Brief Communication

"Defective FoxP3$^+$ Treg cell differentiation in the gut of Type 1 Diabetic patients".

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SUMMARY

Environmental factors that act at the intestinal level such as diet, drugs, and microflora have a high impact on the pathogenesis of autoimmune Type 1 Diabetes (T1D), but it is still unclear how the gut milieu affects autoimmunity outside the intestine. Here we show that peripheral FoxP3^{+} Treg cell differentiation, a mechanism that takes place in the gut and is crucial to maintain systemic immune tolerance, is impaired in T1D patients. These results provide the first evidence that gut mucosa alteration could predispose to autoimmune T1D by affecting systemic immune regulation.

ARTICLE

In recent decades the incidence rates of autoimmune diseases are noticeably rising and nowadays approximately 5% of people in developed countries suffer from one systemic or organ-specific autoimmune disease[1]. Type 1 Diabetes (T1D) is a destructive islet ß-cell specific autoimmune disease resulting from a yet undefined interaction between genetic and environmental factors[2]. The dramatic increase in T1D incidence recorded in western populations in the past forty years, e.g., three-fold increase in developed countries[3] and +30% in the geographically restricted and highly susceptible Sardinian people[4], is unlikely related to genetic variations. Environmental factors such as viral infections (i.e. enteroviruses and rotaviruses)[5,6], reactions to dietary antigens (i.e. cow's milk and gluten)[7-9] and microbiota variations[10] that act at the intestinal level have been observed in association with, or as risk factors for, the development of T1D. In addition, sublinical intestinal abnormalities, such as increased permeability to sugars, have been described in patients with T1D and are detectable even before the clinical onset of the disease, suggesting that these functional abnormalities may be associated with the pathogenesis of T1D[11]. Although existing evidence is suggestive of a causative link between gut environment and the pathogenesis of T1D, it still unclear whether and by which mechanism(s) a dysfunction in the intestine does promote autoimmunity elsewhere, i.e., in the pancreatic β-cells and, if it does, how this process occurs. A fascinating hypothesis is that systemic and organ-specific autoimmune diseases are initiated and, possibly, maintained by virtue of changes in the immune regulatory mechanisms originating in the gut and crucial to maintain immune tolerance toward self-antigens outside the intestine. In facts, the small intestinal mucosa is the preferential site for the extrathymic development of regulatory FoxP3^{+} T cells (Treg cells). Specifically, tolerogenic dendritic cells (DCs) residing in the intestinal lamina propria are responsible for extrathymic FoxP3^{+} Treg cells development and expansion[12-14]. Since FoxP3^{+} Treg cells are instrumental for maintaining peripheral tolerance and preventing autoimmune diseases such as T1D[15], it is likely that defects of peripheral Treg cell differentiation may favor T1D pathogenesis. Considering how the environment impinges on intestinal immunity, it is critical to study immune regulation in the human intestinal mucosa rather than in intestinal samples from animals hosted in SPF facilities and, therefore, not exposed to the environment.

