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CD62L^{neg}CD38⁺ expression on circulating CD4⁺ T cells identifies mucosally differentiated cells in protein fed mice and in human celiac disease patients and controls

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Abstract

Objective—The aim of this study was to identify new markers of mucosal T cells to monitor ongoing intestinal immune responses in peripheral blood.

Design—Expression of cell-surface markers was studied in mice on ovalbumin (OVA)-specific T cells in the gut-draining mesenteric lymph nodes (MLN) after OVA feed. The effect of the local mucosal mediators retinoic acid (RA) and TGF- β on the induction of a mucosal phenotype was determined in *in vitro* T-cell differentiation assays with murine and human T cells. Tetramer

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stainings were performed to study gluten-specific T cells in the circulation of patients with celiac disease, a chronic small intestinal inflammation.

Results—In mice, proliferating T cells in MLN were CD62L^{neg}CD38⁺ during both tolerance induction and abrogation of intestinal homeostasis. This mucosal CD62L^{neg}CD38⁺ T-cell phenotype was efficiently induced by RA and TGF- β in mice, whereas for human CD4⁺ T cells RA alone was sufficient. The CD4⁺CD62L^{neg}CD38⁺ T-cell phenotype could be used to identify T cells with mucosal origin in human peripheral blood, as expression of the gut-homing chemokine receptor CCR9 and β 7 integrin were highly enriched in this subset whereas expression of cutaneous leukocyte associated antigen was almost absent. Tetramer staining revealed that gluten-specific T cells appearing in blood of treated celiac disease patients after oral gluten challenge were predominantly CD4⁺CD62L^{neg}CD38⁺. The total percentage of circulating CD62L^{neg}CD38⁺ of CD4 T cells was not an indicator of intestinal inflammation as percentages did not differ between pediatric celiac disease patients, inflammatory bowel disease patients and respective controls. However, the phenotypic selection of mucosal T cells allowed cytokine profiling as upon restimulation of CD62L^{neg}CD38⁺ cells IL-10 and IFN- γ transcripts were readily detected in circulating mucosal T-cells.

Conclusions—By selecting for CD62L^{neg}CD38⁺ expression which comprises 5–10% of the cells within the total CD4⁺ T-cell pool we are able to highly enrich for effector T cells with specificity for mucosal antigens. This is of pivotal importance for functional studies as this purification enhances the sensitivity of cytokine detection and cellular activation.

Introduction

The mucosal surfaces of our gastrointestinal tract are continuously exposed to foreign antigens. While inflammatory immune responses are needed to eliminate invasive bacteria and protect the body from infection, harmless soluble proteins and commensal flora induce mucosal tolerance (1). Inappropriate regulation of these responses can lead to chronic inflammatory disorders of the intestine such as celiac disease caused by intolerance to the dietary protein gluten and inflammatory bowel diseases (IBD) caused by an aberrant inflammatory response to intestinal microbiota. Despite significant advances in the understanding of celiac disease and IBD ongoing T-cell responses in patients are difficult to study as T cells from mucosal origin are not easily identified in peripheral blood.

Both effector T (Te) cells and regulatory T cells (Treg) differentiate from naive T cells upon antigen presentation by mucosal DC in the Peyer's Patches (PP) and gut-draining mesenteric lymph nodes (MLN) following antigen feed (2–4). Upon activation in the PP or MLN, T cells acquire high expression levels of the integrin $\alpha_4\beta_7$ and the chemokine receptor CCR9 that allow their migration to the small intestine (5–7). Imprinting of this gut-homing phenotype is efficiently induced in response to protein feed regardless of the functional outcome of the T-cell response, as CCR9 is efficiently induced both in the absence and presence of the mucosal adjuvant cholera toxin (CT) (8).

Although the role for CCR9 in migration to the small intestine is well established, the expression of CCR9 is not absolutely required for the localization of CD4⁺ T cells in the small intestine (9). CCR9^{-/-} mice have normal numbers of CD4⁺ T cells in the small intestine whereas the CD8⁺ population is reduced (10–11). In agreement with these findings CD4⁺ T cells in the lamina propria (LP) express a more heterogeneous chemokine receptor profile including CCR9, CXCR3, CXCR6, as well as CCR5 and CCR6 (12). Moreover, expression of CCR9 on human peripheral blood T cells does not seem to be restricted to gut-migrating T cells, as CCR9 expressing naive T cells can be found that lack $\alpha_4\beta_7$ and are presumed to be recent thymic emigrants (13). In addition, a large proportion of $\alpha_4\beta_7$ ⁺ T

cells in blood do not express CCR9 (13). These findings indicate that CCR9 may not exclusively identify all gut-imprinted T cells in peripheral blood.

Hence, we investigated whether mucosal imprinting results in a distinctive phenotype that could be used to identify mucosally-educated T cells in the periphery.

Materials and Methods

For detailed description of antibodies and primers see supplementary data.

Mice

BALB/c mice (Charles River, Maastricht, the Netherlands), DO11.10 transgenic (Tg) and DO11.10Tg $\times$ RAG^{-/-} mice (breeding at ErasmusMC), which have a Tg T-cell receptor (TCR) for the OVA_{323–339}-peptide were kept under routine animal housing conditions. Experiments were approved by the animal experimental committee of the ErasmusMC.

