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"The chondroitin 6-sulfate oligosaccharide unit of human thyroglobulin promotes the induction of experimental autoimmune thyroiditis via CD44-mediated co-stimulation of murine Th1/Th17 cell differentiation"

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Table of contents

LIST OF PUBLICATIONS

1 Zappulla, C., Notartomaso, S., Cetrangolo, G., Fazio, F., Busceti, C., Arcaro, A., Galgani, M., Scarselli, P., **Lepore, A.**, Formisano, S., Nicoletti, F., Gentile, F., and Di Marco, R. (2011) Beneficial effect of mGlu4 receptor activation on multiple animal models of autoimmune disease. In: Proceedings of the 7th International Meeting on Metabotropic Glutamate Receptors, Taormina, October 2-7, 2011, oral presentation. *Current Neuropharmacology* vol. 9: 68-69, Bentham Science Publishers Ltd. (ISSN: 1570-159X)

2 Pizzimenti, S., Ciamporcero, E., Daga, M., Pettazzoni, P., Arcaro, A., Cetrangolo, G., Minelli, R., Dianzani, C., **Lepore, A**., Gentile, F., and Barrera, G. (2013). Interaction of aldehydes derived from lipid peroxidation and membrane proteins. Frontiers in Physiology vol. 4(242): 1-17. doi:10.3389/fphys.2013.00242 (ISSN 1664-042X)

Articles under revision:

1 Arcaro, A., Daga, M., Cetrangolo, G. P., Ciamporcero, E., **Lepore, A.**, Pizzimenti, S., Petrella, C., Ullio, C., Mamone, G., Ferranti, P., Ames, P. R. J., Palumbo, G., Barrera, G., and Gentile, F. Adducts with Heat Shock Protein 60 and CX49orf are formed in HL-60 human myeloid leukemic cells exposed to 4-hydroxy-2-nonenal. A "strange loop" of peroxidative stress and adaptive immunity on the way to atherosclerosis? *Mediators of Inflammation*, *revision pending* (manuscript no. 804531.v1) (ISSN 0962-9351, online ISSN: 1466-1861)

2 Cetrangolo, G. P., Arcaro, A., **Lepore, A.**, Graf, M., Mamone, G., Ferranti, P., Palumbo, G., and Gentile, F. Hormonogenic donor Tyr2522 of bovine thyroglobulin. Insight into preferential T3 formation at the carboxyl terminus of thyroglobulin at low iodination level. *FEBS Letters*, *revision pending* (manuscript no. D-14-00326) (ISSN 0014-5793)

ABSTRACT

The data presented in this work document that the presence of a chondroitin 6-sulphate (CS) oligosaccharide unit linked to Ser2730 of human thyroglobulin (hTg) was associated with a marked increase of the immunopathogenicity of hTg in a model of murine experimental autoimmune thyroiditis (EAT) in CBA/J(H-2^k) and SJL(H-2^s) mice. When used to immunize CBA/J mice, CS-containing hTg (hTgCS) markedly enhanced, in comparison with CS-devoid $hTgCS_0$, the infiltration of murine thyroids by mononuclear cells, the leak of thyroid hormones from thyroid epithelial cells into the bloodstream and the secondary proliferative and secretory responses of splenocytes and isolated $CD4^+$ T cells to both hTgCS and $hTgCS_0$ *in vitro*. These results indicated that the CS oligosaccharide unit of hTg is capable of enhancing markedly the primary sensitization of murine T cells to peptide epitopes shared between hTgCS and $hTgCS_0$ and conserved between human and murine Tg. Moreover, the cytokine secretion patterns obtained with isolated murine $CD4^+$ T cells indicated that the CS unit, both as a part of hTgCS and in the form of a purified, CS-containing nonapeptide isolated to homogeneity from hTgCS (hTgCSgp), was capable of enhancing the differentiation of Tg-reactive T_H1 and T_H17 cells. Furthermore, the response patterns of murine $CD4^+$ T cells to hTgCS and hTgCSgp, also in relation with the responses to anti-CD3 and anti-CD28 antibodies, indicated that the CS unit of hTg did not behave as a pathogenic epitope in itself, but rather as an added co-stimulus for the differentiation of Tg-specific T_H1 and T_H17 cells, thereby promoting strongly thyroid disease. Finally, two-step cross-linking experiments and confocal microscopy of murine $CD4^+$ T cells exposed to the purified hTgCS glycopeptide indicated that the CS oligosaccharide unit of hTg interacted specifically with CD44 of murine $CD4^+$ T cells.

1 INTRODUCTION

1.1 Experimental autoimmune thyroiditis (EAT)

Experimental autoimmune thyroiditis (EAT), a murine model of Hashimoto's thyroiditis, can be induced in mice with haplotypes *H-2*^{s} and *H-2*^{k} by immunization with mouse Tg (mTg) or human Tg (hTg) in complete Freund's adjuvant (CFA) or lipopolysaccharide (Rose *et al.* , 1971). EAT is characterized by the infiltration of thyroid by mononuclear cells, the production of specific antibodies and *in vitro* cell proliferative responses against Tg. EAT is a T cell-mediated disease, which can be transferred from mice immunized with mTg to syngeneic hosts, by injecting mTg-specific T-cell clones and cytotoxic T lymphocytes. $CD4^{+}$ cells play a dominant role in EAT (Flynn *et al.,* 1989). The analysis of the T cell receptor Vβ gene repertoire of thyroid-infiltrating T cells, after immunization with Tg or adoptive transfer of mTg-primed T cells, showed the clonal expansion of $V\beta13^{+}$ T cells (Matsuoka *et al.,* 1994*;* Nakashima *et al.,* 1996). Also the characterization of mTg-primed T cells, expanded in vitro with staphylococcal enterotoxin A, demonstrated the involvement of $V\beta13^{+}$ and $V\beta1^{+}$ T cells in subsequent thyroiditis transfer (Wan *et al.,* 2001). T helper type-1 responses are crucial for thyroiditis development, as underscored by the impaired induction of EAT in interleukin-12-deficient C57BL⁄6 mice (Zaccone *et al.,* 1999) and by the association between the *IFN-γ* gene deletion and the marked reduction of anti-mTg IgG1 and IgG2b production, and thyroid infiltration with T, B and plasma cells in spontaneous autoimmune thyroiditis (SAT) (Yu *et al.,* 2002). Anti-Tg antibodies may also contribute to the pathogenesis of murine autoimmune thyroiditis. Moreover, some murine mAbs facilitated the internalization of mTg by a murine B-cell hybridoma in vitro, and either suppressed or enhanced the presentation of the non-dominant pathogenic epitope 2549–2560 (Dai *et al.,* 1999). In NOD-H-2^{h4} mice, the levels of mTg-specific IgG1 and IgG2b autoantibodies produced correlated closely with the severity of SAT (Braley-Mullen *et al.,* 1999). Moreover, B cell-deficient NOD-H-2^{h4} mice developed minimal SAT, and B cell function could not be replaced by anti-mTg antibodies (Braley-Mullen *et al.,* 2000).

1.2 Genetic regulation of susceptibility to EAT

The development of EAT is under the influence of H-2 molecules of the murine major hystocompatibility complex (Vladutiu 1989*;* Beisel *et al.,* 1992). High susceptibility strains include C3H(*H-2*^{k}) and SJL(*H-2*^{s}), while BALB/C(*H-2*^{d}) and B10(*H-2*^{b}) are relatively resistant. Non-obese diabetic mice (NOD)(*H-2*^{g7}), a strain susceptible to diabetes, are also prone to develop SAT, even though at a very low rate with aging (Damotte *et al.,* 1997). In the context

of the "high-responder" haplotype *H-2*^{k}, the region *H-2A* of the *H-2* complex is the major regulator of the susceptibility to EAT (Beisel *et al.,* 1982), even though the regions *K* and *D* of the *H-2* complex also have influence (Kong *et al.,* 1979). The primary response of mouse lymphocytes to epithelial syngeneic thyroid cells is under a similar control by the *H-2* region, and their proliferation seems to be triggered by the recognition of products of the *I-A* subregion (Salamero and Charreire 1983a and 1983b). Therefore, the recognition of a limited number of thyroiditogenic epitopes of mTg and hTg by HLA-restricted T lymphocytes seems pivotal in the development of EAT. Apparently, H-2 molecules are able to present T cell thyroiditogenic epitopes shared by mTg and hTg.

By using mice characterized by recombination within the *H-2* region, differences in the genetic influence on the induction of EAT by thyroiditogenic peptides of Tg have been observed, in comparison with EAT induction by whole Tg, which is under strong influence by H-2A^{k} products. The rat Tg peptide TgP1 causes EAT with a similar genetic pattern as entire Tg: B10.BR and C3H(*H-2*^{k}) and SJL(*H-2*^{s}) mice are susceptible, while BALB/C(*H-2*^{d}) are partially resistant and B10(*H-2*^{b}) are resistant. At variance from EAT induction by intact Tg, EAT induction by TgP1 (2495-2511), within the susceptible haplotype *H-2*^{k}, requires the expression of *H-2E* products, while the *H-2D* region does not seem to have influence. Such divergences probably reflect differences in antigen processing and presentation between Tg and its peptides. The role of H-2E molecules in the presentation of peptides such as TgP1 could be masked, if most thyroiditogenic Tg epitopes were *H-2A*^{k}-restricted. Moreover, the non-immunodominant peptide TgP1 could be displaced by other peptides of Tg in the interaction with H2-E molecules, or, alternatively, it may not be generated by antigen presenting cells (APC), in the course of Tg proteolysis, whereas it may be generated and presented by intrathyroidal MHC class I- or class II-positive cells (Chronopoulou *et al.,* 1993). Subsequently, it has been demonstrated that the 2496-2504 nonamer was the minimal T-cell epitope in TgP1 and could be presented within the context of the non-isotypic H-2E^{k} molecules of C3H(*H-2*^{k}) mice and H-2A^{s} molecules of SJL(*H-2*^{s}) mice (Rao *et al.,*1994). On the other hand, the genetic pattern of mouse susceptibility can vary, depending on the pathogenic epitope: rTg peptide 2696-2713 (TgP2) caused EAT in SJL(*H-2*^{s}) mice, but not in C3H or B10.BR(*H-2*^{k}), BALB/c(*H-2*^{d}), and B10(*H-2*^{b}) mice (Carayanniotis *et al.,* 1994).

Last, the role of polymorphic genes *HLA-BR1* (*DR3*) in the development of EAT following Tg immunization has been addressed using *HLA-DR* and *HLA-DQ* transgenic mice: immunization of *HLA-BR1*0301(DR3)* transgenic mice with mTg or hTg resulted in severe thyroiditis, while transgenic mice expressing *HLA-BR1*1502(DR2)* gene were resistant to EAT (Kong et *al.,* 1996). The introduction of the *H-2Ea*^{k} transgene into B10(*H2*^{b}) or MHC class II-negative *Ab*^{0} mice, both resistant to EAT, conferred upon them susceptibility

to EAT induced by bovine, porcine or human Tg, but not murine Tg (Wan *et al., 1999).*

1.3 Thyroglobulin

Thyroglobulin is a large homodimeric glycoprotein, with a Mr of 660,000. After being synthesized and assembled into dimers in the endoplasmic reticulum and glycosylated in the Golgi apparatus, it is condensed in secretory granules in the apical cytoplasm of thyroid follicular cells and secreted in a regulated way in the lumen of tyroid follicles. Thyroglobulin is the molecular site of biosynthesis of thyroid hormones, which are synthesized through the iodination and coupling of a small number of specific tyrosyl residues within the context of the polypeptide chains of thyroglobulin. Thyroglobulin is also a major autoantigen, involved in the pathogenesis of thyroid autoimmunity (Gentile *et al.,* 2004).

1.4 Structure and function of human thyroglobulin (hTg)

1.4 1 The Tg gene: structure and evolution

The hTg gene is located on chromosome 8 and it is one of the gratest genes known, encompassing 42 exons and spanning more than 300,000 base pairs (Baas *et al.,* 1986). About two-thirds of Tg at its amino-terminal side consist of tandemly repeated cysteine-rich motifs of various kinds. This portion of the *hTg* gene probably derives from duplication of an ancestral unit made of four exons. The carboxy-terminal third of Tg is homologous with acetylcholinesterase and appears to have been originated from the duplication of an ancestral gene, in common with a superfamily of esterases (Takagi *et al.,* 1991; Krejci *et al.,* 1991). Thus, Tg appears to be made of two mojeties, an amino-terminal one and a carboxy-terminal one, with different evolutionary histories, both being able to support the biosynthesis of thyroid hormones.

1.4 2 Structure of hTg

The full-length sequence of the hTg mRNA has been determined from overlapping cDNA clones (Malthiéry and Lissitzky 1987). It consists of 8448 nucleotides, including an untranslated 5′-end, an open reading frame, and an untranslated 3′-end, and encodes a polypeptide of 2767 amino acids. The first 19 residues probably represent a hydrophobic signal sequence and are absent from the mature protein, which is composed of 2749 residues and has an

expected Mr of 302,773. Two-thirds of the Tg sequence at its amino-terminal side consist of tandemly repeated motifs. The type-1 motif is 60-70-residue long, contains 6 cysteinyl residues, and is repeated 10 times. The type-2 motif consists of 14 to 17 amino acids, 2 of which are cysteines, and is repeated 3 times at the center of Tg. Five type-3 repeats, subdivided into three subtype-3a repeats, with 8 cysteines each, and 2 subtype-3b repeats, with 6 cysteines each, follow in alternating order. The type-1 motif is homologous with a cystein-rich

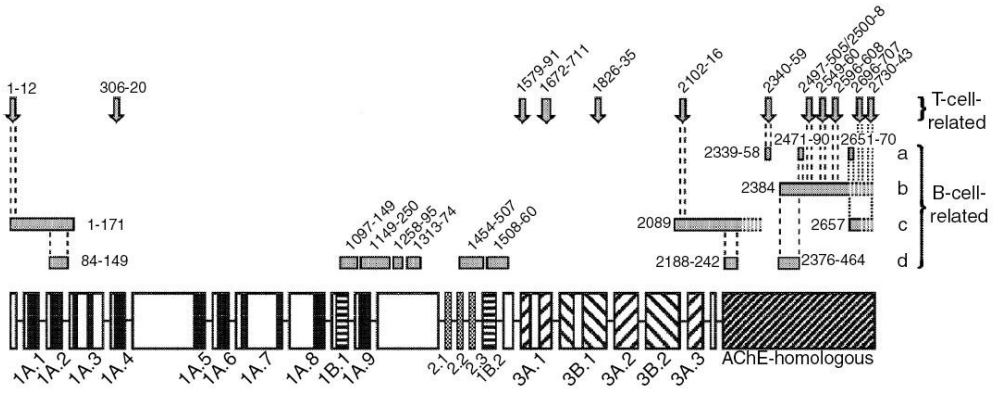

Fig 1. Polipeptide chain of hTg with the localization of the B and T cells epitopes.

motif of the invariant chain associated with the class II major histocompatibility antigen (McKnight *et al.,* 1989). The sequence of 570 amino acids at the COOH-terminus of Tg shows a high degree of similarity (up to 60% in some regions) with those of the members of a superfamily of lipases and esterases, including the acetylcholinesterase of *Torpedo californica,* human serum cholinesterase, and others (Takagi *et al.,* 1991; Krejci *et al.,* 1991). Mature hTg is a mixture of both non-covalent and covalent homodimers, having a molecular mass of 330,000.

1.4 3 Post-translational modifications of hTg: glycosilation

Carbohydrates contribute about 10 per cent of the Tg mass. Two kinds of oligosaccharide units (A and B) are attached by glycosylamine linkages to asparagine residues of Tg. High-mannose A units contain a variable number (7-9) of mannose residues and 2 *N*-acetylglucosamine residues. Complex B units contain 3 mannose residues and a variable number of *N*- acetylglucosamine, galactose, fucose, and sialic acid residues (Arima *et al.,* 1972). Human Tg also contains C and D oligosaccharide units. C units are linked to serine and threonine by O-glycosidic bonds and contain galactosamine; D units are chondroitin-6-sulfate-like oligosaccharides linked to serine and contain a repeating unit of glucuronic acid and galactosamine, plus xylose, galactose,

and sulfate (Spiro *et al.,* 1977). 90 per cent of the [^{35}S]-sulfate incorporated into human Tg is equally distirbuted in: a) biantennary B units, containing galactose-3-sulfate, and tri- and tetra-antennary B units containing galactose-3-sulfate and *N*-acetylglucosamine-6-sulfate; b) chondroitin-6-sulfate-like D units (Spiro and Bhoyroo 1988; Schneider *et al.,* 1988).

1.4 4 Chondroitin 6-sulfate oligosaccharide units

In a previous study (Conte *et al.,* 2006), we characterized in detail the O-linked, type D chondroitin 6-sulfate (CS) oligosaccharide unit of hTg. We developed an ion-exchange chromatography (IEC) method, which permitted us to separate chondroitin 6-sulfate-containing hTg molecules (hTgCS) from the residual hTg molecules (hTgCS$_0$). By exploiting the changes of electrophoretic mobility and staining properties conferred upon the products of limited proteolysis of hTg by chondroitin 6-sulfate units, we first restricted the chondroitin 6-sulfate-containing regions of hTg to a carboxyl-terminal peptide, starting at Thr2514. The subsequent purification of a homogeneous, D-glucuronic acid-containing nonapeptide (hTgCSgp), corresponding to hTg residues 2726-2734, permitted us to establish Ser2730 as the sole site of chondroitin 6-sulfate addition in hTg.
In an ample number of hTg preparations, the fraction of hTgCS in total hTg ranged from 32.0 to 71.6%. The CS unit was composed of a broadly varying number of D-glucuronic acid-N-acetyl-D-galactosamine disaccharide units. In some goiter hTg preparations with high contents of CS, hTgCS heterodimers,

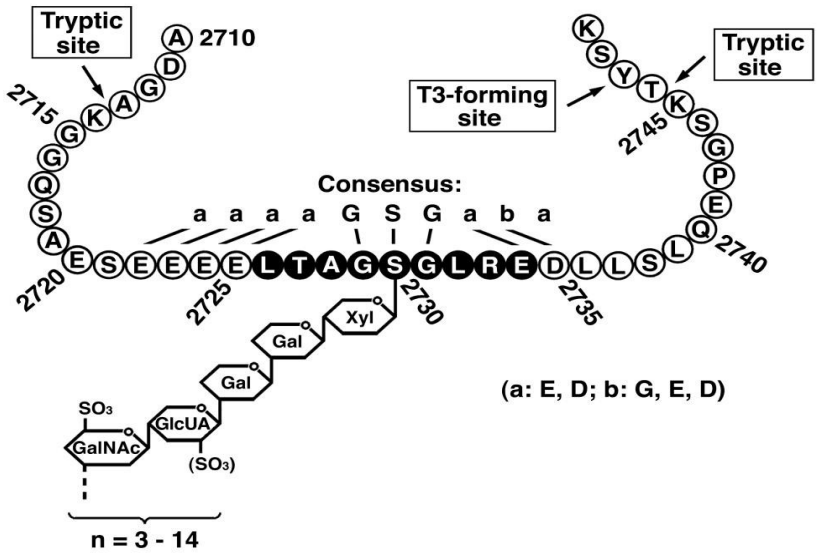

Fig 2 . Structure and localization of chondroitin-6 sulfate oligosacarride unit of hTg: The figure show carbossi-terminal segment (2710-2749) of hTg linked by CS. CS is composed by

xilose, galattose and N-acetilglucosammine disaccharide glucuronic acid sulfate repeated from 7 to 11 times (*Spiro, 1977*).

in which only one hTg subunit contained a CS chain, coexisted with hTgCS homodimers. In the monomers deriving from the dissociation of hTgCS in urea, the net number of disaccharide units per chondroitin 6-sulfate chain did not exceed 14. We also showed that hTgCS has a higher efficiency of hormone formation than $hTgCS_0$, and that the whole unfractionated hTg benefits from this property. Thus, chondroitin 6-sulfate addition represents an ergonomic mechanism, by which the post-translational modification of a fraction of molecules influences the overall function of hTg.
Furthermore, we identified two sites, one between Lys2714 and Gly2715 and the other between Lys2745 and Thr2746, near the site of chondroitin 6-sulfate addition, which were susceptible to limited proteolysis with trypsin. Our data show that the former was protected from proteolysis in hTgCS but not in $hTgCS_0$. We reasoned that the addition of the C6S chain may exert modifying effects in the processing of hTg by antigen-presenting cells and in the ability of hTg to function as an autoantigen. These may go beyond the possible influences on the proteolytic accessibility and/or the recognition of specific T- and B-cell epitopes located in the vicinity of the chondroitin 6-sulfate addition site.

1.5 Effects of chondroitin sulfate proteoglycans

Chondroitin sulfate proteoglycans play important roles in cell adhesion, migration, differentiation and proliferation. Several reports indicate that some chondroitin sulfate proteoglycans affect the proliferation of a number of cell types. Versican 1 and 2 have opposite actions on the proliferation of NIH-3T3 cells: versican 1 induces cell proliferation and inhibits apoptosis, while versican 2 inhibits cell proliferation and does not affect apoptosis (Sheng *et al.,* 2005). TENB2, a chondroitin sulfate proteoglycan identified in prostate cancer, is involved in disease progression and in prostatic cell growth control (Glynne-Jones *et al.,*2001). The degradation of chondroitin sulfates A, B and C chondroitinase reduces the proliferation and invasion capacity of melanoma cells, while inducing caspase-3 activity and thus stimulating apoptosis (Denholm *et al.,* 2001). Endothelial cells, treated with the same enzymes, behave similarly. Some Authors (Yang *et al.,*2004), by studying the chondroitin sulfate proteoglycan of melanoma cells (MCSP), demonstrated that it stimulates proliferation, migration and invasion by tumor cells, by enhancing FAK and ERK activation. By studying globular domains of proteoglycan molecules, specific regions were identified, modulating cellular responses, such as apoptosis or cell proliferation (Cattaruzza *et al.,* 2004).
The action of chondroitin sulfate proteoglycans is often mediated by CD44, the

most important chondroitin sulfate proteoglycan exposed on the leukocyte surface (Taylor and Gallo 2006).
Generally, high levels of proteoglycan chondroitin sulfates are predictive of a poor prognosis and malignant progression of the disease. It is conceivable that the hTgCSgp glycopeptide may be involved in the progression of thyroid tumours and may induce cellular proliferation and inhibition of apoptosis.

1.6 Involvement of CS in Experimental Autoimmune Thyroiditis

CS unit might influence the ability of hTg to induce experimental autoimmune thyroiditis (EAT) in genetically susceptible mice in several ways:
1. The influence of the CS oligosaccharide chain on the proteolytic susceptibility of the extreme carboxy-terminal region of hTg could modify the processing and presentation of hTg by antigen-presenting cells (APCs), particularly because the site of CS addition to hTg is localized within an epitope-rich region, harboring several T cell- and B cell-related epitopes (Gentile *et al.,* 2004). O-linked mono- and disaccharides in tumor-associated glycoprotein MUC1 restricted the repertory of epitopes produced and/or presented in a site-specific manner, either by limiting the accessibility of specific cleavage sites to cathepsin L (Hanisch *et al.,* 2003), or by preventing epitope recognition by a peptide-specific T cell hybridoma. N-linked oligosaccharide chains also inhibited the generation of a self epitope from glutamate receptor subunit 3 (Gahring *et al.,*2001), and of cytotoxic lymphocyte (CTL)-specific epitopes from influenza A nucleoprotein (Wood *et al.,*1998). Conceivably, the CS oligosaccharide unit in hTgCS may hamper the processing by APCs of the surrounding hTg region, within a range which includes the Lys2714-Gly2715 bond, at the amino side, and the Lys2745-Thr2746 bond, at the carboxy side.
2. Direct effects of chondroitin sulfate oligosaccharide units on cellular immune responses have been documented:

- Ii (invariant chain, CD74) is a non-polymorphic glycoprotein that participates in a number of immunological functions. The major functions of Ii are mediated through its association with MHC-II heterodimers (Hiltbolt *et al. ,*2002). In the ER, newly synthesized Ii self assembles into trimers. Three class II heterodimers are sequentially added to one Ii trimer to form a nine-chain complex. In the ER, Ii facilitates MHC-II heterodimer assembly and folding and occupies the MHC-II-binding groove, preventing premature binding of either peptides or unfolded polypeptides. The nonameric complex transits through the Golgi and is transported to late endosomes/lysosomes, where Ii is degraded, allowing MHC-II to be loaded with peptide. However, it has been shown that a small percentage (2-5%) of Ii molecules associated with class II MHC molecules are modified with the addition of a single chondroitin sulfate chain at Ser 291. Chondroitin

sulfate-containing invariant chain complexes (Ii-Cs) are transported rapidly from the *trans*-Golgi network to the cell surface, in spite of the presence of an intact endosomal localization signal. In this form, they remain associated with class II molecules at the surface of APCs (Sant *et al.,*1985;) (Miller *et al.,* 1988), where they act as accessory molecules in antigen presentation, through interaction with CD44 (Naujokas *et al.* ,2003). Recently, it has been demonstrated that the presence of at least one chondroitin sulfate-containing subunit within the context of Ii/MHC-II nonamers is able to direct Ii to the cell surface (Arneson *et al.,* 2007). Enhancing effects of Ii (CD74) on T cell mitogenic and allogenic responses occur through the interaction of Ii-CS (CD74) with CD44 on responding T cells, as they can be inhibited both by anti-CD44 antibodies, and by a soluble form of CD44 (CD44Rg) (Naujokas *et al.,* 1993). Monoclonal antibodies against CD44 were able to trigger the cytotoxic activity of CTL in a TCR-independent manner (Seth *et al.,* 1991). Serglycins, small proteoglycans in secretory granules of hematopoietic cells, activated the CD3-dependent release of cytokines and proteases from CD44-positive CTL clones (Toyama-Sorimachi *et al.,* 1995), by interacting with CD44 through their chondroitin 4-sulfate and 6-sulfate side chains (Toyama-Sorimachi *et al.,* 1997). Moreover, the binding of aggrecan to CD44, through its chondroitin 4- and 6-sulfate side chains, was able to trigger the oligomerization of CD44 molecules and the intracellular signaling (Fujimoto *et al.,* 2001). Both CD44v6 and CD44v7 were transiently up-regulated during lymphocyte activation. Antibodies to CD44s and CD44v7 inhibited antigenic and mitogenic T and B cell responses, while antibodies to CD44v6 selectively inhibited T cell responses (Seiter *et al.,* 2000).

- More recently, Ii-CS (called CD74) has been identified as the cell-surface receptor for Macrophage migration inhibitory factor (MIF) (Leng, 2003). MIF promotes pro-inflammatory cytokine production by macrophages, triggers proliferation of T cells, and induces the release of nitric oxide, matrix metalloproteases, COX-2, and prostaglandin E2. MIF binds to Ii by a high-affinity interaction and induces the serine phosphorylation of the CD74 intracytoplasmic domain in a CD44-dependent manner. CD44 might be necessary for many, if not all, of MIF's signal transduction properties. By using COS-7/M6 (deficient in both CD74 and CD44) cell lines engineered to stably express combinations of CD74, CD44, and CD44D67, some Authors found that CD74 alone imparts MIF binding to cells and that the presence of CD44 did not confer additional binding activity over that of CD74 alone. There are not evidences for a specific interaction between MIF and CD44; however, MIF-mediated ERK1 and ERK2 activation required the expression of a full length CD44. MIF stimulation was associated with the PKA-dependent serine phosphorylation of CD74 and CD44. Investigations with siRNA and

the kinase inhibitor PP2 indicate that MIF-induced ERK activation proceeded via the Src tyrosine kinase, which was previously shown to associate physically with the CD44 intracytoplasmic domain (Taher *et al.,* 1996).

- Finally, it has been demostrated that p41 fragment of invariant chain (containing an additional 64-amino acid sequence in the lumenal domain) is a potent inhibitor of cathepsin L. The additional segment (AS) is cysteine rich, and shares significant homology with the type-1 repetitive sequence of thyroglobulin (Bevec *et al.* ,1996). These data suggest that p41 may enhance Ag presentation, by reducing the proteolytic activity of the Ag-processing compartment, thus protecting a subset of antigenic epitopes from excessive degradation (Fineschi *et al.* ,1996).