Hence, to assess whether the gut environment increases susceptibility to autoimmune diabetes by altering the peripheral differentiation of FoxP3$^+$ Treg cells, we analyzed the immune regulatory cell compartment of the small intestinal mucosa from patients with T1D and controls. We obtained duodenal biopsies and blood samples from 53 individuals, including healthy controls, patients with T1D and patients with celiac disease (a chronic inflammatory disease of the gut). Age, gender and clinical information for each group are summarized in **Supplementary Table 1**. A Multiparametric FACS analysis (see **Supplementary Fig. 1 and Methods**) showed a significant reduction in the percentage of CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ Treg cells in the gut mucosa but not peripheral blood of T1D subjects compared to healthy controls (**Fig. 1a**). Since five out of twelve (40%) T1D patients were also affected by celiac disease (see **Supplementary Table 2**), we performed a multiple groups comparison of gut Treg cell percentages in patients affected by T1D only, T1D in association with celiac disease, individuals with celiac disease only and healthy controls. As shown in **Fig. 1b**, the celiac disease did not significantly alter the gut Treg cell percentages both in diabetic and non-diabetic individuals and the Treg cell reduction was exclusively linked to the diabetic condition (*P*<0.001 with ANOVA statistical test). We verified that the gut CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ T cells that we enumerated belong to the human Treg cell subset by documenting their suppressive capacity upon T effector (Teff) cell proliferation (**Fig. 1c**). Interestingly, Treg cells inhibited more efficiently the proliferation of Teff cells from the same tissue, i.e., gut Treg cells suppressed gut Teff cells more efficiently than blood Teff cells and viceversa (see **Supplementary Fig. 2**). Importantly, in the gut mucosa of patients with T1D, the quantitative defect regarded exclusively the FoxP3$^+$ Treg cell subset while the percentages of total CD4$^+$ T cells as well as other immune populations including NK cells, DCs and $\gamma\delta$ T cells were similar to those of patients with celiac disease and healthy controls (**Fig. 1d**).

Next, we tested whether the reduced number of gut Treg cells in T1D patients is due to their defective differentiation in the intestinal mucosa. A subset of lamina propria DCs (LPDCs) expressing the integrin $\alpha E\beta7$ (also called CD103) has the unique capacity to drive the peripheral differentiation of FoxP3$^+$ T regulatory cells[12-14]. Hence, we analyzed the number and functional properties of CD103$^+$CD11c$^+$ DCs isolated from gut biopsies of T1D patients and controls. We did not detect any difference in the absolute percentage of CD103$^+$CD11c$^+$ DCs (**Fig. 2a**), nor in the percentages of DCs

that expressed the CD103 marker between the three groups (**Fig. 2b**). Then, we derived CD103$^+$CD11c$^+$ DCs from the intestinal mucosa of T1D patients and controls (**Supplementary Fig. 3**) and assessed their ability to induce Treg cell differentiation. CD11c$^+$CD103$^+$ LPDCs from controls were able to convert CD4$^+$CD25$^-$ T cells into CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ Treg cells more efficiently than blood monocyte-derived DCs (BMDCs) (**Fig. 2c**). Strikingly, LPDCs from T1D patients completely lacked that function and failed to induce FoxP3$^+$ Treg cell differentiation (**Fig. 2c**). TGF-β was required for LPDCs to induce Treg cell differentiation in control individuals (see **Supplementary Fig. 4**), however it did not restore the capacity to generate FoxP3$^+$ Treg cells in LPDCs of T1D patients (**Fig. 2c**).

The identification and characterization of a specific defect of the gut immune regulation compartment in patients with T1D contributes to the current understanding of the pathogenesis of the disease. Gut T cells continuously travel from the intestinal mucosa to mesenteric and pancreatic lymph nodes (PLN)[16]. Hence, the defective gut differentiation of FoxP3$^+$ Treg cells may directly influence immune tolerance towards self-islet antigens in PLN. Further studies are necessary to dissect whether the functional defect of LPDCs found in patients with T1D is genetically determined or induced by environmental factors. The enteric commensal microflora critically modulates the immune system and immune regulation by affecting LPDC function and Treg cells differentiation in the gut[17], either directly or through epithelial cells[18]. This raises the hypothesis that the defect of gut immune regulatory mechanisms that we have shown in T1D patients may be due to environmental factors, such as diet and infections, which alter the gut microflora.

Studies were approved by the Ethics Committee of the San Raffaele Scientific Institute, Milan, Italy. All participants signed an informed consent before any data collection or study procedure.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

E.B. isolated gut and blood lymphocytes, performed flow cytometry analyses and conversion assay experiments. C.S. performed conversion assays. A.M., L.M. and A.M.B. collected blood samples and duodenal biopsies. M.C. design research, performed flow cytometry analysis and reviewed the manuscript, M.S. performed statistical analysis and reviewed the manuscript, E.B. contributed to supervising the project and reviewed the manuscript, M.F. designed the experiments, supervised the project and wrote the manuscript.

