IMMUNE REGULATORY PLASMACYTOID DENDRITIC CELLS SELECTIVELY ACCUMULATE IN PERITHYROIDAL LYMPH NODES OF PATIENTS WITH GRAVES DISEASE: IMPLICATIONS FOR THE UNDERSTANDING OF AUTOIMMUNITY

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(Abstract): Endocrine orbitopathy (EO) is the most common extrathyroidal manifestation of Graves disease (GD) but the involvement of the underlying immunological dysregulations remains largely unknown. The major source for IFNα-production, plasmacytoid dendritic cells (PDC) are fundamentally involved in the integration of TH1 and TH2 immune responses but also implicated in the pathogenesis of autoimmune diseases such as lupus. 

Aim: To establish whether PDC may play a role in GD autoimmune reaction and in the pathogenesis of EO.

Material and methods: In a series of six sequential patients with GD as well as six further patients with multinodular goiter, cervical lymph nodes (LN) were sampled and preserved in the setting of thyroid resection. In parallel, peripheral blood samples were collected. The frequency of PDC from the peripheral blood and lymph nodes were determined. Mononuclear cells (MC) were enriched from lymph nodes and peripheral blood using Fiquoll-hypaque density gradient centrifugation. Mononuclear cells were stained using BDCA-2-PE and CD123-FITC monoclonal antibodies and their frequency subsequently analyzed using fluorescence activated cell sorting (FACS). Dead cells were excluded from analysis by appropriate gating strategies and propidium iodide (PI) staining.

Results: In all patients with GD (with or without EO), PDC frequency was significantly increased in perithyroidal LN as compared to LN of patient not suffering from autoimmune diseases, e.g. patients with multinodular goiter (p < 0.01). The number of PDC infiltrating lymph nodes (LN-PDC) was also higher when compared to peripheral blood PDC (pB-PDC) of patients with multinodular goiter (p < 0.05). Finally, LN-PDC counts in perithyroidal lymph nodes of patients with GD were substantially increased when compared to pB-PDC of the same patients (p < 0.01) indicating a migration and accumulation of PDC in the draining LN.

Conclusions: We found evidence that PDC selectively accumulate in perithyroidal LN of patients with GD, but not other thyroid diseases such as multinodular goiter. Based on their central importance in the pathogenesis of autoimmune processes such as in lupus erythematosides, we suggest
that the migration and accumulation of PDC in an anatomical joining point between the thyroid gland and orbita may be of critical importance for the initiation and maintenance of a chronic autoimmune stimulation. This implies a so far unknown role for PDC in GD and as putative cellular targets for new therapeutic approaches. **Keywords:** GRAVES DISEASE, IMMUNOREGULATORY PLASMACYTOID DENDRITIC CELLS, ENDOCRINE ORBITOPATHY, SURGERY

A common part of Graves’ disease (GD) clinical picture (90–95 %), the endocrine orbitopathy (EO) appears less frequently in Hashimoto’s thyroiditis and rarely unrelated to other thyroid conditions (0,5-5 %). About half of the GD patients have clinically obvious ocular signs, mostly of mild to medium severity, while only 3–5 % of the cases develop a severe EO, with orbital nerve compression (1). Typically the EO occurs in the first 6 months from the onset of the thyroid hyperfunction (46-60%) although in 30% of the cases the ocular signs may appear after years, frequently associated with a relapse of the hyperthyroidism. This correlation between the onset of hyperfunction and EO suggests a common pathogenetical mechanism. The pathogenesis of EO comprises an increase of retroocular fibroadipose tissue and swelling of extraocular muscles and orbital connective tissues, due to an infiltration with lymphocytes (T cells), mastocytes and macrophages. A cytokine-related activation of the local connective tissue cells generates an increased production of glycosaminoglycans responsible for the appearance of edema as well as an increased adipogenesis, both resulting in an enlargement of the orbital tissue volume. An overexpression of TSH-R during the differentiation of preadipocytes was noted, underlying the role of TSH-R as an autoantigen in the orbita, which was confirmed by a number of studies on animal models (2).

