Analysis of circulating nutritional antigen-specific T-cells in celiac disease and inflammatory bowel disease

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Abstract

Background: The present study aims to detect, quantify and analyze circulating nutritional antigen-specific T-cells in patients with celiac disease (CeD) as well as inflammatory bowel disease (IBD), thus comparing the specific T-cell response following barrier disruption and antigen translocation. Methods: The antigen-reactive T-cell enrichment (ARTE) technique was applied allowing for a phenotypical and functional flow cytometric analysis of rare nutritional antigen-specific T-cells, including the CeD-causing gliadin (gluten), in the peripheral blood. Results: Our study indicates that by applying the ARTE technique, differences of gluten-specific T-cells as well as the differential cytokine expression between the patient groups can be detected, even without the burdening gluten re-exposure of the patients. CeD patients, independent from the presence or absence of gluten exposure in their current diet, featured an increase of the frequency of gliadin-specific T-cells, which were characterized by a pro-inflammatory phenotype. However, only for active CeD and a consecutive small intestinal barrier breach, an increase of distinct nutritional T-cells could be detected. Accordingly, frequency as well as pro-inflammatory phenotype of nutritional antigen-specific T cells were highest in Crohn's disease patients with small intestinal inflammation whereas no significant increase was observed in ulcerative colitis. Conclusion: In summary, the ARTE method allows not only for detection but also for functional analysis of these rare cells even in healthy subjects. Applying this method, we were able to demonstrate that for non-CeD-related nutritional antigens, small intestinal barrier breach is mandatory for a peripheral antigen-specific T-cell.

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Running title: Antigen-specific T-cells in IBD

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Contributions : YRS performed and analyzed the experiments, DL and FB provided samples and patient information, YRS, MS, US, BS and RG designed the experiments, interpreted the data and authored the manuscript.

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Results: Our study indicates that by applying the ARTE technique, differences of gluten-specific T-cells as well as the differential cytokine expression between the patient groups can be detected, even without the burdening gluten re-exposure of the patients. CeD patients, independent from the presence or absence of gluten exposure in their current diet, featured an increase of the frequency of gliadin-specific T-cells, which were characterized by a pro-inflammatory phenotype. However, only for active CeD and a consecutive small intestinal barrier breach, an increase of distinct nutritional T-cells could be detected. Accordingly, frequency as well as pro-inflammatory phenotype of nutritional antigen-specific T cells were highest in Crohn's disease patients with small intestinal inflammation whereas no significant increase was observed in ulcerative colitis.

Conclusion: In summary, the ARTE method allows not only for detection but also for functional analysis of these rare cells even in healthy subjects. Applying this method, we were able to demonstrate that for non-CeD-related nutritional antigens, small intestinal barrier breach is mandatory for a peripheral antigen-specific T-cell.

Keywords:

antigen-specific T-cells / celiac disease / gluten / IBD / nutritional antigens

word count: 2648

Introduction

Antigen-specific T-cells play a central role in the adaptive immune system, promoting specific acute immune responses and the formation of immunological memory. Analyzing not only frequency, but also phenotype and function of these rare cells, represents a critical step to understand the mechanisms of adaptive immunity in general, but also to determine the specific immune status of the individual patient or to diagnose infectious or auto-immune diseases. The high diversity of the T-cell receptor, which allows for recognition of billions of different antigens, leads to an extremely low frequency of T-cells, specific for a single peptide-MHC ligand. This holds true even for pathogen-specific memory compartments in the absence of acute infections, for which specific T-cell frequencies in peripheral blood are typically far below 1%, but all the more for the naive repertoire ($< 0.0005\%$)[1,2]. Hence, the analysis of antigen-specific T-cells demands methods which are

highly specific, but in parallel allow processing of large cell numbers to enable quantification of these rare events. For the last decade, techniques such as antigen-specific proliferation via ^{3}H-thymidine incorporation[3] were applied, where antigen-specific T-cells have been analyzed via bulk stimulation of peripheral blood mononuclear cells (PBMC) with the respective antigen. However, in such assays, it is not feasible to detect the phenotypic and functional properties of the original reactive cells and therefore it is almost impossible to determine the actual frequency and phenotype of these cells in the starting cell sample or to exclude bystander effects. ELISPOT[4], as an additional technical approach, reveals information on a single cell level, but lacks the high throughput capabilities to detect and quantify rare cells to an extent, where for example food antigen-specific T-cells in healthy individuals can be studied. Several studies aimed to characterize the systemic T-cell response to decipher the role of circulating T-cells and their functional impact [5,6]. Until now, the specific analysis of gluten-specific T-cells via flow cytometry depended on tetramer analysis [5], which is limited to the knowledge and availability of the antigen epitope [7], but also the frequency of target cells, therefore suffering from a high variability in cytokine analysis [8].