FIGURE LEGENDS

Figure 1 Selective reduction of FoxP3$^+$ Treg cells in the gut of Type 1 Diabetic patients. **a**, the percentages of CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ Treg cells were measured in the total lymphocyte population isolated from the small intestinal mucosa (GUT) or peripheral blood mononuclear cells (PBMC). Briefly, at the time of an oesophago-gastro-duodenal endoscopy we collected duodenal biopsies from patients with Type 1 Diabetes (T1D, n=12) and healthy controls (HC, n=17). Simultaneously, we collected blood samples from the same patients. The regulatory T cells in gut mucosa or PBMC samples were targeted by Multiparametric FACS analysis. Data are presented as means $^\pm$ SEM of all experiments. **P = 0.003 (non-parametric Mann-Whitney test). NS = not significant. **b**, the percentages of CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ Treg cells were compared between patients with T1D (T1D, n=7), patients with T1D and celiac disease (T1D+CD, n=5), healthy controls (HC, n=17) and patients with celiac disease (CD, n=24). Groups were compared using two-way analysis of variance (ANOVA statistical test, P < 0.0001) **c**, gut CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ T cells were *bona-fide* Treg cells and showed a suppressive capacity similar to that of their blood counterparts. CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ Treg cells or CD4$^+$CD25$^-$ T effector (Teff) cells isolated from the gut mucosa or PBMC of healthy controls were tested in suppression assays. Briefly, Treg or Teff cells were added at 1:1 ratio to responder CSFE-labeled CD4$^+$CD25$^-$ T cells stimulated with coated anti-CD3 monoclonal antibody, soluble anti-CD28 mAb and rhIL-2. The percent of suppression was measured by the percent reduction of proliferation of responder T cells (by mean of CSFE dilution). Data are expressed as means $^\pm$ SEM of four independent experiments. *P = 0.02 (Student *t* test). **d**, Percentages of total CD4$^+$ T cells, NK cells, DCs and $\gamma\delta$ T cells were measured by Multiparametric FACS analysis in the gut mucosa of patients with T1D, healthy controls and patients with celiac disease. Data are means $^\pm$ SEM of all experiments. NS= not significant (Student *t* test).