1.7 CD 44: gene, structure and function

CD 44 is a monomeric ubiquitous transmembrane type 1 protein that is the most important receptor of hyaluronic acid (HA) and other extracellular matrix glycoproteins.

CD44 gene is located at the short arm of 11 choromosome, containing 20 exons spanning some 50 kilobases of DNA. Exons 1÷16 encode extracellular domain, exon 17 encode trasmembrane domain and exons 18, 19 encode for cytoplasmatic domain. Exons 6÷15 are subjected to alternative splicing with the formation of ten alternative splicing forms of the CD44 protein named V1 ÷ V10 that have different and specifics functions (Ponta *et al.*, 2003).

CD44 structure consist of:

1-Extracellular ammino-terminal domain that contains globular acid hyaluronic binding domain (HBD). This domain has motifs that function as docking sites for several components of ECM. In the ammino-terminal globular region of CD44, a stretch of 90 amino acid (residues 32-123) shows considerable homology with both the cartilage link protein and with the proteoglycan core protein. This domain is known as the "link" domain, that enable CD44 to bind to hyaluronan. HBD of CD44 smallest isoform (CD44s) is separated from the plasma membrane by a short stem structure (46 amino acid). This stem contains putative proteolytic cleavage sites. The stem structure is enlarged by sequences that are encoded by the alternatively spliced exons of CD44 (Ponta *et al.* ,2003);

2- Transmembrane domain composed of 21 hydrophobic amino acids and a cysteine residue which seem to partecipate in the formation of CD44 oligomers. The transmembrane domain might be responsible for the association of CD44 proteins with LIPID RAFTS (Ponta *et al.* ,2003);

3-Cytoplasmatic domain composed of 72 amino acids, that is the target of PKC phosphorylation in serine residue. Members of 4.1 superfamily, in particular the so-colled ERM (ezrin, radixin, moesin) proteins and ankyrin, a protein that

mediates contact with the cytoskeletal component spectrin, interact with a basic amino acids motifs of cytoplasmatic domain (Ponta *et al.* ,2003).
Structural and functional diversity of CD44 is generated by alternative splicing and post-trascritional glycosilation. HBD domain is highly glycosilated in serine, proline and treonine residues. In this region there are five conserved residues for N-linked glycosilation and four serine-glycine (SG) peptides that are linked to glycosaminoglycans. All CD44 isoforms are modified with chondroitin sulfate (CS). The chondroitin sulfate is added to a serine-glycine (SG) site in exon 5, while heparan sulfate and chondroitin sulfate are added to the SGSG site in exon V3, (Wolff *et al.* , 1999). CD44 is involved in leukocyte adhesion, rolling and migration. The binding of CD44 to hyaluronan is induced on T lymphocytes after activation by antigen and on monocytes after stimulation by inflammatory agents. Under inflammatory conditions, CD44 on endothelial cells presents hyaluronan to CD44 on activated T lymphocytes and mediates a rolling interaction under flow conditions. This rolling interaction, together with chemokine signaling upregulates integrin mediated adhesion, induces cell arrest and leads to subsequent migration to the inflammatory site. Studies with monoclonal antibodies against CD44 in mouse models of chronic inflammatory disease showed reduced disease severity attributed to reduced

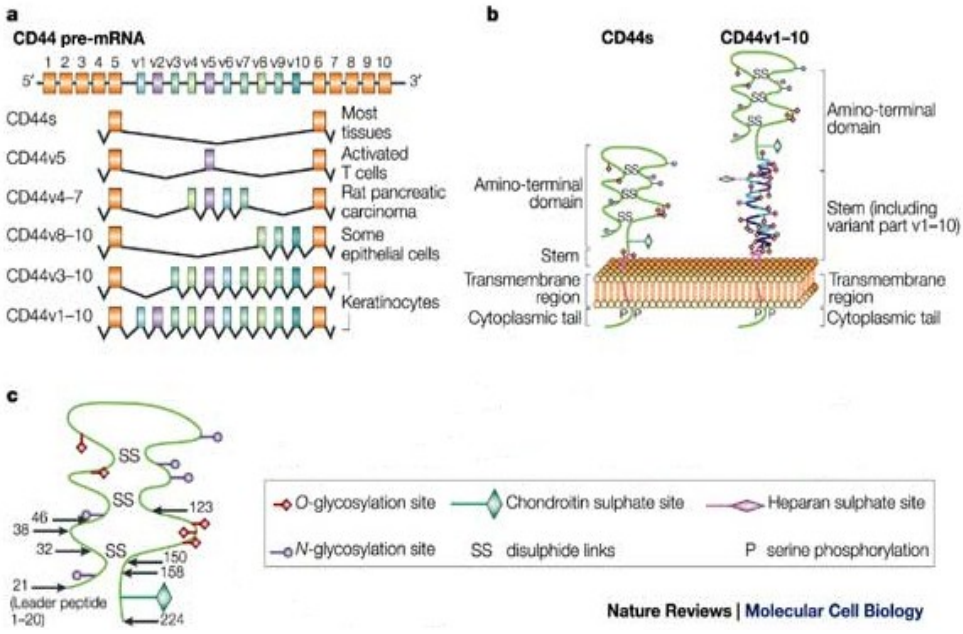

Fig 3. CD44 transcripts and proteins: a CD44 pre-mRNA is encoded by 20 exons, 10 of which can be regulated by alternative splicing (variant or 'v' exons). The smallest CD44 isoform, which is known as CD44 standard (CD44s), is ubiquitously expressed in vertebrates in developing and adult organisms whereas the larger variant isoforms are expressed in only a few epithelial tissues, mainly in proliferating cells, and in several cancers. **b** The protein structure of CD44s is compared with that of the largest variant isoform CD44v1–10, which

shows that the sequences encoded by the variant exons are in the stem region. **c** The amino-terminal domain of CD44 proteins contains the hyaluronan-binding motifs, which are in the 'link domain' (amino acids 32–123), and a basic motif that is outside the link domain (amino acids 150–158). Of key importance for the stability of the link module are four highly conserved cysteine residues that form interchain disulphide links (Ponta, 2003)**.**

leukocyte recruitment (Johnson *et al.* ,2008). Sulfation is required for selectin mediated leukocyte-endothelial cell interactions and inducible sulfation, also, was shown to regulate CD44 mediated leukocyte adhesion at inflammatory sites (Johnson *et al.* ,2000). Moreover, CD44 mediates the binding of lymphoid cells to CS under static condition, and this interaction is sufficient to resist hemodynamic shear forces and support the rolling of the cells on CS under physiological flow conditions (Murai *et al.* , 2004). Recent studies have shown other important implications of CD44 in different biological processes such as the co-stimulatory activity in adaptive and innate immune responses and the mitogenetics antiapoptotic responses too.

1.8 CD44 as co-stimulatory molecule in adaptive and innate immune responses

T cell activation requires two signals: a signal from the TCR and a co-stimulatory signal provided by antigen-presenting cells (APC). CD28 has been recognized as the principal co-stimulatory receptor for T cell activation. In addition to CD28, multiple molecules on the T cell have been described to deliver co-stimulatory signals. CD44 is a good co-stimulatory molecule candidate, that provide T cells activation in a CD28 independent manner co-stimulation. There are many evidence of this. For example the interaction between CD40 ligand and CD40 is critical for activation of T and B cells *in vivo*. This interaction rapidly induces a novel co-stimulatory activity CD44H mediated, distinct from B7 and independent of CD28. Thus, CD44H, rapidly induced by CD40L, co-stimulates T cell proliferation by a CD28-independent mechanism (Guo *et al.* ,1996). Interaction between HA and CD44 can regulate murine B-cell effector functions and this interactions may play a critical role during normal or autoimmune responsiveness of B cells (Rafi *et al.* ,1997). When CD4 and CD8 T cells were divided based on expression levels of CD44, which distinguishes naıve cells (CD44 low) versus those that are antigen-experienced (CD44 hight), IL2 production by and proliferation of CD4 CD44low naıve cells and both sub-populations of CD8 T cells were clearly Bcl10-dependent, whereas these same functional properties of CD4 CD44hight T cells occurred largely independent of Bcl10. CD4 CD44hight T cells did not activate the NF-B pathway in the absence of Bcl10; These CD4CD44 hight antigen-experienced T cells efficiently secreted IL2 after T cell receptor stimulation in NF-kB-independen manner (Zeng *et al.* ,2008). Stimulation of CD44 through Ab cross-linking or binding to its natural ligands hyaluronan and

osteopontin induced NKT cells to secrete cytokines, up-regulate activation markers, undergo morphological changes, and resist activation-induced cell death, whereas conventional T cells only exhibited changes in morphology and protection from activation-induced cell death. This CD44-specific stimulation of NKT cells correlated with their ability to bind hyaluronan. Thus, fundamental differences in CD44 function between these lymphocyte subsets suggest a biological role for CD44 in the innate immune response (Larkin *et al.* ,2006). CD44 co-stimulation promotes FoxP3 regulatory T cell persistence and function via production of IL-2, IL-10, and TGF-1 (Bollyky *et al.* ,2009). In the end, artificial proteoglycans containing the extracellular domain of the cell adhesion protein lymphocyte function-associated antigen-3 (LFA-3) (CD58) and CD44 motifs modified with CS or a combination of CS and HS showed that it retains the ability to engage and trigger the function of its natural ligand CD2, resulting in T cell activation. In addition, the glycosaminoglycan-modified artificial proteoglycan is capable of binding the chemokine RANTES (regulated upon activation, normally T cell expressed and secreted) and delivering it to human T cells, resulting in enhanced T cell activation (Wolff *et al.* ,1999).

1.9 CD44 as mitogenic and antiapoptotic molecule in adaptive immune responses

Regulation of adaptive immune responses is obtained by a good balance of mitogenic and pro-apoptotic factors, CD44 is implicated in the regulation of this homeostasis in different ways. In fact CD44 engagement, either by hyaluronic acid (HA) or anti-CD44 MoAbs, inhibits DNA fragmentation and apoptosis induced by DEX and anti-CD3 MoAbsi n lymphoid cells (Ayroldi *et al.* ,1995). Optimal immunity to micro-organisms depends upon the regulated death of clonally expanded effector cells and the survival of a cohort of cells that become memory cells. After activation of naive T cells, CD44, is upregulated. High expression of CD44 remains on memory cells and despite its wide usage as a ‘‘memory marker,’’. Moreover, CD44 was essential for the generation of memory T helper 1 (Th1) cells by promoting effector cell survival. This dependency was not found in Th2, Th17, or CD8+ T cells despite similar expression of CD44 and the absence of splice variants in all subsets. CD44 limited Fas-mediated death in Th1 cells and its ligation engaged the phosphoinositide 3-kinase-Akt kinase signaling pathway that regulates cell survival (Baaten *et al.* ,2009). Hyaluronan, exists in a high molecular weight (native) form and lower molecular weight form (HMW- and LMW-HA, respectively). These different forms of hyaluronan bind to CD44 but elicit distinct effects on cellular function. The binding of HMW-HA to CD44 inhibits cell cycle progression, whereas the binding of LMW-HA to CD44 stimulates cell cycle progression of vascular smooth muscle cells. HMW-HA binding to

CD44 selectively inhibits the GTP loading of Rac and Rac-dependent signaling to the cyclin D1 gene, whereas LMW-HA binding to CD44 selectively stimulates ERK activation and ERK-dependent cyclin D1 gene expression (Kotapalli *et al.* ,2008). CD44v6 is transiently expressed during T cell activation. CD44 standard form (CD44s) cross-linking induced ERK1/2, JNK, c-jun, and IkBα phosphorylation only in the context of TCR engagement on the contrary CD44 variant 6 (CD44v6) cross-linking induced ERK1/2, JNK, c-jun, and IkBα phosphorylation in a TCR indipendent manner. This event promotes T cell proliferation and protects them from apoptosis. Furthermore, a CD44-associated 85-kDa protein became hypophosphorylated only after CD44v6 cross-linking. Threonine hypophosphorylation of this protein coincided with the activation of MAP and SAP kinases (Marhaba *et al.* ,2004). Finally, CD44v6 and CD44v9 isoforms exhibit an antiapoptotic effect because can block Fas-mediated apoptosis, indeed, colocalize and interact with Fas and an anti-CD44v6 antibody can abolish his antiapoptotic effect (Mielgo *et al.*,2006).

2 AIM OF THE STUDY

The regional location of the chondroitin 6-sulfate oligosaccharide unit of hTg coincides with a cluster containing some of the best characterized T cell-dependent epitopes in murine models of autoimmune thyroid disease. It's known that chondroitin sulfate-containing molecules, exert effects on immune cell responses, so chondroitin 6-sulfate oligosacchride unit of hTg may determining and controlling the development of immune responses to hTg.
The aim of this study was to determine whether and by which mechanisms the chondroitin 6-sulfate oligosaccharide unit of human thyroglobulin may modulate the immunopathogenicity of hTg in a murine model of experimental autoimmune thyroiditis (EAT).

3 MATERIALS AND METHODS

3.1 Preparation of hTg and isolation of hTgCS from $hTgCS_0$

hTg was prepared as described (Gentile and Salvatore, 1993) from informed euthyroid patients, hemilaryngectomized for non-thyroidal disease, and patients undergoing thyroidectomy for non-familial, simple or multinodular goiter. Protein concentration was assayed by measuring the optical absorbance at 280 nm. Iodine content was assayed as described, using L-thyroxine as the standard (Palumbo *et al.,*1982). HTg molecules containing type-D (chondroitin 6-sulfate) oligosaccharide units (hTgCS) were separated from residual hTg molecules, devoid of type-D units ($hTgCS_0$), by ion-exchange chromatography on trimethylamino-substituted Q-Sepharose (Q-IEC), using 5-mL HiTrap™ Q-Sepharose HP columns, equilibrated in 0.025 M Tris/HCl, pH 7.4 (buffer A). Up to 20 mg of hTg in buffer A, plus 0.05 M NaCl, were applied to a column. After washing with buffer A, a linear gradient from 0 to 100% of buffer B (1.2 M NaCl in buffer A) was developed in 24 min, at the flow rate of 2.5 mL/min. One-mL fractions were analyzed, or stored at -80 °C until use. All the solutions for preparation of hTg and isolation of hTgCS from $hTgCS_0$ were filtered by using steritop™ filter Merk Millipore. The concentration of bacterial endotoxin in hTg, hTgCS and $hTgCS_0$ preparations to be used both for mice immunization and secondary splenocyte proliferation assays was measured by the Limulus amebocyte lysate test. Sample purity threshold for use with cell cultures was set at < 0.01 Endotoxin Units (EU)/mL.

3.2 Purification, derivatization and *p*-Azidobenzoyl hydrazid cross linking of the hTgCSgp glycopeptide

The chondroitin 6-sulfate-containing glycopeptide of hTg (hTgCSgp) was purified as previously described (Conte *et al.,* 2006). A 40-mg aliquot of hTgCS was denatured and reduced, in 15 mL of 0.3 M Tris/HCl, pH 8.0, 6.0 M guanidine/HCl, 1 x 10^{-3} M EDTA, 0.01 M dithiothreitol, at 37 °C for 2 h. The reduced protein was carboxymethylated with a 5-fold molar excess of iodoacetamide, with respect to total –SH groups, at room temperature for 30 min in the dark. Alkylation was stopped with excess dithiothreitol. The sample was dialyzed against 0.05 M Na phosphate, pH 7.8, and digested with endoproteinase Glu-C (protease V8) from *Staphylococcus aureus*, at the enzyme:substrate weight ratio of 1:100, at 37 °C for 18 h. The sample was adjusted with concentrated solutions to 0.025 M TrisHCl, 0.1 M NaCl, 2.0 M urea, pH 7.4 (buffer C), and loaded onto a 5-mL HiTrap™ Q-Sepharose HP column, equilibrated in the same buffer. After washing with buffer C, a

gradient was started, from 0 to 100% of buffer D (1.2 M NaCl in buffer C) in 55 min, followed by 100% buffer D for 10 min, at the flow rate of 1 mL/min. One-mL fractions were monitored for the optical absorbance at 280 nm and D-glucuronic acid content. A single D-glucuronic acid-containing peak was subjected to size exclusion chromatography on a 1.5-by-100-cm column of Bio-Gel P-2, in 0.01 M NH_4HCO_3. A D-glucuronic acid-containing peak, eluted in the void volume, was lyophilized and further purified by gel chromatography on a 0.5-by-40-cm column of Sephadex G-50 fine, in 0.01 M NH_4HCO_3. One-mL fractions were monitored for peptide content, by measuring the optical absorbance at 220 nm, and D-glucuronic acid content, and a single peptide- and D-glucuronic acid-containing peak was lyophilized. Aliquots of 150 nmoles of hTgCS-terminal peptides were dissolved in 0.1 mL of PBS, pH 7.2. Then, 45 µL of 20 mM sulfosuccinimidyl-6-(biotin-amido) hexanoate (EZ-Link® Sulfo-NHS-LC-Biotin, Pierce) were added to each sample (biotin:peptide molar ratio = 6:1), and mixtures were kept on ice for 2 h. Products were filtered on a 1.5-by-100-cm P2 column in 10 mM NH4HCO3, in order to remove unreacted biotin. 2-mL fractions were monitored at 220 nm and peaks were pooled and liophylized. Samples were assayed with the Lowry protein assay. Liophylized, biotinylated hTgCS-terminal peptides, resuspended in 0,5 ml 0,1 M sodium acetate, was oxidated by using 0,5 ml 20mM sodium metaperiodate in the dark on ice for 30 minutes. Products were filtered on a 1.5-by-100-cm P2 column in 33 mM PBS 50 mM NaCl pH 7,2, in order to remove the excess of sodium metaperiodate. 1-mL fractions were monitored at 220 nm and peaks were liophylized to 3 ml. After oxidation, biotinylated hTg CS was cross-linked with 150 µl of 50 mM p-Azidobenzoyl hydrazid (ABH) photoactivatable (Pierce) in DMSO for 2 hour in the dark at ambient temperature. Products were filtered on a 1.5-by-100-cm P2 column in 33 mM PBS 50 mM NaCl pH 7,2, in order to remove the excess of p-Azidobenzoyl hydrazid. 1-mL fractions were monitored at 220 nm and peaks were pooled and liophylized.

3.3 Experimental animals

All experiments were conducted using groups of five female, 4-week-old CBA/J(*H-2*^{k}) and SJL mice genetically susceptible to EAT induction, purchased from Jackson laboratories (Bar Harbor, ME).

3.4 EAT induction

Experimental groups of five female, SJL (H-2^s) and CBA/J(H-2^k) mice were immunized at day 0 with 100 micrograms of $hTgCS_0$, hTgCS (positive controls) and PBS (negative controls) in Freund's complete adjuvant (CFA), containing 3.5 µg/mL of *Mycobacterium tuberculosis*, in 50 µl of PBS, in the

dorsal thoracic region. On day 7, all animals were given a supplemental dose of 50 μg of the respective immunogen in 25 μl of PBS, emulsionated with the same volume of incomplete Freund's adjuvant (IFA). After 30 days, mice were sacrificed by excess anesthesia, and blood, spleens and thyroids immediately collected. Moreover, groups of five famale 4-week-old SJL(H-2^s) and CBA/J(*H-*2^k) were immunized with hTg, $hTgCS_0$ or hTgCS and the selective mGluR4 enhancer (N-fenil-7-idroxyamincyclopropan[b]cromen-carboxamide [PHCCC] Tocris Cockson Bristol, UK). PHCC was dissolved in sesame oil (vehicle) and administrated daily to the experimental groups at the dose of 0,03 - 0,3 - 3 - 300 $mg\backslash kg^{-1}$. After 21 days, the mice were sacrificed by excess anesthesia, and blood, spleens and thyroids immediately collected.

3.5 Evaluation of EAT

Thyroids were fixed, sliced and stained with May-Grünwald-Giemsa for histological examination, or embedded in paraffin for immunoistochemical characterization. Mononuclear infiltration of the thyroid was evaluated histologically, according to Verginis et al. (2002), in at least 30 sections per thyroid lobe per animal, and scored as follows: 0 = no infiltration; 1 = small interstitial accumulation between two or more follicles; 2 = one or two foci of inflammatory cells more than the size of one follicle; 3 = 10-40% of field area occupied by inflammatory cells; 4 = 40-80% of field area infiltrated.

3.6 Assay of T4 and T3 in mice sera

The concentrations of fT4 and fT3 in mice sera were determined on 10-100 μL-aliquots of sera, using solid-phase radio-immunometric assays (Medical Systems), with reference curves of 0.3-7.7 pmol for T4, and 30-920 fmol for T3.

3.7 Secondary proliferative and secretory responses of splenocytes in vitro

Murine spleens were collected in 5 mL of RPMI 1640 medium, containing Na pyruvate, L-glutamine, penicillin and streptomycin. Splenocytes were obtained by mechanical disruption of spleens with the upper extremity of a disposable syringe plunger and transferred into sterile 50 mL Falcon tubes. In order to remove red cells and dead cells, spleen cells were layered onto a cushion of Lympholyte-M (Cedarlane) and centrifuged at 1000 *g* for 20 minutes. Live cells were carefully collected at the interface with Lympholyte-M, transferred to new tubes and resuspended by the addition of 5 mL of medium, diluted 1:100 with PBS and counted in a Burker's cell. After centrifugation, medium was aspirated and cells were resuspended in complete

RPMI 1640 medium, containing 2% fetal bovine serum (FBS), so to obtain a cell density of 2.5 x 10^6 cells/mL. 200 µL aliquots of cell suspension (5 x 10^5 cells/100µL/well) were cultured for proliferation assays in flat-bottom 96-well plates and incubated at 37°C, in a 5% CO_2/90% air-humidified incubator in the absence or presence of graded doses (30 µg) of $hTgCS_0$ or hTgCS, or purified hTgCSgp glycopeptide. After 48 h of incubation, 50-µL aliquots of supernatants were removed from each well for cytokine assay. 1 µCi of [^{3}H]-thymidine was added to each well and incubation prolonged for 16 h, after which the cells were blotted onto glass-fiber filters and radioactivity counted in beta counter. Same procedure was adoptetd in the mice CBA/J and SJL immunized with antigens hTg, $hTgCS_0$, hTgCS and treated with PHCC. All assays were performed in triplicate and results were expressed as Stimulation Index (S.I.), defined as follows: cpm in the presence of antigen/cpm in the absence of antigen. The concentrations cytokines in the supernatants of splenocyte proliferative reactions were measured, using a multiparametric bead-based flow-cytometric Analyte Detection System (FlowCytomix Mouse TH1/TH2 10plex, Bender MedSystems®). Measurements were performed using a Dako flow cytometer.

3.8 Serum antibody assays

Total IgG, IgG1, IgG2a and IgG3 concentrations in mice sera were measured by ELISA assay in 96-well plates coated with 1 µg of $hTgCS_0$, hTgCS or hTgCSgp as the capture antigen. IgG were assayed on 1:6000 dilutions of sera, using biotinylated goat anti-Ig γ chain $F(ab')_2$ secondary antibodies. IgG1, IgG2a and IgG3 were assayed on 1:20000, 1:500 and 1:500 dilutions of sera, respectively, using biotinylated rat monoclonal anti-mouse IgG1, IgG2a and IgG3 antibodies (LO-MG_1, LO-MG_{2a} and LO-MG_3 clones, Sigma). Detection was performed with streptavidine-peroxidase conjugates and orthophenylenediamine.

3.9 Isolation of murine $CD4^+$ cells

The purification of $CD4^+$ T lymphocytes was performed with the MACS CD4 isolation kit (Miltenyi Biotec). The cell mixture derived from splenic tissue in RPMI supplemented with 2 % FBS at a cell density of 2.5x10^6 cells/ml, was centrifuged for 10 minutes at a speed of 300 rcf. The supernatant was removed and the pellet resuspended in 15 ml falcon with PBS pH 7.2, 0.5 % BSA , 2 mM EDTA (buffer A), at a dilution of cell 10^7 cells/40 µl at 4 °C. 10 µl of a mixture of monoclonal antibodies biotinylated directed against CD8a (Ly -2) (rat IgG2a), CD11b (Mac -1) (rat IgG2b), CD45R (B220) (rat IgG2a), DX5 (rat IgM) and Ter -119 (rat IgG2b) (Biotin- Antibody Cocktail, No. 130-090-860 , Miltenyi Biotec) were added to each 10^7 cells. The reaction mixture

was incubated for 10 minutes at 4-8 °C. Subsequently were added 30 µl of buffer A and 20 µl of antibody monoclonal anti-biotin bound to magnetic beads (Anti -Biotin Microbeads , No. 130-090-860 , Miltenyi Biotec), incubating for 15 minutes at 4-8 °C. After two washes with buffer A, the pellet was resuspended in 500 µl of the same buffer for a dilution of 2x108 cells/ml. The separation of $CD4^+$ lymphocytes was completed using a column LS MACS (Miltenyi Biotec , No. 130-042-201) equilibrated with buffer A. The column was placed in a magnetic field in this way all non $CD4^+$cells were retained, while the $CD4^+$ lymphocytes were eluted and collected in tubes collectors (MS columns plus tubes , No. 130-041-301 Miltenyi Biotec).

3.10 Secondary proliferative and secretory responses of lymphocytes $CD4^+$ in vitro

Secondary proliferative and secretory responses of lymphocytes $CD4^+$ in vitro experiments were performed as indicate in secondary proliferative and secretory responses of splenocytes in vitro experiments previously described.

3.11 Cross-linking of hTgCSgp to T cells receptors and Western blot

24 µg of hTgCSgp biotinylated and conjugated with ABH, was diluted to a final volume of 3.0 ml with pyrogen-free water, 365 µl of his solution (~ 3 g) were added to the 6-well plate containing the isolated $CD4^+$ T cells (10^7 cells/well). After incubation for 30 minut-es at 37 °C each culture medium, was transferred to run the washing procedure for the elimination of excess hTgCSgp - ABH not bound. The washing was performed by centrifugation at a speed of 1600 rpm for 5 min, at the end of centrifugation, the supernatant was removed and the pellet resuspended in serum-free medium. Finally the cells were transferred into a 6-well culture plate and irradiated with a UV lamp to a length wavelength of 365 nm, in the dark, for 15 minutes. After irradiation, the cells were centrifuged at 1600 rpm for 5 minutes, the supernatant was removed and the pellet resuspended in 5 ml of sterile D-PBS. This solution were collected :

1. 10 µl for the treatment with 4 % paraformaldehyde (PFA), to perform the confocal microscopy.
2. 4.80 ml were used to prepare the cell lysate.

Cells were lyseted in 0.5 ml of lysis buffer (50 mM Tris/HCl pH 7.4 , 150 mM NaCl, 5 mM EDTA, 1 % Nonidet P-40, 1 mM sodium orthovanadate and 1 X Mix Antiprotease) and centrifuged at a speed of 10,000 rcf for 10 minutes. The supernatant was transferred in conical tubes centrifuge on ice. 100-200 µg of total cell lysate were immunoprecipitated by adding ~ 0.4 µg of primary anti-

biotin antibody (Biotin-39 15D9: sc-57636) 1 h at 4 °C then, 20 µl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, INC. SC-2003). Sample was incubated O.N. at 4 °C with shaking on a shaker rotating plate vertical. After 3 wash at 1000 rcf for 5 minutes at 4 °C with PBS, the immunoprecipitate were resuspended in 40 µl of Laemmli 1X for the SDS-PAGE and western blot analisis.
The SDS-PAGE was performed as described by Leammeli (Leammeli, 1970), setting up a gradient of 8-18 % acrylamide in buffer 0.375 M Tris/Hcl pH 8.8, 0.1% SDS, and using an electrophoretic Amersham sistem. The electrophoresis was conducted at 10 mA for 16 hours.The western blot was performed with a semi-dry blotting American Bionetics (model SDB -1000) using as transfer buffer 25 mM Tris base and 10 mM glycine, at a constant current of 0.8 mA/cm 2 for 90 minutes. Blotted Immobilon-P membranes (Millipore) was blocked with PBS-Tween-Milk for 60 minutes at room temperature (PBS pH 7.5, 0.2 % Tween-20, 5% Milk powder Bio-Rad). Immobilon-P membranes was washed 3 times with PBS-Tween and incubated O.N. at 4 °C with the monoclonal primary antibody anti -biotin (Biotin - 39 15D9 : sc- 57636) in a ratio of 1/100 (v/v) of PBS -Tween, 3 %BSA. Finally, after 3 wash wih PBS-Tween, immobilon-P membranes was incubated with secondary antibody anti-mouse IgG HRP (horseradish peroxidase) conjugate, in relation 1/20.000 (v/v) with PBS Tween, 3% BSA followed by washing. Immunoreactive bands were visualized with ECL Plus (GE Healthcare).