Adoptive transfer and CFSE labelling

BALB/c mice received an intravenous (i.v.) injection of 6–10·10⁶ 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE)-labelled DO11.10 cells as described previously (2, 14). The next day, mice received 70mg 98% pure OVA (Sigma Aldrich, Zwijndrecht, the Netherlands) intragastrically (i.g.) or 400µg OVA (Calbiochem, San Diego, CA) intra-muscularly (i.m.). To break tolerance, mice received 70mg OVA with 20µg CT (Sigma) i.g.. At 72h after OVA, cells from draining LN and spleen were stained for CD4, CD38, CD62L, Foxp3 and DO11.10 Tg TCR and measured by flowcytometry. Gating of CD38 on CD4⁺KJ1.26⁺ cells was set according to expression on CD38^{int} lymphocytes (mainly B cells) in the same LN. CD38^{int} was defined as a level of expression with fluorescence intensity in between CD38^{neg} and CD38^{hi} lymphocytes (Supplementary Figure 1).

In vitro T-cell differentiation assays (mouse)

For polyclonal T-cell differentiation experiments, CFSE⁺CD4⁺KJ1.26⁺ cells were obtained as described above. CD25⁺ cells were depleted with PC61 antibody (kindly provided by Dr. J. den Haan, Amsterdam, the Netherlands). 5×10⁵ CFSE⁺CD4⁺KJ1.26⁺ T cells were stimulated with plate-bound anti-CD3e (145-2C11, 10µg/ml, BD) and soluble anti-CD28 (37.51, 10µg/ml, BD) for 72h.

DC from MLN, PLN and spleens for OVA-specific T-cell differentiation assays were obtained as described previously (15). Naive CD4⁺KJ1.26⁺ T cells were obtained from LN and spleens of DO11.10Tg $\times$ RAG^{-/-} mice by positive selection using biotinylated KJ1.26 and the CELLection biotin-binder kit (Invitrogen). 2×10⁴ DC were cultured with 5×10⁵ CFSE-labelled CD4⁺KJ1.26⁺ cells, OVA_{323–339} peptide for 96h and/or 20ng/ml rhTGF- β (Preprotech, Rocky Hill, NJ) and 10nM RA (Sigma). Concentrations of rhTGF- β and RA were determined in a dose response experiment and chosen based on optimal induction of Foxp3 (not shown). In some experiments recIL-4 (300ng/ml, R&D systems, Abingdon, UK), LPS (E. coli 0111:B4, 100ng/ml, Difco), RA receptor antagonists LE540 (10µM, Wako Chemical, Osaka, Japan) or LE135 (10µM, Tocris Bioscience, Bristol, UK) were added.

Peripheral blood

Healthy controls—Peripheral blood cells from healthy volunteers were stained for CD4, CD38, CD62L, CD45RA, Foxp3, CCR9, CLA, β 7-integrin and isotype control. PBMC were isolated and stimulated with CD3/CD28 T-cell expander beads (Dyna) in the presence of

20ng/ml rhTGF- β and/or 10nM RA. After 96h the cells were analyzed by flow cytometry. All participants gave written informed consent for the study

Pediatric celiac disease and IBD patients—Peripheral blood cells were obtained from pediatric patients who underwent an esophago-gastro-duodenoscopy (EGD) with suspicion of celiac disease in the Erasmus MC – Sophia Children’s Hospital, Rotterdam, the Netherlands. Children with biopsy-proven celiac disease were included in the patient group (34 patients) whereas children with a normal intestinal histology were included in the control group (21 controls). In addition, peripheral blood from IBD patients was analyzed. All IBD patients (29 patients) were diagnosed by endoscopy, histopathological and clinical characteristics according to the Porto criteria (16). Age matched orthopedic patients (14 patients) with no underlying inflammatory or intestinal diseases served as controls. Written informed consent from the parents was obtained for each patient.

Adult celiac disease patients—For detection of gluten-specific T cells in blood, six celiac disease patients on gluten free diet were recruited (study approved by the Regional Ethics Committee for Southern Norway). The patients ate four slices of standard white bread daily for three days (approximately 160g, Bakers AS), thereafter continued their gluten-free diet. On day 6, blood was drawn and gluten-reactive T cells were stained with tetramers as described below and reported previously (17).

Biopsies

Duodenal biopsy specimens were obtained from patients who underwent gastroendoscopy for their diagnostic work-up for abdominal pain and diarrhoea (2 patients) or Barret esophagus (one patient). Histology revealed normal mucosal architecture. Epithelium was removed by incubation in 2mM EDTA (2 $\times$ 30 min.), followed by digestion of the tissue in 1mg/ml collagenase H (Sigma). Cells were stained with antibodies to CD45, CD62L, CD4, CD38 and TCR $\alpha\beta$ and 0.2 μ g/ml propidium iodide to exclude dead cells. Collection of clinical samples was approved by the regional ethics committee for Southern Norway and the patients gave written informed consent.

Gliadin specific T-cell staining using α -gliadin tetramers

Water-soluble DQ2 (DQA1*0501/DQB1*0201) molecules with three different peptides were produced as previously described (17); two gliadin-tetramers with deamidated T-cell epitopes of α -gliadin (i.e., DQ2- α I tetramer and DQ2- α II tetramer) and one control tetramer containing the invariant chain peptide CLIP2 (18). Tetramers were made by conjugating DQ2-peptide complexes with streptavidin-PE (Molecular Probes, Eugene, OR). Specificity of the tetramers was assessed after conjugation by staining two T-cell clones TCC 380.E.2 (specific for the DQ2- α -I epitope) and TCC 412.5.28 (specific for the DQ2- α -II epitope) with all three DQ2 tetramers.