Figure 2. Lamina propria dendritic cells are present in the small intestine of T1D patients but fail to induce Treg cell differentiation. **a**, percentages of CD103$^+$CD11c$^+$ DCs in the lamina propria of patients with T1D, healthy controls and patients with celiac disease. **b,** percentages of cells that express the gut
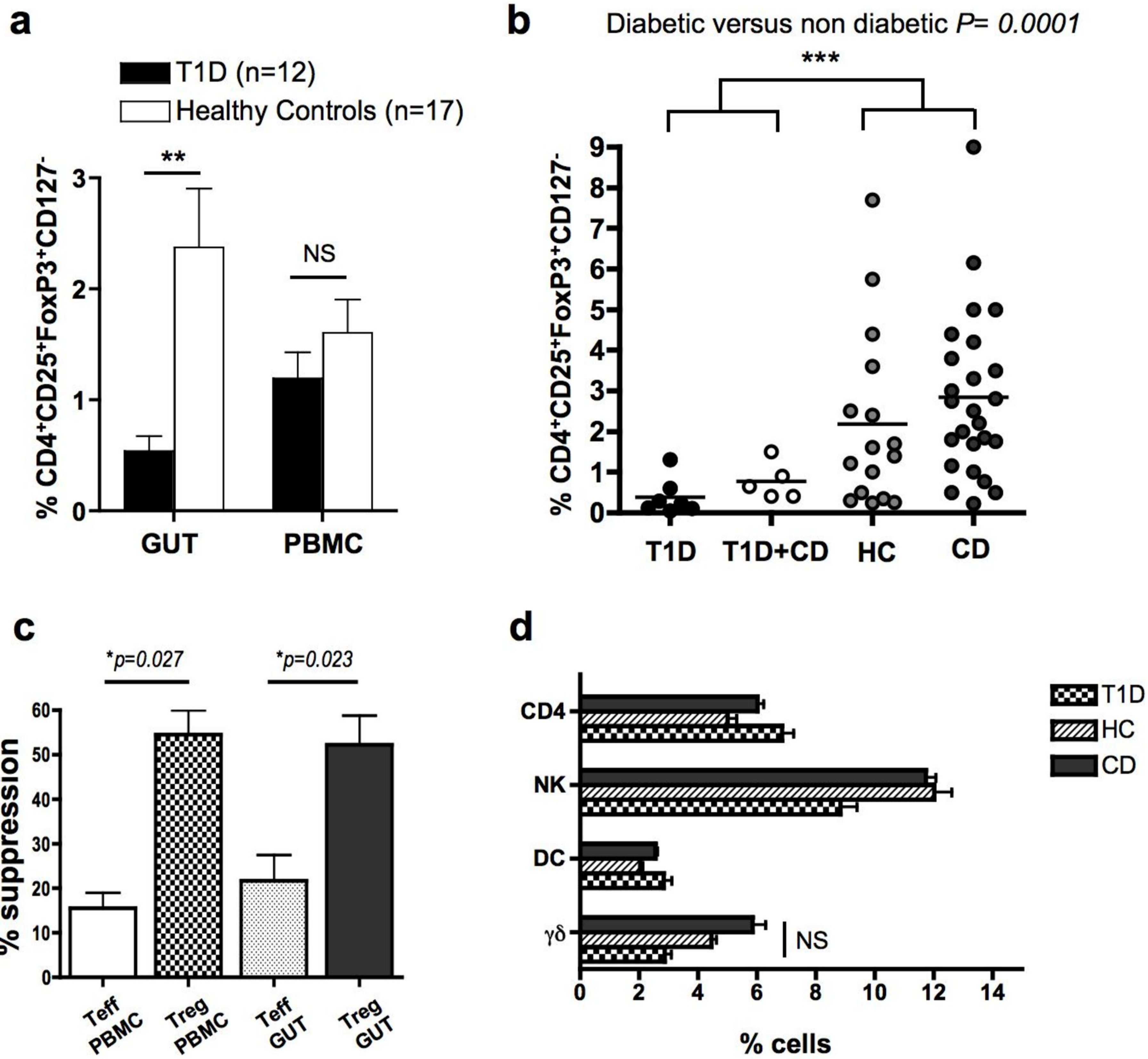
homing receptor CD103 among different gut lymphocyte subsets in the three groups. **c**, the capacity of lamina propria CD11c$^+$CD103$^+$ DCs of T1D patients and controls to induce Treg cell differentiation was evaluated in conversion assays**.** Blood monocyte-derived DCs (BMDCs) or CD11c$^+$CD103$^+$ lamina propria DCs (LPDCs) were obtained from T1D patients (T1D) and controls (CTR) and added to autologous naive CD4$^+$CD25$^-$ T cells isolated from PBMC and stimulated with soluble anti-human CD3 mAb in the presence of hrIL-2 and hrTGF-β. Cells were collected after 5 days and FACS analyzed to measure percentages of CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ Treg cells. One representative experiment is shown on the left inset (cells gated on CD4$^+$CD25$^-$CD127$^-$ cells). The right inset shows the mean $\pm$ S.E.M. of CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$Treg cell percentage obtained with different DCs in three (T1D) or four (CTR) independent experiments. *P = 0.028 (non-parametric Mann-Whitney statistical test).