3.12 Confocal microscopy

The cells ($2x10^4$) were spread on a glass slide, fixed for 15 minutes in 4% formaldehyde, washed twice with PBS, permeabilized with 1% Triton X-100 for 30 minutes at room temperature and washed again with PBS. Subsequently the slides were incubated with 1% BSA in PBS for 30 minutes at room temperature, after which, were incubated ON with primary antibodies anti-biotin and anti-CD44 (1/100 in 1% BSA dissolved in PBS) at 4 °C. The slides were washed, and incubated for 60 minutes at room temperature with secondary antibodies conjugated with fluorochromes, phycoerythrin (PE) for anti-biotin and fluorescein isothiocyanate (FITC) for anti-CD44 (1/100 in 0.1% BSA dissolved in PBS), washed again and mounted with a coverslip. The slides were examined under a Leica TCS SP2 confocal microscope equipped with an objective to water immersion HCX Apo 0.8.

3.13 Statistical analysis

Data are expressed as mean ± SD, unless otherwise specified. The statistical significance between data obtained in experiments of splenocyte, lymphocytes proliferations, cytokine assas and serum immunoglobulin

concentrations was determined by *t* paired or two-tailed Mann-Withney test, as indicated. A value of $p < 0.05$ was considered to be statistically significant. Hystological evaluation of thyroid infiltration was analyzed by the non-parametric rank-sum Wilcoxon test.

4. RESULTS

4.1. hTgCS is a stronger inducer of EAT compared with $hTgCS_0$ in CBA/J(H-2^k) mice

In order to evaluate the ability of $hTgCS_0$ and hTgCS to induce EAT in genetically susceptible CBA-/J(H-2^k) mice, two groups of experimental animals were immunized subcutaneously with the respective immunogens in complete (sensitization) and incomplete (boosting) Freund's adjuvant. Immunization with hTgCS resulted in the induction of a more severe thyroiditis than immunization with $hTgCS_0$, as judged from histopathological and biochemical indices of thyroid organ disease.

Figure 4 and Table 1 show the average disease scores assigned to 61 mice in 4 immunization experiments, by the histological examination of 25-30 seriate tissue sections per thyroid lobe per animal. The large majority of scores 2 and all scores 3, corresponding, respectively, to one or more foci of infiltration more than the size of a thyroid follicle, and 10-40% of field area occupied by mononuclear cells, were assigned to mice immunized with hTgCS. In contrast, mice immunized with $hTgCS_0$ were either unaffected or showed very limited thyroid involvement.

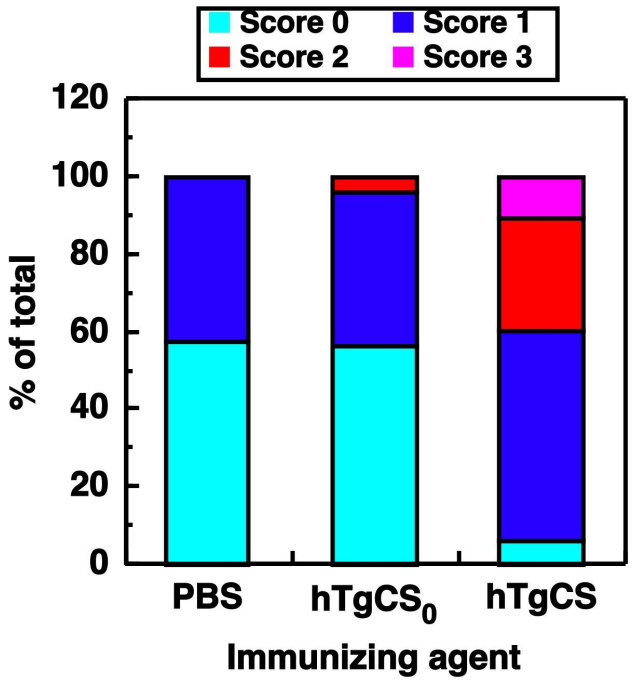

Fig. 4. Histological EAT score, indicating the extent of mononuclear cell infiltration of the thyroid glands of CBA/J(H-2^k) mice immunized with the two different forms of hTg. EAT score was calculated in accordance with the criteria by Verginis *et al.* (2002) infiltration; 1 =

limited interstitial infiltration of mononuclear cells between two or more follicles; 2 = one or two foci of infiltrating mononuclear cells more than the medium size of one follicle; 3 = 10-40% of field area occupied by infiltrating cells; 4 = 40-80% of field area infiltrated.

Table 1. Number and percent of CBA/J(H-2^k) mice assigned disease scores from 0 to 4 in four different immunization experiments with $hTgCS_0$ and hTgCS. For score codes, refer to the legend to Figure 1.

Immunizing agent	Score 0	Score 1	Score 2	Score 3	Number of mice
PBS	57,69%	41,31%	0%	0%	13
$TgCS_0$	56,25%	39,58%	4,17%	0%	24
TgCS	6,25%	54,17%	29,17%	10,42%	24

Figure 5 shows a typical aspect of thyroid infiltration with mononuclear cells in EAT, distinctly more marked in CBA/J mice immunized with hTgCS (*score 3*), compared with mice immunized with $hTgCS_0$ (*score 1*).

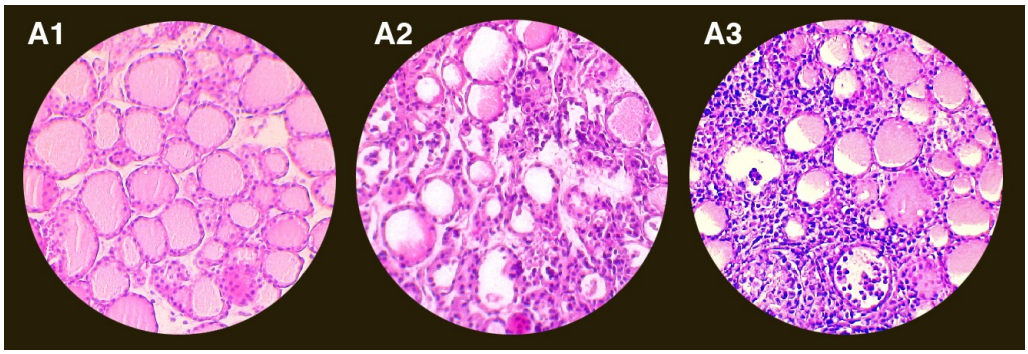

Fig. 5. Representative aspects of thyroid mononuclear infiltration in EAT. The thyroid section shown in A3 was obtained from a CBA/J(H-2^k) mouse immunized with hTgCS and was assigned *score 3*, while the section shown in A2 was from a mouse immunized with $hTgCS_0$ (*score 1*). A normally appearing thyroid section from a control animal is shown in A1 (*score 0*).

The increased severity of thyroid tissue involvement detected by histological examination was reflected in the increases in serum concentrations of 3,5,3',5'-tetraiodothyronine (T4) and 3,5,3'-triiodothyronine (T3), which were significantly different in mice immunized with hTgCS, in comparison with both control mice and mice immunized with $hTgCS_0$.

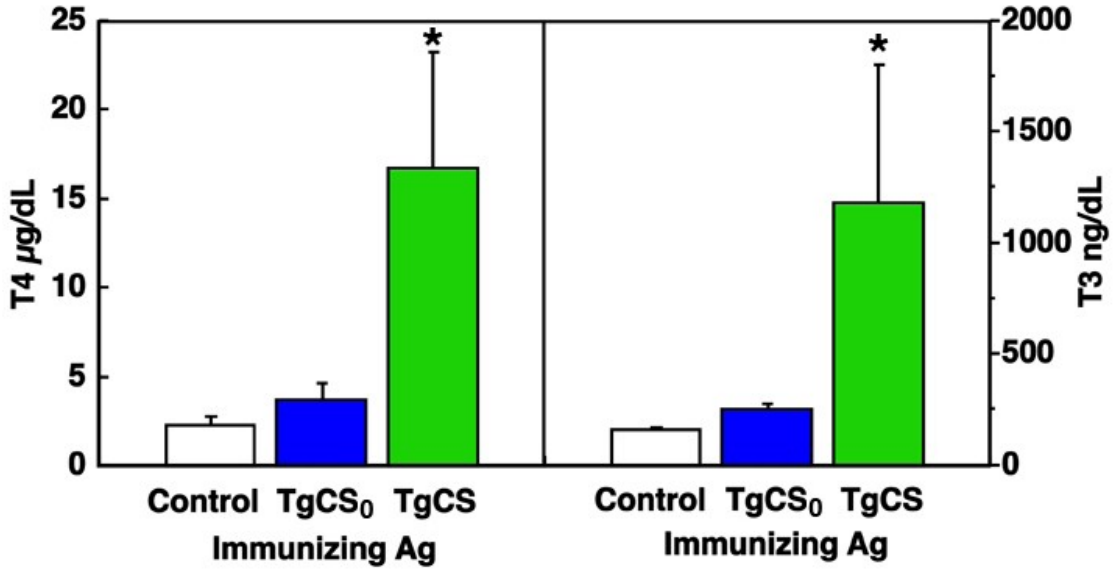

Fig. 6. Serum concentrations of T3 (3,5,5'-triiodothyronine) and T4 (thyroxine) of CBA/J(H-2^k) mice immunized with hTgCS$_0$ or hTgCS. Results are expressed as means ± S.D. * indicates the statistical significance between hTgCS and both mice groups immunized with PBS (negative control) and hTgCS$_0$ ($p < 0,05$).

We have shown that hTg is regularly composed of a mixture of hTgCS$_0$ and hTgCS, in variable proportions among different individuals. From the data here reported, it appears that the induction of autoimmune thyroid disease in experimental animals depends prevailingly on the hTgCS fraction of hTg. Because investigators of EAT have been using so far unfractionated hTg for their experiments, it appears that the variable severity of EAT observed in many studies was influenced by the composition of the hTg preparations used.

Moreover, the relative proportion of heterodimeric and homodimeric hTgCS and the average chain length of the chondroitin 6-sulfate chain linked to hTg might influence EAT severity. Further studies in regard are warranted.

4.2 hTgCS is more effective than $hTgCS_0$ in promoting the primary sensitization *in vivo* of murine splenic lymphocytes to shared epitopes of $hTgCS_0$ and hTgCS

Secondary proliferative assays were performed *in vitro* by stimulating murine splenocytes with $hTgCS_0$ and hTgCS. Proliferative responses were markedly higher in mice immunized with hTgCS than in mice immunized with $hTgCS_0$. On the other hand, the splenocytes from both groups exhibited responses of comparable magnitude to both forms of hTg, i.e., $hTgCS_0$ and hTgCS, even though the responses to $hTgCS_0$ were blunted at the highest doses, whereas those to hTgCS kept increasing with the dose (Figure 7). Overall, these results clearly indicate that the differences in immunopathogenic potential between $hTgCS_0$ and hTgCS reside in the greater ability of hTgCS to promote the primary sensitization of murine T cells to shared epitopes between $hTgCS_0$ and hTgCS, these being, in turn, also shared between hTg and murine Tg (Simon *et al.*, 1986). These epitopes are most likely of peptidic nature, whereas the chondroitin 6-sulfate oligosaccharide unit of hTgCS appears to be acting as a promoter of the breaking of tolerance to these epitopes, without being an immunopathogenic epitopes of its own.

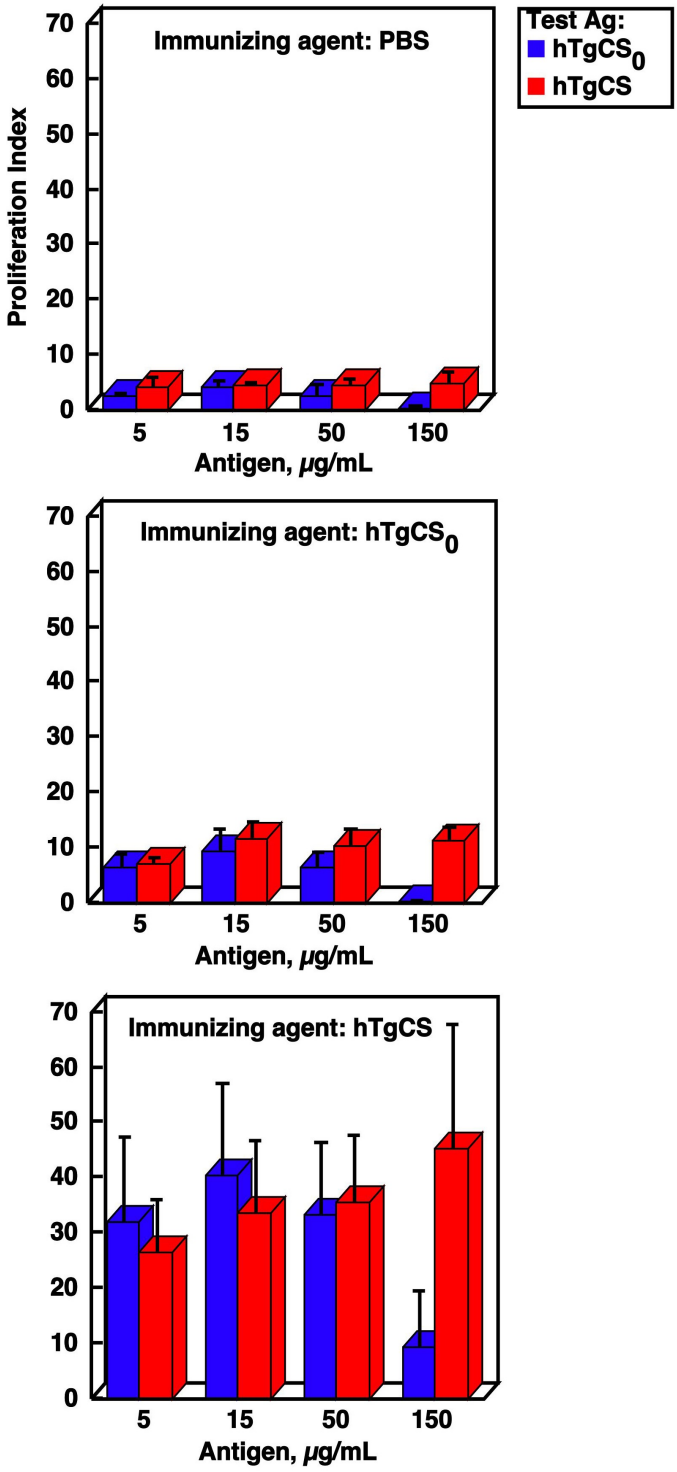

Fig. 7. Proliferative responses of splenic lymphocytes from CBA/J(H-2^k) mice immunized

and restimulated *in vitro* with the two forms of hTg. Mice were immunized with $hTgCS_0$ or hTgCS in CFA, as described in the Methods. Splenic lymphocytes were restimulated *in vitro* with both antigens, at the doses indicated. Results are expressed as means ± S.D.

4.3 Enzymatic dechondroitination deprives hTgCS of its enhanced ability to sensitize murine lymphocytes to shared hTg epitopes

To prove the role of the CS oligosaccharide unit of hTg in the observed increased ability of hTgCS to promote the sensitization *in vivo* and the proliferative responses *in vitro* of murine T cells to shared antigenic determinants of $hTgCS_0$ and hTgCS, we determined the proliferation indices of splenocytes from mice immunized with hTg subjected to enzymatic dechondroitination with ABC chondroitinase from *Proteus vulgaris*($hTgCS_{de}$), restimulated with the various forms of hTg. As shown in Figure 8, the proliferation indices obtained in mice immunized with $hTgCS_{de}$ upon restimulation with $hTgCS_0$, hTgCS and $hTgCS_{de}$ were similar to the responses obtained in mice immunized with $hTgCS_0$, paralleling those observed in negative control mice. On the other hand, the splenocytes from mice immunized with hTgCS proliferated in response to $hTgCS_{de}$, with a similar pattern as observed with $hTgCS_0$, in that both responses tapered down as the dose of the test antigen increased, whereas the responses to hTgCS kept increasing with the dose. These results indicate that the differences observed in the proliferative responses of splenocytes from mice immunized with $hTgCS_0$ and hTgCS were solely dependent upon the CS oligosaccharide unit of hTgCS.

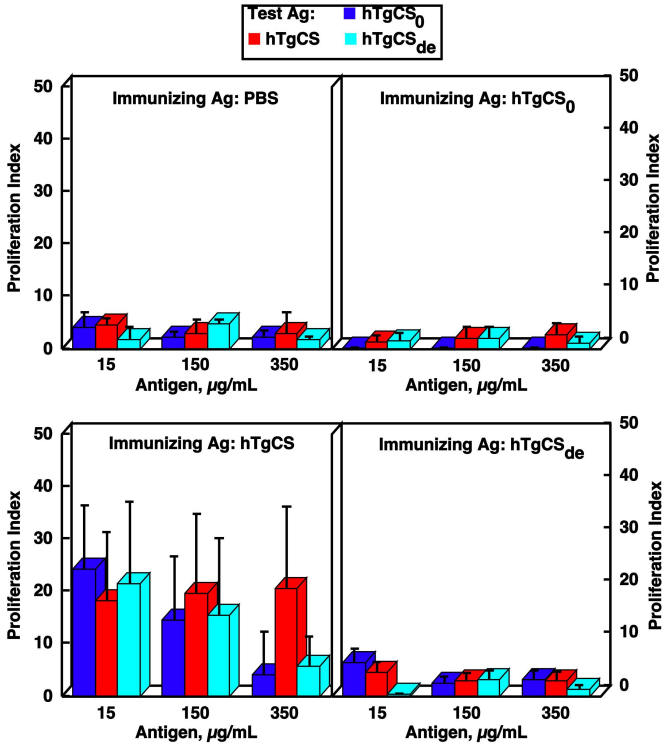

Fig. 8. Proliferative responses of splenic lymphocytes from CBA/J(H-2^k) mice immunized and restimulated *in vitro* with hTgCS$_0$, hTgCS and enzymatically dechondroitinated hTg (hTgCS$_{de}$): Mice were immunized with hTgCS$_0$, hTgCS or hTgCS$_{de}$ in CFA, as described in the Methods. Splenic lymphocytes were restimulated *in vitro* with each of the three antigens, at the doses indicated. Results are expressed as means ± S.D.

4.4 Chondroitinated nonapeptide hTgCSgp isolated from hTgCS displays mitogenic activity towards naïve and hTg-sensitized murine splenic lymphocytes

Next, we investigated whether the improved mitogenic ability exhibited by hTgCS, especially at high doses, in comparison with hTgCS$_0$, in secondary proliferative responses of splenic lymphocytes could be actually attributed to its chondroitin 6-sulfate oligosaccharide unit. To this aim, we isolated and purified to homogeneity the sole CS oligosaccharide unit of hTg, linked to Ser2730, in the most elementary form obtainable with the least possible contribution by the polypeptide mojety of hTg, i.e., in the form of the chondroitin sulfate-containing nonapeptide hTgCSgp. The latter was produced by the extensive digestion of reduced and alkylated hTgCS with staphylococcal V8 protease (endoproteinase Glu-C) and purified to homogeneity, as described in detail in the Methods. The results of the restimulation of splenic lymphocytes from mice immunized with PBS, hTgCS$_0$ and hTgCS with hTgCSgp are shown in Figure 8. The CS-containing glycopeptide stimulated

the proliferation not only of splenocytes from immunized mice, but also from negative control animals, at variance with the homologous synthetic, non-glycosylated hTg peptide 2726-2734, with the LTAGSGLRE sequence, which proved ineffective (Figure 9).

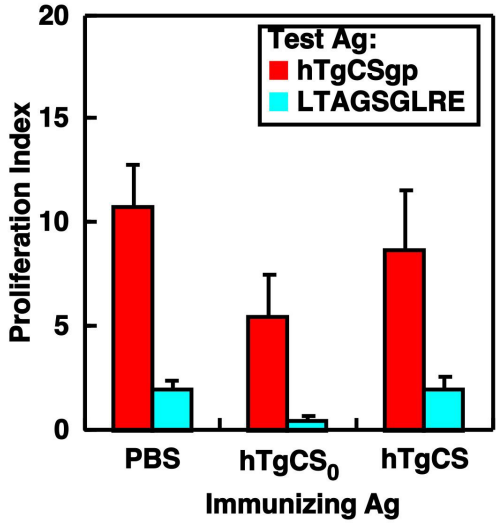

Fig. 9. Proliferative responses of splenic lymphocytes from CBA/J(H-2^k) mice immunized and restimulated *in vitro* with hTgCS$_0$ or hTgCS and restimulated *in vitro* with the CS-copnatining purified glycopeptide hTgCSgp: Mice were immunized as described in the Methods. Splenic lymphocytes were restimulated *in vitro* with 2 μg/mL of the purified CS containing glycopeptide hTgCSgp or its synthetic, non-glycosylated homologous nonapeptide LTAGSGLRE. Results are expressed as means ± S.D.

4.5 The CS unit of hTg, both as a mojety of hTgCS and in the form of hTgCSgp glycopeptide, synergizes CD28-mediated costimulation of the proliferation of CD4$^+$ T cells in response to anti-CD3 Abs

We first investigated the proliferative responses of CD4$^+$ T cells isolated from the spleens of CBA/J mice immunized with hTgCS$_0$ or hTgCS to the C6S unit of hTg, as a mojety of hTg-CS or in isolated form (hTgCSgp), alone or in combination with monoclonal anti-CD3 and anti-CD28 antibodies at optimal concentrations (Figure 10). Marginal proliferation was observed in T cells stimulated with anti-CD3 alone, whereas simultaneous anti-CD3 and anti-CD28 stimulation in CD4$^+$ T cells from negative control (PBS-immunized) mice and hTg-immunized mice was marked by proliferation indexes around 20 and 40-45, respectively, as one might expect.

Complementation of the stimulation by anti-CD3 antibodies with the addition of hTgCS or hTgCSgp was associated with significant, comparable

increases of the proliferation of $CD4^+$ T cells from hTg-immunized mice, more marked in those which appeared to have been sensitized more effectively as a consequence of the immunization with hTgCS rather than with $hTgCS_0$. Limited proliferative responses, with a similar pattern as just described, followed the administration of hTgCS and hTgCSgp, even in the absence of anti-CD3 antibodies, and may reflect a TCR-independent, although limited mitogenic potential of CS in activated $CD4^+$ T cells. Such effects of hTgCS were replicated by hTgCSgp, but not by $hTgCS_0$ and were not seen with $CD4^+$ T cells from negative control animals. Compared with the costimulation afforded by anti-CD28 in addition to anti-CD3 antibodies at optimal concentration, the costimulation provided by hTgCS or hTgCSgp alone was partial, more so in $hTgCS_0$-immunized mice than in hTgCS-immunized mice. Notably, at variance with CS-dependent costimulation, CD28-mediated costimulation induced the proliferation of $CD4^+$ T cells not only from hTg-immunized mice, but also from negative control animals (likely reflecting the novel sensitization of naïve T cells), even though to a smaller extent. However, in hTg-immunized mice, the costimulation afforded by the CS unit was additive (see the effect of hTgCS) or actively synergistic (see the effect of hTgCSgp) with CD28-mediated costimulation, particularly in $CD4^+$ T cells from hTgCS-immunized mice.

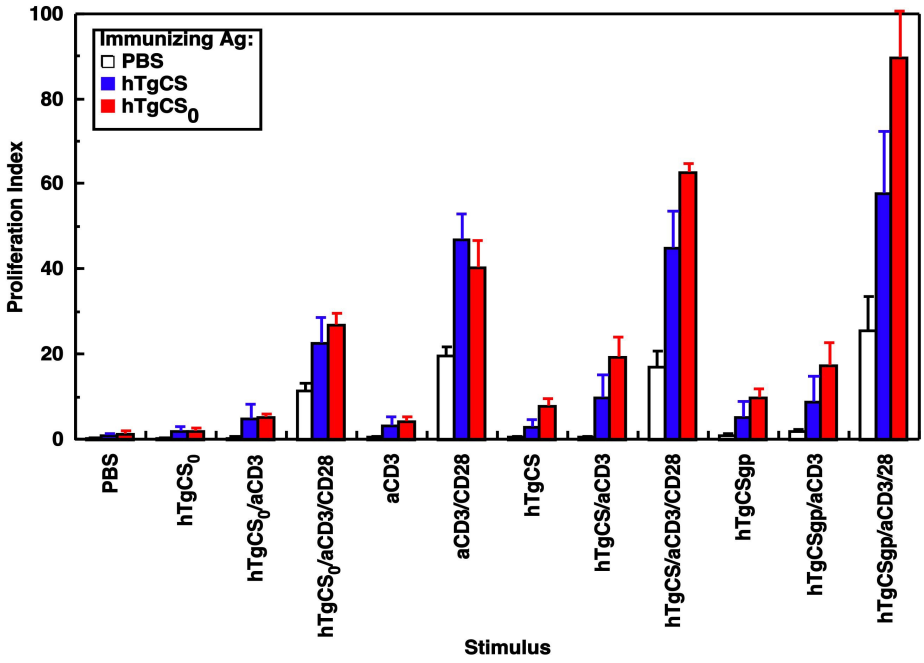

Fig 10. Secondary proliferative responses of $CD4^+$ T cells isolated from splenic lymphocytes from CBA/J mice immunized with $hTgCS_0$ or hTgCS and restimulated *in vitro* with $hTgCS_0$. hTgCS or hTgCSgp, with or without the addition of anti-CD3 and

anti-CD3/CD28 antibodies. Test antigen concentrations were: 50 μg/mL for hTgCS and $hTgCS_0$, 1.15 μg/mL for hTgCSgp. Results are expressed as means ± S.E.M.

4.6 Cytokine secretion patterns in secondary responses of isolated murine $CD4^+$ T cells reveal the costimulatory role of the CS unit of hTg and its variable impact on the differentiation and phenotypic expression of distinct T_H cell subsets

IL-2 secretion (Figure 11) was elicited in positive controls by the combined addition of anti-CD3 and anti-CD28 antibodies, as expected. The comparable levels of the IL-2 secretory responses of negative control mice and hTg-immunized mice suggests that the T cells responsible for the observed IL-2 production were mostly naïve T cells, in keeping with the role played by IL-2 production in the early phase of T_H0 differentiation. Accordingly, the study of IL-2 production might permit us to gain some insight into the role played by the CS unit of hTg in the *de novo* sensitization of $CD4^+$ T cells, even within the context of secondary proliferative responses. The CS unit, both as a mojety of hTgCS and in isolated form (hTgCSgp), was unable to elicit IL-2 secretion, either by itself or in combination with anti-CD3 antibodies. However, the addition of the hTgCS or, to an even larger extent, the addition of hTgCSgp, to anti-CD3/CD28-stimulated $CD4^+$ T cells was accompanied by marked increases of IL-2 secretion. This is reminiscent of what was reported by Galandrini et al. in 1993 for the interaction of hyaluronic acid (HA) with CD44 and suggests that the costimulatory effects of the CS unit on IL-2 production were accessory with respect to the costimulatory action of CD28 and someway dependent upon it (Galandrini *et al.*, 1993). This, in turn, may imply that also the effects of the CS unit on $CD4^+$ T cell proliferation described above (see paragraph 4.5) were complex and included: a) effects upon the proliferation of *de novo* sensitized hTg-specific naïve $CD4^+$ T cells, which the CS unit was able to exert only conditionally, by enhancing the effects of CD28-mediated costimulation; b) effects upon the re-expansion of hTg-specific, effector memory $CD4^+$ T cells, which the CS unit was able to exert in a CD28-independent way, by activating the expression of typical phenotypic markers of definite T_H cell subsets, starting from the secretion of cytokines caoable of sustaining such re-expansion. The data that follow will show how, in turn, some of these effects were also independent of the stimulation of TCR, like the secretion of IFN-γ and TNF-α by T_H1 cells, whereas other effects were dependent upon it, like the secretion of IL-17 by T_H17 cells.

