homeostasis. *Science* **275**, 1937-1939.

Wang, J., Griggs, N. D., Tung, K. S. K. and Klein, J. R. (1998). Dynamic regulation of gastric autoimmunity by thyroid hormone. *Int. Immunol*. **10**, 231-236.

Wang, H. C., Zhou, Q., Dragoo, J. and Klein, J. R. (2002). Most murine CD8+ intestinal intraepithelial lymphocytes are partially but not fully activated T cells. *J. Immunol*. **169**, 4717-4722.

Wang, H. C., Dragoo, J., Zhou, Q. and Klein, J. R. (2003). An intrinsic thyrotropin-mediated pathway of TNFα production by bone marrow cells. *Blood* **101**, 119-123.

Wilders-Trushnig, M. M., Kabel, P. J., Drexhage, H. A., Beham, A., Leb, G., Eber, O., Hebenstreit, J., Loidolt, D., Dohr, G., Lanzer, G. and Krejs, G. (1989). Intrathyroidal dendritic cells, epitheloid cells, and giant cells in iodine deficient goiter. *Amer. J. Pathol.* **135**, 219-225.

Wu, L., Vermec, D., Ardavin, C., Winkel, K., Suss, G., Georgiou, H., Maraskovsky, E., Cook, W. and Shortman, K. (1995). Mouse thymus dendritic cells: kinetics of development and changes in surface markers during maturation. *Eur. J. Immunol.* **25**, 418-425.

Zhou, Q., Wang, H. C. and Klein, J. R. (2002). Characterization of novel anti-mouse thyrotropin monoclonal antibodies. *Hybridoma Hybridomics* **21**, 75-79.