CD4⁺ lymphocytes were isolated from PBMC by positive selection (Invitrogen) and stained as described previously (17) with slight modifications. Briefly, 1 to 4 million CD4⁺ T cells were stained in 100 μ l with 10 μ g/ml tetramer at 37°C, cooled and stained with specific antibodies: CD62L, CD38, CD11c, CD45RA, CD4 and CCR9. At least 0.8 million cells were analyzed by flow cytometry.

Q-PCR

RNA was purified from purified murine CD4⁺ T cells or T-cell subsets using the Nucleospin RNA-XS kit (Macherey-Nagel, Düren, Germany) and cDNA was synthesized. Real-time quantitative PCR was performed using SYBRgreen (Finnzymes, Espoo, Finland) on an AbiPrismR7900 Sequence Detection System (PE Applied Biosystems, CA, USA). Relative

expression to cyclophilin was calculated as follows: relative expression = $2^{-\Delta C_t}$, whereby $\Delta C_t = C_{t_{\text{target}}} - C_{t_{\text{housekeeping}}}$. Primers are specified in supplementary methods.

Statistics

Data are expressed as the mean $\pm$ SD and analyzed either using Student's t-test or ANOVA. $p < 0.05$ was considered significant.

Results

Under homeostatic conditions mucosal T cells have a CD62L^{neg}CD38^{int} phenotype

Previously we have shown that within 48h after OVA feed, proliferating antigen-specific T cells in the MLN are CD62L^{neg} and express CD38 (2). To assess whether CD62L^{neg}CD38⁺ expression is characteristic for gut imprinted cells *in vivo*, we compared the T-cell response in the gut draining MLN after OVA feed with the T-cell response in popliteal and inguinal LN referred to as peripheral LN (PLN) after i.m. OVA injection. Expression of CD62L rapidly decreased upon differentiation of tolerogenic OVA-specific T cells in the MLN after OVA feed, whereas expression levels remained high on differentiating T cells in the PLN after OVA i.m. (Figure 1A). The differentiating CD62L⁺ T cells in the PLN co-expressed high levels of CD25 (not shown). Moreover, the CD62L⁺ phenotype remained upon injection of OVA in incomplete Freund's adjuvant, indicating that absence of CD62L downregulation is not due to suboptimal T-cell activation (not shown). As a result, the percentage of cells that had become CD62L^{neg} in the MLN was significantly higher in comparison with the PLN (Figure 1B). Downregulation of CD62L on differentiating T cells in the MLN was not influenced by the APC–T-cell ratio, as adoptive transfer of different numbers of CD4⁺KJ1.26⁺ T cells resulted in an equal percentage of CD62L^{neg} cells (Supplementary Figure 2).

The mean fluorescence intensity (MFI) of CD38 on dividing T cells in the MLN gradually increased upon division towards a CD38^{int} phenotype, whereas in PLN most dividing cells were CD38^{neg} (Figure 1A–B). A small proportion of the OVA-specific T cells expressed CD38 at high levels. As these cells were CD62L⁺ this phenotype may reflect precursors for central memory cells, as CD62L⁺CD38^{hi} cells are also observed during a secondary flu infection in the lung (Frances Lund). Taken together, the CD62L^{neg}CD38^{int} phenotype is characteristic for T cells that differentiate in the MLN after protein feed (Figure 1C). Differentiating MLN T cells elicited by OVA feed are Tregs that suppress an inflammatory delayed-type hypersensitivity response upon adoptive transfer (2). A proportion of these Treg *de novo* expresses Foxp3 ((15) and Figure 1D). To assess whether the CD62L^{neg}CD38^{int} phenotype correlated with the differentiation of Foxp3⁺ Tregs, we analyzed dividing Foxp3⁺ and Foxp3^{neg} T cells in the MLN. Both Foxp3⁺ and Foxp3^{neg} T cells in the MLN were predominantly CD62L^{neg} (Figure 1E). In contrast, the majority of Foxp3⁺ T cells that differentiated in PLN were CD62⁺ (Supplementary Figure 3). The percentage of CD38⁺ cells was significantly higher in the Foxp3⁺ T-cell population when compared with Foxp3^{neg} cells in both MLN (Figure 1E) and PLN (Supplementary Figure 3), indicating that CD38 expression correlates with the *de novo* induction of Foxp3. In sum, during mucosal tolerance induction, antigen-specific T cells preferentially have a CD62L^{neg}CD38^{int} phenotype.

TGF- β and retinoic acid induce differentiation of CD62L^{neg}CD38⁺T cells

To determine whether the CD62L^{neg}CD38^{int} phenotype is driven by factors in the gastrointestinal microenvironment, we stimulated naive CD4⁺ T cells with α CD3 α CD28, TGF- β and RA. Without TGF- β and RA, T-cell stimulation resulted in CD62L^{hi} expression for most activated T cells. Upon the addition of rhTGF- β the expression of CD62L was

rapidly lost (Figure 2A). As rhTGF- β inhibited T-cell activation, these results demonstrate that loss of CD62L does not exclusively reflect T-cell activation. Addition of RA to TGF- β stimulated T cells only slightly further increased the percentage of CD62L^{neg} cells (Figure 2A–B) while culturing with RA alone also induced a loss of CD62L (not shown).