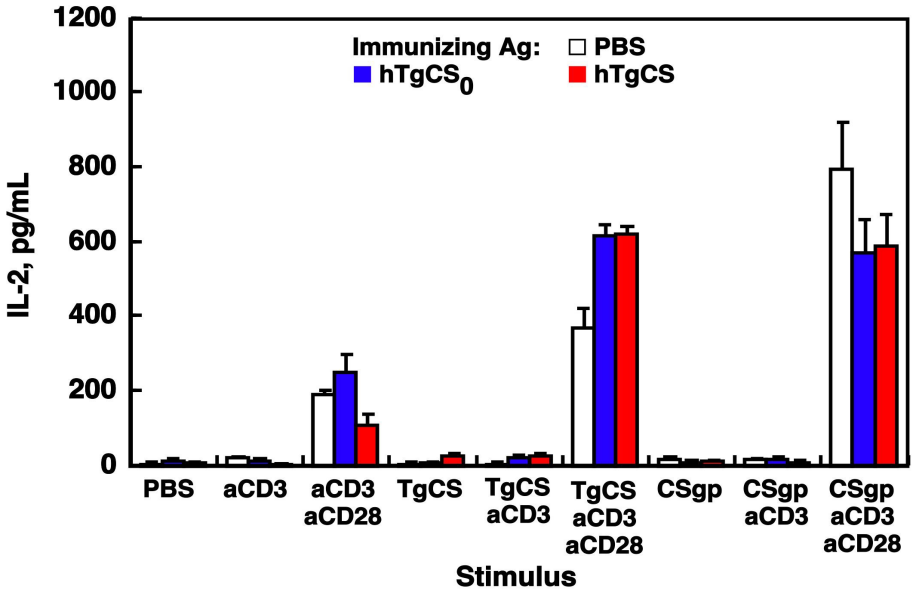

Fig 11. IL-2 secretion in the media of secondary proliferative responses of $CD4^+$ T cells isolated from splenic lymphocytes from CBA/J mice immunized with $hTgCS_0$ or hTgCS and restimulated *in vitro* with $hTgCS_0$. hTgCS or hTgCSgp, with or without anti-CD3 and anti-CD3/CD28 antibodies. Test antigen concentrations were: 50 μg/mL for hTgCS and $hTgCS_0$, 1.15 μg/mL for hTgCSgp. Results are expressed as means ± S.E.M.

Stimulation with the CS unit, either in the form of hTgCS or hTgCSgp, elicited significant IFN-γ (Figure 12) secretion in $CD4^+$ T cells from hTgCS-immunized mice, and minor secretory responses in $hTgCS_0$-immunized animals. The addition of anti-CD3 antibodies to hTgCS or hTgCSgp had statistically non-significant additive effects. Instead, costimulation with anti-CD3/CD28 antibodies, in addition to the CS unit, elicited significant, proportional increases of IFN-γ secretion by $CD4^+$ T cells not only from immunized mice, but also from negative control mice. Notably, CD28-mediated costimulation appeared to be conditional for the expression of the IFN-γ secretory ability by $CD4^+$ T cells from control mice, likely reflecting the T_H1 differentiation of *de novo* sensitized hTg-reactive $CD4^+$ T cells. Instead, hTgCS and, to a larger extent, hTgCSgp exhibited the ability to elicit significant IFN-γ secretion by the $CD4^+$ T cells of hTgCS-immunized mice and minor responses by the $CD4^+$ T cells of $hTgCS_0$-immunized mice, independent of any adjunct stimulation, even of the TCR. Also, the addition of the CS unit, particularly in the form of hTgCSgp, effectively synergized CD28-mediated costimulation of newly sensitized $CD4^+$ T cells from negative control animals. Thus, the large increases of IFN-γ secretion observed with $CD4^+$ T cells from $hTgCS_0$- and, to a larger extent, from hTgCS-immunized mice, likely reflected a combination of: 1) the independent action of the CS unit on the expression of

the IFN-γ secretory ability by hTg-reactive effector TH1 cells (especially those more effectively sensitized via the immunization with hTgCS); 2) the synergysm between CS-dependent and CD28-mediated costimulation in the *de novo* sensitization of naïve hTg-reactive $CD4^+$ T cells.

The data reported above seem to indicate that IFN-γ secretion by naïve $CD4^+$ T cells was strictly dependent, like proliferation, upon CD28-mediated costimulation, which could be enhanced, but not substituted for by CS-dependent costimulation. In contrast, CS-dependent stimulation was absollutely required and sufficient for hTg-reactive effector TH1 cells to express their IFN-γ secretory ability (a typical marker of the TH1 differentiated phenotype), even in the absence of anti-CD3-mediated TCR stimulation. This is reminiscent of what was observed in experimental autoimmune encephalomyelitis (Guan *et al.*, 2011).

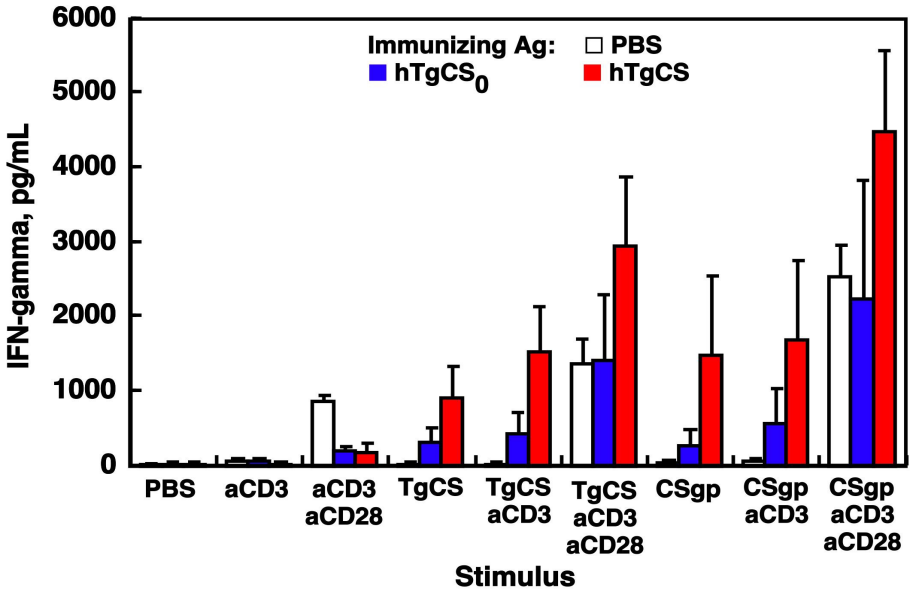

Fig 12. IFN-γ secretion in the media of secondary proliferative responses of $CD4^+$ T cells isolated from splenic lymphocytes from CBA/J mice immunized with $hTgCS_0$ or hTgCS and restimulated *in vitro* with $hTgCS_0$. hTgCS or hTgCSgp, with or without anti-CD3 and anti-CD3/CD28 antibodies. Test antigen concentrations were: 50 μg/mL for hTgCS and $hTgCS_0$, 1.15 μg/mL for hTgCSgp. Results are expressed as means ± S.E.M.

TNF-α secretion in response to the CS unit, alone or in combination

with anti-CD3/CD28 antibodies, conformed to a similar overall pattern as just described for IFN-γ. However, the secretory activity of naïve $CD4^+$ T cells from control mice, whose *de novo* sensitization appeared to be dependent on CD28-mediated costimulation, was minor and the addition of the CS unit did not appear to synergize appreciably the effects of CD28-mediated costimulation on TNF-α secretion in these cells (Figure 13).

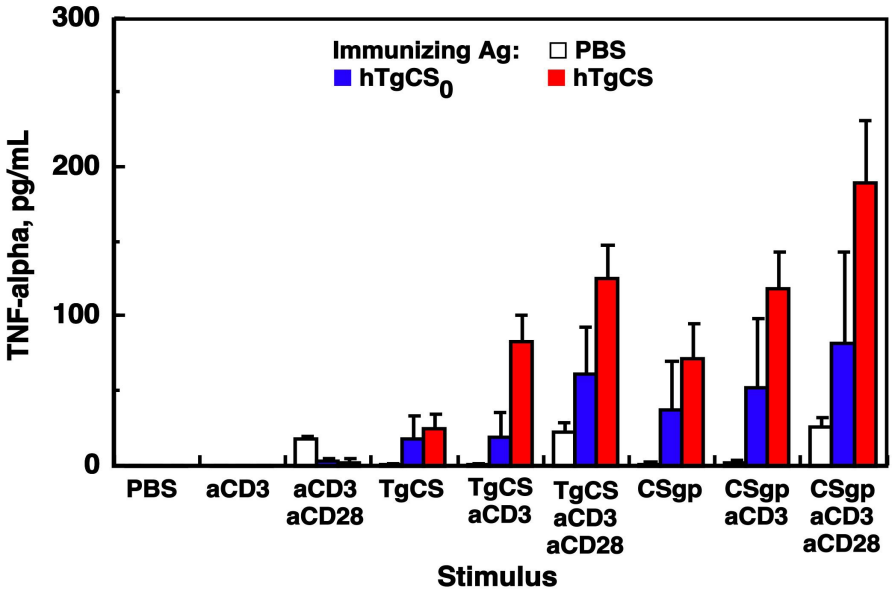

Fig 13. TNF-α secretion in the media of secondary proliferative responses of $CD4^+$ T cells isolated from splenic lymphocytes from CBA/J mice immunized with $hTgCS_0$ or hTgCS and restimulated *in vitro* with $hTgCS_0$. hTgCS or hTgCSgp, with or without anti-CD3 and anti-CD3/CD28 antibodies. Test antigen concentrations were: 50 μg/mL for hTgCS and $hTgCS_0$, 1.15 μg/mL for hTgCSgp. Results are expressed as means ± S.E.M.

The patterns of IL-17 (Figure 14) secretory by isolated $CD4^+$ T cells were peculiar in that: a) the combined use of anti-CD3/CD28 antibodies did not elicit any detectable secretion of IL-17, not even by naïve $CD4^+$ T cells from negative control animals; significant, although minor amounts of IL-17 were secreted by the $CD4^+$ T cells of the control group of mice only in response to the combined administration of hTgCS or hTgCSgp and anti-CD3/CD28 antibodies; b) with $CD4^+$ T cells from hTgCS-immunized mice, maximal IL-17 secretion was observed upon combined stimulation with hTgCS or hTgCSgp and anti-CD3 antibodies, independent of CD28-mediated costimulation; c) like IL-17 secretion by $CD4^+$ T cells from control mice, significant IL-17 secretion also by poorly sensitized $CD4^+$ T cells from $hTgCS_0$-immunized animals required the simultaneous costimulation with anti-CD28 antibodies and CS, in

adjunct to TCR stimulation with anti-CD3 antibodies. This seems to indicate that hTg-reactive, effector $CD4^+$ T cells effectively sensitized by hTgCS did not have a requirement for CD28-mediated costimulation, other than restimulation of their TCRs. This is in keeping with the observation that serglycins, small proteoglycans stored in secretory granules of hematopoietic cells, activated the CD3-dependent release of cytokines and proteases from CD44-positive cytotoxic lymphocytic clones (Toyama-Sorimachi *et al.*, 1995) by interacting with CD44 through their chondroitin 4-sulfate and 6-sulfate side chains (Toyama-Sorimachi *et al.*, 1997). Unlike effector $CD4^+$ T cells, hTg-reactive $CD4^+$ T cells in the naïve status or less effectively sensitized by hTg-CS_0 had complex costimulation requirements for the expression of the T_H17 phenotype (marked by IL-17 secretion), which included the simultaneous costimulation by CS and CD28 ligands. On one hand, with these naïve T cells, CS-dependent costimulation seemed to be strictly required, to the same extent as and in combination with CD28-mediated costimulation, in order to prompt the T_H17 differentiation of hTg-specific naïve $CD4^+$ T cells. On the other hand, the expression of the T_H17 differentiated phenotype by effector memory $CD4^+$ T cells had completely different requirements for costimulation with respect to naïve cells, being: a) rigorously CS-dependent, but completely independent of CD28-mediated costimulation; b) rigorously conditioned to the simultaneous TCR engagement.

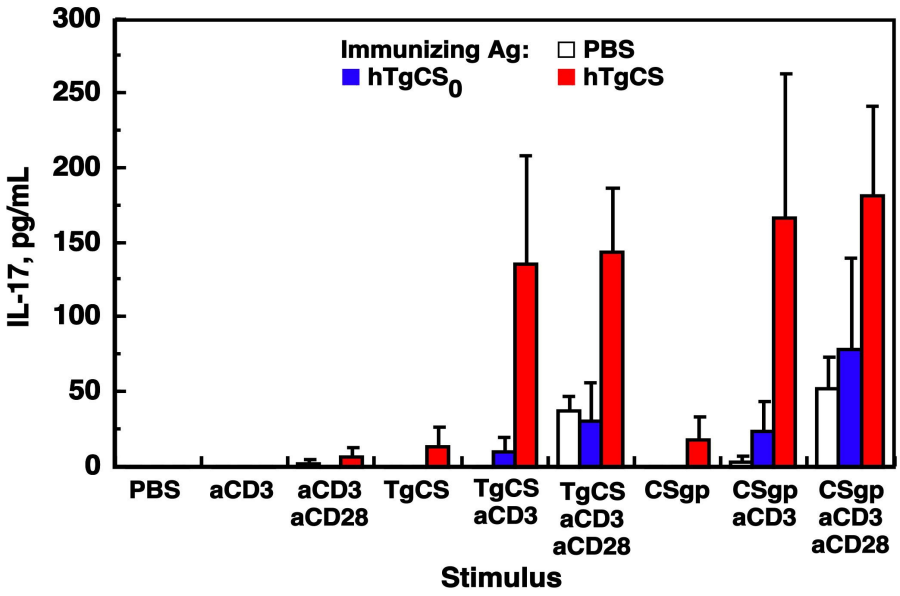

Fig 14. IL-17 secretion in the media of secondary proliferative responses of $CD4^+$ T cells isolated from splenic lymphocytes from CBA/J mice immunized with $hTgCS_0$ or hTgCS and restimulated *in vitro* with $hTgCS_0$. hTgCS or hTgCSgp, with or without anti-CD3 and anti-CD3/CD28 antibodies. Test antigen concentrations were: 50 μg/mL for hTgCS and $hTgCS_0$, 1.15 μg/mL for hTgCSgp. Results are expressed as means ± S.E.M.

Finally, considerable IL-6 (Figure 15) secretion was evoked by hTgCS and, to a larger extent, by hTgCSgp in $CD4^+$ T cells isolated from the spleens of mice immunized with hTgCS. Proportionally smaller responses were elicited by the same additives in $CD4^+$ T cells from $hTgCS_0$-immunized mice. Such responses were totally independent of concomitant CD3- and CD28-mediated stimulation and costimulation, respectively. No IL-6 secretion at all was incited either by the CS unit, or by anti-CD3/CD28 antibodies in $CD4^+$ T cells of control mice.

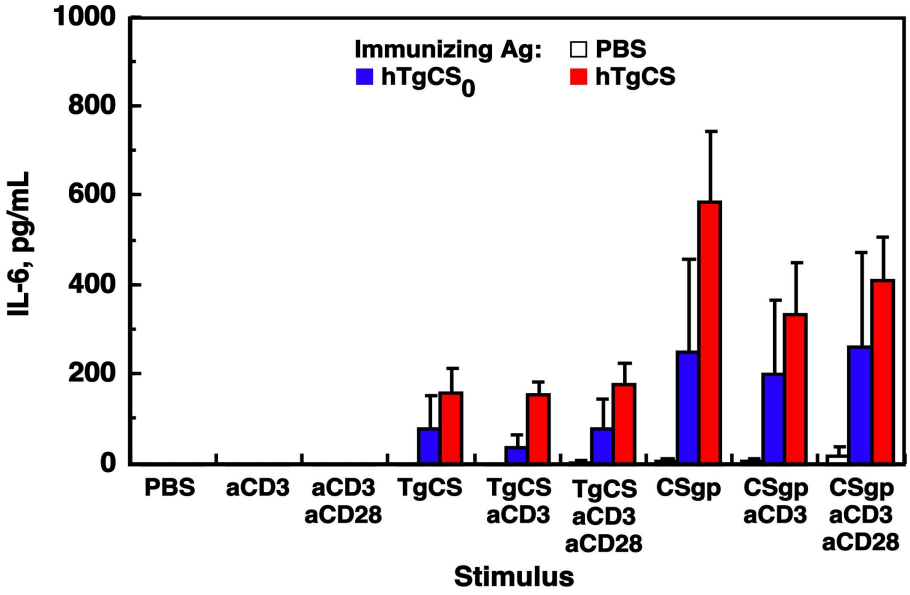

Fig 15. IL-6 secretion in the media of secondary proliferative responses of $CD4^+$ T cells isolated from splenic lymphocytes from CBA/J mice immunized with $hTgCS_0$ or hTgCS and restimulated *in vitro* with $hTgCS_0$. hTgCS or hTgCSgp, with or without anti-CD3 and anti-CD3/CD28 antibodies. Test antigen concentrations were: 50 μg/mL for hTgCS and $hTgCS_0$, 1.15 μg/mL for hTgCSgp. Results are expressed as means ± S.E.M.

4.7 EAT induction in CBA/J(H-2^k) and SJL(H-2^s) mice is suppressed by PHCCC, a selective inhibitor of T_H17 cell differentiation

The data reported above indicated that EAT induction in CBA/J(H-2^k) mice depend prevailingly or solely on the hTgCS fraction of hTg. In particular, the CS oligosaccharide unit of hTgCS played a pivotal role not only in the expression of the differentiated phenotype by effector T_H1 and T_H17 cells, but also in the sensitization of hTg-specific $CD4^+$ T cells into armed effector T_H cells and in the polarization of their differentiation in T_H cells of both kinds. In particular, the costimulatory action of the CS unit was shown to be accessory and synergistic with respect to CD28-mediated costimulation of the *de novo*

sensitization of TH1 cells, while being capable of stimulating the phenotypic expression of differentiated effector TH1 cells in a CD28-independent manner. Instead, CS-dependent costimulation turned out to be as necessary as CD28-mediated costimulation for the differentiation of TH17 cells, while its ability to stimulate the phenotypic expression of differentiated effector TH17 cells was fully CD28-independent, yet strictly dependent upon TCR stimulation by anti-CD3 antibodies. Therefore, it appears that the CS unit conferred upon hTg a quantitative immunopathogenic advantage, as for the differentiation of TH1 effector cells, together with a unique qualitative advantage, as far as the differentiation of Th17 effector cells was concerned. Thus, it may be expected that a selective strategy for inhibiting the differentiation of autoreactive, hTg-specific TH17 cells might suffice to counteract most of the enhancement of EAT induction brought about by hTgCS. To this aim, we adopted an experimental strategy based upon the usage of *N*-phenyl-7-(hydroxyimino)-cyclopropa[b]chromen-1a-carboxamide (PHCCC). Four groups of mice were immunized, respectively, with PBS, $hTgCS_0$, hTgCS and hTgCS plus PHCCC. PHCCC is a selective agonist of metabotropic glutamate receptor 4 (mGluR4) enhancer, which was proven to suppress highly effectively the development of experimental autoimmune encephalomyelitis (EAE) in mice, by negatively affecting the TH17 differentiation of naïve antigen-specific T cells (Nicoletti *et al.,* 2007; Fallarino *et al.*, 2010).

We performed two experiments of this kind in two strains of genetically susceptible mice, namely CBA/J(H-2^k) and SJL(H-2^s) mice. Initial exploratory experiments were performed not only with fractionated $hTgCS_0$ and hTgCS, but also with unfractionated hTg (hTg), which contains both. The daily administration of PHCCC to mice of bith strains immunized with hTg for the entire period preceding sacrifice was associated with the development of a very mild autoimmune thyroiditis, in comparison with the more severe disease which developed in mice immunized with hTg alone. Also the histological disease scores recorded in experimental animals immunized with hTgCS were distinctly higher than those recorded in mice immunized with hTgCS, in association with the treatment with PHCCC (Figure 16-17). In addition, the average histological scores detected in mice immunized with hTgCS plus PHCCC were similar to the average disease scores detected in mice immunized with $hTgCS_0$. These evidences indirectly confirmed the essential costimolatory role played by the CS oligosaccharide unit of hTg in the induction of the TH17 differentiation of hTg-specific $CD4^+$ T cells, and the strict requirement for such subset of autoreactive TH cells for the development of EAT.

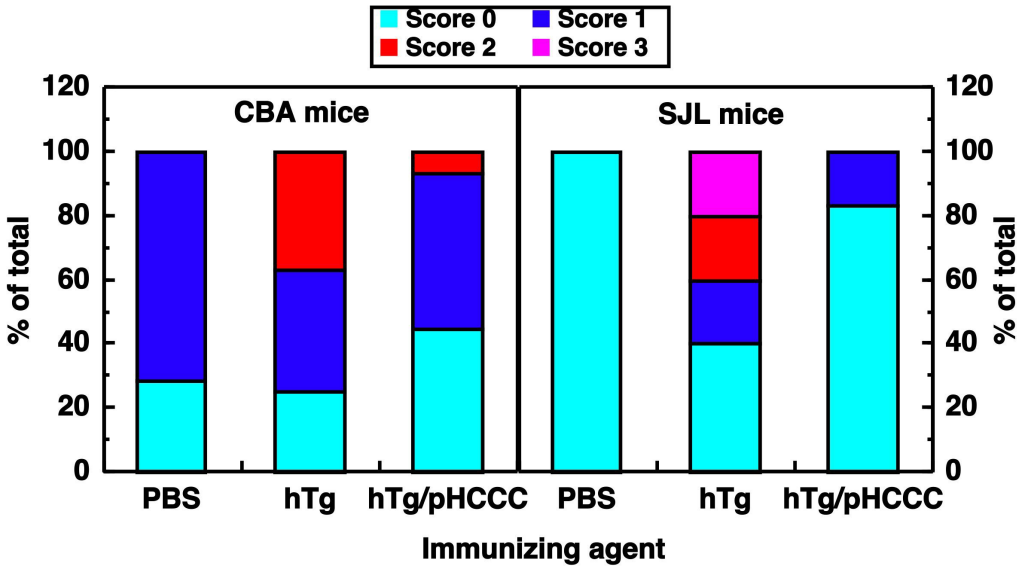

Fig 16. Histological EAT scores, indicating the extent of mononuclear cell infiltration of the thyroid glands of CBA/J(H-2^k) and SJL(H-2^s) mice immunized with unfractionated hTg, with or without added treatment with PHCCC. The EAT score was calculated in accordance with Verginis *et al.* (2002). Negative control mice were immunized with PBS only.

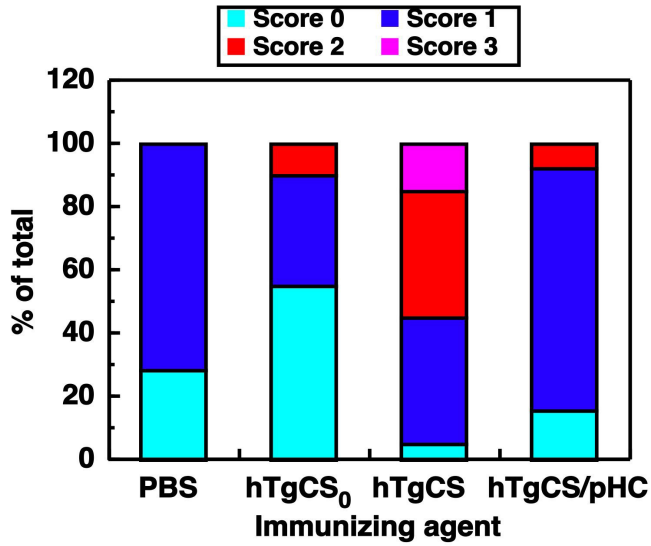

Fig 17. Histological EAT score, indicating the extent of mononuclear cell infiltration of the thyroid glands of CBA/J(H-2^k) mice immunized with hTgCS$_0$ or hTgCS, with or without added treatment with PHCCC. The EAT score was calculated in accordance with Verginis *et al.* (2002). Negative control mice were immunized with PBS only.

Figure 18 shows typical aspects of thyroid infiltration with mononuclear cells, distinctly more marked in both CBA/J(H-2^k) mice (*score 2*) and SJL(H-2^s) mice (*score 3*) immunized with hTg, compared with mice immunized with hTg with added PHCCC treatment (*score 1*). Figure 19, instead, shows the contrast between the milder and more severe thyroiditis which developed in CBA/J mice after immunization with hTgCS with and without added PHCCC treatment, respectively, and the similarity between the milder diseases ensuing either the immunization with $hTgCS_0$, or with hTgCS plus PHCCC treartment.

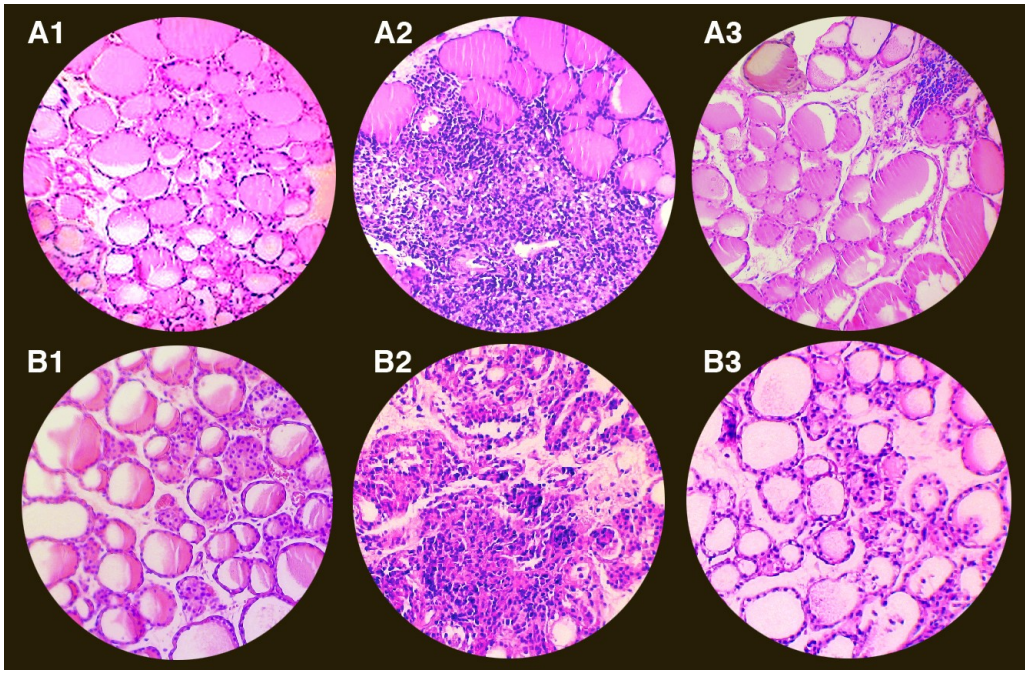

Fig. 18. Representative aspects of thyroid mononuclear infiltration in EAT. The thyroid section shown in A2 was obtained from a SJL(H-2^s) mouse immunized with hTg and was assigned *score 4*, while the section shown in A3 was from a SJL(H-2^s) mouse immunized with hTg and also treated with PHCCC (*score 1*). A normally appearing thyroid section from a control animal is shown in A1 (*score 0*). The thyroid section shown in B2 was obtained from a CBA/J(H-2^k) mouse immunized with hTg (*score 3*), while the section in B3 was from a CBA/J(H-2^k) mouse immunized with hTg, which also received PHCCC treatment (*score 1*), normally appearing thyroid section from a control animal is shown in B1 (*score 0*).