In contrast to other regions of the body, the orbital fibroblasts express CD40. Normally present on the surface of B-cells, macrophages and dendritic cells, CD40 binds to the CD40-ligands (CD154) activated T-cells and subsequently stimulates the production of cytokines by macrophages. CD40-igation determines orbital fibroblasts to express IL6 and IL8 (with subsequently enhanced chemotaxis and migration of immune competent cells in the orbita) as well as to activate inflammatory cyclooxygenases (by production of prostaglandine E) and raise the production of hyaluronic acid. Considering that cervico-regional lymph nodes are almost constantly enlarged in cases of Graves’ disease, the aim of this pilot study was to investigate the role of the plasmacytoid dendritic cells (PDC) from the perithyroidal lymph nodes, as antigen presenting cells and major IFNα source, in the pathogenesis of autoimmune mechanism of the disease and particularly of EO.

**MATERIAL AND METHODS**

A series of 10 consecutive patients with GD as well as a series of 10 patients with multinodulareuhyroid goiter were enrolled in this study. In all cases, besides thyroidectomy, perithyroidal cervical lymph nodes (LN) were sampled. In the mean time, peripheral heparine-blood samples (20 ml) were collected. Fiquoll-hypaque density gradient centrifugation was used in order to isolate multinuclear cells (MNC) from LN and blood. To obtain LN tissue, the LNs were previously pressed on ice through a mesh, always on the day of the operation,
to avoid the loss of cells.

By fluorecence-activated cell sorter (FACS) following a staining of the PBMC with two monoclonal antibodies (BDCA-2-PE und CD123-FITC), PDC were identified as a clearly defined and highly quantifiable population. The percentage of double positive cells was referred to percentage of MNC. Dead cells were excluded by appropriate gating techniques and propidium iodide (PI) staining. When sufficient material was available, a separation of PDC from MNC was performed by magnetic automated cell sorting (MACS), in order to provide RNA for further genetic testing (cDNA arrays). However, the purity after BDCA-4 MACS was approximately 30% only. The staining of the MNC followed by monoclonal antibodies as previously mentioned. For immunostaining of TSH receptor in the LN of GD patient samples were fixed in 10% neutral buffered formalin for 24 hours then embedded in paraffin as a routine procedure. Sections were immunostained with a 1:250 diluted polyclonal anti-TSH Ab (DakO, Glostrup, Denmark). Detections were carried out using biotin-streptavidin and counterstained with Hämalaun for 10 sec to visualize nuclei.

RESULTS

Immunohistochemistry showed a powerful expression of TSH-receptors in the perithyroidal LN of the patients with GD and EO (fig. 1).

In all GD patients, irrespective of EO, the frequency of PDC was significantly higher in perithyroidal LN compared to normal cervical LN, sampled from patients with euthyroid goiter (p < 0.01).

**Fig. 1.** Positive anti TSH-Receptor immunostain in perithyroidallymphnodes of patients with GD and EO

In a series of healthy patients and normal LN, the initial frequency of PDC was mean 0.4 % of MNC in blood compared to 0.2–0.3 % of MNC in LN. Higher frequencies of lymph nodes-PDC (LN-PDC) were always recorded in GD patients compared to peripheral blood (pB-PDC) (p < 0.05). And, above all, LN-PDC was significantly higher than pB-PDC in GD patients (p < 0.01)(fig. 2, 3).
**DISCUSSION**

As previously noted by Kriss et al. (3), in GD, hyperthyroidism and ocular disease share a common pathogenetical mechanism. They further formulated the hypothesis that regional LN may be the site of antibody presentation and processing. This was supported by the particular observation...
of common lymph drainage pathways of thyroid and orbita. In 1970, Kriss and coworkers published the results of a thyroid 99Tcm colloid scintigraphy study, showing that in patients with GD and EO, compared to patients with other forms of hyperthyroidism or nodular thyroid diseases, a rapid and massive fixation of radionuclide occurs in cervical nodes bilaterally. They also proved the communication between the orbita and cervical LN through the inferior orbital fissure, allowing a deposition of antigen structures and the autoimmune reaction in both cervical LN and orbita. A clear correlation was established between the severity and activity of EO and the involvement of cervical LN, which Kriss thought at that time to be favoured by thyroglobuline (TG) (3). The enlargement of cervical LN by patients with GD has been previously noted by surgeons.