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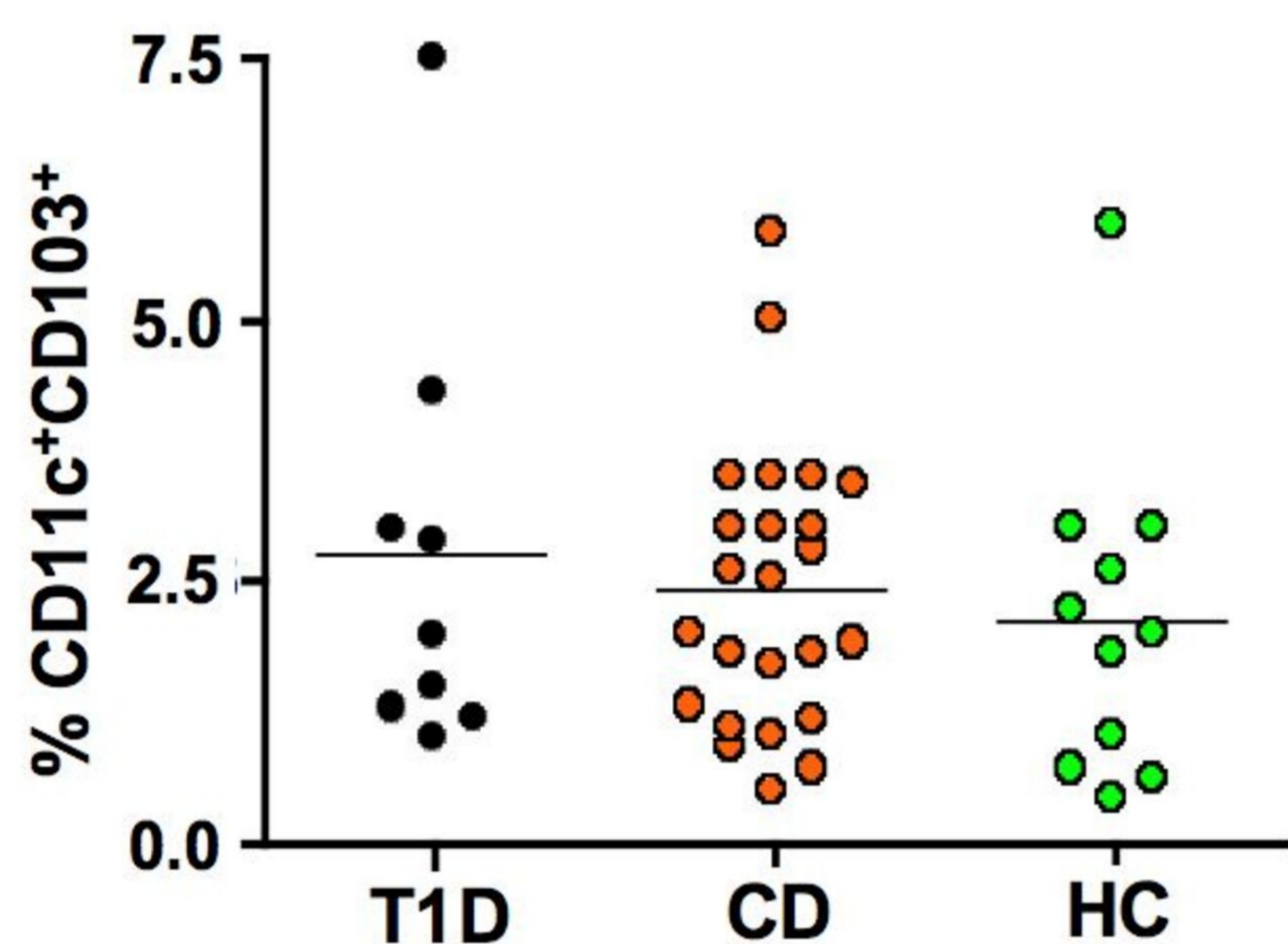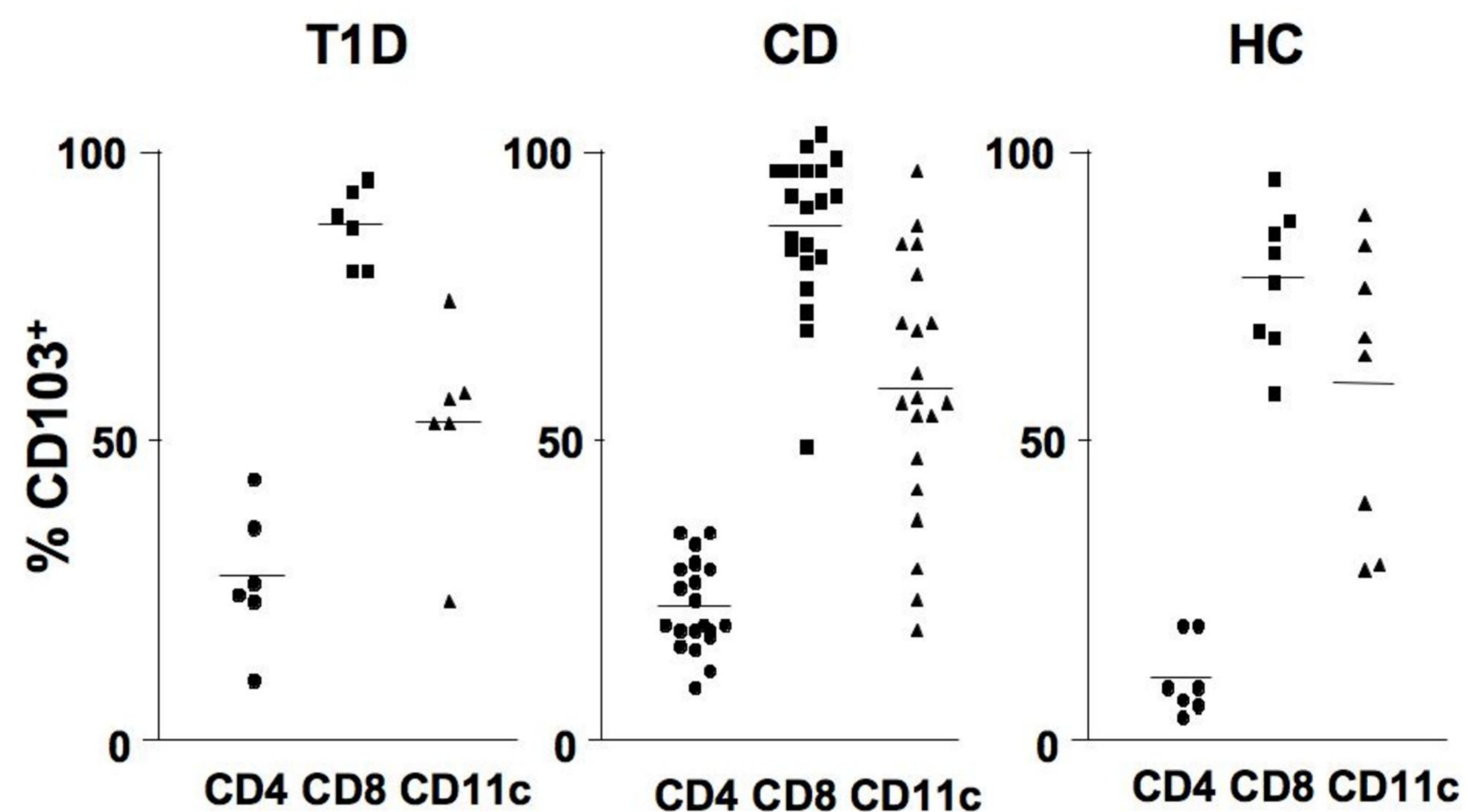18. Artis, D. *Nat. Rev. Immunol.* **8**, 411-419 (2008).

Figure 1

Figure 2

a

b

c