T-cell stimulation without TGF- β and RA did not result in induction of CD38 expression (Figure 2A). In contrast, TGF- β induced a moderate increase in CD38 expression at both protein (Figure 2A–B) and mRNA levels (Figure 2C). This TGF- β -mediated induction of CD38 occurred simultaneously to Foxp3⁺ Treg differentiation (Figure 2A). Culture with RA and TGF- β together further increased the induction of CD38 and Foxp3. In particular, culture with RA and TGF- β induced a three-fold increase in the number of CD38⁺ cells (Figure 2B) and a four-fold increase in CD38 mRNA levels compared with polyclonal stimulation alone (Figure 2C). No effect on CD38 expression was observed when RA was used alone (not shown). Thus, RA and TGF- β drive reduction of CD62L and increase CD38 expression on differentiating mucosal Tregs.

Next, we assessed whether mucosal DC, that are able to convert vitamin-A into RA (19), are more efficient in inducing a CD62L^{neg}CD38⁺ phenotype *in vitro* when compared with non-mucosal DC. Thereto, OVA-specific T cells were stimulated with OVA-loaded MLN-DC, PLN-DC or splenic DC. As previously reported, CD62L expression rapidly decreased upon stimulation with DC, irrespective of their origin (5), and the downregulation of CD62L was further enhanced by TGF- β (not shown). No differences in CD38 expression could be detected between T cells cultured with MLN-DC, PLN-DC or splenic DC (not shown). Addition of TGF- β significantly increased CD38 expression on T cells stimulated by MLN-DC but not by PLN-DC or splenic DC (Figure 3A, Supplementary Figure 4A). To establish that the imprinting of CD38 by MLN-DC could be explained by their synthesis of RA, we added selective RA-receptor antagonists LE135 and LE540. Both antagonists inhibited the induction CD38 by MLN-DC (Figure 3B), demonstrating that synergy between endogenous RA and TGF- β was responsible for imprinting of CD38 expression. Moreover, interference with RA signaling resulted in a reduction of CD62L^{neg} cells (Figure 3B).

Addition of exogenous RA together with TGF- β to T cells cultured with OVA-loaded MLN-DC further increased the expression of CD38 (Figure 3C, Supplementary Figure 4B). Furthermore, exogenous RA and TGF- β abolished the differences between mucosal and non-mucosal DC in the imprinting of CD38 expression on T cells (Figure 3D). In sum, in the presence of TGF- β , mucosal DC efficiently induce expression of CD62L^{neg}CD38⁺ on differentiating T cells in an RA-dependent manner.

CD38 is expressed irrespective of regulatory or effector T-cell function

To test whether the imprinting of the CD62L^{neg}CD38⁺ phenotype by MLN-DC is maintained upon inhibition of adaptive Treg induction we cultured T cells with MLN-DC in the presence of RA, TGF- β and rIL-4, which abolishes the differentiation of mucosal Foxp3⁺ Tregs (15, 20–21), or lipopolysaccharide (LPS). Both rIL-4 and LPS decreased Foxp3 mRNA levels. However, the suppression of Foxp3⁺ Treg differentiation did not affect the imprinting of the CD62L^{neg}CD38⁺ phenotype (Figure 4). No differences in the downregulation of CD62L were observed (not shown). The CD38⁺ phenotype was maintained in the absence of Foxp3⁺ expression, however the percentage of CD38⁺ cells was slightly decreased by both rIL-4 and LPS (Figure 4B–C). Strikingly, rIL-4 regulated the expression of CD38 as the MFI of CD38⁺ cells and CD38 mRNA levels were increased (Figure 4D). In agreement with previous studies (22), rIL-4 also induced the expression of CCR9 on T cells cultured with MLN-DC in the presence of RA and TGF- β (not shown). *In vivo*, the CD62L^{neg}CD38⁺ phenotype was also maintained upon abrogation of tolerance

with CT (Supplementary Figure 5A–C). In conclusion, these data suggest that the CD62L^{neg}CD38⁺ phenotype is maintained upon inhibition of Foxp3⁺ T-cell differentiation.

CD62L^{neg}CD38⁺ phenotype can be used to identify mucosal T cells in human peripheral blood of healthy donors

We hypothesized that the CD62L^{neg}CD38⁺ phenotype can be used to identify T cells from mucosal origin in humans. First, we determined the expression of CD62L and CD38 on normal small-intestinal biopsy specimens. Almost all CD4⁺ T cells in human duodenum are CD62L^{neg}CD38⁺ (Figure 5A). Next, we assessed whether RA and TGF- β could induce the CD62L^{neg}CD38⁺ phenotype on human CD4⁺ T cells. Thereto, PBMC were stimulated with anti-CD3, anti-CD28 in the presence of RA, TGF- β or medium. Stimulation in the presence of RA induced an on average three-fold increase of the CD62L^{neg}CD38⁺ T-cell subset (fold increase 3.22 ± 0.60 relative to anti-CD3, anti-CD28-treated medium control, Figure 5B), resulting in CD62L^{neg}CD38⁺ expression on approximately 50% of CD4⁺ T cells. Addition of TGF- β to the RA cultures did not further enhance the percentage of CD62L^{neg}CD38⁺ T cells (fold increase compared with anti-CD28-treated medium control 2.68 ± 0.60 , Figure 5B). Stimulation in the presence of TGF- β did not enhance the expression of CD38 on human CD4⁺ T cells (Figure 5B). Thus, upon polyclonal stimulation in the presence of the mucosal factor RA, human CD4⁺ T cells acquire a CD62L^{neg}CD38⁺ phenotype.