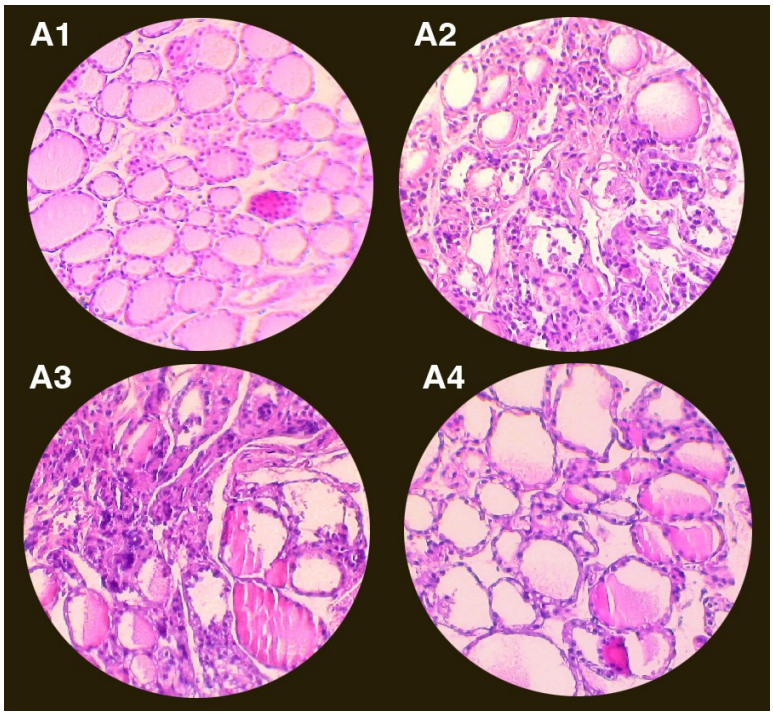

Fig. 19. Representative aspects of thyroid mononuclear infiltration in EAT. The thyroid section shown in A2 was obtained from a CBA/J(H-2^k) mouse immunized with $hTgCS_0$ (*score 1*), while the section in A3 was from a CBA/J(H-2^k) mouse immunized with hTgCS. Instead, the section in A4 was from a CBA/J(H-2^k) mouse immunized with hTgCS, which also received PHCCC treatment (*score 1*). A normally appearing thyroid section from a control animal is shown in A1 (*score 0*).

The decreased severity of thyroid tissue infiltration and destruction detected by histology in CBA/J mice receiving pHCCC treatment throughout immunization with hTg was reflected in the lower serum concentrations of

3,5,3',5'-tetraiodothyronine (T4) and 3,5,3'-triiodothyronine (T3). These were significantly higher in mice immunized with hTg, in comparison both with control mice and with mice immunized with hTg plus PHCCC (Figure 20).

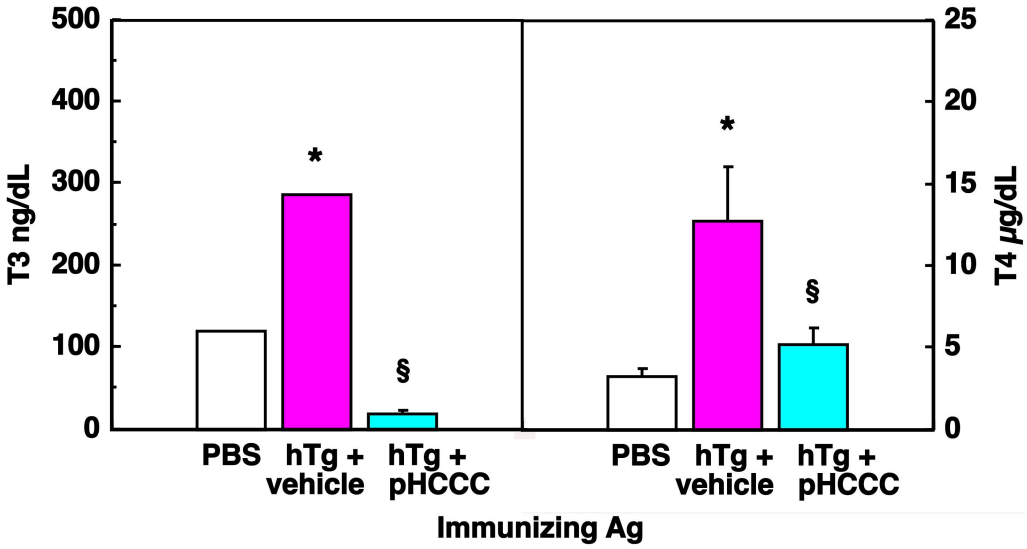

Fig. 20. Serum concentrations of T3 (3,5,5'-triiodothyronine) and T4 (thyroxine) in the sera of CBA/J(H-2^k) mice immunized with hTg and hTg plus PHCCC. Results are expressed as means ± S.D. * indicates the statistical significance between hTg and each of the control and hTg plus PHCCC condition (*p <0,05*).

4.8 Treatment with PHCCC does not abolish secondary proliferation of CD4$^+$ T cells from SJL mice *in vitro* in response to hTgCS$_0$ and hTgCS

Secondary assays of splenocyte proliferation *in vitro* were performed with the splenocytes of both susceptible mice strains immunized with PBS, hTg or hTgCS *plus* or *minus* PHCCC and restimulated in vitro, after sacrifice, with the same antigens. Figure 21 shows the results obtained in SJL mice immunized with hTg. Similar results were obtained also in CBA/J mice (data not shown). Cpm counts (reflecting the incorporation of [^{3}H]-thymidine) reached high levels in both groups of mice immunized with hTg and hTg plus PHCCC, in comparison with negative control mice immunized with PBS only. This result indicated that both groups of mice immunized with hTg and hTg plus PHCCC were effectively sensitized against hTg. Nonetheless, mice immunized with hTg *plus* PHCCC did not develop thyroid organ disease, as detected by the histological scoring of EAT (Figure 19).

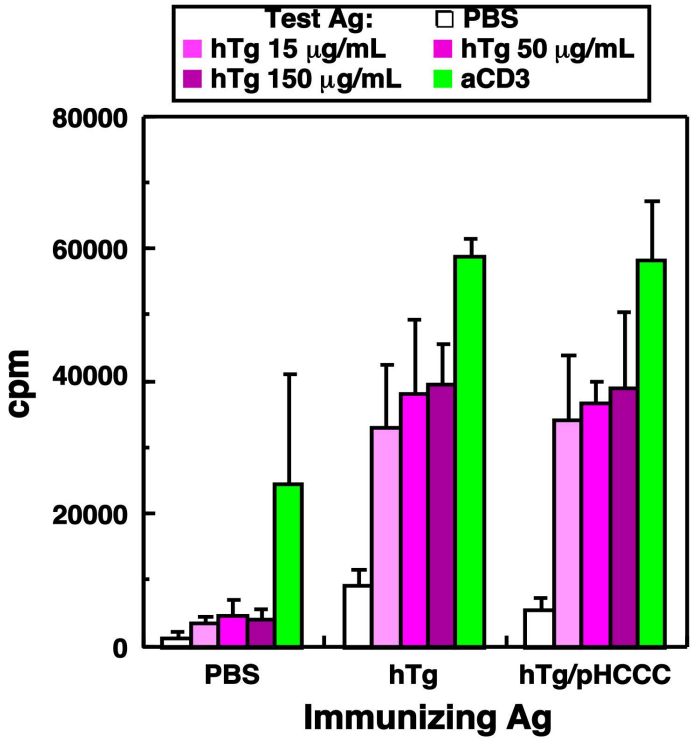

Fig. 21 Proliferative responses of splenic lymphocytes from CBA/J(H-2k) mice immunized with hTg, without or with added PHCCC treatment, and restimulated *in vitro* with hTg. Mice were immunized and PHCCC treatment was conducted as detailed in the Methods. Negative controls received PBS only. On day 30, mice were sacrificed, spleens removed and splenocytes were restimulated with the indicated doses of hTg. Anti-CD3 antibodies were used as positive controls. Results are expressed as means ± S.D.

4.9 Cytokine secretion patterns in secondary proliferations of splenic lymphocytes from mice immunized with hTg with added PHCCC treatment reveal a selective impairment of Th17 cell differentiation

In order to evaluate the effects of the treatment with PHCCC on the polarization of TH cell differentiation, the concentrations of IL-2, INFγ, IL-6 and IL-6, IL-17 and were measured in the supernatants of the secondary proliferations of splenic lymphocytes from CBA/J(H-2^k) mice immunized with hTg, with or without added treatment with PHCCC (Figure 22). As shown in figure 18,, IL-2 and IFN-γ were produced at comparably high levels in the supernatants of splenocytes from CBA/J mice immunized with hTg, both with and without the addition of PHCCC treatment, whereas they were lacking in negative control mice. In contrast, high levels of IL-6 and IL-17 were detected only in the supernatants of splenocytes from mice immunized with hTg, in the absence of PHCCC treatment, whereas significant decreases in the

concentrations of this cytokines were detected in the supernatants of splenocytes from both control mice immunized with PBS and mice immunized with hTg with added PHCCC treatment. Thus, the selective inhibition of differentiation of hTg-specific TH17 effector cells by PHCCC was sufficient to cause the effective suppression of EAT, even though the differentiation and phenotypiic expression of TH1 effector cells did not seem to be affected. This result outlines the determinant role of the participation of effector TH17 cells to the autoimmune response towards the thyroid gland, for the establishment of thyroid organ disease. These results represent also an indirect confirmation of the causative role of the selective enhancement of Th17 differentiation and phenotypic expression determined by the CS unit of hTg in the improved induction of EAT by hTgCS, as observed in the assays of cytokine production by $CD4^+$ T cells isolated from the spleens of CBA/J(H-2^k) mice immunized and restimulated with hTgCS, in comparison with $hTgCS_0$ (Figures 22).

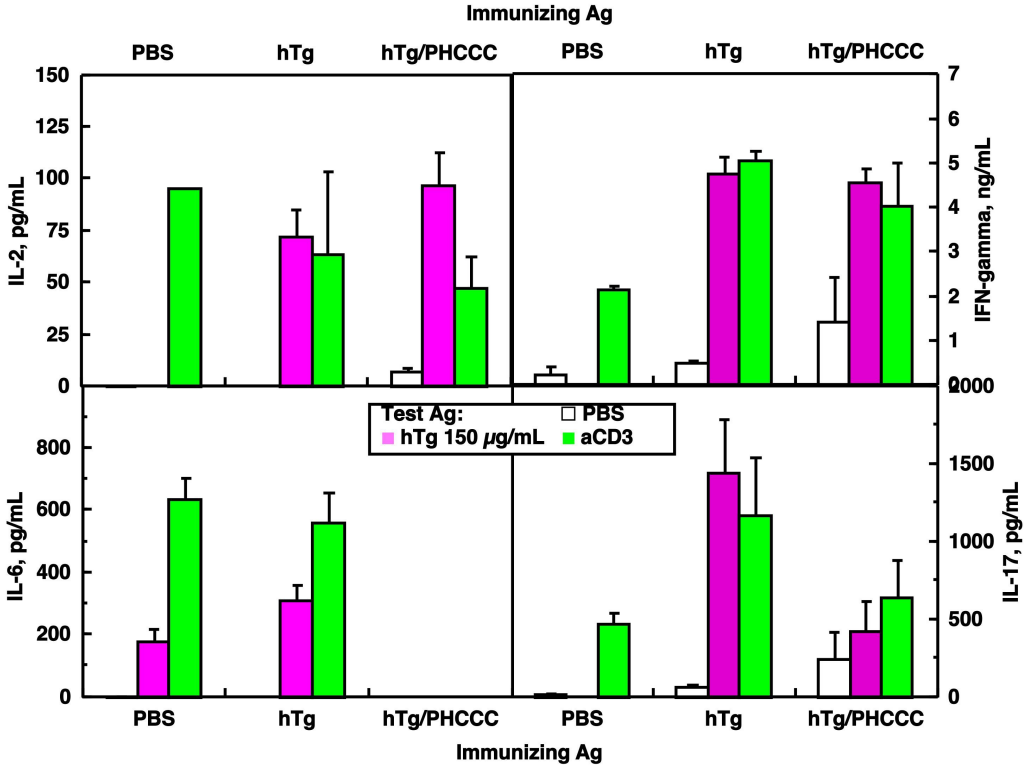

Fig 22. Assays of cytokine secretion in the supernatants of secondary proliferative responses by splenocytes of CBA/J mice immunized with hTg, without or with added PHCCC treatment. The levels of IL-2, IL-6, IL-17, INF-γ were mesured in the supernatants of secondary proliferative responses of splenocytes from CBA mice immunized with hTg plus the sole vehicle (sesame oil) of PHCCC administration, or hTg plus added PHCCC treatment, after restimulation with hTg at the dose of 150 µg/mL. Anti-CD3 antibodies were used as positive controls. Results are expressed as means ± S.D.

4.10 CD44 is the receptor of the CS oligosaccharide unit of hTg at the surface of murine $CD4^+$ T cells

As previusly indicate in the introduction, CD44 was showed to be implicated in the development of adaptive and innate immune responses. In addiction many evidences showed both that CS can bind CD44, and that this interaction can influence the CD44 functions on the adaptive and innate immune responses. To evaluate if CS unit of hTg effectivily, was bounded to CD44 at the surface of lymphocyte T $CD4^+$ we performed an SDS PAGE experiment. Lymphocytes T $CD4^+$ from mice immunized with hTgCS were stimulated *in vitro* with anti-CD28 plus anti-CD3 and incubated with biotinilated hTgCSgp glycopeptide linked to bifunctional p-Azidobenzoyl hydrazid photo-activable cross linker. After irradiation at 365 nm and wash procedure, biotinilated hTgCSgp glycopeptide linked to bifunctional p-Azidobenzoyl hydrazid photoactivable cross linker, resulted covalently linked to the receptor recognized at the surface of lymphocyte T $CD4^+$. Finally, cellular lysis procedure, samples were immunoprecipitated with anti biotin antibody and immunobloted with anti-biotin and anti-CD44. As shown in figure 23. The immunoblot showed that biotinilated hTgCSgp glycopeptide linked to bifunctional p-Azidobenzoyl hydrazid photoactivable cross linker, was bounded to CD44 at the surface of lymphocyte T $CD4^+$.

This result was confirmed by confocal microscopy experiment effetuated on the same cells which were used for the immunoblot analisis. As showed in figure 24, the colocalization between hTgCSgp glycopeptide biotinilated and linked with bifunctional and photo-activable p-Azidobenzoyl hydrazid cross linker with CD44 recertor was an other prove af this interaction.

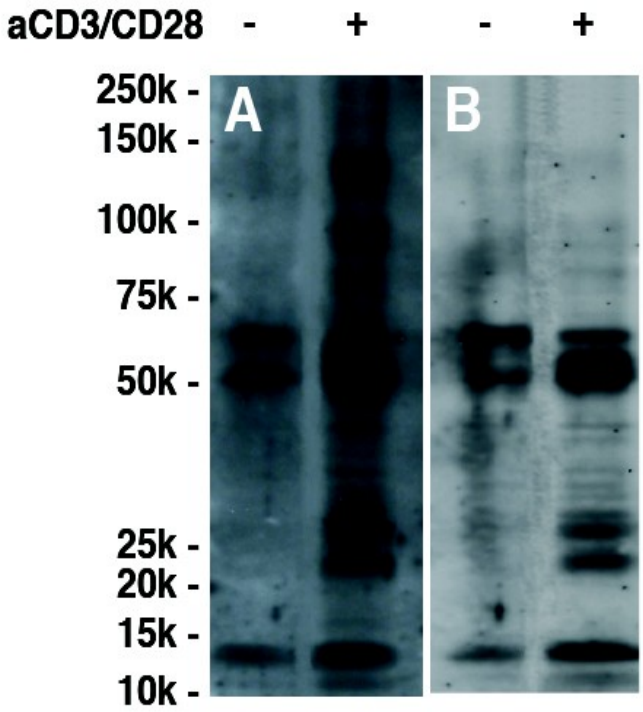

Fig 23. Immunoblots of lysates of CD4$^+$ T cells isolated from the spleens of CBA/J(H-2^k) mice, incubated with the biotinylated hTgCSgp glycopeptide covalently linked to the bifunctional cross-linker *p*-azidobenzoyl hydrazide and immunoprecipitated with anti-biotin or anti-CD44 antibodie. Lanes 1-3: unstimulated CD4$^+$ T cells. Lanes 2-4: CD4$^+$ T cells stimulated with aCD3/aCD8 antibodies. All samples were incubated with the biotinylated hTgCSgp glycopeptide covalently linked to the photoactivatable, bifunctional cross-linker *p*-azidobenzoyl hydrazide.

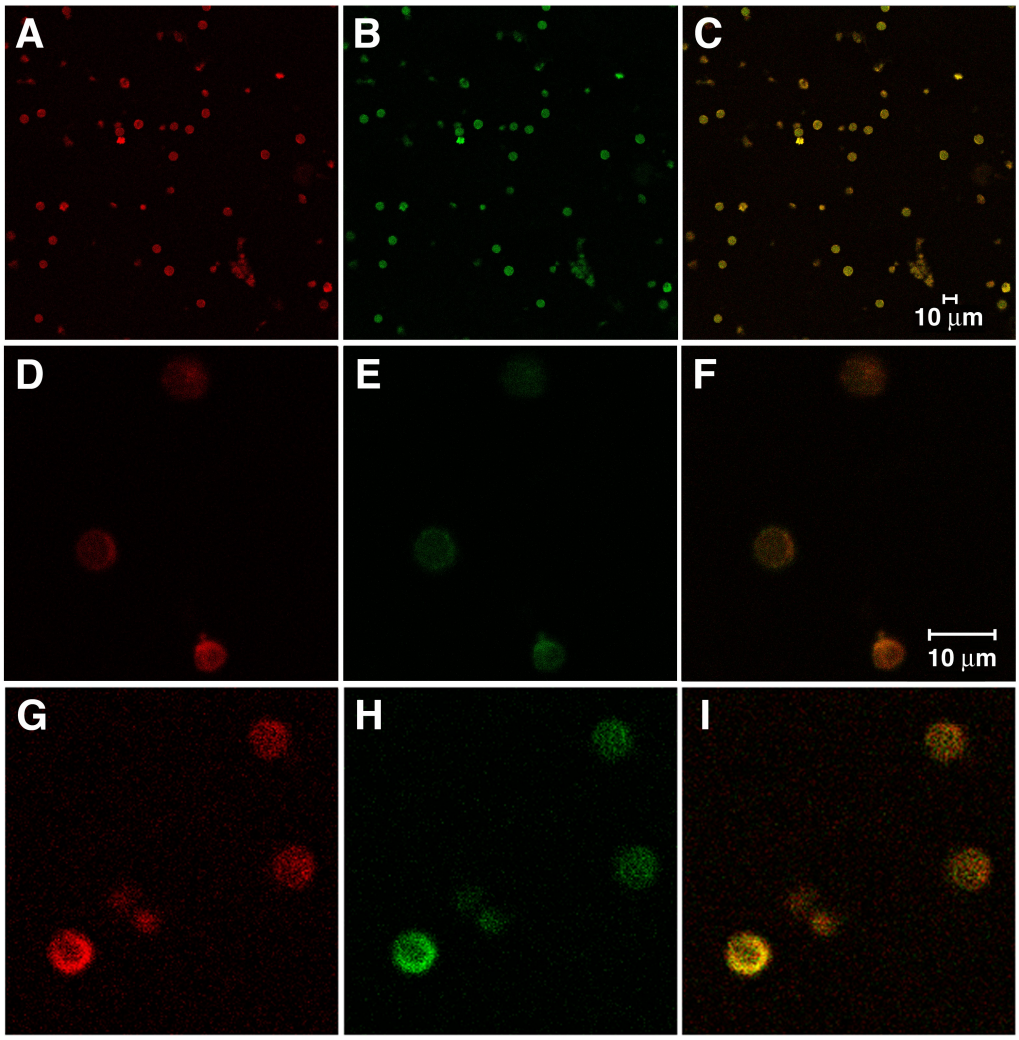

Fig 24. Confocal microscopy of CD4$^+$ cells isolated from the spleens of CBA/J(H-2^k) mice and incubated with the biotinylated hTgCSgp glycopeptide covalently linked to the photoactivatable, bifunctional cross-linker *p*-azidobenzoyl hydrazide. A, D, G, CD4$^+$ cells exposed to anti-biotin-PE; B, E, H, CD4$^+$ cells exposed to anti-CD44-FITC; C, F, I, image merge.

5 DISCUSSION

The data presented in this work document that the presence of a chondroitin 6-sulphate (CS) oligosaccharide unit linked to Ser2730 of human thyroglobulin (hTg) (Conte *et al.*, 2006) was associated with a marked increase of the immunopathogenicity of hTg in a model of murine experimental autoimmune thyroiditis (EAT) in CBA/J(H-2^k) and SJL(H-2^s) mice. When used to immunize CBA/J mice, CS-containing hTg (hTgCS) markedly enhanced, in comparison with CS-devoid $hTgCS_0$, the infiltration of murine thyroids by mononuclear cells, the leak of thyroid hormones from thyroid epithelial cells into the bloodstream and the secondary proliferative and secretory responses of splenocytes and isolated $CD4^+$ T cells to both hTgCS and $hTgCS_0$ *in vitro*. These results indicated that the CS oligosaccharide unit of hTg is capable of enhancing markedly the primary sensitization of murine T cells to peptide epitopes shared between hTgCS and $hTgCS_0$ and conserved between human and murine Tg. Moreover, the cytokine secretion patterns obtained with isolated murine $CD4^+$ T cells indicated that the CS unit, both as a part of hTgCS and in the form of a purified, CS-containing nonapeptide isolated to homogeneity from hTgCS (hTgCSgp), was capable of enhancing the differentiation of Tg-reactive T_H1 and T_H17 cells. Furthermore, the response patterns of murine $CD4^+$ T cells to hTgCS and hTgCSgp, also in relation with the responses to anti-CD3 and anti-CD28 antibodies, indicated that the CS unit of hTg did not behave as a pathogenic epitope in itself, but rather as an added co-stimulus for the differentiation of Tg-specific T_H1 and T_H17 cells, thereby promoting strongly thyroid disease. Finally, two-step cross-linking experiments and confocal microscopy of murine $CD4^+$ T cells exposed to the purified hTgCS glycopeptide indicated that the CS oligosaccharide unit of hTg interacted specifically with CD44 of murine $CD4^+$ T cells.

The above observations invite a number of comments:

1.

EAT has been known so far for being characterized by low-grade, focal mononuclear infiltration of thyroid glands, even in genetically susceptible murine strains. Investigators working in the field were used to observe even lower-grade mononuclear cell infiltration into the thyroids of experimental animals, when human Tg (hTg) was used as the immunizing agent, instead of murine Tg (mTg), even though it is generally accepted that EAT results from the recognition by murine autoreactive T cells of epitopes which are conserved between mTg and hTg. We have shown previously that hTg is regularly composed of a mixture of hTgCS0 and hTgCS, in broadly variable proportions among different individuals (Conte *et al.*, 2006). In the light of the data here reported, it appears that the induction of autoimmune thyroid disease in

experimental animals depends prevailingly or solely on the hTgCS fraction of hTg. Because investigators in the field have been using so far unfractionated hTg for the purpose of inducing EAT, it appears that the variable severity of EAT observed in many studies was deeply influenced by the composition of the particular hTg preparations used, not only with regard to the percent abundance of hTgCS, but possibly also to the average chain length of the chondroitin 6-sulfate chain linked to each hTg subunit and to the relative proportion of heterodimeric and homodimeric hTgCS.

2.

The data presented here highlight the essential contribution of the TH17 polarization of the differentiation of effector T helper cells in the pathogenesis of EAT. They also indicate the inhibition o the differentiation of TH17 cells as the most probable mechanism of action of the immunomodulator drug PHCCC, as suggested by other observations collected in the murine models of experimental autoimmune encephalomyelitis (EAE) (Fallarino *et al.*, 2010) and experimental autoimmune neuritis (EAN) (Zappulla *et al.*, 2011).

The primary role of IL-17 and IL-17-secreting T cells was already appearing from the experimentl strategy employed for the induction of murine experimental granulomatous thyrodiditis (G-EAT), by the use of anti-IL-2R antibodies or IFN-γ, in addition to T cells sensitized *in vivo* and re-exposed *in vitro* to Tg (Braley-Müllen *et al.*, 1991). In fact, IFN-γ secreted by TH1 cells inhibits the IL-23-dependent expansion of TH17 cells, while IL-2 abolishes IL-17 production and induces IFN-γ production by the same cells. It was hypothesized that TH17 cells may participate in the autoimmune granulomatous reaction by supporting, on one hand, the inflammatory response, via the release of pro-inflammatory cytokines (TNF-α, IL-6 and IL-17) and, on the other hand, by recruiting neutrophile granulocytes and macrophages, via the release of chemokines and growth factors (CCL2/MCP-1, CCL3/MIP-1, G-CSF) (Chen et *al.*, 2005). TH17 are known as a source of pro-inflammatory cytokines IL-17 (IL-17A), IL-17F, IL-22, IL-6 e TNF-α, as well as GM-CSF. By binding to IL-17R (IL-17RA) receptors on fibroblasto, macrohages, astrocytes, epithelial and endothelial cells, IL-17 orchestrates tissue inflammation, inducing the expression of cytokines, such as IL-6 and TNF-α, chemokines, such as CCL2 (MCP-1, *macrophage chemotactic protein 1*) and CCL3 (MIP-1), and matrix metalloproteases, which mediate the infiltration and tissue destruction. IL-17 also induces the production of G-CSF, which promotes the development and the differentiation of neutrophile granulocytes and cooperates in T cell activation and DC maturation. The data reported in the present study document distinct associations between the increases in the production of IL-17 and IL-6 and the more efficient induction of EAT by hTgCS, both as an immunizing agent in primary sensitization *in vivo* and a restimulating antigen in secondary proliferative responses *in vitro*. A substantial contribution of the amelioration

of the responses of Tg-specific TH1 cells (both at the stage of differentiation and of activation of their phenotypic expression) by the use of chondroitinated form of hTg (hTgCS) to the development of EAT also emerged from the study of the patterns of cytokine secretion associated with the proliferative responses *in vitro* of T cells from the various experimental mice groups. Taken together, the data presented here indicate that an interplay between the responses of antigen-specific effector TH1 and TH17 cells seems to be necessary for the development of autoimmune thyroid disease, far beyond the experimental model of G-EAT, to include classical forms of chronic lymphocytic thyroiditis, such as EAT, and that the balance between these two components of the effector response might determine the severity and the progression rate of organ disease, by modulating the extent and intensity of those aspects of the immune response wich are more connected with the inflammatory reaction, such as the homing of antigen-specific armed effector T cells in target tissues and organs. The data presented permit a finer dissection between different modalities by which the CS unit exerts its enhancement of TH1- and TH17-mediated responses. The cooperation to the differentiation of antigen-specific naïve T cells into effector TH1 cells seemed to occur in the form of an accessory costimulation, mediated by and synergistic with CD28 costimulation. However, simultaneous CD28-mediated costimulation did not appear to be conditional for the (re)activation of effector memory TH1 cells by the CS unit. This may not surprise, as a relative independence from classical costimulation is a hallmark of effector memory T cells. However, it is of importance to notice that, in our experimental setting, the sensitivity to CS-dependent costimulation appeared to be expressed in a privileged and almost exclusive manner by T cells which had been sensitized *in vivo* in the presence of the CS unit (i.e., in mice immunized with hTgCS) and seemed to provide a pathway for the reactivation of these cells, which was largely TCR-independent. Such a peculiar feature of the differentiation of effector TH1 cells induced or modulated by the CS unit may provide a potent mechanistic rationale for the maintenance of the autoimmune reaction towards the thyroid, well beyond the episodical settings and circumstances in which the breaking of the tolerance to self antigens had become possible. On the other hand, the contribution of the CS unit to the differentiation of antigen-specific naïve T cells into effector TH17 cells, even though also synergistic with CD28-mediated costimulation, seemed to be also conditional for the latter, which was deprived of effect in the absence of simultaneous stimulation by CS. That is to say that the CS unit provided a unique, qualitative contribution to the enforcement of a TH17 component in the induction of EAT in hTgCS-immunized mice. As in the case of TH1 cells, simultaneous CD28-mediated costimulation did not appear to be required, instead, for the (re)activation of effector TH17 cells by the CS unit either. As with TH1 cells, the sensitivity of effector TH17 cells to CS-dependent (re)activation also was expressed almost exclusively by T cells from mice

immunized with hTgCS. However, at variance with TH1 cells, the activation of these cells was strictly dependent also upon the stimulation of TCR. Such a phenotypic feature of the TH17 cells differentiated in response to the *in vivo* sensitization to hTgCS may provide a mechanistic explanation for the pathogenetic role commonly attributed to TH17 cells in cytotoxc cell-mediated hypersensitivity and autoimmune reactions, that is, to set up local conditions within the context of target tissues and organs, i.e., in coincidence of the source of target antigens, for the extravasation, invasion, cell and tissue damaging actions of TH1, $CD8^+$ cytotoxic cells and activated macrophages.