A single center two-armed prospective randomized study was published in 1998, comparing the results of subtotal thyroidectomy with or without cervical lymphadenectomy in GD and EO. In a follow-up period of three years, the authors monitored the titer of antibodies, thyroid function and development of EO by orbital MRT. A significant reduction of EO was noted for patients with thyroid resection and lymphadenectomy compared to patients with thyroidectomy only. Objectively assessing the severity of EO the volume of extraocular muscles, significant improvement was observed postoperatively in patients with thyroidectomy and lymphadenectomy (p<0.001) compared to patients with thyroidectomy only (p=0.2) (4). Moreover, in this study (and the following studies also), a reduction of serum standard immunological parameters to undetectable titers was achieved in patients with lymphadenectomy. It is worth mentioning that, in a previous paper, the same authors reported a significant (but not complete) reduction of TS-Ab after subtotal thyroidectomy, allowing them to state that additional lymphadenectomy generated a drastic reduction of TS-Ab.

Preliminary results of our team showed an intense expression of TSH-receptors in the perithyroidal LN of the patients with GD and EO. This drove us to the assumption that cervical LN plays a significant role in retrobulbar immune processes. Moreover, the confirmed systematical isolation of activated PDC in the LN draining the thyroid and orbita supports our opinion that regional LN are involved in chronic stimulation of immune system, possibly by pleiotrofic effects. It is a known fact that PDC, after initial activation in peripheral blood (e.g. after viral infections), migrate in the LN and present antigens to both B- and T-cells. Immune response may also be augmented by regulation of T-cells. Systemic lupus is probably the most researched autoimmune disease in regard to the role of PDC, which congregate in the skin and generate the autoimmune effect. Therefore, it is a logical assumption that accumulation of PDC in the regional perithyroidal LN is involved in the pathogenesis of the GD. In the view of both literature and our data, we consider that regional LN play a major role in the induction and development of extrathyroidal antigens processing, persistence and autoimmune stimulation in the frame of GD autoimmune hyperthyroidism. For this reason, it is to be proved by further studies if additional cervical modified lymph node dissection should be a standard procedure in the treatment of GD, aiming to significantly improve the outcome of ocular disease.
REFERENCES


NEWS

MICRORNA-27A INHIBITS HEPATITIS C VIRUS REPLICATION

A study by Shirasaki et al. on microRNAs expressed in hepatitis B and hepatitis C virus-infected liver, found that miR-27a, which regulates lipid metabolism, is preferentially expressed in HCV-infected liver. miR-27a was found to repress the expression of lipid metabolism-related genes and ApoA1, ApoB100 and ApoE3, essential for production of viral infectious particles. Repression of miR-27a increased the cellular lipid content, viral replication and infectivity, while miR-27a overexpression decreased viral infectivity and enhanced in vitro interferon signaling. The study showed that patients who expressed high levels of miR-27a had a favorable response to pegylated IFN and ribavirin therapy. Expression of miR-27a is up-regulated by HCV infection and lipid overload, and in turn it represses HCV infection and lipid cell storage. The negative feedback contributes to the maintenance of a low viral load and can help the virus to escape the host immune surveillance and cause a persistent chronic infection (Shirasaki T, Honda M, Shimakami T, Horii R, Yamashita T, Sakai Y, et al. MicroRNA-27a regulates lipid metabolism and inhibits hepatitis C virus replication in human hepatoma cells. J Virol. 2013 Feb 28. [Epub ahead of print]).

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