Among autoimmune diseases, celiac disease (CeD) represents a model disease, since gluten has been identified as the disease-causing antigen and elimination of gluten results in regeneration of the duodenal mucosa and consecutive wellbeing of the patient [9]. However, the diagnosis in CeD patients who are already on a gluten-free diet (GFD) remains challenging. Under a gluten-free diet (GFD), tissue-transglutaminase (tTG) antibodies normalize and the small intestinal villus atrophy regenerates. Until now, a burdening re-challenge of the patients to gluten is mandatory for a valid diagnosis. Yet, translocation of nutritional (and pathogenic) antigens due to intestinal barrier breaches is described not exclusively for CeD, but also for inflammatory bowel diseases (IBD). Although there are first studies connecting GFD to improvement of patient wellbeing[10] and even microbiota composition[11] in IBD, until now, circulating nutritional antigen-specific T-cells have not been analyzed in these patients. Since the small intestine is the primary contact surface for food antigens and hence for the immunological response, we analyzed the specific nutritional T-cell response in the peripheral blood of patients with small intestinal Crohn´s disease (CD), celiac disease as well as ulcerative colitis (UC), respectively. To exclude the influence of a non-intestinal inflammation, rheumatoid arthritis patients (RA) were included as control.

We here applied the novel antigen-reactive T-cell enrichment (ARTE)[7] technology to determine the specific nutritional effector T-cell response in the peripheral blood. The ARTE technique is based on the stimulation of PBMC with a defined antigen and the subsequent up-regulation of the activation marker CD154^{+}, which is exclusively expressed on antigen-specific CD4^{+} T-cells [12]. This method permits the detection of the entire antigen-specific CD4^{+} T-cell response just by adding the antigen of choice directly to PBMC without the need of in-vitro expanding the reacting cells. The subsequent enrichment of CD154^{+}cells enables further in-depth phenotyping of this rare cell population. Thus, the ARTE technique allows a direct *ex vivo* cytometric - and hence functional - analyses of gluten-specific, but also even rarer nutritional antigen-specific T-cells.

Methods

PBMC from CeD, CD, UC and rheumatoid arthritis patients as well as healthy controls (**Table 1**) were cultured for 6 h in the presence of defined antigens followed by magnetic enrichment of activated CD154^{+} T-cells (as marker for antigen-specific T-cells) [7] (**Figure 1A, B**).

Blood donors and PBMC isolation

Peripheral blood samples were obtained from healthy donors and patients of the Charité - Universitätsmedizin Berlin, Medical Department, Division of Gastroenterology, Infectiology and Rheumatology. All blood donors gave informed consent and the study was approved by the ethical committee of the Charité - Universitätsmedizin Berlin. PBMC were freshly isolated from 20 mL blood by density gradient centrifugation (Biocoll; Biochrom, Berlin, Germany). Heparinized whole blood was layered on the Biocoll Separation Solution and centrifuged at 1200 g for 25 min at 21 °C. PBMC were collected from the interphase, washed and resuspended in RPMI1640 (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 5% human AB-serum (Sigma-Aldrich, St. Louis, MO, USA).

Patients

PBMC of CD patients with small intestinal manifestation and UC patients with either active disease (defined by Harvey Bradshaw Index (HBI)) or remission (defined by HBI; pMayo) [13,14] were analyzed. Additionally, CeD patients on a GFD for at least one year, newly diagnosed active CeD patients still exposed to gluten (aCeD), or GFD-refractory CeD patients (Refr) were included. Moreover, healthy first-degree relatives (FDR) on a regular diet without symptoms were included. The diagnosis of CeD was based on the presence of tTG antibodies in the serum and characteristic histopathological features in duodenal biopsies (Marsh score >1). Refractory celiac disease diagnosis was based on the presence of a Marsh III enteropathy and clinical malabsorption in spite of consumption of a gluten-free diet for at least one year. Clonality analysis as done by PCR of the CDR3 region of the TCR was performed. Detection of a clonal T-cell population and aberrant lymphocytes by immune phenotyping of duodenal tissue allowed for diagnosis of Refr. type II. All other Refr. cases were diagnosed as RCD type I[15]. HLA-DQ status could not be determined for IBD patients and controls. As additional non-intestinal inflammatory control group PBMC from rheumatoid arthritis (RA) patients were analyzed.

