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Letter to the Editor

ATP-binding cassette transporter 1 and Transglutaminase 2 act on the same genetic pathway in the apoptotic cell clearance

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Dear Editor,

The clearance of cells undergoing apoptosis remains the less characterised phase of the apoptotic program. In contrast to the removal of pathogens or necrotic cells, the phagocytosis of apoptotic cells removes cell bodies in the absence of both inflammatory response and tissue damages. The efficiency of clearing is extremely high because *in vivo* free apoptotic corpses are rarely observed.[1]

The clearance of apoptotic cells can be considered an active anti-inflammatory process,[2] in that defective removal is danger both directly via local tissue injury and indirectly via the triggering of autoimmune responses.[3] In fact, apoptotic cells, when uncleared, undergo secondary cytolytic events directly ensuing in inflammatory responses. Extensive post-translational modifications of cellular proteins occur during apoptosis resulting not only in profound structural changes but also in modulation of cellular antigenic properties.[4–5] Over the last few years, it has became clear that a defect in the clearance owing to the ablation of genes involved in phagocytic pathways may lead to the development of autoimmune diseases.[6] In humans, an impaired clearance of dying cells is considered a major event in the etiopathogenesis of systemic lupus erythematosus.[7]

Extensive mutagenesis studies in *Caenorhabditis elegans* led to the identification of seven genes required for an efficient engulfment of dying cells: *ced-1,-2,-5,-6,-7,-10,-12*. These act along two distinct, but partially redundant pathways. One involves *ced-2,-5,-10* and *-12*, the other *ced-1, -6 and -7*. The mammalian orthologs of the ced-2, -5, -10, -12 proteins are the adapter protein CRKII and DOCK180, the Rho family GTP-binding protein Rac and ELMO, respectively. The homologous protein in the second pathway are the engulfment adaptor protein GULP for ced-6, and the ATP-binding transporter, ABCA1, for ced7. No clear ortholog to ced1 has been defined so far, although a number of candidates have been proposed.[8] As a counterpart to the seminal work on the netamode system, the recent availability of genetically manipulated mouse models carrying deletion of engulfment genes has allowed both to confirm the involvement of putative intermediates, and to identify new players in the phagocytic process.

The ATP-binding cassette transporter 1 (ABCA1) is the mammalian ortholog of Ced-7,[9] and its role in phagocytosis has been demonstrated by both *in vivo* and *in vitro* studies. In *ABCA1* knockout (KO) embryos at stage E13.5, a transient accumulation of apoptotic corpses in the limb buds is detected in spite of a normal local recruitment of macrophages. Also peritoneal macrophages from adult *ABCA1* KO mice showed a 50% reduction in their capacity to engulf dead cells whereas their phagocytic competence towards other preys is unaltered.[10] Experimental evidences indicate that *ABCA1* in mammals controls the physicochemical properties of the plasma membrane acting as a translocator of lipids between the two membrane leaflets. The resulting modifications of membrane fluidity may well affect the lateral mobility of proteins and thus facilitate the spatial recruitment of the receptor recognizing the apoptotic cell. Furthermore, changes in the distribution of the lipid across the bilayer may *per se* generate forces favouring or hindering membrane bending and budding. Thus, *ABCA1* may favour engulfment via both protein and lipid–mediated mechanisms.[11]

Tissue transglutaminase (TG2) is a versatile multifunctional protein ubiquitously expressed in mammalian tissues and involved in a variety of cellular processes at various cellular locations. TG2 catalyses Ca^{2+}-dependent crosslinking reactions resulting in oligomerisation of substrate proteins that acquire the features of resistance to breakage and chemical attack.[12] TG2 has been widely related to programmed cell death[13] and recently it has been shown that TG2 is required both *in vitro* and *in vivo* for the efficient clearance of apoptotic cells.[14] In *TG2* KO mice, the clearance of apoptotic cells is defective during the involution of thymus elicited by dexamethasone, anti-CD3 antibody, or γ-irradiation.[14] The reduced clearance of apoptotic cells has been associated with an inflammatory infiltrate in the liver and this is in line with previous studies showing that the clearance efficiency of apoptotic cells is a key factor in the suppression of tissue inflammation.[15] In *ex vivo* studies, although *TG2* KO macrophages were competent for the phagocytosis of apoptotic thymocytes, they showed a reduced capacity to engulf dead thymocytes. The defect can be considered macrophage-specific since uninfluenced by the genotype of the apoptotic thymocytes. Additionally, the KO deficit is restricted to the phagocytosis of apoptotic cells as *TG2* KO macrophages ingest normally both bacteria and yeast.[14]