In this perspective, the data reported in the present study, besides confirming the immunomodulatory properties of PHCCC and its potential utility in the control of autoimmune diseases, also shed light onto general pathogenetic mechanisms of autoimmunity, both within and outside the thyroid field, with special regard to the respective functions of TH1 and TH17 cells and their cooperation. The role of TH1 cells in autimmunity has been known for a long time. In murine models of autoimmune diseases, such as type 1 diabetes and EAE, $CD4^+$, IFN-γ-producing T cells were capable of inducing the disease in syngeneic hosts by adoptive transfer. Moreover, TH1-type cytokines are found in inflammatory lesions of the CNS in correspondence with acute disease phases and their disappearance is related with clinical healing. Experimental animals with defects of transcription factors STAT-4 and T-bet do not produce IFN-γ and are resistant to the induction of EAT. However, the observation that IFN-γ-defective experimental mice lacking IFN-γ, IFN-γR or transcription factor STAT-1 are even more prone to develop autoimmunity suggested the existence of a distinct, pathogenetically important T cell population, subsequently identified as TH17 cells (Bettelli *et al.*, 2007). The deficit of IL-17 or its treatment with antagonists of IL-17R prevent the development of murine adjuvant-induced arthritis, whilst IL-17-blocking antibodies prevent the development of EAE. The latter has a slower and milder course in IL-17-defective mice. Also IFN-γ protects mice from EAE. Moreover, murine defects of IL-27R are associated with severe autoimmune pathologies and increases in the production and homing to target tissues of TH17 cells, while, in the presence of STAT-1 defects, exhacerbations of EAE are observed, with increased recruitment of macrophages and neutrophils into the CNS. These observations support a critical pathogenetic role of TH17 cells in autoimmune diseases, considering that IL-23 promotes the expansion of differentiated TH17 cells, while IL-27 and IFN- suppress, respectively, the differentiation of TH17 cells and their IL-23-driven expansion, both in a STAT-1-dependent manner. It appears that both TH1 e TH17 cells are involved in the induction of autoimmunity, which is further supported by the observation that mice lacking TH1 cells (due to defects of STAT-4 and Tbet) are resistant to EAE, even though they have an excess of TH17 cells. In fact, T cells and cytokines of both types are found in murine CNS during the EAE climax and

develop from transgenic T cells, with myelin-reactive TCRs, capable of inducing EAE by adoptive transfer. The data presented here are fully concordant with this scenario and add further insight into the vision, according to which it is probable that effector TH17 cells are produced earlier in the autoimmune process and direct the early phase of acute, immune-driven inflammation, whereas TH1 cells prolong and perpetuate the autoimmune adaptive response (Bettelli *et al.*, 2007).

3.

The data discussed so far, concerning the critical importance of the CS unit of hTg in the development of EAT and the role of TH17 cells and their interplay with TH1 cells in mediating the actions of the CS unit are further supported and strengthened by our identification of CD44 as the receptor of the hTgCSgp glycopeptide on murine $CD4^+$ T cells. The importance of CD44 and the multiplicity of its roles within the context of innate and adaptive immune responses was extensively accounted for in the introductory part of this thesis. CD44 was repeatedly shown to act as a costimulatory molecule and an antiapoptotic effector. Our results indicate that CS exerts strong actions on the development of EAT by the means of a costimulatory influence upon the primary sensitization of Tg-specific $CD4^+$ T cells. Such costimulation was synergistic with CD28-mediated csotiumulation. However, while it was accessory in nature with regard to the differentiation of TH1 cells, it appeared to be conditional and indispensabile for TH17 differentiation. In both cases, the effects of CS appeared to be mediated by CD44. Our observations are consistent with recent work by several investigators, although in different contexts of autoimmune disease. Guan and coworkers showed that targeted deletion of CD44 attenuated myelin oligodendrocyte glycoprotein (MOG) peptide-induced EAE, through novel regulatory mechanisms affecting TH cell differentiation. CD44 deficiency on $CD4^+$ T cells conferred protection against EAE induction. CD44 was shown to play a crucial role in TH cell differentiation, inasmuch as deletion of the *CD44* gene inhibited TH1/TH17 differentiation, while simultaneously enhancing TH2/TREG cell differentiation (Guan *et al.*, 2011). The expression of CD44 promoted TH1/TH17 differentiation. In fact, when osteopontin and hyaluronic acid, the two major CD44 ligands, were tested for their role in TH cell differentiation, osteopontin (Opn), but not hyaluronic acid, promoted TH1/TH17 differentiation. Furthermore, activation of $CD44^+$ encephalitogenic T cells with MOG peptide led to demethylation at the *ifn-γ/il17a* gene promoter region, while promoting hypermethylation at the *il4/foxp3* gene promoter. Therefore, in encephalitogenic T cells, CD44 plays a crucial role in the differentiation of TH cells, through epigenetic regulation, namely, DNA methylation of TH1/TH17 and TH2 cytokine genes (Guan *et al.*, 2011). Moreover, Opn inhibited IL-10 production via the CD44 receptor CD44R and anti-Opn treatment reduced the

clinical severity of EAE by reducing IL-17 production (Murugaiyan *et al*., 2008).

As for the role of CD44 as a costimulatory molecule, a number of non-CD28 costimulatory molecole, such as CD5, CD9 and CD2, including CD44, were shown to be present in the detergent-insoluble glycolipid-enriched (DIG) fraction/raft of the T cell surface, which is rich in TCR signaling molecules and generates a TCR signal upon recruitment of the TCR complex (Ohtani *et al.*, 2000). Moreover, certain anti-CD44 mAbs can activate human resting T cells and mouse cytotoxic T cells in the absence of anti-CD3 or anti-CD2 mAb. An anti-CD44 mAb triggered proliferation of human $CD3^+/CD4^+$ T cell clones in a similar fashion as observed with mAbs to CD3 (Galandrini *et al.*, 1993). Finally, with regard to the anti-apoptotic role of CD44, it was shown that the CD44v6 and CD44v9 isoforms exhibit anti-apoptotic effects and could block Fas-mediated apoptosis. Importantly, an anti-CD44v6 antibody was able to abolish the anti-apoptotic effect of CD44v6 (Mielgo *et al.*, 2006).

4.

The wide range of variation of the percentage abundance of hTgCS in whole hTg which can be observed in humans (Conte *et al.*, 2006) and the documented effects of the CS oligosaccharide unit upon hTg hormonogenic efficiency (Conte *et al.*, 2006) and the hTg immunopathogenic capacity in the murine model of EAT documented here warrant a systematic investigation of the possible relationships between the percentage amount of hTgCS in thyroid colloid substance and thyroid function and pathology. The measure of hTgCS fraction of hTg may turn out to be a useful marker of prognostic usefulness in the assessment of the risk of autoimmune thyroid disease (AITD) in humans, under conditions which are known to favour it, such as borderline, chronic iodine deficiency, smoking, acute or subacute thyroiditis and genetic polymorphisms of the *Tg*, *CTLR4, HLADRB1 and PTPN22* genes).

5.

The actions documented here by the CS unit of hTg and its receptor CD44 on the development of autoimmune thyroid disease may pave the way to further experimentation, aiming at evaluating the feasibility and possible usefulness, in animal models of autoimmune thyroid disease and possibly in the setting of human disease, to targeted strategies of metabolic and/or genetic interference with the chondroitin sulfate addition to thyroglobulin (by the use of metabolic inhibitors or genetic silencing of xylosyl transferases), or of CD44 expression in the cells of the immune system (by the use of specific miRNAs), for the prevention of autoimmune thyroid disease, in the presence of risk factors, or the immunomodulation of established disease.

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the selectivity at mGlu3 receptor we have used highly purified preparations of NAAG. We also performed experiments in the presence of glutamate-pyruvate transaminase which removes all traces of glutamate. These new results confirm our previous observations that NAAG is an agonist of mGlu3 receptors and that it's activity is not due to contamination with glutamate. We also show that a new compound, 2-hydroxymethylglutamate (HMG), is a selective agonist of mGlu3 receptors. When tested in cell lines expressing the various mGluRs, HMG showed no significant activity at group I mGluRs, while among group III mGluRs it was a very weak agonist of mGlu4, without activity at mGlu6, mGlu7 and mGlu8 receptors. At group II mGluRs, HMG showed an unusual pattern of selectivity activating mGlu3, while inhibiting mGlu2 receptors. HMG was also inactive at ionotropic glutamate receptors (NMDA, AMPA or kainate) in primary cultures of cerebellar neurons.Neuroprotective properties of HMG were tested in three *in vitro* models of neurotoxicity in mixed neuronal-glial mice cortical cultures. Neurotoxicity was evoked by (1) 10 min application of 75 μM NMDA, (2) 60 min glucose and oxygen deprivation, and (3) 48 h application of 25 μM β-amyloid peptide. Cell death was quantified by measurement of lactate dehydrogenase. In all three models HMG showed strong neuroprotective action, although the maximal efficacy varied depending on the model used. Additional experiments showed that the efficacy of HMG depended on the presence of glial cells, suggesting that the neuroprotective mechanism may involve the activation of glial mGlu3 receptors followed by the release of trophic factors. These results are consistent with our previous observations that the neuroprotective action of NAAG is depends on the presence of glial cells. Since the neuroprotective actions of NAAG are similar to the actions of a selective mGlu3 agonist (HMG), these data provide additional evidance that NAAG is a selective endogenous agonist of mGlu3 receptors. *Supported by NIH grant NS37436.*

187.Unique inhibitory Synapse with Particularly Rich Endocannabinoid Signaling Machinery on Pyramidal Neurons in Basal Amygdaloid Nucleus

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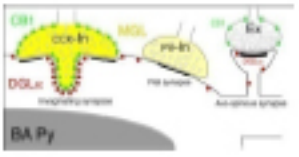
2-Arachidonoylglycerol (2-AG) is the endocannabinoid that mediates retrograde suppression of synaptic transmission in the brain. 2-AG is synthesized in activated postsynaptic neurons by *sn*-1-specific diacylglycerol lipase (DGL), binds to presynaptic cannabinoid CB_1 receptors, suppresses neurotransmitter release, and is degraded mainly by monoacylglycerol lipase (MGL). In the basolateral amygdala complex, CB_1 is particularly enriched in axon terminals of cholecystokinin (CCK)-positive GABAergic interneurons, induces short- and long-term depression at inhibitory synapses, and is involved in extinction of fear memory. Here we clarified a unique molecular convergence of DGLα, CB_1, and MGL at specific inhibitory synapses in the basal nucleus (BA), but not lateral nucleus, of the basolateral amygdala. The synapses, termed invaginating synapses, consisted of conventional symmetrical contact and unique perisynaptic invagination of nerve terminals into perikarya. At invaginating synapses, DGLα was preferentially recruited to concave somatic membrane of postsynaptic pyramidal neurons, while invaginating presynaptic terminals highly expressed CB_1, MGL, and CCK. No such molecular convergence was seen for flat perisomatic synapses made by parvalbumin-positive interneurons. On the other hand, DGLα and CB_1 were expressed weakly at axo-spinous excitatory synapses. Consistent with these morphological data, thresholds for DGLα-mediated depolarization-induced retrograde suppression were much lower for inhibitory synapses than for excitatory synapses in BA pyramidal neurons. Moreover, depolarization-induced suppression was readily saturated for inhibition, but never for excitation. These findings suggest that perisomatic inhibition by invaginating synapses is a key target of 2-AG-mediated control of the excitability of BA pyramidal neurons. In the talk, we will also introduce some other brain regions with such intensive convergence of endo cannabinoid signaling molecules.



188.Histamine: an Endogenous Modulator of Metabotropic Glutamate Receptors

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The metabotropic glutamate receptors (mGluRs) are a group of Family C G-protein-coupled receptors that are activated by glutamate. As a member of the group III mGluRs, mGluR4 is expressed predominantly presynaptically and has been shown to rectify inappropriate GABA release at the striatopallidal synapse in Parkinson's Disease. While synthetic small molecules have been discovered that specifically potentiate the mGluR4 activity, it is also possible that endogenous compounds also exist which can modulate receptor activity in a positive or negative fashion. We screened a series of neurotransmitters to determine if these endogenous ligands could modulate glutamate activity at mGluR4. Interestingly, the neurotransmitter histamine appeared to potentiate mGluR4 activity with similar efficacy when compared to known small molecule positive allosteric modulators of mGluR4. These data suggest that histamine could serve as an endogenous modulator that plays an important role in regulating mGluR4 function. Ongoing studies are focused on developing a detailed understanding of the mechanism by which histamine potentiates mGluR4 responses and how this impacts different aspects of mGluR4 signaling. *Supported by grants from the NIH. Vanderbilt is a Specialized Chemistry Center in the MLPCN.*

189.Beneficial Effect of mGlu4 Receptor Activation on Multiple Animal Models o Autoimmune Disease

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The mGlu4 receptor, is gaining increasing interest for its immunomodulatory properties. We show here the effects of the treatment with a selective mGlu4 receptor enhancer, (-)-N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), on two models of Experimental Autoimmune Neuritis (EAN), a T-cell-mediated autoimmune disease reminiscent of the clinical, pathological and electrophysiological features of the human Guillain-Barre syndrome (GBS). Acute EAN induced in Lewis rats by active immunization leads to a monophasic disease with transient flaccid paresis of the tail and limbs due to an acute and mainly demyelinating inflammatory polyradiculoneuropathy. A new and biphasic form of EAN is described in dark agouti rats (DA rats) those develop mild episodes of disease; DA-EAN may serve as a model for relapsing inflammatory demyelinating polyneuropathies such as chronic inflammatory demyelinating polyneuropathy (CIDP). In both experimental models we treated animals with PHCCC (3 mg/kg, s.c., once a day), either under prophylactic regimen (from the day of immunization) or therapeutically (at the onset of symptoms). Both treatments, when compared to the respective control groups, were associated with significant improvements in the clinical scores, as well as in the histological hallmarks of neuroinflammation, and with reductions in the number and severity of relapses in DA-EAN models. In order to assess whether similar effects could be reproduced on different preclinical models of autoimmunity, we extended the study to experimental autoimmune thyroiditis (EAT) in CBA/J(H-2k) mice, a murine model of Hashimoto's thyroiditis, which can be induced in mice with H-2k and H-2s haplotypes by immunization with mouse thyroglobulin (mTg) or human Tg (hTg) in complete Freund's adjuvant (CFA). EAT is characterized by the infiltration of thyroid by mononuclear cells, the production of specific antibodies and secondary proliferative responses of lymphocytes against Tg *in vitro*. Once immunized with 100 μg of hTg in CFA subcutaneously, mice were treated daily with PHCCC (3mg/kg). Boosting on day 10 was accomplished with 50 μg of hTg in IFA s.c. Histological examination of thyroid lobe showed decreased mononuclear infiltration of thyroid tissue in PHCCC treated mice, compared with untreated mice. In addition, the analysis of multiple cytokine concentrations revealed marked decreases in the secretion of inflammatory cytokines, such as IL-6 and IL-17, but not IFN-γ, in mice treated with PHCCC, compared with untreated mice. IL-2 secretion was not affected by treatment. These data indicate a role of mGlu4 receptors in the pathophysiology of inflammatory/autoimmune diseases and suggest a potential role of mGlu4 receptor enhancers in the treatment of experimental neuroinflammation.

190.Role of Group II Metabotropic Receptors in Long-Term Plasticity of Presynaptic Vesicular Release at Glutamatergic Synapses

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Long-term plasticity of synaptic transmission can consist of multiple changes and loci, both presynaptic and postsynaptic. Different forms of long-term potentiation (LTP) and long-term depression (LTD) of glutamatergic synaptic transmission can be induced by activation of either N-methyl-D-aspartate (NMDAR) or group I metabotropic (mGluR) glutamate receptors. Utilizing two-photon laser scanning microscopic (TPLSM) imaging of presynaptic terminals labelled with fluorescent indicators, we discovered that NMDAR-dependent LTP and LTD can be expressed as a combination of alterations in both presynaptic glutamate release and postsynaptic sensitivity to glutamate. TPLSM imaging of vesicular release using either FM1-43 or the pH-sensitive vesicular release marker SynaptopHluorin reveals components of LTP and LTD expressed as selective changes in vesicular release from the rapidly-recycling vesicle pool loaded by action potentials or hypertonic shock. We found that activation of either presynaptic group II mGluRs or A1 adenosine receptors, when paired with elevation of intracellular [cyclic GMP], is sufficient to induce LTD. These G protein-coupled receptors, when activated, release $Gi_2\alpha$ and Gβγ in the presynaptic terminal. $Gi_2\alpha$ inhibition of adenylate cyclase promotes, but does not induce, LTD. Gβγ is known to both inhibit voltage-dependent calcium channels, and to bind directly to the C-terminus of the SNARE protein SNAP-25. We discovered that, at Schaffer collateral-CA1 synapses in the hippocampus, the C terminus of SNAP-25 is necessary for the expression of LTD, but *not* LTP. Using type A botulinum toxin (BoNT/A) to enzymatically cleave the 14 amino acid C-terminus of SNAP-25 eliminated the ability of low-frequency synaptic stimulation to induce LTD. Presynaptic infusion by electroporation of CA3 pyramidal neurons with either the 14 amino acid C-terminus of SNAP-25, or the Gβγ scavenging peptide mSIRK, also blocked the induction of LTD, without persistently altering presynaptic calcium influx. Taken together, our data show that involvement of presynaptic group II mGluRs is a necessary, but not sufficient, condition for long-term plasticity of vesicular transmitter release. The larger view of studies of activity-dependent synaptic plasticity shows us that persistent changes in both transmitter release and postsynaptic receptor sensitivities are regulated by patterns of synaptic activation, and that they can even shift in opposing directions as a function of transsynaptic signaling molecules that produce a bidirectional dialogue across the synapse.

191.Loss of mGluR-Dependent Long-Term Depression in the ACC after Digit amputation

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ACC is a crucial cortical region involved in brain-related physiological and pathological conditions such as emotional fear and chronic pain. Both long-term depression (LTD) and long-term potentiation (LTP) have been reported in the ACC of adult animals. Using traditional single recording electrode approach, we reported previously that peripheral injury (digit amputation) caused loss of LTD induced by low-frequency stimulation. Due to the limit of the recording method, it is unknown if such loss of LTD is wide-spreading within the ACC. In the current study, we used a multi-electrode array (MED64) system to map cortical LTD in a spatiotemporal manner. The MED64 system enabled us to observe the network properties of ACC by stimulating deep layer V and detecting field excitatory postsynaptic potentials (fEPSP) from other 63 sites within the ACC. We found that low frequency stimulation (1Hz) applied on deep layer V induced LTD in nearby layers II/III and layers V/VI regions of the ACC. Pharmacological results showed that L-type voltage-gated calcium channels, metabotropic glutamate receptors (mGluRs) especially mGluR1 are required for the induction of ACC LTD. Blocking NMDA receptors also reduced LTD. Amputation of the distal tail impaired LTD that persisted for at least two weeks. Our results provide the first study on the network properties of ACC LTD in adult mice, and demonstrate that tail amputation causes LTD impairment within the ACC circuit.

192.Role of Optineurin and Huntingtin in the Regulation of mGluR5 signalling

Stephen S. G. Ferguson and J. Allyn Taylor

frontiers in
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Interaction of aldehydes derived from lipid peroxidation and membrane proteins

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A great variety of compounds are formed during lipid peroxidation of polyunsaturated fatty acids of membrane phospholipids. Among them, bioactive aldehydes, such as 4-hydroxyalkenals, malondialdehyde (MDA) and acrolein, have received particular attention since they have been considered as toxic messengers that can propagate and amplify oxidative injury. In the 4-hydroxyalkenal class, 4-hydroxy-2-nonenal (HNE) is the most intensively studied aldehyde, in relation not only to its toxic function, but also to its physiological role. Indeed, HNE can be found at low concentrations in human tissues and plasma and participates in the control of biological processes, such as signal transduction, cell proliferation, and differentiation. Moreover, at low doses, HNE exerts an anti-cancer effect, by inhibiting cell proliferation, angiogenesis, cell adhesion and by inducing differentiation and/or apoptosis in various tumor cell lines. It is very likely that a substantial fraction of the effects observed in cellular responses, induced by HNE and related aldehydes, be mediated by their interaction with proteins, resulting in the formation of covalent adducts or in the modulation of their expression and/or activity. In this review we focus on membrane proteins affected by lipid peroxidation-derived aldehydes, under physiological and pathological conditions.

Keywords: lipid peroxidation, aldehydes, membrane proteins, human diseases, signal transduction

INTRODUCTION: LIPID PEROXIDATION-DERIVED ALDEHYDES

Reactive intermediates produced under conditions of oxidative stress cause the oxidation of polyunsaturated fatty acids (PUFAs) in membrane lipid bilayers, leading eventually to the formation of aldehydes (Esterbauer et al., 1991). Among these, the most abundant aldehydes are 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), while acrolein is the most reactive one (Esterbauer et al., 1991). HNE is the lipoperoxidation product which has displayed the highest biological activity and, for this reason, has been most intensively studied. On the other hand, acrolein, which is the most electrophylic compound, has received less attention, because it is scarcely represented among lipoperoxidation products. Both acrolein and HNE are α,β-unsaturated electrophilic compounds, which preferentially form 1,4-Michael type adducts with nucleophiles, such as proteins and DNA. Even though MDA shows little reactivity under physiological conditions, at low pH its reactivity increases, when beta-hydroxyacrolein becomes the predominant species and, analogously to acrolein and HNE, it can form 1,4-Michael type adducts with nucleophiles (Esterbauer et al., 1991). Even though it was demonstrated that MDA does not react with glycine and GSH, and reacts slowly with cysteine (Esterbauer et al., 1991) under physiological conditions, cellular proteins are much more readily modified by MDA (Chio and Tappel, 1969).

Due to the high chemical reactivity of aldehydes, mammals have evolved a battery of enzymes which convert these compounds to less reactive chemical species. The main reactions of aldehydes are the adduction with glutathione (GSH), which can either occur spontaneously or be catalyzed by glutathione S-transferases (GSTs), the reduction to alcohol by aldo–keto reductases (AKRs) or alcohol dehydrogenase and the oxidation to acid by aldehyde dehydrogenases. The metabolism of aldehydes has been reviewed in excellent mode by Esterbauer and collaborators (1991). More recent reviews were focused on the biochemistry of lipid peroxidation products (Guéraud et al., 2010) and acrolein biotransformation (Stevens and Maier, 2008). The catabolic rates of the various aldehydes contribute, together with their rates of production from lipid peroxidation, in determining their steady-state intracellular concentrations. At high concentrations, all these aldehydes were found to play a role in the toxic effects of lipid peroxidation. Aldehyde toxicity is mainly due to the alterations of several cell functions, which mostly depend on the formation of covalent adducts with cellular proteins (Grimsrud et al., 2008). Due to their amphiphilic nature, aldehydes can easily diffuse across membranes and can covalently modify any protein

in the cytoplasm and nucleus, far from their site of origin (Negre-Salvayre et al., 2008). Similarly, the aldehydes formed outside the cells (i.e., in a site of inflammation or in plasma), can react with adjacent cells, even in cases when they are not primary sites of lipid peroxidation. In the latter instance, plasma membrane proteins represent the first targets for adduct formation. Exogenous or endogenous aldehydes can react also with nuclear proteins, thus modulating protein expression through their reaction with transcription factors or other regulatory elements (Jacobs and Marnett, 2010). The targets of lipid peroxidation-derived aldehydes are cell-type specific and dependent both on the pattern of proteins expressed by the cell and the aldehyde concentration. Moreover, the modification of a specific protein can have different biological consequences, in relation to its specific cell function. However, at low concentration, HNE in particular can play an important role in signal transduction and exert antiproliferative and anti-invasive actions toward cancer cells, by interfering with the modulation of gene expression via the formation of protein and/or DNA adducts (Gentile et al., 2009; Barrera, 2012).

The presence of aldehyde-protein adducts has been demonstrated in a wide range of physiological and pathological conditions. Those among the latter in which aldehyde-protein adducts, in particular HNE-protein adducts, have been most intensively studied are neurodegenerative diseases and atherosclerosis. Recently, a role has emerged for aldehyde-protein adducts in autoimmune diseases, since the covalent alteration of protein structure can bring about a sufficient modification of a self antigen for it to break the immunological tolerance of autoreactive T and/or B cells. In the following sections, we shall examine the mechanisms of formation of aldehyde-protein adducts and the main biological consequences of the formation of aldehyde adducts with membrane proteins in neurodegenerative diseases, atherosclerosis, autoimmune diseases and in relation with the functions played by cell proteins at the plasma membrane level. The chemical structures of HNE, MDA and acrolein are illustrated in **Figure 1**

CHARACTERISTICS OF ALDEHYDES AND THEIR PROTEIN ADDUCTS

4-HYDROXYNONENAL (HNE)

4-Hydroxynonenal (HNE) is an aldehyde highly represented among the products of lipid peroxidation, which displays high biological activity. This aldehyde has three main functional groups: the aldehyde group, the C=C double bond and the hydroxyl group, which can participate, alone or in sequence, in chemical reactions with other molecules (Esterbauer et al., 1991). Due to its strong hydrophobic nature, HNE is mostly associated with the membranes where it is produced, but it can also diffuse to different cellular compartments (Butterfield and Stadtman, 1997). HNE is a highly electrophilic molecule that easily reacts with glutathione, proteins and, at higher concentration, with DNA. HNE forms adducts with three different amino acyl side chains, namely of Cys, His, and Lys residues, via Michael addition either to thiol (−SH) or to amino ($-NH_2$) groups. Cys residues display the highest reactivity with HNE, even though Cys residues are not always the preferential targets of HNE, because the tertiary structure of the protein can condition their accessibility and, therefore, their reactivity toward exogenous chemicals. No reaction of HNE was detected with Glu (Doorn and Petersen, 2003). Besides by the simple formation of Michael adducts to lysyl, histidyl, and cysteinyl residues (Esterbauer et al., 1991), HNE can modify protein structure through Schiff base formation with lysyl residues, leading to pyrrole formation (Sayre et al., 1996). In addition, HNE modification can result in the cross-linking of two lysyl residues through reversibly formed Schiff base Michael adducts (Parola et al., 1999; Xu et al., 1999), as well as irreversibly formed 2-hydroxy-2-pentyl-1,2-dihydropyrrol-3-one iminium moieties (Parola et al., 1999; Dianzani, 2003; Barrera et al., 2008). The target proteins of HNE adduct formation *in vitro* and *in vivo* have been reviewed in great detail by Poli et al. (2008).

HNE has been detected *in vivo* in several pathological conditions characterized by increased lipid peroxidation, including inflammation, atherosclerosis, chronic degenerative diseases of the nervous system, and chronic liver diseases (Moreau et al., 2005).

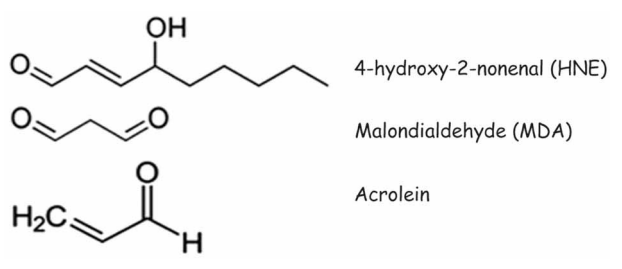

FIGURE 1 | Structures of 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and acrolein.