Antigen-reactive T-cell enrichment

Identification and enrichment of antigen-reactive T-cells was performed by applying the recently described ARTE technique [7]. Briefly, 0.5 –1 x10^7 PBMC were cultured in RPMI1640 supplemented with 5% human AB-serum and stimulated for 6 h with 1 µg/mL CD40 (Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence or absence of the pepsin-trypsin digested 33-mer gliadin peptide (200 µg/mL) (Sigma- Aldrich), OVA-peptide (Invitrogen) or soybean or peanut extract (200 µg/mL) (Greer Laboratories). For the last 2 h, 1 µg/mL brefeldin A (Sigma- Aldrich) was added. Cells were indirectly labeled with anti-CD154-biotin antibody, followed by anti-biotin MicroBeads (CD154 MicroBead-Kit, Miltenyi Biotec) and magnetically enriched using MS columns (Miltenyi Biotec).

Flow cytometric cell analysis

Surface staining was performed on the MS column (first panel: Brilliant violet 510 anti-human CD4; RPA-T4, Brilliant Violet 421 anti-human CD197 (CCR7); G043H7, PE/Cy7 anti-human CD45RA; HI100, second panel: Brilliant violet 510 anti-human CD4; PE/Cy7 anti-human CD29/ß1; TS2/16, PE anti-human ß7; FIB504, all from BioLegend (Koblenz, Germany), VioBlue anti-human CD49d/α4; MZ18-24A9 from Miltenyi Biotec. (Bergisch Gladbach, Germany) and human FC block (CSL Behring, Marburg, Germany)). The enriched cell fraction was fixed using eBioscience, FoxP3 staining buffer, Thermo Fisher Scientific, Waltham, MA USA) and intracellular staining was performed (APC anti-human IFNγ· 4S.B3, APC/Cy7 anti-human IL-17A; BL168, PerCP/Cy5.5 anti-human TNFα· MAb11, all from BioLegend and FITC anti-human CD154 (5C8) from Miltenyi Biotec. Flow cytometry analysis was performed on the FACS Canto II device (BD Bioscience, Heidelberg, Germany). Data were analyzed with FlowJo analysis software (Ashland, OR, USA).

Statistics

Statistical analysis was performed using Prism software (GraphPad Software). Significance was determined using Mann-Whitney-U-Test as indicated. *P>0.05, **P>0.01, ***P>0.001.

Results

Circulating gliadin-specific T-cells are increased in active disease with ileal inflammation

ARTE technology was applied to all blood samples (**Figure 1A**) for various nutritional antigens, including controls for antigen-specific T-cell enrichment and T-cell activation. Moreover, we could clearly demonstrate the necessity for T-cell enrichment to allow for deeper cell analysis and therefore the advantage of this method over direct staining protocols for rare antigen-specific cell populations (**Figure 1B**). With the overall frequency of $CD4^+$ T-cells remaining stable in the various disease conditions (**Figure 1C**), the frequency of gliadin-specific $CD154^+$ T-cells among $CD4^+$ T-cells in PBMC was expectedly highest in active CeD (aCeD), i.e. without GFD, as well as in refractory CeD patients (Refr). aCeD are rare patients as we did

not actively initiate a gluten-re-challenge. Moreover the frequencies were also significantly increased in CeD patients on a GFD without clinical symptoms, when compared to healthy controls. Remarkably, a similar frequency to active CeD patients was observed in active CD patients with ileal inflammation (**Figure 2A**).

The frequency was significantly lower in CD patients in remission, in UC patients, independent of their inflammatory state and in healthy controls. Interestingly, first-degree relatives (FDR) of CeD patients, considered healthy by standard diagnostics, revealed a significant increase in the frequency of gliadin-specific T-cells compared to controls without familiar predisposition of CeD. Of notice, RA as auto-inflammatory control without intestinal inflammation, did not differ from healthy controls.