Based on the similarity in the phagocytic defects observed in the *ABCA1* and *TG2* KO mice, we decided to study the functional relationships between these two genes in the control of engulfment. To this aim, we have generated double KO mice and analysed *in vitro* and *in vivo,* their phenotypic features related to the engulfment of apoptotic cells.

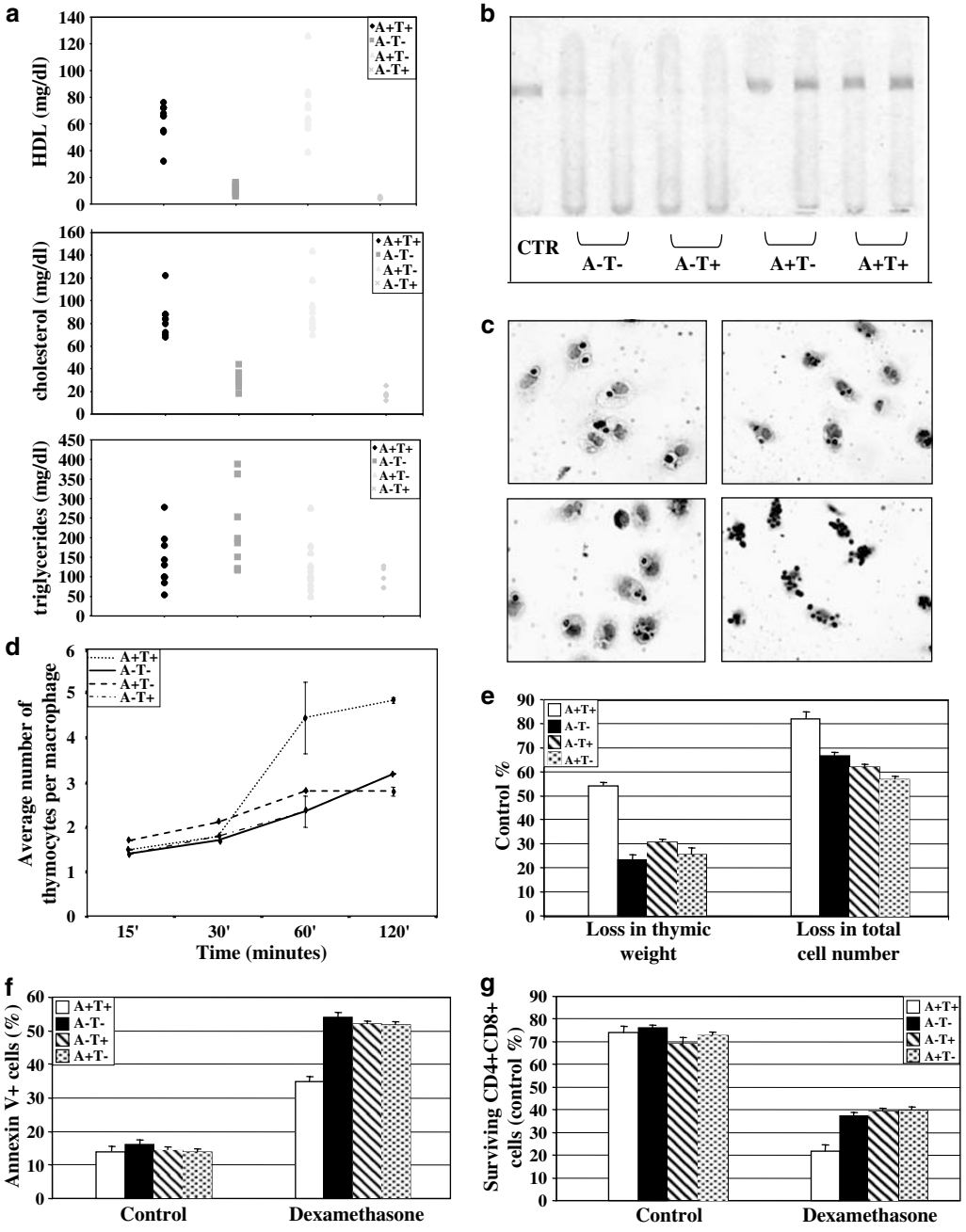

Figure 1 Characterisation of *ABCA1* and *TG2* KO mice. (**a**) Plasma lipids and lipoprotein analysis: all the phenotypic analyses were performed in 10 double KO and 10 control adult fasting mice. Mouse plasma was isolated from retroorbitally collected blood. HDL, cholesterol, and tryglicerides levels were determined by using enzymatic colorimetric assay (Modular PP automated analyzer, Roche) or (**b**) by immunofixation assay (Rabbit antibody to Mouse Apolipoprotein A1, Biodesign). (**c**) *In vitro* phagocytosis assays: phagocytosis analysis of double KO (upper panel) and wild-type (lower panel) peritoneal macrophages cocultured with apoptotic thymocytes for 15 (left) and 60 (right) min. Resident macrophages were harvested from adult mice by peritoneal lavage and seeded on two well chamber slides (3×10^5). Isolated thymocytes were seeded (10^6 cells/ml) and treated with 0.1 μM dexamethasone. Apoptosis was determined by flow cytometry (Beckton Dickinson) of PI/Annexin V-stained samples. Apoptotic thymocytes were placed on top of the macrophages at a concentration of 1.5×10^6/ml. After 15, 30, 60 and 120 min of coculture, thymocytes that had not been taken up were washed away, whereas the adherent cells were fixed and stained with ematossilin and eosin. (**d**) Preparations were observed by light microscopy and the number of apoptotic thymocytes per macrophage was determined (400 macrophages that have internalised one or more apoptotic thymocytes were counted). Only thymocytes clearly within the perimeter of the macrophage