ACROLEIN

Acrolein is a little aldehyde with three carbon atoms and a double bond. Besides being formed endogenously during lipid peroxidation, this aldehyde is inhaled with cigarette smoke and is present in cooked oils and other foods (Stevens and Maier, 2008). Acrolein is the strongest electrophile in the α,β-unsaturated aldehyde series; its reaction with the thiol group of cysteine was about 110–150 times faster than that of HNE (Esterbauer et al., 1991; Witz, 1997). The toxicity of acrolein is related to its ability to deplete glutathione (Kehrer and Biswal, 2000), and to form DNA and protein adducts (Esterbauer et al., 1991; Sanchez et al., 2005; Feng et al., 2006). Potential targets of acrolein in proteins include the side chains of cysteinyl, histidyl, and lysyl residues, as well as free N-terminal amino groups (Cai et al., 2009). Cysteine is widely accepted as the most likely site of acrolein adduct formation. The sulfhydryl group of a cysteinyl residue is the most reactive nucleophile in proteins and the thiol adducts with acrolein are considerably more stable than the adducts formed by other α,β-unsaturated aldehydes (Esterbauer et al., 1991; Witz, 1997). Cysteinyl residues are located at the active sites of several proteins and are often involved in the catalytic activity of enzymes, thus the formation of acrolein-cysteine adducts has broad functional implications. It has been reported that the modification of cysteinyl residues by acrolein leads to the inactivation of enzymes, such as aldose reductase (Srivastava et al., 1999) and protein

tyrosine phosphatase 1B (Seiner et al., 2007). However, no cysteine adducts of acrolein have been identified *in vivo*. Other Authors have shown that acrolein generated during lipid peroxidation may primarily react with histidyl residues of proteins, to form Nτ-(3-propanal)-histidine and that acrolein-histidine is the major adduct formed with proteins in *in vitro* studies (Maeshima et al., 2012).

Elevated plasma concentrations of acrolein are detected in patients with chronic renal failure, and the abundance of the proteins adducts of acrolein is increased in tissues obtained from patients with Alzheimer's disease, Parkinson's disease, atherosclerosis and chronic obstructive lung disease (Uchida et al., 1998a; Shamoto-Nagai et al., 2007; Stevens and Maier, 2008; Moretto et al., 2012).

MALONDIALDEHYDE (MDA)

Malondialdehyde (MDA) is widely used as a marker for the peroxidation of ω3 and ω6 fatty acids, measured by the chemical determination of thiobarbituric acid reactive substances (TBARS) (Negre-Salvayre et al., 2010), although the latter provides an incomplete perspective, as MDA derives from the decomposition of only certain lipid peroxidation products and is neither the sole end product, nor one of lipid peroxidation only (Halliwell and Whiteman, 2004). At neutral pH, MDA is present as enolate anion, with low chemical reactivity (Esterbauer et al., 1991). Nevertheless, it is able to interact with nucleic acid bases to form several different adducts (Marnett, 1999). MDA has been reported to react *in vivo* with primary amines, to form Nε-(2-propenal) lysine and generate lysine-lysine cross-links with 1-amino-3-iminopropene and pyridyledihydropyridine type bridges (Uchida, 2000). These reaction products have been detected in Apo B fractions of oxidized lipoproteins (LDL) and are thought to be involved in the impaired interaction of modified lipoproteins with macrophages (Palinski et al., 1994). Mooradian and coworkers have reported that protein glycosylation and the presence of acetaldehyde enhance MDA modification of proteins (Mooradian et al., 1996, 2001). Moreover, MDA and acetaldehyde can form stable adducts (MAA) (Tuma et al., 1996) and can react covalently and synergistically with proteins, forming MAA–protein adducts. The latter can be pro-inflammatory and pro-fibrogenic and are capable of inducing strong immune responses (Tuma, 2002).

PHOSPHATIDYL γ-HYDROXYALKENALS (PC-HAs)

Phosphatidylcholine γ-hydroxyalkenals (PC-HAs) are the most abundant and biologically relevant compounds in the class of γ-hydroxyalkenal phospholipids, deriving from the peroxidation of polyunsaturated fatty acids (PUFAs) esterified to phosphoglycerides at the *sn*-2 position of phosphatidylcholine (PC). β-Scission of an alkoxyl radical derived from dihydroperoxide produces two γ-hydroxy-α,β-unsaturated aldehydes, i.e., a methyl-terminal HNE molecule and a mirror image of HNE, still esterified to PC (namely, 9-hydroxy-12-oxo-10-dodecenoic acid [HODA] or its PC ester from linoleate and 5-hydroxy-8-oxo-6-octenoic acid [HOOA] or its PC ester from arachidonate). Because they possess a γ-hydroxy-α,β-unsaturated terminal aldehyde like HNE, PC-hydroxyalkenals are expected to form Michael adducts with primary amino groups of lysyl residues and thiol groups of cysteinyl residues, as well as pentylpyrrole adducts, incorporating the ε-amino groups of lysyl residues (**Figure 2**). γ-Hydroxyalkenal phospholipids contribute strongly in the pathogenesis of the atherosclerotic disease. ω-Carboxyalkylpyrrole modifications of proteins, after lypolysis of intermediate phospholipid adducts, are of pathogenetic importance in age-related macular degeneration, autism and cancer, and promote wound healing. In regard, the reader is referred to the excellent reviews by Salomon et al. (2011), and Salomon and Gu (2011).

ALDEHYDE-PROTEIN ADDUCTS IN NEURODEGENERATIVE DISEASES

Central nervous system (CNS) is one of the major targets of lipid peroxidation. The brain is highly sensitive to oxidative stress because it consumes about 20–30% of inspired oxygen and contains high levels of PUFAs. In particular, high levels of the markers of lipid peroxidation have been found in brain tissues and body fluids in several neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), Huntington disease (HD) and Down syndrome (DS) (Sajdel-Sulkowska and Marotta, 1984; Butterfield et al., 2010; Ruiperez et al., 2010; Lee et al., 2011; Shichiri et al., 2011). We focus here on the adducts of lipid peroxidation-derived aldehydes with protein targets whose oxidative modifications might be relevant for the neuronal dysfunctions observed in Alzheimer's disease.

ALZHEIMER'S DISEASE (AD)

Oxidative damage occurs in early stages of Alzheimer's disease (AD) (Butterfield et al., 2006a; Reed et al., 2008, 2009a; Mangialasche et al., 2009). Several Authors, using redox proteomic approaches, have undertook the task of compiling inventories of cellular proteins modified as a consequence of increased oxidation, glycoxidation, or lipoxidation in the course of oxidative and/or nitrosative stress (Pamplona et al., 2005; Butterfield et al., 2006b; Newman et al., 2007; Reed et al., 2008, 2009a,b; Perluigi et al., 2009). Other searches focused upon proteins modified by tyrosine nitration (Castegna et al., 2003; Sultana et al., 2006a; Reed et al., 2008) and S-glutathionylation (Newman et al., 2007). A number of comprehensive reviews are available (Mangialasche et al., 2009; Martínez et al., 2010; Reed, 2011; Sultana et al., 2012). Like in other neurodegenerative diseases with proteinaceous deposits, vulnerable proteins could be assigned to a few distinct functional groups with crucial roles in plasma membrane ion and nutrient transport, energy metabolism (glycolysis, mitochondrial electron transport and oxidative phosphorylation), cell signaling, cytoskeletal organization, antioxidant defenses, cellular stress responses, protein synthesis, signal transduction, and regulation of neurotransmission (**Table 1**).

HNE-Amyloid β peptide adducts

Amyloid β (Aβ) peptide is the major protein component of amyloid plaques and one of the main components of neurofibrillary tangles (NFTs), hallmarks of AD. This molecule is a 40-to-42-amino acid peptide derived from the integral membrane Amyloid Precursor Protein (APP), through sequential proteolytic cleavages

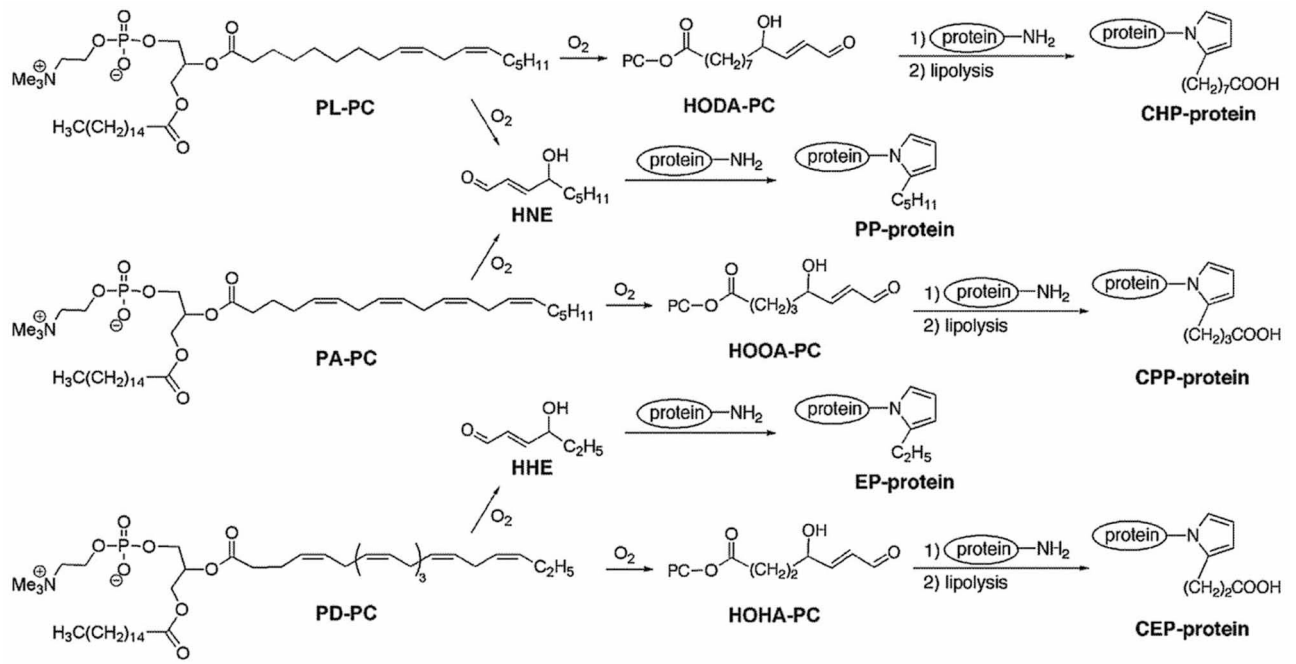

FIGURE 2 | Structures and reactions of phosphatidylcholine γ-hydroxyalkenals (PC-HAs). In addition to 2-pentylpyrrole-modification of proteins by the electrophilic addition of HNE to ε-amino lysyl groups, carboxyalkylpyrrole-modified proteins are also formed by adduct formation with other γ-hydroxyalkenals (mirror images of esterified HNE) also formed, at the time of HNE formation, from the oxidation of PUFA-containing phospholipids. Legend: HHE, 4-hydroxy-2-hexenal; HNE, 4-hydroxy-2-nonenal; PL-PC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PA-PC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PD-PC, 1-palmitoyl-2-docosahexanoyl-*sn*-glycero-3-phosphocholine; HODA-PC, 9-hydroxy-12-oxo-10-dodecenoyl-phosphatidylcholine (this compound may also derive from 1-linoleoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, LA-PC); HOOA-PC, 5-hydroxy-8-oxo-6-octenoyl-phosphatidylcholine; HOHA-PC, 4-hydroxy-7-oxo-5-heptenoyl-phosphatidylcholine; PP-protein, 2-pentylpyrrole-modified protein; EP-protein, 2-ethylpyrrole-modified protein; CPP-protein, carboxypropylpyrrole-modified protein; CEP-protein, carboxyethylpyrrole-modified protein [Reprinted with permission from Salomon et al. (2011)].

by β-secretase (BACE) and γ-secretase (Hardy and Selkoe, 2002). HNE can react directly with the Aβ peptide. This process was reported to exacerbate the formation of toxic Aβ-dependent diffusible oligomers and insoluble aggregates, which, in turn, enhanced oxidative stress, fromation of lipid peroxidation products, such as HNE, and Aβ oligomerization and toxicity (Siegel et al., 2007). HNE modified the three histidyl residues of Aβ, so that HNE-modified Aβ molecules had increased affinities for membrane lipids and adopted a similar conformation as mature amyloid fibrils (Murray et al., 2007; Liu et al., 2008).

HNE-α-enolase adducts

α-Enolase (non neural enolase, ENO1) is a multiform, multifunctional protein. In the cytoplasm, it is a 48-kDa enzyme, catalyzing 2-phospho-D-glycerate dehydration to phosphoenolpyruvate. At the cell surface of neutrophils, B and T cells, monocytes, epithelial, endothelial cells and neurons, it serves as a plasminogen receptor, involved in fibrinolysis (Pancholi, 2001) and in neutrophil and monocyte recruitment (Busuttil et al., 2004; Wygrecka et al., 2009). Binding to α-enolase protects plasmin from inactivation by α2-antiplasmin (Bergman et al., 1997). Alternative translation of α-enolase mRNA produces a 37-kDa protein, preferentially located in the nucleus (myc Binding Protein-1, MBP-1), with *c-myc* gene promoter-binding and tanscription repressing activity (Feo et al., 2000; Subramanian and Miller, 2000). In addition, α-enolase was reported to be a hypoxic stress protein (Graven and Farber, 1998). A regulatory circuit between c-myc, MBP-1, and α-enolase was described, connecting cell energy metabolism and proliferation (Sedoris et al., 2007). Redox proteomic studies identified α-enolase as a target of oxidative modification in all stages of Alzheimer's disease, undergoing the formation of carbonyl groups (Castegna et al., 2002; Butterfield et al., 2006b; Sultana et al., 2006b), MDA adducts (Pamplona et al., 2005), 4-HNE adducts (Reed et al., 2008, 2009a; Perluigi et al., 2009), tyrosine nitration (Castegna et al., 2003; Sultana et al., 2006a; Reed et al., 2009b) and S-glutathionylation (Newman et al., 2007). A typical metabolic feature of AD is the reduced rate of glucose metabolism, as seen in positron-emission tomography with 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG/PET) (de Leon et al., 2001). Despite compensatory increases of α-enolase expression in AD (Sultana et al., 2007) and even though enzymatic activity was not assayed, it was suggested that the loss of function associated with the oxidative modifications of α-enolase might render neurons prone to apoptosis, by dysrupting their energy metabolism (Sultana et al., 2012). As peptide Aβ(1-42), by aggregating in cross-β-structured fibrils, with a similar conformation as fibrin peptides, could substitute for fibrin in the activation of tissue plasminogen activator (tPA) (Kingston

Table 1 | HNE-protein adducts detected in Alzheimer's disease, in relation with disease progression[a].

Protein	AD stage[a]	Function	References
Aldolase	PAD, LAD	Energy metabolism	Perluigi et al., 2009
Triose phosphate isomerase (TPI)	EAD	Energy metabolism	Reed et al., 2009a
Phosphoglycerate kinase (PGK)	MCI	Energy metabolism	Reed et al., 2008
α-Enolase (non neural enolase, ENO1)[b]	MCI, EAD, LAD	Energy metabolism	Reed et al., 2008, 2009a; Perluigi et al., 2009
Pyruvate kinase (PK) M2 isoform	PAD, MCI	Energy metabolism	Reed et al., 2008
Lactate dehydrogenase B (LDHB)	MCI	Energy metabolism	Reed et al., 2008
Aconitase	PAD, LAD	Energy metabolism, mitochondrial function	Perluigi et al., 2009
Malate dehydrogenase, mitochondrial	EAD	Energy metabolism, mitochondrial function	Reed et al., 2009a
ATP synthase α subunit	PAD, MCI, EAD, LAD	Energy metabolism, mitochondrial function	Reed et al., 2008, 2009a; Perluigi et al., 2009
Mn Superoxide dysmutase (SOD2)	EAD, LAD	Mitochondrial function, antioxidant defense	Perluigi et al., 2009; Reed et al., 2009a
Carbonyl reductase 1	MCI	Antioxidant defense	Reed et al., 2008
Peroxiredoxin VI (Phospholipase A2)	LAD	Antioxidant defense	Perluigi et al., 2009
Heme oxygenase 1 (HO-1)	MCI, LAD	Antioxidant defense	Sultana et al., 2012
70-kDa heat shock protein (HSP70)	MCI	Stress response	Reed et al., 2008
Pleckstrin homology-like domain, family A, member 2 (IPL)		Signal transduction	Reed et al., 2008
β-Actin	MCI	Cytoskeleton	Reed et al., 2008
α-Tubulin	LAD	Cytoskeleton	Perluigi et al., 2009
Elongation factor Tu (EF-Tu)	PAD, MCI	Protein synthesis	Reed et al., 2008
Initiation Factor α (eIFα)	MCI	Protein synthesis	Reed et al., 2008
Glutamine synthetase	LAD	Excitotoxicity	Perluigi et al., 2009
Neuropolypeptide h3	PAD, MCI	Neuronal communication	Reed et al., 2008
Collapsin response mediated protein 2 (CRMP-2)[c]	EAD, LAD	Neuronal communication	Perluigi et al., 2009; Reed et al., 2009a

[a] *Clinical stages of Alzheimer's disease (AD) progression, in chronological order: PAD, preclinical AD; MCI, mild cognitive impairment; EAD, early stage AD; LAD, late stage AD.*

[b] *Integral or perypheral membrane proteins of plasma or organelle membranes are underlined.*

[c] *Also known as dihydropirimidinase-related protein 2 (DRP-2).*

et al., 1995), and because plasmin cleaved peptide Aβ1-40 into a truncated form, with potent stimulatory activity toward tPA (VanNostrand and Porter, 1999), it was proposed that the loss of plasminogen-binding activity of HNE-α-enolase might foster apoptosis in AD, by hindering Aβ peptide degradation (Sultana et al., 2012).

Such a scenario is supported by a functional study of HNE-α-enolase adducts in HL-60 leukemic cells (Gentile et al., 2009). α-Enolase was among a few proteins recognized by anti-histidine-HNE antibodies, after 15 min of exposure to 1–10 μM HNE. HNE–α-enolase adducts were detected early on the surface of HL-60 cells, indicating a high degree of α-enolase exposure. HNE treatment did not alter α-enolase expression or enzymatic activity. The low-level expression of an anti-α-enolase Ab-reactive 37-kDa peptide, possibly corresponding to MBP-1, did not vary after HNE treatment. The main functional alteration of HNE-α-enolase concerned its plasminogen-binding ability. Treatment with 1 μM HNE strongly inhibited plasminogen binding to α-enolase at the cell surface and consequently reduced HL-60 cell adhesion to human umbilical venous cells (HUVECs), suggesting that HNE and other inhibitors of plasminogen binding to α-enolase may be of use in the control of tumor invasion. α-Enolase emerged from this study as a protein most susceptible to HNE adduct formation, in keeping with various proteomic studies, in the context of neurodegeneration, cited in section Alzheimer's Disease (AD), which pinpointed a limited number of protein targets of oxidative modification, including α-enolase, grouped in selected functional subsets (Martínez et al., 2010; Reed, 2011; Sultana et al., 2012). Some of these proteins targets were also identified as autoantigens frequently recognized by autoantibodies in autoimmune diseases. α-Enolase, in particular, was recurrently indicated as a novel autoantigen in systemic lupus erythematosus (SLE), systemic sclerosis (SSc) (Moscato et al., 2000; Pratesi et al., 2000; Mosca et al., 2006; Bussone et al., 2011), SSc with interstitial lung fibrosis (Terrier et al., 2010), rheumatoid arthritis (Goëb et al., 2009; Saulot et al., 2002), mixed cryoglobulinemia (MC) with nephropathy (Sabbatini et al., 1997; Moscato et al., 2000; Pratesi et al., 2000), pulmonary arterial hypertension (Bussone et al., 2012), giant-cell arteritis (Régent et al., 2011), Behçet's disease (Lee et al., 2003) and inflammatory bowel disease (IBD) (Roozendaal et al., 1998). Anti-α-enolase autoantibodies isolated from patiens with SLE, SSc and MC recognized membrane-associated α-enolase and inhibited plasminogen binding to it (Moscato et al., 2000). It is tempting to speculate

that the high susceptibility of α-enolase to modification by HNE and other aldehydes might be instrumental for its involvement in autoimmunity as an oxidatively modified self antigen, capable of breaking the immunological tolerance of autoreactive T and B cells. This is in keeping with the identification of α-enolase among the proteins undergoing carbonyl addition and HNE adduction in heart homogenates and cardiomyocytes oxidized *in vitro* with 4-HNE or H_2O_2. Oxidative modifications correlated with increased recognition of α-enolase by serum antibodies of rodents and humans affected with Chagas' disease, which is characterized by increased production of ROS of inflammatory and mitochondrial origin (Dhiman et al., 2012).

HNE adducts with other neuronal enzymes, transporters, and receptors

Inducible ***heme oxygenase 1 (HO-1)*** catalyzes heme conversion to biliverdin-IXa, which is further reduced to antioxidant bilirubin-IXa (Mancuso and Barone, 2009). The expression of the *HO-1* gene is redox-regulated by an antioxidant responsive element in its promoter. Activation of HO-1 contributes to the adaptive response to oxidative stress in AD (Poon et al., 2004). Increased levels of HO-1 were observed in association with neurofibrillary tangles (NFTs) and senile plaques (Takeda et al., 2000) and in hippocampal neurons of AD patients, together with increases of serine phosphorylation, tyrosine nitration and 4-HNE modification of HO-1, as though adaptive increases in HO-1 expression and activation were counteracting the structural and functional impairment of HO-1, via tyrosine nitration and HNE-HO-1 adduct formation.

Collapsin response mediator protein 2 (CRMP2). Participates in axon guidance and synapse maturation, by mediating the transduction of reelin (Yamashita et al., 2006) and semaphorin 3A signals (Uchida et al., 2009). Sultana et al. (2012) has proposed that the HNE-CRMP2 adducts (Reed et al., 2008; Perluigi et al., 2009) might be of pathogenic importance for neurite shortening and the loss of synapses, early features of AD (Hensley et al., 2011; Scheff et al., 2011), and that Aβ peptide-induced oxidation of peptidylprolyl cis/trans isomerase (Pin1) (Butterfield et al., 2006b; Sultana et al., 2006b) may be responsible for the dysregulation of glycogen synthase kinase-3β (GSK-3β) and cyclin-dependent kinase 5 (CDK5) and for the hyperphosphorylation of tau proteins and of colocalized CRMP2 within NFTs (Williamson et al., 2011).

Reduced glucose utilization and energy production (Rhein and Eckert, 2007) are early occurrences in AD. They may be explained by the reported formation of HNE adducts with neuronal ***glucose transporter GLUT3*** in rat hippocampal neurons (Mark et al., 1997a) and with the ***mitochondrial ATP synthase α subunit*** in human AD brains (Reed et al., 2008; Perluigi et al., 2009; Terni et al., 2010). Decreased levels of ATP synthase activity were also reported in AD (Schagger and Ohm, 1995). Soluble Aβ peptide oligomers were responsible for electron-transport chain dysruption, enhanced ROS generation, mitochondrial fragmentation, and synaptic damage (Reddy et al., 2010), as well as for enhanced HNE production (Mark et al., 1997b). ATP synthase α subunit was colocalized with CRMP2 within NFTs (Sergeant et al., 2003). In AD brains, LDL receptor-related protein 1 (LRP-1), a membrane receptor involved in Aβ peptide removal, was also covalently modified by HNE, which might contribute to the extracellular deposition of amyloid substance (Owen et al., 2010).

Acrolein-protein adducts

Acrolein is neurotoxic *in vitro*. Moreover, in Alzheimer's brains, high levels of acrolein were detected in hippocampus and temporal cortex, where oxidative stress is high (Dang et al., 2010). Thus, several studies addressed the mechanism of acrolein neurotoxicity. Dang and coworkers (2010) showed that, in neuronal primary cultures of hippocampal cells, acrolein exerted more toxic effects than HNE. This might depend on the higher reactivity of acrolein, which was an initiator of oxidative stress by forming adducts with cellular nucleophilic groups in proteins, lipids, and nucleic acids. Indeed, it was documented that in synaptosomal proteins, exposed to high concentrations of acrolein, a loss of thiol group content occurred, due to Michael adduct formation between acrolein and thiol groups of proteins (LoPachin et al., 2007). Moreover, such adduct formation led also to protein cross-linking. Based on the cited evidence, LoPachin et al. (2002, 2003) proposed that nerve terminals were the primary sites of acrolein action and that synaptic dysfunction was a necessary step in the production of neurotoxicity. In support of this hypothesis, *in vivo* and *in vitro* studies showed that exposure to acrolein was associated with reduced presynaptic neurotransmitter release. This effect involved inhibition of key proteins, which regulate membrane–vesicle fusion, such as N-ethylmaleimide-sensitive fusion protein (NSF) and synaptosomal-associated protein of 25 kDa (SNAP-25) (Barber and LoPachin, 2004; LoPachin, 2004). Acrolein also inhibited presynaptic membrane neurotransmitter uptake and vesicular storage *in vivo* and *in vitro* (LoPachin et al., 2006; LoPachin, 2004). Proteomic analyses showed that these dysfunctions were associated with the formation of adducts with the ***dopamine transporter*** and ***v-ATPase***, respectively Barber and LoPachin, 2004; LoPachin, 2004; LoPachin et al., 2007; Barber et al., 2007. *In vitro* studies showed that acrolein and HNE disrupted synaptosomal membrane protein conformation and phospholipid asymmetry (Subramaniam et al., 1997; Pocernich et al., 2001; Castegna et al., 2004), reduced glutamate uptake and GLUT3-mediated glucose transport in synaptosomes and cultured nerve cells (Keller et al., 1997a,b; Lovell et al., 2000), reduced respiration and induced oxidative stress in synaptosomal mitochondria (Humphries et al., 1998; Morel et al., 1999; Picklo et al., 1999; Picklo and Montine, 2001; Luo and Shi, 2005; Raza and John, 2006), inhibited membrane Na^+ and Ca^{2+} ion pumps and dysrupted ion regulation in cultured nerve cells (Keller et al., 1997b; Mark et al., 1997b).

MDA-protein adducts

A largely coincident protein repertoire as the one delineated by anti-HNE antibodies was compiled by the immunochemical detection of Nε-MDA-lysine. It included: α- (non neural) and γ-enolase (neural), glutamic acid dehydrogenase I, creatin kinase B chain (CKB), ubiquinol-cytochrome c reductase complex core protein I, ATP synthase β subunit, glutathione synthase (GS), 60-kDa heat shock protein (HSP-60),

guanine nucleotide-binding protein G(I)/G(S)/G(T) β subunit 2 (GNB2), β- and γ-actin, α- and β-tubulin, vimentin, neurofilament L, glial fibrillar acidic protein (GFAP), collapsin response mediator protein 2, CRMP2, DRP-2) (Pamplona et al., 2005), and 14-3-3 protein ζ (HYWAZ) and γ (HYWAG) isoforms (Santpere et al., 2007).

A redox model of Alzheimer's disease pathogenesis

Far beyond individual protein dysfunctions, the generation of markers of lipid peroxidation in AD appears to be associated with the progressive endangerment of vital processes, such as energy metabolism, antioxidant defenses, signal transduction, axonal transport, and synapse conservation. A redox model of Alzheimer's disease pathogenesis (Sultana et al., 2012) is depicted in **Figure 3**.