Phenotyping circulating gliadin-specific T-cells

Further phenotyping of gliadin–specific $CD4^+CD154^+$ T-cells in effector memory ($CD45RA^-$ $CCR7^-$), central memory ($CD45RA^-CCR7^+$) or naïve ($CD45RA^+CCR7^+$) cells revealed a shift towards the memory types in the groups with active small intestinal inflammation. Remarkably, effector memory T-cells outnumbered naïve T-cells (ratio>1, **Figure 2B**) only in aCeD, Refr and CD patients. Moreover, gliadin-specific $CD4^+CD154^+$ T-cells, positive for the small intestinal homing marker $\alpha4\beta1$, but not for $\alpha4\beta7$, a general gut homing marker, were increased in aCeD patients (**Figure 2C, D**), further strengthening the connection of peripheral nutritional antigen-specific T-cells to small intestinal inflammation.

Pro-inflammatory cytokines of circulating gliadin-specific T-cells

The subsequent functional analysis of the antigen-specific T-cells after gliadin stimulation (**Figure 2E, F; S. Figure 1**) revealed highest production of the pro-inflammatory cytokines IFNγ, IL-17A and TNFα in cells from aCeD, Refr, but also from CD patients with small intestinal involvement. Remarkably, antigen-specific T-cells of first-degree relatives of CeD patients (FDR) presented with higher frequencies of gliadin-specific T-cells and an increased TNFα expression, and were thus comparable to aCeD patients. TNFα-positive $CD154^+$ cells were most discriminative when comparing active and inactive CeD to healthy controls.

Further food antigen-specific T-cells are increased in active disease with ileal inflammation

To dissect a sole barrier defect, as it is present in small intestinal CD, from the disease-driving gliadin-reactivity in CeD, we included soybean protein, peanut protein and OVA-peptide in our analysis. In fact, an increased frequency of antigen-specific $CD4^+$T-cells was exclusively observed in the presence of small intestinal inflammation, namely CD and aCeD. CD patients in remission as well as UC patients independent of their inflammatory state did not differ from healthy controls. Furthermore, CeD patients on GFD, although being highly reactive to gliadin, showed no reaction to other nutritional antigens (**Figure 3A, B**).

Discussion

So far, the published data for ARTE have been focusing on bacteria- or fungi-specific antigens as well as house dust mites[16]. However, this methods allows also to study even rarer nutritional antigen-specific T-cells without in-vitro expanding of the reacting cells and without re-challenging the patients. Therefore, we apply this method for detection of the rare nutritional antigen-specific T-cells in peripheral blood, in order to analyze antigen reactivity for different clinical subgroups of CeD and IBD patients.

Recently, peripheral gluten-specific $CD4^+$ T-cells were analyzed applying HLA-DQ2:gluten tetramers, thus identifying an increase of gluten-specific $CD4^+$ T-cells in aCeD[5,6]. However, the here applied ARTE allows for a deeper analysis of the respective specific $CD4^+$T-cells to distinguish different disease states of CeD. The diagnosis of CeD in patients who already follow a GFD is challenging, since tTG antibodies under GFD normalize and histological markers, as the small intestinal villous atrophy, regenerates. This clinical need is even growing, giving the increasingly popular gluten-free life style in the western world [17,18], or for first degree relatives, who frequently initiate a GFD when a household member is diagnosed with CeD. For the latter, the high risk of developing CeD has been proven in many studies [19,20] and surveillance for CeD is even recommended for first-degree relatives of a diagnosed patient where carriage of a risk gene has not been

excluded [21,22]. Work herein might be the first step towards identifying such cases, without a conventional burdening gluten re-challenge, since characteristic changes in cytokine expression in gliadin-specific $CD4^+$ T-cells in the peripheral blood are present. For the rare subgroup of Refr patients, especially for type I, the specific immunological nature remains unclear. Diagnosis is still based on histopathology alone, while recent studies suggest a heterogeneous composition of different pathologies to be merged under this term. In this respect, the ARTE technique for gliadin-specific T-cells represents a unique research tool for future studies that has the potential to contribute to a subclassification of this disease group. Furthermore, our data reveal a specific immunological phenotype of gliadin-specific $CD4^+$ T-cells from FDR regarding the hypersensitivity towards gluten, even if diagnosed as healthy due to their tTG status (without GFD). By demonstrating an active immune response against the pathogenic antigen, identification and even phenotyping of gluten-reactive T-cells from peripheral blood might represent an alternative, all the more in pediatric cases, where first time diagnosis is common, while invasive endoscopy is meant to be avoided. Thus, this novel approach could fulfill the clinical need for a noninvasive marker of CeD activity as clinical and research tool [23].