were counted. Experiments were performed in triplicate. The resulting mean was expressed as 'average number of thymocytes per macrophage'. (**e**) Phagocytosis analysis *in vivo*. Loss in thymic weight and in total cell number 24 h after injection of 0.2 mg of dexamethasone acetate (Sigma) in double KO and control 4-week-old mice. (**f**) Percentage of Annexin-V-positive cells determined by flow cytometry of all thymic cells, FITC Annexin-V stained (Sigma). (**g**) Percentage of CD4 + CD8 + cells that survived in wild type and double KO thymi after treatment. Cells were stained with PE-labelled anti-CD4 and APC-conjugated anti-CD8 (PharMingen)

The analysis of the *ABCA1−/− TG2−/−* offsprings showed an evident shifting from the expected mendelian frequency owing to *ABCA1* ablation and unchanged by the further ablation of *TG2*.

As *ABCA1* KO mice are faithful phenocopies of Tangier disease patients,[9] and display dramatic alterations in lipoproteins metabolism,[16] we checked whether the concomitant deletion of *ABCA1* and *TG2* had any visible effect on the metabolic profile characteristic of this disease. We compared high-density lipoprotein (HDL), cholesterol and triglycerides levels of double-KO mice and *ABCA1* KO in adult fasting mice. Double-KO mice showed near complete loss of HDL, reduced levels of cholesterol, and normal levels of triglycerides similar to the *ABCA1* single-KO mice, whereas no alterations in their levels were observed in the counterpart *TG2* single-KO mice (Figure 1a). Similarly, the circulating levels of Apo-A1 analysed by immunofixation confirmed (Figure 1b) that the concomitant deletion of TG2 did not modify substantially the near complete lack of circulating Apo-A1 observed in the absence of *ABCA1*. In conclusion, our results indicated that, in spite of a similar impact on the phagocytosis process, *TG2* does not share with *ABCA1* a function in lipoproteins synthesis.