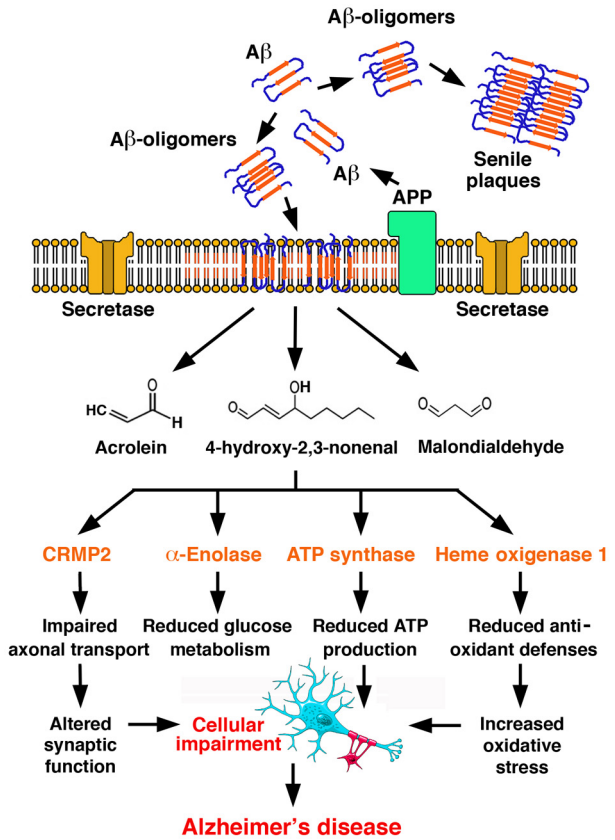

FIGURE 3 | A redox model of Alzheimer's disease pathogenesis. Amyloid β-peptide (Aβ) is generated by proteolytic cleavage of Amyloid Precursor Protein (APP) by secretases. Aβ undergoes aggregation, with the formation of oligomers, which undergo a conformational transition to β-structured diffusible oligomers and eventually deposit as amyloid plaques in the ECM. Aβ oligomers insert in the plasma membrane, where they initiate lipid peroxidation, leading to the formation of reactive aldehydes, such as acrolein, MDA and HNE. Adduct formation compromises the function of critical proteins in a number of functional subsets of neurotransmission, energetic metabolism, mitochondrial function, antioxidant defenses, represented here by collapsin response mediated protein 2 (CRMP2), α-enolase, ATP synthase α subunit and heme oxygenase 1. Such process is self-feeding and ultimately leads to Alzheimer's disease [Redrawn with permission from Sultana et al. (2012)].

ALDEHYDE-PROTEIN ADDUCTS IN ATHEROSCLEROSIS

The potential role of reactive aldehydes in the pathogenesis of atherosclerosis was suggested by their increases in plasma in association with extensive aortic atherosclerosis and the high levels of aldehydes generated during the oxidation of phospholipids in LDLs (Salomon et al., 2000). The observed consequences of LDL oxidation by aldehydes *in vitro* are described below. The formation of protein-bound lipid peroxidation products in atherosclerotic lesions was also repeatedly reported.

ALDEHYDE-LDL ADDUCTS

Early studies of the contribution of aldehyde-protein adducts to atherogenesis provided evidences that modification of LDL by aldehydes enhanced their recognition and uptake by macrophages (Hoff et al., 2003). The formation of aldehyde adducts with apolipoprotein B (Apo B) in LDL converted the latter to an atherogenic form that was taken up by macrophages, leading to the formation of foam cells (Steinberg et al., 1989; Steinberg, 1995). The adduction products detected in Apo B of oxidized LDL included: (a) acrolein derivatives, such as N-(3-methylpyridinium)lysine (MP-Lys) (Obama et al., 2007) and the 3-formyl-3,4-dehydropiperidino adduct (FDP-lysine) formed by the addition of two acrolein molecules to one lysyl side chain (Uchida et al., 1998a,b); (b) HNE adducts, such as the enaminal-type HNE-histidine and HNE-lysine adducts (Uchida et al., 1994); (c) MDA adducts, such as Nε-(2-propenal)-lysine (Uchida et al., 1997), and 1-amino-3-iminopropene-type MDA-lysine cross-links (Requena et al., 1997). The formation of aldehyde-LDL adducts could alter the binding of LDL to membrane scavenger receptors at the surface of endothelial cells and activated macrophages. The participation of reactive aldehydes in LDL-receptor interactions was documented by several immunohistochemical analyses of atherosclerotic lesions from human aorta, using antibodies against various aldehyde adducts, such as HNE-histidine (Uchida et al., 1995), Nε-MDA-lysine (Uchida et al., 1997), and Nε-acrolein-lysine (FDP-lysine) (Uchida et al., 1998a), in which intense positivities were associated with cells, primarily macrophages. It was recently reported that HNE–histidine Michael adducts had significant affinities and interacted with LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1), an important scavenger receptor mediating endothelial oxLDL uptake. HNE-modified proteins strongly inhibited the uptake of acetylated LDL (AcLDL). In human aortic endothelial cells, the binding of HNE-histidine adducts to LOX-1 stimulated ROS formation and activated extracellular signal-regulated kinase 1/2 (ERK 1/2) and NF-κB (Kumano-Kuramochi et al., 2012).

Using recombinant human Apo E (an exchangeable antiatherogenic apolipoprotein) and immunoblotting with acrolein-lysine-specific antibodies, other Authors (Tamamizu-Kato et al., 2007) showed that acrolein severely compromised the functional integrity of Apo E, as for heparin, lipid, and LDL receptor binding. These results were in agreement with previous observations of acrolein being widely present in atherosclerotic lesion, as revealed by the use of anti-acrolein antibodies (Uchida et al., 1998a). Nε-(3-methylpyridinium)-lysine (MP-Lys), an acrolein derivative, was detected in Apo B of native LDL (Obama et al., 2007). Moreover, acrolein-LDL induced foam

cell formation from macrophages, suggesting that acrolein maight contribute to LDL modification, foam cell formation and atherogenesis (Watanabe et al., 2013).

PHOSPHATIDYLCHOLINE γ-HYDROXYALKENALS AND ATHEROSCLEROSIS

Starting from the early observation that proteins modified by 2-pentylpyrrole incorporation of lysyl ε-amino groups, upon covalent addition of HNE, accumulated in the blood of individuals with atherosclerosis and in brain neurons of patients with Alzheimer's disease (Sayre et al., 1996), it became evident that γ-hydroxyalkenal phospholipids and their ω-carboxyalkylpyrrole derivatives contributed strongly in the pathogenesis of atherosclerosis. This was the subject of recent reviews (Salomon and Gu, 2011; Stemmer and Hermetter, 2012). Antibody-based studies revealed the presence of carboxyethylpyrroles (CHPs) and carboxypropylpyrroles (CPPs) in oxLDL (Kaur et al., 1997). Also the CHP immunoreactivity, reflecting the presence of protein adducts of 9-hydroxy-12-oxo-10-dodecenoic acid (HODA) or its phosphatidylcholine ester in human plasma, was significantly higher in the plasma of patients with atherosclerosis and end-stage renal disease than in healthy controls (Kaur et al., 1997). HODA-protein adducts were produced *in vivo* from 9-hydroxy-12-oxo-10-dodecenoyl-phosphatidylcholine (HODA-PC), one of the oxidized lipids derived from 1-palmityl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PA-PC), altogether referred to as oxPA-PC. Chemically synthesized 5-hydroxy-8-oxo-6-octenoyl-phosphatidylcholine (HOOA-PC) exhibited properties of a chemical mediator of chronic inflammation. It activated, in a dose-dependent manner, human aortic endothelial cells to bind monocytes and to secrete increased levels of monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8), two chemokines promoting monocyte entry into chronic lesions. It also inhibited LPS-induced expression of E-selectin, an adhesion molecule mediating endothelial-neutrophil interactions (Subbanagounder et al., 2002). HOOA-PC was found unbound and in pyrrole adducts in lipid extracts of oxLDL and human atheromas (Podrez et al., 2002; Hoff et al., 2003). A scenario emerged from these studies, in which atherogenesis might involve myeloperoxidase-initiated, free radical-induced production of oxPC, which promoted subendothelial monocyte infiltration and endocytosis of oxLDL by macrophages, accompanied by conversion into foam cells and atheroma formation (Salomon and Gu, 2011). Thereafter, it was shown that scavenger receptor CD36, another mediator of oxLDL uptake by macrophages, at variance with the LDL receptor, bound oxidized lipid derivatives within oxLDL, including the derivatives of 1-palmitoyl-2-arachidonoyl-glycerophosphocholine (oxPA-PC), such as HOOA-PC, and of 1-linoleoyl-2-arachidonoyl-glycerophosphocholine (oxLA-PC), such as HODA-PC (**Figure 2**). These γ-oxygenated-α,β-unsaturated aldehydes, collectively referred to as $oxPC_{CD36}$, were potent activators of the CD36-mediated endocytosis of oxLDL by macrophages, promoting the cytotoxic effects of the formation of protein adducts of electrophilic oxidized derivatives of cholesterol and phospholipids (Salomon and Gu, 2011). As an instance, the formation of Michael or pyrrole adducts of HOOA-PC or HODA-PC to a cysteinyl thiol group of lysosomal cathepsin B reduced the ability of mouse macrophages to degrade internalized macromolecules (Hoff et al., 2003). OxLDL and individual $oxPC_{CD36}$ also interfered with the binding of HDL to scavenger SR-B1 receptors of hepatocytes, thus inhibiting the HDL-mediated delivery of cholesteryl esthers to the liver (Ashraf et al., 2008).

HNE-SCAVENGER RECEPTOR B1 ADDUCTS AND KERATINOCYTE HDL UPTAKE

Scavenger Receptor B1 (SR-B1), also known as HDL receptor, is expressed in cells of the epidermal *stratum corneum*. In cultured human keratinocytes, exposure to cigarette smoke caused the translocation and eventual loss of SR-B1, driven by the activation of cellular NADPH oxidase (NOX) and the enhanced H_2O_2 production. Cigarette smoke also caused the formation of acrolein-SR-B1 and HNE-SR-B1 adducts and increased SR-B1 ubiquitination. It was proposed that such oxidation-dependent modifications of SR-B1 subcellular localization and stability might affect the physiological uptake of cholesterol by SC epidermal cells, which, in turn, might compromise their lipid composition and barrier function in the course of oxidative stress (Sticozzi et al., 2012).

ALDEHYDE-PROTEIN ADDUCTS IN AUTOIMMUNITY

Modification of self antigens in the course of oxidative stress, by adduct formation with reactive products of lipid peroxidation, HNE being one of the most commonly involved, is generally regarded to as a mechanism by which concomitant modification of self and neoantigen formation may lead to the breaking of tolerance to self antigens and, thus, to the pathogenesis of autoimmune disease. Indeed, it was known for a long time that abnormally high levels of HNE-protein adducts can be detected in the sera of children affected by autoimmune diseases (Grune et al., 1997). According to this view, cross-linking of HNE with self antigens would be instrumental in creating neoantigens from formerly tolerated autoantigens and, thus, initiating autoimmunity.

HNE-PROTEIN ADDUCTS IN SJÖGREN'S SYNDROME (SS) AND SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Sjögren syndrome (SS) is an autoimmunity-driven chronic infammatory disorder, characterized by infiltration and destruction of lacrimal and salivary glands by effector $CD4^+$ and $CD8^+$ T cells and activated macrophages, resulting in keratoconjunctivitis with dry eyes and xerostomia (dry mouth). Secondary SS can also add to the clinical picture of other autoimmune diseases, such as systemic lupus erythematosus (SLE). Among autoimmune diseases, SS is second only to rheumatoid arthritis (RA) in prevalence (1%), with affected females outnumbering males by 9–1. Antibodies to self antigens, such as anti-nuclear antibodies (ANA), are characteristically found in SS, some of them being in common with other autoimmune diseases, such as SLE, RA, and systemic sclerosis (SSc). Typical ANA targets in SS include the SS-A/Ro and SS-B/La proteins. The former include a 52-kDa form, located in nucleus and in cytoplasm, (SS-A1/Ro52; TRIM21) and a 60-kDa cytoplasmic form (SS-A2/Ro60; TROVE2). Both are components of Ro ribonucleoprotein (RNP) particles, in which they associate with short non-coding, histidine-rich RNAs

(hY-RNAs). The 48-kDa SS-B/La antigen is a transcription termination factor for RNA Polymerase III, transiently associated with hY-RNAs in ribonucleoprotein particles involved in tRNA processing and histonic mRNA stabilization. Systemic lupus erythematosus (SLE) is a multisystemic disease characterized by a polyclonal B cell activation, leading to the differentiation of plasma cells producing autoantibodies toward a broad range of autoantigens. ANA are found in 95% of patients with SLE, as well as in patients with other autoimmune diseases. They are heterogeneous and include antibodies toward: double stranded (ds) DNA; histones; ribonucleoproteins (RNP), such as the Smith (Sm) antigen (corresponding to the common core proteins of spliceosomal small nuclear RNPs), and the SS-A/Ro and SS-B/La antigens. The formation and deposition of immune complexes and complement in the wall of small arteries, at the dermo-epidermal junction and in the glomerular basal membrane (GBM) is responsible, respectively, for the diffuse necrotizing vasculitis, the cutaneous lesions of erythematous, bullous, and ulcerative kind, and the nephritis associated with SLE.

Notwithstanding their nuclear and/or cytoplasmic location, Ro and La antigens appeared to become exposed at the cell surface in the course of apoptosis. Epitopes expressed at the surface of apoptotic cells are named "apotopes." After the first observations of the clustering of cytoplasmic and nuclear antigens, including SS-Ro and SS-La antigens, in two types of blebs at the surface of apoptotic cells (Casciola-Rosen et al., 1994), the accessibility of SS-A/Ro and SS-B/La antigens at the surface of apoptotic cells was further confirmed (Miranda-Carús et al., 2000; Ohlsson et al., 2002). In SLE and SS, both the number of circulating apoptotic leukocytes and the susceptibility of lymphocytes to activation-induced apoptosis *in vitro* increased (Emlen et al., 1994; Georgescu et al., 1997; Zeher et al., 1999; Ren et al., 2003). Impaired efferocytosis (clearance of apoptotic cells) by macrophages also contributed to the higher degree of exposure to autoantigens determined by the increased rate of apoptosis in SLE (Ren et al., 2003). It was speculated that both factors may trigger autoimmunity (Savill et al., 2002). It was proposed (Casciola-Rosen et al., 1994) that the breaking of tolerance to autoantigens at the surface of apoptotic cells might be favored by oxidative modifications occurring as a result of the oxidative stress that characterizes apoptosis (Hockenberry et al., 1993).

The contribution of the formation of HNE adducts to the modification of self antigens, such as SS-A2/Ro60, in Sjögren's syndrome was explored by Scofield and coworkers. They hypothesized that modification of SS-A2/Ro60 with HNE might facilitate the breaking of tolerance to the self antigen. After immunizing rabbits with either HNE-modified or unmodified SS-A2/Ro60, they observed that autoimmunity was established faster and more strongly in animals immunized with HNE-modified SS-A2/Ro60 (Scofield et al., 2005). In an extension of this model, an SS-like condition, with anti-SS-A2/Ro60 antibodies, decreased salivary flow and salivary gland mononuclear infiltrates, could be induced in BALB/c mice by immunization with a peptide of SS-A2/Ro60 (Kurien et al., 2011). Efficient production of anti-SS-A2/Ro60 and anti-SS-B/La autoantibodies ensued immunization with SS-A2/Ro60, both as such and modified with increasing concentrations of HNE (0.4, 2, or 10 mM). However, antibody production was faster after low- and medium-level modification of SS-A2/Ro60 with HNE. Differential use of unmodified or HNE-modified SS-A2/Ro60 as the solid-phase substrate in ELISAs for autoantibodies revealed, among the antibodies produced by mice immunized with HNE-modified SS-A2/Ro60, an additional subpopulation of antibodies, which recognized HNE or HNE-SS-A2/Ro60, but not unmodified SS-A2/Ro60. Most interestingly, immunization with medium-level HNE-modified SS-A2/Ro60 was accompanied by the appearance of anti-dsDNA autoantibodies, which induced the Authors to imply a SLE-like disease, although they did not provide pathological evidence of it. Together with the already mentioned appearance of anti-SS-B/La antibodies, following immunization with SS-A2/Ro60, the occurrence of anti-dsDNA antibodies represented an example of intermolecular epitope spreading. In turn, the ability of HNE to form adducts with a large number of biological macromolecules could be of help in understanding the broad range of autoantibody responses in SLE and SS. Moreover, immunization with high-level HNE-modified SS-A2/Ro60 was associated with weaker antibody responses to unmodified SS-A2/Ro60 and SS-B/La, reduction of salivary flow and lymphocytic infiltration of salivary glands, suggesting a Sjögren's syndrome-like condition. Notably, high-level HNE modification of SS-A2/Ro60 was accompanied by aggregation, which prompted the Authors to interpret the results as due to increasing bifunctional cross-linking of SS-A2/Ro60 and diminished exposure of HNE at the surface of SS-A2/Ro60 molecules (Kurien et al., 2011). A more likely interpretation could be that large, particulate immunocomplexes of aggregated HNE-SS-A2/Ro60 and autoantibodies stimulated the phagocytic and antigen-presenting activity of macrophages, which skewed the autoimmune response toward a prevailingly cytotoxic cell-mediated mechanism.

The molecular mimicry between the adducts of lipid peroxidation products with proteins and nucleic acids, as a possible mechanism initiating the production of anti-DNA autoantibodies, in response to some other modified self antigen, was the subject of interesting studies by Uchida and coworkers. After raising an anti-HNE monoclonal antibody (anti-*R* mAb 310), recognizing enantioselectively (*R*)-HNE-histidine Michael adducts (Hashimoto et al., 2003), they unexpectedly found that the sequence of this anti-HNE mAb was highly similar to those of various clonally related anti-DNA antibodies. Despite these structural similarities, the cross-reactivity of mAb R310 with native dsDNA was limited, but was strongly enhanced by treating DNA with 4-oxo-2-nonenal (ONE), a HNE analog. The 7-(2-oxo-heptyl)-substituted 1,N^2-etheno-type ONE-2'-deoxynucleoside adducts were identified as alternative epitopes of mAb R310 in ONE-modified DNA. On these grounds, these Authors hypothesized that endogenous reactive electrophiles, like HNE, might function as immunologic triggers for human autoimmunity (Akagawa et al., 2006). These Authors further investigated the possible involvement of HNE-modified proteins as the endogenous source of anti-DNA antibodies. They found HNE-specific epitopes in the epidermis and dermis of patients with SLE, pemphigus vulgaris and contact dermatitis, as well as antibodies against HNE-modified bovine serum albumin (BSA) in the sera of patients affected with

SLE, Siögren's syndrome, rheumatoid arthritis, systemic sclerosis and idiopathic inflammatory miopathies, and also in the sera of diseased MRL/*lpr* mice. Upon repeated immunization with HNE-modified KLH, mice developed also a subpopulation of B cell clones recognizing native DNA, but not HNE-BSA. In agreement with previous results, the reactivity of anti-HNE B cell clones toward DNA was greatly enhanced by DNA modification with ONE. On the other hand, anti-DNA mAbs cross-reacted with ONE-modified BSA. These data suggested that HNE-specific epitopes produced upon physiological generation of HNE in cells might serve as triggering antigens for the development of bispecific antibodies against native DNA and ONE-modified proteins. On the whole, these findings strongly supported the pathogenic role of lipid peroxidation products in autoimmune disease (Toyoda et al., 2007). The pathogenic role of lipid peroxidation in SLE and the potential usefulness of anti-MDA and anti-HNE antibody titers in predicting its progression was underscored also by a report showing that the prevalences and serum levels of MDA- and HNE-protein adducts, as well as of MDA- and HNE-specific antibodies, were significantly higher in SLE patients than in healthy controls, and were in correlation with the SLE Disease Activity Index. The levels of each aldehyde-protein adduct were also in correlation with the titers of the respective antibodies (Wang et al., 2010).

ALDEHYDE-PROTEIN ADDUCTS AND STRUCTURAL INTEGRITY, ION TRANSPORT, AND SIGNAL TRANSDUCTION AT THE PLASMA MEMBRANE LEVEL

HNE is the product of lipid peroxidation which has been shown to be mostly involved in the control of cell functions. Under physiological conditions, HNE can be found at low concentrations in human tissues and plasma (Parola et al., 1998; Okada et al., 1999; Ji et al., 2001; Siems and Grune, 2003), where it participates in the control of signal transduction, cell proliferation and differentiation (Parola et al., 1998). HNE-protein adducts in peripheral blood primarily involve albumin, transferrin and immunoglobins (Barrera et al., 1996). Adducts between HNE and proteins have been detected *in vitro* in various mammalian cell types (Parola et al., 1998; Okada et al., 1999; Ji et al., 2001; Siems and Grune, 2003), in which the percent of total added HNE in HNE-protein adducts was between 1 and 5% (Rinaldi et al., 2001). Some adducts of HNE with cell proteins involved in specific functions at the plasma membrane level were characterized in detail.

HNE-SPECTRIN ADDUCTS AND RED CELL MEMBRANE INTEGRITY

Spectrin is the main component of the submembranous cytoskeleton lining the intracellular side of the plasma membrane of red blood cells, playing a fundamental role in maintaining its stability and strength, via direct interactions with membrane lipids and the actin cytoskeleton. Immunoblotting and mass spectrometric analyses revealed that, in human red cells, α- and β-spectrin were the primary targets of HNE adduction. Exposure of intact red cells to HNE resulted in selective HNE-spectrin adduct formation, with preferential β-spectrin modification and cross-linking of HNE-modified spectrin molecules. The Authors speculated that local spectrin aggregation, by freeing the lipid bilayer from the underlying spectrin-actin cytoskeleton, might lead to membrane surface area loss by extrusion (Arashiki et al., 2010). Together with the reported accumulation of HNE in aging circulating red blood cells (Ando et al., 1995), these observations may be of relevance not only for the physiological destruction of aged red cells, but also for the immune-mediated hemolysis of red blood cells under conditions of enhanced production of lipid hydroperoxides.

HNE-Na^+-K^+-ATPase ADDUCTS

Na^+-K^+-ATPase is an integral plasma membrane protein of great functional importance. Its primary functions are the maintenance of intracellular K^+ ion levels and the excretion of Na^+ ions. It contains 70 cysteinyl residues per molecule. The binding of HNE at 1–10 μmolar concentration to Na^+-K^+-ATPase was rapid and was accompanied by a decrease in measurable SH-groups and an irreversible loss of enzyme activity (Siems et al., 1996). Na^+-K^+-ATPase could be attacked by HNE formed both intra- and extracellularly, due to the free access of HNE to integral plasma membrane proteins. These Authors suggested that the reduction of Na^+-K^+-ATPase activity upon covalent HNE binding might represent an important form of secondary oxidative cell damage. Their findings were confirmed by the demonstration that in cultured hippocampal neurons HNE impaired Na^+-K^+-ATPase activity and induced increases of intracellular Ca^{2+} ion concentration (Mark et al., 1997b).

HNE ADDUCTS WITH TYROSINE KINASE RECEPTORS

Tyrosine kinase receptors (RTKs), such as the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR), are transmembrane glycoproteins, displaying tyrosine kinase activity in their cytoplasmic domains. Stimulation of RTKs by ligand-dependent or -independent mechanisms (radiation, metal ions, ROS) induces receptor dimerization and autophosphorylation of tyrosyl residues, followed by catalytic activation, whereas downregulation of RTKs is mediated by internalization and dephosphorylation (Pawson and Scott, 1997). Oxidized LDL (but not native LDL) and free HNE induced in living cells the formation of HNE-EGFR and HNE–PDGFR adducts, evidenced by the binding of anti-HNE-protein antibodies and by the loss in free $-NH_2$ group content (Suc et al., 1998; Hubbard and Till, 2000; Escargueil-Blanc et al., 2001). At physiological or moderate HNE concentrations (0.1 μM and 1–10 μM), the formation of HNE-EGFR and HNE-PDGFR adducts resulted in sustained RTK activation (Suc et al., 1998; Escargueil-Blanc et al., 2001). A short incubation of vascular smooth muscle cells (SMCs) with a low concentration of HNE (0.1–1 μM) induced the derivatization and autophosphorylation of RTKs, with the consequential activation of the phosphatidylinositol 3-kinase (PI3K)/Akt-mediated survival pathway and of the mitogenic response of SMCs (Auge et al., 2002). On the other hand, high concentrations of HNE, for longer incubation times, inhibited EGFR- and PDGFR-mediated cell proliferation (Liu et al., 1999; Vindis et al., 2006), through inhibitory effects on RTK signaling (Negre-Salvayre et al., 2003). High doses of HNE exerted similar negative effects on proteasomes (Okada et al., 1999; Vieira et al., 2000), mitochondrial transition pores (Irwin et al., 2002), glyceraldehyde-3-phosphate dehydrogenase

(Uchida and Stadtman, 1993) and cathepsin B activities (Crabb et al., 2002). The inhibitory effect of 4-HNE on growth factor-dependent cell proliferation was in agreement with the progressive desensitization of PDGFR β subunit to its ligand PDGF B-chain in SMCs (Vindis et al., 2006). In other cell types, low HNE concentrations (1 μM) did not cause RKT activation. In human hepatic stellate cells (hHSC), 1 μM HNE rather inhibited tyrosine autophosphorylation of PDGFR β induced by the PDGF BB isoform, which resulted in the inhibition of the mitogen-activated protein kinase (MAPK) and PI3K cascades and a consequential decrease of PDGF-dependent DNA synthesis (Robino et al., 2000). Acrolein was also shown to be a potent inactivator of protein tyrosine phosphatase 1B (PTP1B), a member of an important class of cysteine-dependent enzymes, working in tandem with protein tyrosine kinases in the regulation of a number of signal transduction pathways (Seiner et al., 2007).

HNE ADDUCTS WITH PROTEINS IN THE INSULIN SIGNALING CASCADES

The regulation of insulin signaling starts with the binding of insulin to its receptor, whose tyrosyl residues are rapidly phosphorylated. This permits the recruitment of adaptor proteins, such as insulin receptor substrates (IRSs) and Src homology-2-containing (Shc) proteins, which transmit the insulin signal down the PI3K cascade for glucose, lipid, and protein metabolism and the MAPK cascade for cell proliferation and differentiation (Saltiel and Kahn, 2001; Van Obberghen et al., 2001; White, 2002; Taniguchi et al., 2006). Reductions in the levels of IRSs and insulin-induced IRSs and a decrease in insulin receptor β phosphorylation were observed upon exposure to HNE at non-toxic concentrations (Demozay et al., 2008). Such effects could be due to the formation of HNE-IRS adducts, likely impairing IRS function and favoring IRS degradation. The downstream signaling cascades, involving PI3K and protein kinase B (PKB), were also down-regulated upon exposure to HNE, which resulted in blunted metabolic responses. The Authors of this study hypothesized that HNE build-up in diabetic rats (due to increased lipid peroxidation and altered clearance of its products by detoxifying enzymes) might be a cause of signaling dysfunction, hindering insulin action.

CONCLUSIONS

The adducts of reactive aldehydes with membrane proteins participate in physiological, as well as pathological processes and can determine variable functional consequences, in relation with the protein targets of adduction and their functional roles. Polyclonal and monoclonal antibodies directed against protein-bound aldehyde adducts have been of great help in exploring the aldehyde-related modifications of the cell proteome, while mass spectrometry-based techniques have been playing a key role in elucidating the stoichiometry and sites of covalent protein modification with reactive aldehydes. Nonetheless, the inventory of aldehyde-modified membrane proteins detected so far is probably still largely incomplete, when compared with the plethora of biological effects displayed by these molecules. Quantitative technical limitations in the individuation of aldehyde-protein adducts are being gradually overcome by the increases in sensitivity, molecular specificity and tolerance to impurities of spectrometric instrumentation and techniques (Wu and Vogt, 2012). Current challenges include: (1) characterizing the functional consequences of cell protein modification with aldehydes, which was not addressed by most redox proteomic studies published until now. This may involve major efforts of expression, reconstitution, modification and activity/interactivity assays of protein targets of aldehyde modification *in vitro*, as well as innovative approaches of protein-specific tracking and functional characterization at the cellular level; (2) clarifying the sources, sites and circumstances of increased lipid peroxidation in cells and the topological/functional relationships (e.g., in terms of subcellular compartmentalization and regulation of gene expression and gene product activity) linking the increased generation of reactive aldehydes with the modifications of specific cell membrane proteins.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Membrane_Physiology_and_Membrane_Biophysics/10.3389/fphys.2013.00242/abstract

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