With regard to IBD, which shares the characteristics of barrier disruption [24] and subsequent intestinal inflammation in the lamina propria, we detected increased levels of gliadin-specific T-cells in the peripheral blood of active CD patients with concurrent small intestinal inflammation, paralleled by the highest frequency of antigen-specific T-cells expressing pro-inflammatory cytokines. This distinct occurrence suggests the small intestinal barrier disruption as major cause for the observed T-cell activation, since these cells express small intestinal homing markers. The homing to the ileum ($\alpha 4\beta 1$) was described as essential pathway in CD [25]. In line, only in these three patient groups of active small intestinal inflammation, the effector-memory T-cells outnumbered the naïve phenotype among gliadin-specific T-cells in the peripheral blood. Furthermore, peripheral T-cells from CD patients with small intestinal inflammation proved to be responsive to other major nutritional antigens[26], while neither active UC nor CD or UC in remission showed any reaction. Again, only antigen-specific T-cells from active CeD, but not GFD patients demonstrated similar properties, corroborating on the one hand the leaky barrier of the affected small intestine as the site of food antigen translocation and subsequent T-cell activation. On the other hand, the significant effect of gliadin, but no other food antigen, in the GFD group further confirmed the singular antigen-driven nature of CeD. Nevertheless, based on surveys, it has been suggested, that long-term GFD improves gastrointestinal symptoms in active IBD patients [10]. With the present study, we are able to convey cellular and functional data by demonstrating an enhanced gliadin-specific response of pro-inflammatory cytokines towards gliadin for CD patients

Our data strongly suggest, that small intestinal inflammation is key for the development of a nutritional antigen-specific T-cell response. Therefore, ARTE allows to distinguish CD with small intestinal inflammation from UC and CD in remission by a unique profile of circulating antigen-specific T-cells, raising the question, if a well-defined nutritional regimen (e.g. GFD) might have therapeutic potential in the setting of IBD. Hence, based on the analysis of the systemic immune response, an "anti-inflammatory" diet could be developed and monitored. In addition, this technique allows detailed analyses of gliadin-specific T-cells in such a high resolution that even healthy first-degree relatives can be discriminated and might thus provide a novel non-invasive diagnostic tool to identify symptom-free CeD patients on a gluten-free diet.

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Table 1

	Non-relative controls (n=24)	**First-degree relatives** (n=6)	**Celiac disease on GFD** (n=24)	**Active celiac disease** (n=9)	**Refractory celiac disease*** (n=11)
Age (mean ± SD)	33.3 ± 9.4	34.5 ± 9.5	43.9 ± 16.8	47.3 ± 12.4	61.1 ± 11.6
Female [%]	55	83	80	66	82
tTG (mean ± SD) [U/mL] #	1.6 ± 0.7	1.4 ± 0.4	6.3 ± 4.8	114.2 ± 70.9	21.0 ± 27.2
[CE]	-	-	-	3428.1 ± 1313.7	-
Marsh grade - IIIa **at first diagnosis** - IIIb - IIIc	-	-	10 9 4	3 3 2	6 2 2
RCD type I /II [%]	-	-	-	-	63.6/36.4

	Non-relative controls (n=24)	**Rheumatoid arthritis** (n=5)	**Crohn´s disease** (n=19+13)	**Crohn´s disease (remission)** (n=10+4)	**Ulcerative colitis** (n=12+7)	**Ulcerative colitis (remission)** (n=9+2)
Age (mean ± SD)	33.3 ± 9.4	49.4 ± 10.8	36.2 ± 9.3	41.4 ± 13.9	41.0 ± 14.9	42.0 ± 15.1
Female [%]	55	67	50	57	37	64
Clinical score - HBI	-	-	5.1 ± 2.7	0.5 ± 1.2	-	-
- partial Mayo	-	-	-	-	3.9 ± 1.9	1.0 ± 1.0