Next, we determined whether the RA-induced CD62L^{neg}CD38⁺ T-cell population can be identified in fresh peripheral blood. A clear CD62L^{neg}CD38⁺ cell subset was observed consisting of 5.69 ± 2.17 (n=9) percent of total CD4⁺ lymphocytes (Figure 5C). Distribution of the other CD62L/CD38 subsets was as follows: CD62L^{neg}CD38^{neg}: 10.66 ± 4.23 , CD62L⁺CD38^{neg}: 26.44 ± 7.91 and CD62L⁺CD38⁺: 57.65 ± 10.90 (n=9). To determine the proportion of naive and memory cells within these subsets we co-stained for CD45RA. Naive CD45RA⁺ T cells were found mainly in the CD4⁺CD62L⁺CD38⁺ cell subset (Figure 5D). Most cells within both CD4⁺CD62L^{neg} subsets were CD45RA^{neg} (Figure 5D). Moreover, both CD62L^{neg}CD38⁺ and CD62L^{neg}CD38^{neg} contained similar number of CD25⁺ cells and had the capacity to secrete IFN- γ upon restimulation indicating that both populations consist mainly of effector memory cells (not shown). In order to determine whether the CD4⁺CD62L^{neg}CD38⁺ subset could be used to identify T cells with mucosal origin in the periphery, we measured expression of CCR9 and β 7-integrin as indicative for mucosal imprinting. There was a pronounced enrichment for CCR9⁺ cells and β 7-integrin⁺ cells within the CD4⁺CD62L^{neg}CD38⁺ population (Figure 5D). Conversely, measurement of cutaneous leukocyte-associated antigen revealed that these non-mucosal cells are underrepresented in the CD4⁺CD62L^{neg}CD38⁺ subset (Figure 5D). Foxp3⁺ cells were present within the CD4⁺CD62L^{neg}CD38⁺ subset, however there was no enrichment of Foxp3⁺ cells when compared to the CD4⁺CD62L^{neg}CD38^{neg} subset (Figure 5D). This may reflect that peripheral Foxp3 induction occurs in mucosally imprinted (CD38⁺) as well as non-mucosally imprinted (CD38^{neg}) effector T cells (CD62L^{neg}). In contrast, the Foxp3⁺ population in the CD62L⁺CD38⁺ subset would consist of naturally-occurring Foxp3⁺ T cells.

Gluten-specific T cells are mainly detected in the CD62L^{neg}CD38⁺ CD4⁺ T-cell subset

Finally, we wished to examine a human disease to demonstrate that the CD62L^{neg}CD38⁺ T-cell phenotype can be used to detect gut-imprinted T cells. Gluten-specific T cells, restricted by the disease-associated DQ2 molecule and likely involved in the formation of celiac lesion in the small intestine, can be cultured from the duodenal mucosa of celiac patients but not of healthy individuals (23). In blood, these gluten-specific T cells can only be detected after a short dietary gluten challenge in celiac disease patients that have been restricted to a gluten-free diet (17, 24). To establish that the CD62L^{neg}CD38⁺ T-cell population indeed contains

gluten-specific T cells, six treated adult celiac patients were recruited and ate four slices of gluten-containing bread daily for 3 days. At day 6, CD4⁺ PBMC were isolated and stained with both control- and gluten-tetramers (DQ2- α I and DQ2- α II), as well as antibodies against CD62L, CD38 and CCR9. In agreement with earlier observations (17), in four of the 6 patients, 0.01–0.1 % of the CD4⁺ T cells bound the tetramers (Figure 6A). In one patient, no gluten-specific cells were detected by tetramer staining. In another patient, the background staining with the control tetramer was very high precluding proper analysis. The majority of the tetramer⁺ T cells had a CD62L^{neg}CD38⁺ phenotype, as demonstrated in the analysis of these cells from the four remaining patients (Figure 6B–C). Surprisingly, although most gluten-specific T cells were shown to express the β_7 integrin (17), CCR9 was expressed by only 10–30% of tetramer⁺ cells (Figure 6C). Taken together, these results suggest the use of the CD62L^{neg}CD38⁺ T-cell phenotype to characterize ongoing mucosal immune responses in peripheral blood of patients with chronic intestinal disease.

No difference in the percentage of circulating CD62L^{neg}CD38⁺ CD4⁺ T cells in patients with chronic intestinal disease

To examine whether the percentage of circulating CD62L^{neg}CD38⁺ CD4⁺ T cells changed in patients with chronic intestinal disease we first included 34 patients with active celiac disease and 21 control patients with normal duodenal histology. Celiac disease patients had a Marsh score of Marsh I (4 patients), Marsh IIIA (8 patients), Marsh IIIB (15 patients) or Marsh IIIC (7 patients). Patients who were diagnosed with other diseases were excluded from analysis. There were no differences in age and gender between pediatric celiac disease patients and control patients. Upon analysis of the CD62L^{neg}CD38⁺ CD4⁺ mucosal T-cell population no differences in the percentages of the cells were found between pediatric celiac disease patients and controls (Figure 7A). This finding may have been expected as previous studies have shown that circulating gliadin reactive T cells can only be detected in patients that have received a gluten challenge (17, 24).

Similarly, the percentage of CD62L^{neg}CD38⁺ CD4⁺ mucosal T-cells was also determined in peripheral blood of IBD patients. As such 21 Crohn's disease patients, 8 Ulcerative Colitis patients and 14 age matched orthopedic control patients with no underlying inflammatory or intestinal diseases were investigated. In agreement with the data from the celiac disease patients no significant differences in the percentage of CD62L^{neg}CD38⁺ CD4⁺ mucosal T-cells were observed (Figure 7B).

From these data we conclude that the total percentage of circulating CD62L^{neg}CD38⁺ in the CD4⁺ T cell pool is not an indicator of intestinal inflammation. This absence of quantitative differences in circulating lymphocytes is a phenomenon that is not restricted to intestinal disease but also seen in other chronic mucosal disease such as chronic pulmonary disease. However, in chronic obstructive pulmonary disease (COPD) significant advantages have been made by correlating peripheral T-cell cytokine profiles with severity of disease (25). Therefore, we examined whether sorted CD62L^{neg}CD38⁺ CD4⁺ T cells from healthy donors exhibited detectable cytokine profiles by quantitative PCR. Indeed, in healthy controls IL-10 and IFN- γ transcripts were readily detected in highly purified CD62L-CD38⁺ CD4⁺ T-cells demonstrating that functional analysis is feasible (Figure 7C). Moreover, the new phenotype allowed us to compare CD62L^{neg}CD38⁺ mucosal effector T-cell function with peripheral CD62L^{neg}CD38^{neg} effector T-cell responses within the same individual as upon analysis CD62L^{neg}CD38^{neg} CD4⁺ T cells also contained IFN- γ after restimulation.

Discussion

Here we identify the CD62L^{neg}CD38⁺ phenotype as a subset of CD4⁺ T cells that have received mucosal imprinting. Our data demonstrate that endogenous RA produced by MLN-

DC is, together with TGF- β , a crucial factor in the differentiation towards the CD62L^{neg}CD38⁺ phenotype. Using a mouse model of mucosal tolerance we show that dividing T cells in the MLN are CD62L^{neg}CD38⁺, whereas this phenotype is not induced upon T-cell activation in PLN. This restricted population of mucosally-activated T cells was found to be conserved between mice and humans, as RA also induces the CD62L^{neg}CD38⁺ phenotype on human PBMC. The analysis of CD4⁺CD62L^{neg}CD38⁺ T cells in human peripheral blood revealed a strong enrichment of CCR9⁺ cells confirming mucosal imprinting. Moreover, in CD patients gliadin-specific T cells predominated in the CD4⁺CD62L^{neg}CD38⁺ T-cell pool. These data reveal that combined cell-surface expression of CD62L^{neg} and CD38 can be used to identify mucosal CD4⁺ T cells in peripheral blood.

Increased prevalence of T cells with either a CD62L^{neg} or a CD38⁺ phenotype in the gut has been observed in several studies (26–30). For example, it was previously shown that all intraepithelial lymphocytes in the murine small intestine, but not in the colon, have a CD62L^{neg} phenotype (26). Regarding CD38, it has been reported that human LP T cells express CD38 at higher levels than circulating T cells (27). In addition, the majority of CD4⁺CD25⁺ T cells in the LP, but not in the MLN or the spleen, lack expression of CD62L (27). It is unlikely that the CD62L^{neg} phenotype of lymphocytes in mucosal tissues is the result of continuous activation as we show that T-cell activation induced by i.m. protein injection does not induce this phenotype. For CD8⁺ T cells, CD62L expression can depend on the initial frequency of naive T cells and on the strength of the T-cell activation (31). In our *in vivo* model transfer of various numbers of T cells, resulting in different APC–T-cell ratios, did not affect CD62L expression. We argue that the mucosal microenvironmental factors RA and TGF- β , that can be secreted by mucosal DC (19, 32), modulate the expression of CD62L upon intestinal T-cell activation. In particular, Seibold *et al.* have demonstrated that microenvironmental factors induce shedding of CD62L in the small intestine (26).

CD38 expression could reflect a regulatory T-cell phenotype, as it is induced by RA and TGF- β , two factors that also mediate the differentiation of Foxp3⁺ Tregs. Indeed, we demonstrate that in mice, expression of CD38 correlates with that of Foxp3, as the percentage of CD38⁺ cells is increased in *de novo* induced Foxp3⁺ Tregs when compared with Foxp3⁻ cells. In agreement, CD38⁺ T cells have suppressive capacity *in vitro* (29, 33). Moreover, in NOD $\times$ CD38^{-/-} mice a modest reduction was detected both in the number of CD4⁺CD25⁺ Tregs and Foxp3 mRNA (34). However, our data show that CD38 does not exclusively identify mucosal Tregs as the CD62L^{neg}CD38⁺ phenotype is maintained when Foxp3 expression is inhibited. In particular, *in vivo* abrogation of mucosal tolerance by administration of OVA together with the adjuvant CT significantly reduced the number of Foxp3⁺ T cells in the MLN. *In vitro* Treg-differentiation assays confirmed this finding as the proportion of TGF- β and RA-induced CD38⁺ T cells did not decrease upon complete inhibition of Foxp3⁺ expression. Taken together, our data indicate that expression of CD38 reflects TGF- β and RA-dependent imprinting by mucosal APC rather than a regulatory T-cell function. As Foxp3 was normally induced on polyclonally stimulated CD4⁺ T cells from CD38^{-/-} mice by TGF- β and RA (not shown), it seems unlikely that CD38 is essential for the generation of Foxp3⁺ Tregs *in vitro*.

Although an association between RA and CD38 was reported for human leukemia cell lines and in B cells (35–39), we are the first to report that RA induces CD38 expression in primary T cells. The role of CD38 in T-cell differentiation is unclear. While there are no published reports demonstrating a functional role for CD38 on T cells, unpublished findings show that T cells from CD38^{-/-} mice do exhibit intrinsic functional defects (Frances Lund). Nevertheless, there is no evidence that these mice develop an intestinal pathology. It has been demonstrated that CD38 plays an important role in B-cell responses (40). Furthermore,

CD38 plays a crucial role in the migration of DC (41) and therefore one could question a role for CD38 in the migration of RA and TGF- β -imprinted CD62L^{neg}CD38⁺ T cells to the small-intestinal mucosa. Moreover, the strength of the APC–T-cell interaction may be influenced by the expression levels of CD38 on T cells, as human CD38 associates with the T-cell receptor (42) and CD38 can be recruited to the immunologic synapse upon antigen-mediated T-cell activation (43).

Possibly, CD62L^{neg}CD38⁺ imprinting extends to other mucosal tissues as CD62L^{neg}CD38⁺ T cells were detected in human tonsils. Moreover, in mice four months after mucosal influenza infection all influenza-specific T cells were CD62L^{neg}CD38⁺ (Frances Lund). Additional research will address this issue and focus on the expression CD62L^{neg}CD38⁺ on CD8⁺ T-cell responses.

The value of defining this new subset lies in lowering the threshold for detection of luminal antigen specific T cells within the circulating CD4⁺ T-cell pool. By selecting for CD62L^{neg}CD38⁺ expression which comprises 5–10% of the cells within the total CD4⁺ T-cell pool it is now possible to highly enrich for effector T cells with specificity for mucosal antigens. This is of pivotal importance for functional studies as this method removes 90–95% of CD4⁺ non-gut imprinted effector T cells and naive T cells from the analysis. Indeed, in healthy controls IL-10 and IFN- γ transcripts were readily detected in highly purified CD62L^{neg}CD38⁺ CD4⁺ T cells demonstrating that functional analysis is feasible. Moreover, the new phenotype allows us to compare CD62L^{neg}CD38⁺ mucosal effector T-cell function with peripheral CD62L^{neg}CD38^{neg} effector T-cell responses within the same individual as upon analysis CD62L^{neg}CD38^{neg} CD4⁺ T cells also contained IFN- γ after restimulation. We propose that future analysis of the anti-inflammatory and proinflammatory cytokine profile of this cellular subtype in different patient cohorts should ameliorate disease profiling in chronic intestinal disease. Currently most of these studies are performed with total PBMC. By preselecting for gut-imprinted cells instead of restimulating total PBMC the threshold of detection of T-cell responses to luminal antigens is lowered and the specificity of the readout is enhanced.

Overall better functional analysis of mucosally imprinted T-cells in the periphery may have important consequences for subtyping IBD and celiac disease patients and help evaluating therapy responsiveness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Study Highlights

What is the current knowledge?

- It is difficult to characterize defects in mucosal T-cell responses that contribute to chronic intestinal diseases as no ideal markers are available to monitor ongoing intestinal immune responses in peripheral blood.
- Mucosal dendritic cell derived factors TGF- β and vitamin-A steer the mucosal T cell response.

What is new here?

- Intestinal factors TGF- β and vitamin-A derivative retinoic acid induce CD62L^{neg}CD38⁺ expression on CD4⁺ T cells in mouse and man.
- CD62L^{neg}CD38⁺ expression reflects mucosal imprinting irrespective of regulatory or inflammatory function.
- After a gluten challenge in celiac disease patients, the majority of the gluten-specific T cells were detected in the small CD4⁺CD62L^{neg}CD38⁺ peripheral blood T-cell subset establishing that the latter phenotype better defined a mucosally imprinted population than the known markers CCR9 and $\alpha_4\beta_7$.

How might it impact on clinical practice in the foreseeable future?

- The value of defining this new subset lies in lowering the threshold for detection of luminal antigen specific T cells within the circulating CD4⁺ T-cell pool. By selecting for CD62L^{neg}CD38⁺ expression which comprises 5–10% of the cells within the total CD4⁺ T-cell pool it is now possible to highly enrich for effector T cells with specificity for mucosal antigens. This is of pivotal importance for functional studies as this method removes 90–95% of CD4⁺ non-gut imprinted effector T cells and naive T cells from the analysis.

Figure 1. Mucosal T cells express CD62L^{neg}CD38^{int} under homeostatic conditions
BALB/c mice received CFSE-labelled CD4⁺KJ1.26⁺ cells and OVA i.g. or OVA i.m.. At 72h after OVA, cells from gut-draining MLN- (OVA i.g.) and thigh muscle draining popliteal and inguinal LN cells denoted as PLN (OVA i.m.) were analyzed by flow cytometry. (A) CD62L or CD38 expression gated on CFSE⁺KJ1.26⁺ cells. (B) Percentage CD62L^{neg} and CD38^{int+hi} cells of CD4⁺KJ1.26⁺ T cells in each division peak (n=3, mean $\pm$ SD) (C) Combined expression of CD38 and CD62L and CD62L^{neg}CD38^{int} T cells as percentage of CD4⁺KJ1.26⁺ T cells (n=3, mean $\pm$ SD). (D) Representative dotplots showing Foxp3 staining of CFSE⁺CD4⁺KJ1.26⁺ T cells in draining LN 72h after OVA i.g. and OVA i.m. (E) Quantitative analysis of CD62L^{neg} and CD38⁺ cells within Foxp3⁺CD4⁺KJ1.26⁺ and Foxp3^{neg}CD4⁺KJ1.26⁺ gated cells (n=3, mean $\pm$ SD). *(p<0.05) Plots are representative of n=3.

Figure 2. TGF- β and RA induce a CD62L^{neg}CD38⁺ phenotype on activated T cells
 CFSE-labelled murine CD4⁺ T cells were stimulated with α CD3 α CD28 +/- rhTGF- β and/or RA. **(A)** Flow cytometry of CD62L, CD38 and Foxp3. **(B)** Quantitative data of flow cytometry (n=3, mean $\pm$ SD). **(C)** CD38 mRNA expression (n=3). *(p<0.05)

Figure 3. Expression of CD38 is preferentially induced by mucosal DC in a TGF- β and RA-dependent manner

OVA-loaded murine DC and CFSE⁺CD4⁺KJ1.26⁺ cells were cultured in different conditions. At 96h relative CD38 mRNA expression and flow cytometry analysis was performed on the T cells (A) culture with TGF- β . CD62L and CD38 MFI expression shown per peak of T-cell division and mRNA expression (n=3, mean $\pm$ SD). (B) MLN-DC and T cells cultured with TGF- β and RA-receptor antagonists LE135 and LE540. Percentage CD38⁺ and CD62L^{neg} cells and CD38 mRNA levels (n=3, mean $\pm$ SD). (C) MLN-DC and T cells cultured with TGF- β and TGF- β +RA. MFI and mRNA expression of CD38 on T cells (n=3, mean $\pm$ SD). (D) DC from MLN, PLN and spleen cultured with TGF- β and RA. MFI of CD38 and mRNA expression. (n=3, mean $\pm$ SD) *(p<0.05)

Figure 4. TGF- β and RA-induced CD38 is maintained when Foxp3 conversion is reduced
 OVA-peptide loaded murine MLN-DC and CFSE⁺CD4⁺KJ1.26⁺ T cells were cultured with TGF- β and RA. rIL-4 and LPS were added to inhibit Foxp3⁺ differentiation. **(A)** Relative expression of Foxp3 mRNA (n=3, mean $\pm$ SD). **(B)** CD38 expression by flow cytometry. **(C)** The percentage of CD38⁺ cells (n=3, mean $\pm$ SD). **(D)** MFI of cells within the CD38⁺ gate and relative expression of CD38 mRNA (n=3, mean $\pm$ SD). *(p<0.05)

Figure 5. CD62L^{neg}CD38⁺ identifies human antigen-experienced mucosal T cells

(A) Duodenal CD62L and CD38 expression in individuals with normal histology. Representative dotplot gated on total CD4⁺ T cells. (B) PBMC from healthy individuals (n=8, mean $\pm$ SD) were cultured with anti-CD3/anti-CD28 in the presence of TGF- β , RA or medium. The percentage of CD62L^{neg} CD38⁺ within the CD4 population is shown. Baseline percentages of CD62L^{neg} CD38⁺ before the start of the culture were (mean $5.69 \pm$ SD 2.17). (C) Flow cytometry of CD62L and CD38 in freshly isolated (non-stimulated) peripheral blood cells (gated on CD4⁺ cells). (D) Percentages of CD45RA⁺ (n=9), CCR9⁺ (n=9), Foxp3⁺ (n=9), β 7 integrin⁺ (n=8) and cutaneous leukocyte-associated antigen (CLA⁺) cells (n=8) within CD4⁺ T-cell gate and CD62L/CD38 CD4⁺ T-cell subsets. Error bars indicate SD, * (p<0.05).

Figure 5. Gluten-specific T cells are mainly detected in the CD4⁺CD62L^{neg}CD38⁺ subset
 Blood was collected from six adult celiac disease patients after a 3-day bread challenge. CD4⁺ PBMC were isolated and gluten-specific T cells were detected by tetramer staining. (A) One representative FACS plot showing the percentage of DQ2-control tetramer, DQ2- α I and DQ2- α II positive tetramer cells within CD4⁺ gate. (B) Tetramer-positive cells of four celiac disease patients were analyzed for CD62L and CD38. The percentages of CD62L^{neg}CD38⁺ cells within total CD4⁺ T-cell gate, control tetramer stained cells and DQ2- α I and DQ2- α II tetramer⁺ cells were calculated. (C) Flow cytometry of one representative patient. Staining of CD62L and CD38 of total CD4⁺ PBMC (upper left) and

DQ2- α I tetramer⁺ cells (upper right). Staining of isotype control on total CD4⁺ PBMC (lower left), CCR9 of total CD4⁺ PBMC (lower middle) and DQ2- α I tetramer⁺ cells (lower right). *(p<0.05)

Figure 7. No difference in the percentage of circulating CD62L⁻CD38⁺ CD4⁺ T cells in patients with chronic intestinal disease

Peripheral blood from pediatric celiac disease patients (A) IBD patients (B) and controls was, stained for CD4, CD62L and CD38 and subsequently analyzed by flow cytometry. (A) The percentage of CD62L^{neg}CD38⁺ mucosal CD4⁺ T cells in peripheral blood of celiac disease patients (n=34) and controls (n=21). (B) The percentage of CD62L^{neg}CD38⁺ mucosal CD4⁺ T cells in peripheral blood of Crohn's disease (n=21), Ulcerative colitis patients (n=8) and controls (n=14). (C) Peripheral blood CD4⁺ cells from a healthy donor were cell sorted into four CD62L/CD38 subsets and restimulated with PMA (0.05 μ g/ml) and ionomycin (0.5 μ g/ml) during 5h. IL-10 (left) and IFN- γ (right) expression was determined relative to GAPDH. n.s. denotes p>0.05.