To study the effect of the simultaneous *TG2* and *ABCA1* ablation on the phagocytosis of apoptotic cells by professional macrophages, we investigated the ability of *ABCA1−/− TG2−/−* peritoneal macrophages to clear apoptotic thymocytes *in vitro*. Resident peritoneal macrophages were isolated from double-KO mice and the respective controls mice and exposed to apoptotic thymocytes for various time intervals. In order to mimic the *in vivo* event, we used apoptotic thymocytes in the early stages of apoptosis as indicated by the annexin V positive and propidium iodide negative staining (data not shown). The quantitative evaluation of the number of internalised thymocytes per phagocyte showed that, after 15–30 min, macrophages have internalised an average of 1–2 apoptotic cells. At 60–120 min, this increases to 4–5 cells for WT macrophages, whereas the number of apoptotic cells in double-KO macrophages remains constant (Figure 1c and d). Importantly, the defect of double KOs is similar to what observed for the *TG2* or *ABCA1* single-KO macrophages, indicating that the concomitant deletion of the two genes does not lead to a more severe phenotype. These data thus indicate that in the context of engulfment, *TG2* and *ABCA1* act along the same genetic pathway.

To further confirm this conclusion and quantify the engulfment competence of double-KO macrophages *in vivo,* we induced massive apoptotic involution of the thymus by injecting dexamethasone in 4-week-old double-KO and the respective control mice. To evaluate the apoptotic event, we measured the variation of thymic weight and cellularity, whereas to evaluate phagocytosis, we compared the percentage of annexin V-positive cells out of all thymic cells (Figure 1e–g).

In accordance with the phagocytosis tests *in vitro,* the results obtained for the double-KO mice and for *TG2* or *ABC1* single-KO mice were essentially superimposable confirming that the combined deletion of *ABC1* and *TG2* genes does not exacerbate the defect observed in the single KO and further supporting the conclusion that TG2 acts on the same regulatory pathway of ABCA1.

What is the role of TG2 in the Ced1/Ced6/ced7 pathway? It has been described that ABCA1 controls the dynamic equilibrium of lipids in the membrane. This event can affect both the lateral mobility of proteins, facilitating the spatial recruitment of the receptors recognizing the apoptotic cell, as well as the generation of lipid-determined directional forces, sufficient to promote membrane sprounting around the particle to be ingested.[11] In this scenario, it is possible that the transporter makes at least transient contacts with the Ced1 ortholog in mammals. However, no evidence addressing this point has been reported so far. It is worth noting that the reconstitution of the molecular events leading to engulfment via this pathway is still rather scarce. One of the candidate Ced-1 ortholog, CD91,[17] has indeed been found able to bind to the adapter molecule GULP, but the signals activated by this molecular interaction lie still unknown. The activation of 'Rac like' GTPase is likely, as growing evidences suggest that members of this family play a crucial role in the reorganisation of the actin cytoskeleton during different types of phagocytosis.[18] TG2 could be involved in this pathway at various levels: (1) acting as G-protein, it could take part in transmitting signals downstream of GULP; (2) through transamidation of Rho A, a member of Rho GTPases, TG2 could play a role in cytoskeleton rearrangements; (3) TG2 can also interact and modify major component of cytoskeleton such as actin, beta-tubulin and the microtubule binding protein tau.[12,13]

The comprehension of role played by TG2 in the clearance of apoptotic cells will contribute to elucidate important aspects of the molecular mechanisms controlling this process, which may be relevant in the onset of pathologies caused by alterations of apoptotic cell removal such as autoimmunity.

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