	Non-relative controls (n=24)	**Rheumatoid arthritis** (n=5)	**Crohn´s disease** (n=19+13)	**Crohn´s disease (remission)** (n=10+4)	**Ulcerative colitis** (n=12+7)	**Ulcerative colitis (remission)** (n=9+2)
Montreal classification - A1 <17 years - A2 17-40 years - A3 >40 years Crohn´s disease - L1 ileal - L3 ileocolonic - L4 upper GI Ulcerative colitis - E1 proctitis - E2 distal UC - E3 extensive UC	-	-	0 23 9 11 12 6	0 7 7 4 8 2	0 7 12 0 8 10	0 6 5 0 8 3

HBI, Harvey-Bradshaw index

Figure legends

Figure 1 . *Enrichment of food antigen-specific T-cells.*(**A-C**) Peripheral blood mononuclear cells (PBMC) were stimulated with various food antigens, magnetically enriched for CD154 and analyzed by flow cytometry. (**A**) Methodology and (**B**) exemplary density plots of $CD154^+$ T-cell enrichment after stimulation with gliadin or control antigen are shown. (**C**) Frequencies of $CD4^+$ T-cells in PBMC were determined from healthy controls (non-relatives, NR), patients with active Crohn´s disease (CD) or ulcerative colitis (UC), or each of these entities in remission (-R), celiac disease patients (CeD) ± gluten-free diet (GFD, aCeD) or refractory (Refr) patients, first degree relatives of CeD patients (FDR) and rheumatoid arthritis patients (RA). Data are shown as median with 95% CI.

Figure 2 . *Phenotyping gliadin-specific T-cells.*(**A-F**) Peripheral blood mononuclear cells (PBMC) were stimulated with various food antigens, magnetically enriched for CD154 and analyzed by flow cytometry. (**A**) Frequencies of $CD154^+$cells among $CD4^+$ T-cells in PBMC from healthy controls (non-relatives, NR), patients with active Crohn´s disease (CD) or ulcerative colitis (UC), or each of these entities in remission (-R), celiac disease patients (CeD) ± gluten-free diet (GFD, aCeD) or refractory (Refr) patients, first degree relatives of CeD patients (FDR) and rheumatoid arthritis patients (RA) are shown. (**B**) Ratio of gliadin-specific effector memory $CD4^+CD154^+$ T-cells ($CD45RA^-CCR7^-$) and naïve T-cells ($CD45RA^+CCR7^+$) from controls and disease groups are shown. (**C**) Exemplary dot plots of α4β1 within $CD4^+$ and $CD4^+CD154^+$ T-cells are shown. (**D**) Frequencies of $CD4^+$ T cells and $CD4^+$ $CD154^+$ T cells, positive for integrins α4β1 and α4β7 are shown. (**E**) Frequencies of gliadin-specific IFNγ$^+$, IL-17A$^+$ and TNFα$^+$ cells within $CD154^+$ T-cells in between the patient groups are shown. (**F**) Exemplary dot plots of IFNγ$^+$, IL-17A$^+$ and TNFα$^+$ $CD4^+CD154^+$gliadin-specific T-cells are shown. Data are shown as median. Significance was determined using Mann-Whitney-U-Test. *P>0.05, **P>0.01, ***P>0.001. Statistically significant differences were calculated in comparison to healthy non-relatives, if not indicated otherwise.

Figure 3 . *Frequencies of food antigen-specific T-cells.*(**A-B**) Peripheral blood mononuclear cells (PBMC) were stimulated with various food antigens, magnetically enriched for CD154 and analyzed by flow cytometry. (**A**) Exemplary dot plots of $CD154^+$ T-cell enrichment of indicated nutritional antigens with absolute numbers and frequencies are shown. (**B**) Frequencies of $CD154^+$ cells among $CD4^+$ T-cells after stimulation with

soybean, peanut and OVA-peptide from healthy controls (non-relatives, NR), patients with active Crohn´s disease (CD) or ulcerative colitis (UC), or each of these entities in remission (-R), celiac disease patients (CeD) ± gluten-free diet (GFD, aCeD) or refractory (Refr) patients are shown. Data are shown as median. Significance was determined using Mann-Whitney-U-Test. *P>0.05, **P>0.01, ***P>0.001. Statistically significant differences were calculated in comparison to healthy non-relatives, if not indicated otherwise.

Figure 1

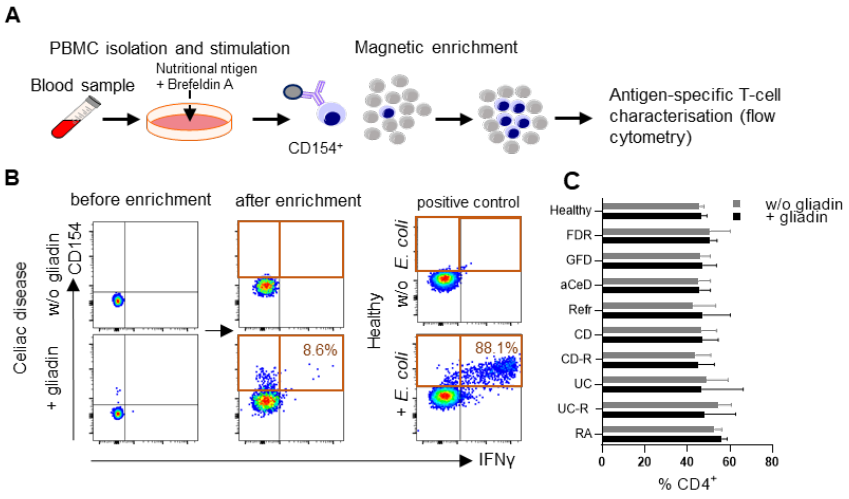

Figure 2:

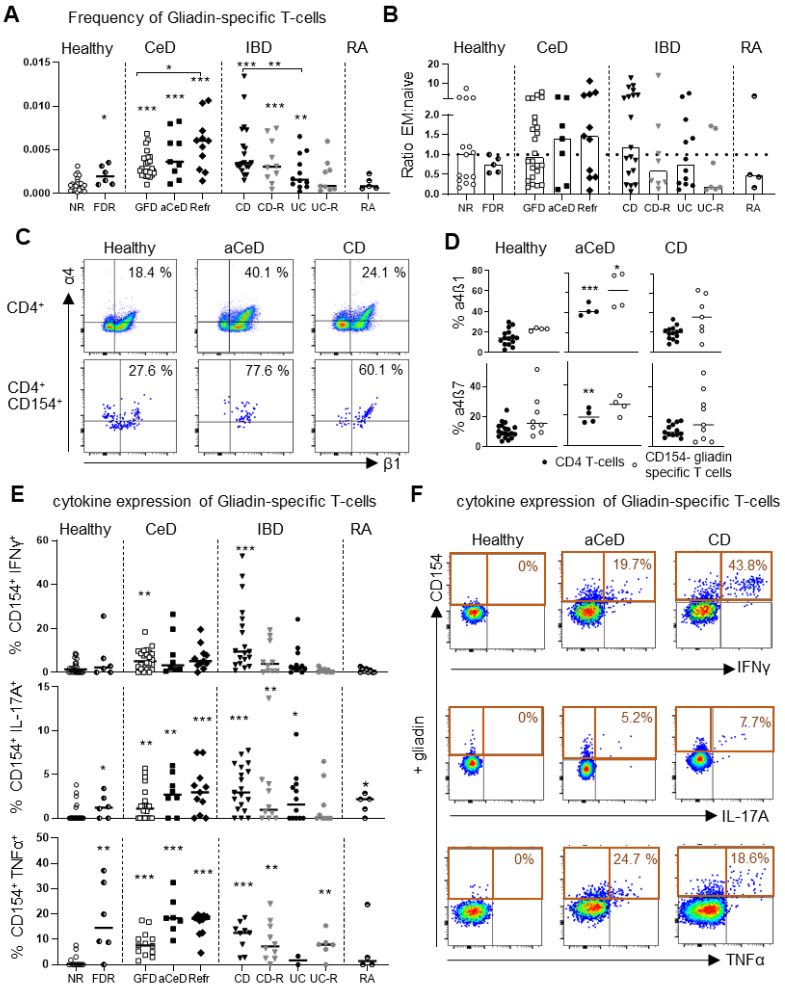

Figure 3: