Histol Histopathol (2009) 24: 1439-1449

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

The MHC-related protein 1 (MR1) is expressed by a subpopulation of CD38+, IgA+ cells in the human intestinal mucosa

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Summary. MHC-related 1 (MR1) molecule is a non-classical member of the MHC class I family of proteins. The sequence homology between classical MHC class I molecules and MR1 is very high, although the MR1 gene is not polymorphic and is highly conserved between species. MR1 is the restriction molecule of a sub-population of T lymphocytes, which are CD4-,CD8- and display conserved TCR α chain. The function of these cells is currently unknown, but they are believed to have regulatory properties similar to those of the CD1d restricted NKT cells.

The MR1 gene is ubiquitously transcribed; however it is unknown what types of cells express the MR1 protein "in vivo". In the present work we analyzed the expression of the MR1 protein using specific antisera and monoclonal antibodies in different human cell lines, in primary cells and in mucosal tissues. We found some lymphoid cell lines that express MR1 on the cell surface but at levels much lower than the MR1 transfected cell lines. In addition, we observed that expression of MR1 in the mucosa is restricted to a subpopulation of plasma cells or plasmablasts, CD38+ or CD138+ and IgA+, located in the human intestinal mucosa. This suggests a function for MR1 in the development of IgA producing plasma cells.

Keywords: Human, MHC, MR1, Mucosa, Plasma cells, IgA

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Introduction

Classical HLA class I genes encode for highly polymorphic, ubiquitously expressed molecules (Bjorkman and Parham, 1990), which are able to bind and present short peptides to TCRα/ß cytotoxic CD8+ T lymphocytes. While non-classical HLA molecules are similar in structure to classical HLA molecules, they display a variety of functions and generally are non-polymorphic (Rodgers and Cook, 2005). Human MR1 (MHC-related 1) is a non-classical HLA class I molecule encoded by a gene located on chromosome 1 (Hashimoto et al., 1995). Orthologous genes to human MR1 have been described in rat (Walter and Gunther, 1998), mouse (Yamaguchi et al., 1997), chimpanzee, and orangutan (Parra-Cuadrado et al., 2001). The MR1 gene is highly conserved between species, is ubiquitously transcribed (Riegert et al., 1998) and is non-polymorphic (Parra-Cuadrado et al., 2000).

A sub-population of T lymphocytes named MAIT (Mucosal-associated invariant T cells), are restricted by MR1 (Treiner et al., 2003). MAIT cells have been found in human, mice and cattle. They are CD4-,CD8- lymphocytes which display a TCR α/ß with a conserved TCR α chain (V α7.2-Jα33 in humans, Vα19-Jα33 in mice and cattle) (Tilloy et al., 1999). In mice, it has been found that the development of MAIT cells is dependent on the expression of MR1, intestinal flora and the thymus, as well as on the existence of B cells (Treiner et al., 2003; Hansen et al., 2007). The function of MAIT cells is currently unknown. Recently, a transgenic mouse model for TCR Vα19 that has high numbers of MAIT

Abbreviations: MR1, MHC-related 1; MAIT, Mucosal-associated invariant T, NKT; Natural Killer T cells.

cells was generated (Kawachi et al., 2006). In this model Kawachi et al. observed that a high proportion of MAIT cells express the NK1.1 marker and most of them had a phenotype similar to that of Vα14 iNKT cells, which are restricted by CD1d. The MAIT cells were able to produce IFN-γ, IL-4, IL-5 and IL-10 upon TCR ligation, suggesting that these cells may have regulatory functions similar to those of NK T cells (Kawachi et al., 2006).

Croxford et al. found that T cells expressing the Vα19i TCR transgene inhibited the induction and progression of EAE, a murine model of Multiple Sclerosis (Croxford et al., 2006). These authors also found that EAE was exacerbated in MR1 knock-out mice, which lack Vα19i T cells. Studies in humans have shown an accumulation of Vα7.2- Jα33 invariant T cells in the central nervous system of patients with Multiple Sclerosis and in nerve biopsy samples from chronic inflammatory demyelinating polyneuropathy patients (Illes et al., 2004).

It is well established that MR1 binds to ß2m (Yamaguchi and Hashimoto, 2002; Miley et al., 2003). Also, when MR1 is overexpressed in transfected cells it has been found to be associated to proteins from the HLA class I peptide loading-complex, including calnexin, calreticulin, ERp57, TAP and tapasin (Miley et al., 2003). However, development of MAIT cells in TAP-/- mice appears to be normal (Treiner et al., 2003). More recently, Huang et al. (2008) reported that MR1 localizes in multivesicular endosomes in MR1 transfected cells and that the protein uses an endocytic pathway to activate MAIT cells. Most of the data accumulated until recently described expression of MR1 on the cell surface as being possible, but limited by a putative unidentified ligand. Huang et al. have developed monoclonal antibodies against MR1 (Huang et al., 2005) and using these reagents they described that MR1 can be detected in an open or folded conformation and that only the folded version of the protein was able to stimulate MAIT hybridoma cells. In addition, they used a panel of MR1 mutants in experiments which suggested that MR1 interacts with a putative ligand that controls its expression and interaction with MAIT cells (Huang et al., 2005). It has been recently described that α-mannosyl glycolipids can be presented by MR1 to invariant Vα19-Jα33 T-cells (Okamoto et al., 2005; Shimamura et al., 2007a,b).

Since the kind of cell that expresses the MR1 protein is unknown, the goal of the study presented here was to analyze endogenous MR1 protein expression in cell lines, as well as primary cells and tissues of human origin.

Materials and methods

MR1 cloning and expression

Total RNA was isolated from human PBLs using the ULTRASPEC RNA isolation method (Bioteck Laboratories, Houston, TX). The first strand cDNA was synthesized from total RNA using a first strand cDNA synthesis kit (Roche Biochemicals-Boehringer Mannheim, Mannheim, Germany). Full-length MR1 cDNA was obtained after PCR amplification using primers MR1UP (5'):GGGCGTCGACGGACTA TGGGGGAACTGATG and MR1LO (3'):CCGCAA GCTTAGAGGAAGGAGAACTGGAAA containing *Sal*I and *Hind*III recognition sites at 5' ends. The PCR products were sequenced and cloned into the pSRαNeo vector. The plasmid was transfected by electroporation in the HLA class I-deficient lymphoblastoid cell lines C1R (Edwards et al., 1982) and L721.221 (Shimizu and Demars, 1982) as described (Campos-Martin et al., 2004). Cells expressing high levels of MR1 were selected using the anti ß2m monoclonal antibody BBM1.1 with Dynabeads M-450 (Dynal Biotech, Great Neck, NY) or the antibody 12.2 (monoclonal antibody anti-MR1, gift from Dr. T. H. Hansen, Department of Genetics, Washington University School of Medicine, St. Louis, MO, USA) with MicroBeads (Miltenyi Biotec, Cologne, Germany). The cell lines were cultured in RPMI 1640 and 10% FCS (Harlan Sera-Lab, Indianapolis, IN).

The cDNA encoding the soluble MR1 form was obtained after PCR amplification using the previous plasmid pSRαNeo-MR1 as template and the following primers: (5'):GGGAAGCTTATGGGGGAACTGAT GGC and (3'):CAAGGATCCCGTCGATCTGGT GTTGG containing EcoRI recognition site at 5' end. The human leader peptide was substituted by the mouse one obtained synthetically (without template) by PCR amplification with the following primers (5'):TTTA AGCTTGCCGCCACCATGGATTTTCTGGTACAAAT TTTCAGCTTCCT containing *Hind*III recognition site at 5' end and (3'):AGTGCGTCCGTCCCCTGCTCA TTGCTACGCTTGCGCTAATAAGAAGGAAG. Finally, both cDNA (MR1 and leader peptide) were attached with a PCR without primers, because both templates (MR1 and the leader peptide) have overlapping ends.

The ß2-microglobulin cDNA was obtained after PCR amplification using the following primers: (5'):GAGCAGGGGAATCCAGCGTACTACTCCAAA GATT and (3'):TTTCCCGGGTTACATGTCTCGAT CCCACTTA containing XmaI recognition site at the 5' end. The human leader peptide was also substituted by the mouse one obtained synthetically (without template) by PCR amplification with the following primers (5'):TTTAAGCTTGCCGCCACCATGGATTTTCTGGT ACAAATTTTCAGCTTCCT containing HindIII recognition site at 5' end and (3') TACGCTGGATTCC CCTGCTCATTGCTACGCTTGCGCTAATAAGAAGG AAG. Both cDNA (ß2m and leader peptide) were attached with a PCR amplification without primers.

The PCR products MR1 and ß2m were sequenced and cloned into plasmids of GS system (Lonza, Basel, Switzerland) pEE6.4 and pEE12.4 respectively. pEE6.4 was digested with NotI and BamHI and ligated into pEE12.4, obtaining a plasmid with both cDNAs. The

plasmid was transfected by electroporation into the NS0 cell line.

The cDNA of the recombinant MR1-FLAG protein was obtained after PCR amplification using the previous plasmid pSR·Neo-MR1 as template and the following primers: (5'):TTTTAAGCTTGCCGCCACCGGACTAT GGGGGAACTG containing a HindIII recognition site at 5' end and (3'):TTTTGAATTCCTACTTGTCGTC GTCGT containing an EcoRI recognition site and FLAG at 5' end. The cDNA was also cloned into plasmids of GS system as described above.

Recombinant protein MR1-FLAG production

A clone of NS0-ß2m-MR1-FLAG cells was selected and bred in DMEM medium glutamine free (JRH Biosciences, Hampshire, UK) and 10% FCS (Invitrogen Life Technologies, Carlsband, CA). The MR1-FLAG protein was purified by affinity chromatography with M2 antibody.

Cell lines

The following cell lines were maintained in RPMI 1640 (Invitrogen Life Technologies, Carlsband, CA), and 10% FCS (Harlan Sera-Lab, Indianapolis, IN): C1R (Edwards et al., 1982) LCL721.221(Shimizu and Demars, 1982), JY (Terhorst et al., 1976), Daudi (Klein et al., 1968), Jurkat (Schneider et al., 1977), K562 (Lozzio and Lozzio, 1975), SupT1 (Smith et al., 1984), NB-4 (lanotte et al., 1991), HL-60 (Collins et al., 1977), Peer (Ravid et al., 1980), U937 (Sundstrom and Nilsson, 1976) and Molt4 (Minowada et al., 1972). The cell line T84 (Murakami and Masui, 1980) was maintained in 50% F12 (Invitrogen Life Technologies Carlsband, CA), 50% Dulbecco's MEM (Invitrogen Life Technologies Carlsband, CA), and 5% FCS. Caco2 (Fogh et al. 1977) was maintained in Dulbecco's MEM, 20% FCS. NS0 [39] was maintained in DMEM (Invitrogen Life Technologies) with 10% FCS.

Flow cytometry

The antibodies used were anti-human MR1 12.2 and 26.5 (mouse IgG2a) (gift from Dr. T. H. Hansen), anti-human ß2m, BBM1.1 (mouse IgG2b) (from American Type Culture Collection (ATCC), Manassas, VA), anti-FLAG M2 (mouse IgG1) (Sigma-Aldrich, Oakville, Ontario, Canada); PE- and fluorescein-coupled polyclonal goat anti-mouse IgG (H+L; Caltag Laboratories) were used as secondary Abs in FACS analysis. An Epics Elite cytometer (Beckman Coulter) was used in the flow cytometry analysis.

Immunoprecipitation

Recombinant MR1 proteins were immuno-precipitated from supernatants of transfected NS0 cells with antibodies BBM1.1 or M2 and protein A- or G-Sepharose, respectively, and the immuno-precipitated material was separated in a SDS-PAGE electrophoresis.

SDS-PAGE and Western-Blot

One dimensional polyacrylamide gel electrophoresis (PAGE) was carried out using Mini-PROTEAN II Electrophoresis Cells (Bio-Rad, Hercules, CA) gel. Gel and sheet were previously equilibrated in transference buffer (25 mM Tris-HCl, 185 mM Glicina, 2.5 mM SDS, 20% Metanol) and the transfer was performed in a Trans-Blot (BioRad, Hercules, CA). PVDF papers were treated with a blocking solution and then incubated with primary antibodies M2 (Sigma-Aldrich), polyclonal anti-ß2m (Sigma-Aldrich) or RAMRE-1 diluted to 1/50 in the blocking solution (with 0.5% BSA). Specifically, bound antibodies were detected with anti-rabbit (Sigma-Aldrich) or anti-mouse (Amersham Biosciences) antibodies alkaline phosphatase conjugated which were diluted 1:60000 or 1:10000 respectively, in the blocking solution for 30 min. After rinsing 3 times in TBS, the blots were incubated with ECL reactive and exposed to radiography.

Rabbit immunization and antisera characterization

Two male NZ rabbits were subcutaneously immunized with 100 μg MR1-FLAG in the presence of Freund's adjuvant, complete or incomplete, five times over two months.

The sera were purified by affinity chromatography with MR1 immobilized in sepharose beads. After this, the sera were incubated with human platelets in order to remove the reactivity against human ß2m. Finally, the sera obtained were tested by Western-blot, flow cytometry and immunofluorescence.

Histology and immunofluorescence

Human biopsy specimens of macroscopically normal ileum (n=3), appendix (n=3) and colon (n=3) were used. Specimens, within 15 min of sampling, were embedded in OCT compound (Miles, Elkhart, IN), snap frozen in liquid nitrogen and stored at -70º. Cryosections (6 μm thick) were air-dried for 2 h at room temperature and fixed in acetone for 10 min. Nonspecific binding of antibodies and endogenous biotin was blocked by incubation with avidin-biotin, Biotin Blocking System (Vector Laboratories, Burlingame, CA). After PBS washing the slides of tissue sections were incubated overnight at 4°C with PBS containing 5% BSA (IgG free, protease free, Jackson Immunoresearch Laboratories, West Grove, PA). Next, tissue sections were sequentially incubated with anti MR1 monoclonal antibodies (12.2 or 26.5) or anti MR1 rabbit antiserum (RAMRN-2) generated in our laboratory, 1 hr at room temperature, followed by biotin conjugated F(ab´)2 of goat anti IgG (H+L) mouse or rabbit (Jackson ImmunoResearch Laboratories), followed by

Streptavidin-Texas Red (Jackson ImmunoResearch Laboratories). Controls were irrelevant isotype-matched primary antibody (Purified IgG 2a, κ; BD-Pharmingen) or preimmune rabbit serum. Multicolor immunostaining for phenotype cell population was performed by adding FITC conjugated anti human CD antibodies: anti- CD68-FITC (clone: KP1; Dako, Carpinteria, CA), anti- CD19-FITC (clone: HD37; Dako cytomation, Carpinteria, CA), anti- CD38-FITC (clone: T16; Serotec, Oxford, U.K.), anti- CD138-FITC (clone: BB4; Dako, Carpinteria, CA), anti- CD11c-FITC (clone: 3.9; Serotec), anti- HLA-DR-FITC (clone: HL-39; Serotec), anti- IgA-FITC (goat antiserum; Caltag Laboratories), anti- CD3-FITC (clone: S4.1; Caltag Laboratories). Nuclear cell staining was performed with DAPI (Vectashield; Vector). Finally, slides were mounted in PBS-Glicerol 50% (v/v).

Confocal microscopy

Cell morphology and stainings were analysed using confocal fluorescence microscopy.

Fluorescence images were acquired in a confocal microscope (Leica, TCS-SP2 AOBS spectral confocal system, Germany) coupled to an inverted microscope (Leica IRE-2). Fluorochrome excitation laser lines were 405nm (for DAPI), 488 nm (for FITC-coupled Abs) and 594 nm (for TexasRed conjugated Abs). Fluorescence emission was optimally recorded by adjusting spectral detectors to the maximum emission peaks of the corresponding fluorochromes. Fluorochrome excitation and emission detection were carried out sequentially for each fluorochrome in order to avoid fluorescence spillover between them. Scanning resolution was set at 1024x1024 pixels. Z-series confocal images were acquired through optimizing pinhole aperture and z-stepping. Then, either maximum-projections of z-series or individual optical confocal sections were used for image analysis. 60x immersion oil microscope objective was used. Image analysis was carried out with Leica software (Leica TCS).

Results

Expression of an MR1 soluble recombinant molecule and generation of anti MR1 rabbit antisera

We generated two types of secreted recombinant MR1/ß2m molecules expressed in NS0 cells. The first, MR1/ß2m, was formed by the ectodomain of human MR1, including the α1, α2 and α3 subdomains. The second, MR1-FLAG, was identical to the first one, except for the FLAG tail added at the C-terminus. These recombinant proteins were generated by cloning the fraction of cDNA encoding the extracellular portion of MR1 or MR1-FLAG epitope (which we will name MR1-FLAG) into the vector PEE12.4, in which we have previously cloned the cDNA encoding for human ß2m. After this, NS0 cells were transfected, cultured and subsequently cloned. The secretion of MR1 was detected in the NS0 supernatants using a rabbit anti MR1 serum

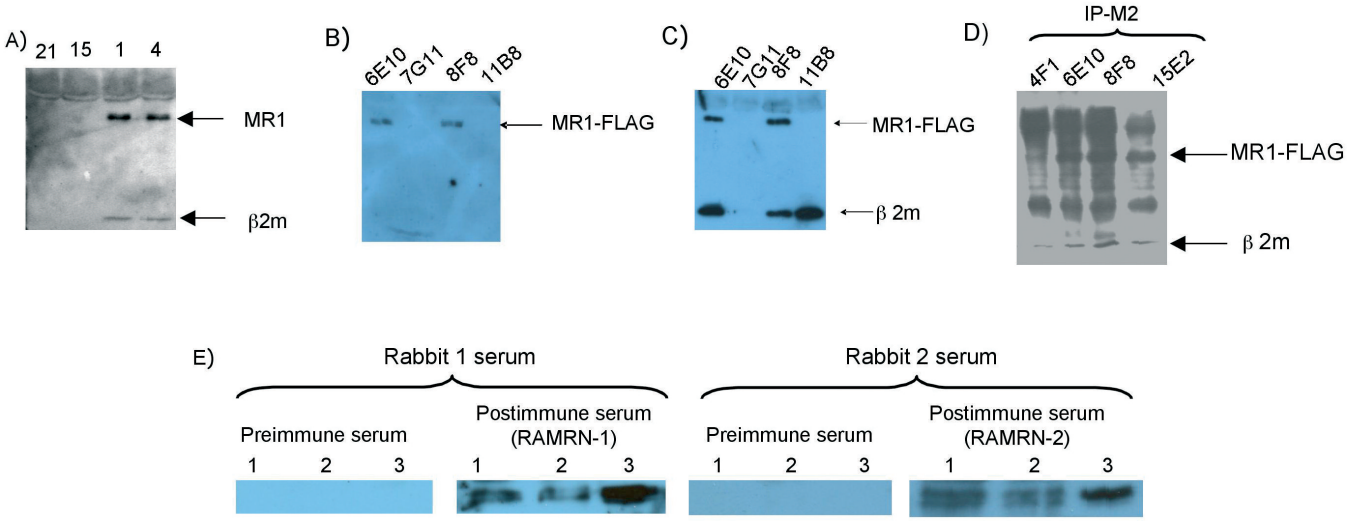

Fig. 1. Expression of MR1 recombinant proteins and rabbit antisera development. Several clones were analyzed for MR1 expression and their names are indicated on the top of the blots. **A.** Secreted MR1 and human ß2m in the supernatants of NS0 transfected cells were detected by Western blot using a rabbit anti MR1 serum (RAMRE-1) and a rabbit anti ß2m antiserum. **B.** The supernatant of NS0 transfected cells was analyzed in order to detect the expression of MR1-FLAG fusion protein by Western blot using an anti FLAG monoclonal antibody (M2). **C.** Detection of MR1-FLAG fusion protein by Western blot using a rabbit anti MR1 serum (RAMRE-1); ß2m detection was carried out with a commercial antiserum. **D.** The NS0 supernatant was immunoprecipitated with the anti FLAG monoclonal antibody (M2), after that MR1-FLAG and ß2m were detected by Western blot as indicated in D. **E.** Detection of MR1-FLAG by Western-blot using the newly developed rabbit antiserum RAMRN-1 and RAMRN-2, lanes 1 and 2: NS0 ß2m-MR1 supernatant, lane 3: purified MR1-FLAG protein.

(RAMRE-1), previously developed in our laboratory against an MR1 recombinant protein expressed in *E. coli* (Parra-Cuadrado, 2002), whereas detection of ß2m was carried out with a commercial antiserum. We obtained several clones secreting both MR1 and human ß2m (Fig. 1A) or MR1-FLAG and human ß2m (Fig. 1B,C). In the case of MR1-FLAG we also performed immunoprecipitation experiments with the anti FLAG monoclonal antibody M2. The material was analyzed in subsequent Western-blot experiments using the RAMRE-1 and anti ß2m antiserum. In these experiments we observed (Fig. 1D) that ß2m was detected in the material immunoprecipitated with M2, indicating that cells were secreting MR1-FLAG bound to human ß2m.

The recombinant MR1-FLAG protein was purified by affinity chromatography with the M2 monoclonal antibody from the NS0 supernatants and subsequently used to immunize two rabbits. The anti-sera generated (RAMRN-1 and RAMRN-2) recognized the MR1 as well as the MR1-FLAG proteins in Western blot experiments, as shown in figure 1E.

Generation of L721.221 and C1R cell lines transfected with MR1

To generate cell lines expressing high levels of MR1, we transfected HLA class I deficient cell lines with the vector pSRαNeo containing full length cDNA encoding for MR1 (MR1.pSR). Both L721.221 and C1R cell lines were transfected with MR1.pSR. The cells were analyzed by FACS using anti ß2m (BBM1.1) and the 12.2 and 26.5 anti-MR1 monoclonal antibodies, as well as our anti MR1 antisera. As shown in figures 2A, the anti MR1 monoclonal antibodies detected high levels of expression on the surface of the transfected cells. The RAMRN antisera also recognized MR1 on the transfected cells, as shown by FACS (Fig. 2B) and immunofluorescence (Fig. 2C) techniques.

The MR1 protein is expressed by B lineage cells located in the intestinal mucosa

The expression of the MR1 protein in vivo has not yet been documented. The RNA for MR1 is ubiquitously expressed and it has been found to be expressed in cell lines of different origin, including T cell lines, B cell lines, and epithelial cell lines (Riegert et al., 1998). For this reason, we decided to analyze MR1 expression on cell lines of different lineage by FACS, using anti MR1 monoclonal antibodies. As observed in figure 3, some cells are able to express modest levels of MR1 on the cell surface but none of them at levels similar to those of

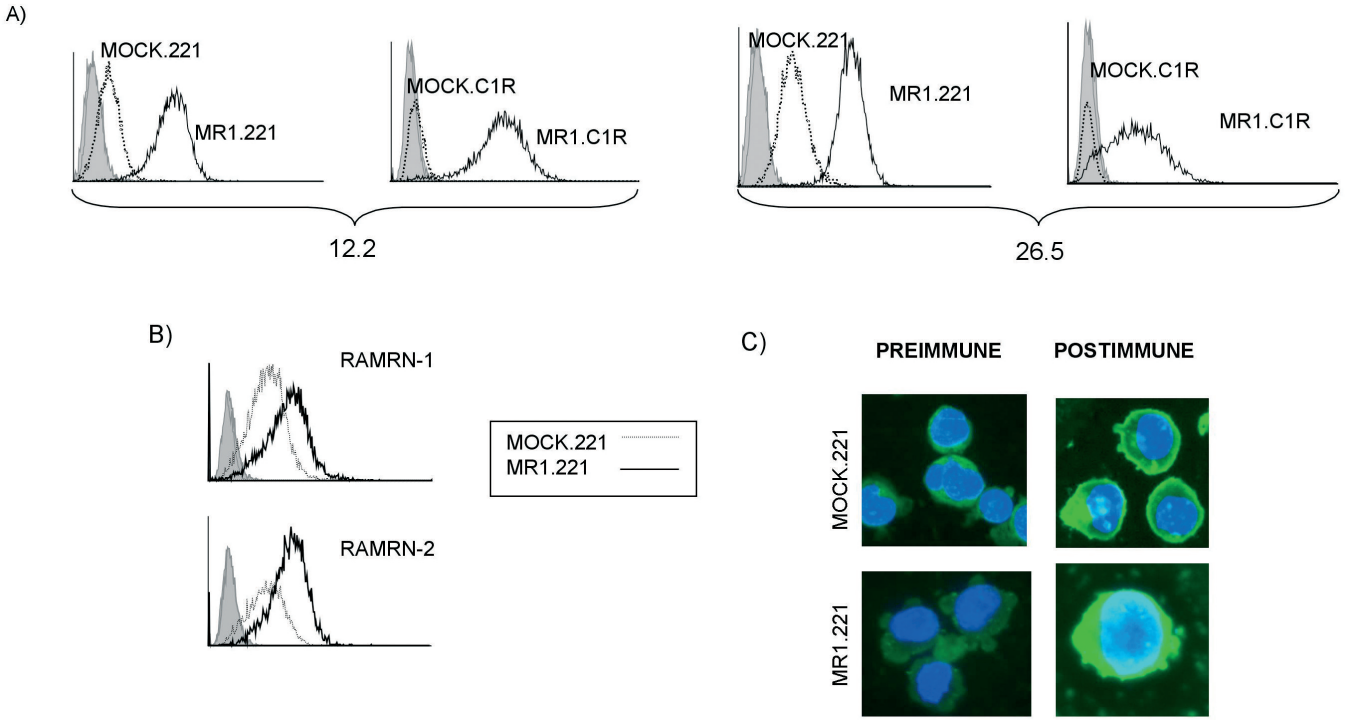

Fig. 2. Generation of MR1 transfected cells. **A.** The expression of MR1 on L721.221 (MR1.221) and C1R (MR1.C1R) transfected cells was analyzed by FACS using the anti MR1 monoclonal antibodies 26.5 and 12.2. **B.** Expression of MR1 on MR1.221 cells was analyzed by FACS using two rabbit anti MR1 sera (RAMRN-1 and RAMRN-2). **C.** Expression of MR1 on MR1.221 cells analyzed by indirect immunofluorescence using anti MR1 serum (RAMRN-1). Isotype control antibody fluorescence is indicated in gray histograms.

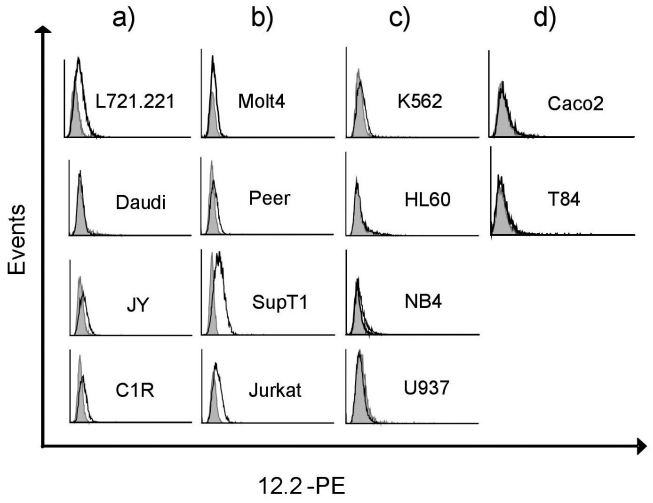

Fig. 3. Expression of MR1 on the surface of different human cell lines was analyzed by FACS using the 12.2 anti-MR1 monoclonal antibodies. Isotype control antibody fluorescence is indicated in gray histograms. **a.** B-cell lines. **b.** T-cell lines. **c.** Mieloblastoid cell lines. **d.** Intestinal epithelial cell lines.

MR1 transfected cell lines MR1.Taking into account that the protein MR1 can be detected in different cell lines we decided to study the expression of MR1 in primary human cells and tissues. First, we analyzed the expression of MR1 on peripheral blood leukocytes by FACS, using the RAMRN-2 and the monoclonal antibodies 12.2 and 26.5. We also analyzed the expression of MR1 in monocyte-derived macrophages and dendritic cells. We did not find detectable MR1 expression in any of these cell types (results not shown).

MAIT cells are mainly located in the intestinal mucosa, which suggests that MR1 protein could also be expressed in these tissues. For this reason we decided to stain sections of normal human intestine by immunofluorescence using anti MR1 monoclonal antibodies and antisera. We found MR1 positive cells in the lamina propria of ileum, appendix and colon (figure 4). These cells were located outside the lymphoid follicles and displayed a non-lymphocyte morphology.

In order to characterize the type of MR1+ cells, we performed double staining with 26.5 and different monoclonal antibodies. Double stainings with monoclonal anti MR1 and anti-CD14 (data not shown), CD68 or CD11c showed a non-overlapping pattern expression as observed in figure 4 (E-H) in which no double labeled cells are present, indicating that MR1+ cells were neither monocytes/macrophages nor dendritic cells. In these stainings we also observed a similar reactivity when comparing the monoclonal antibody 26.5 and our antiserum RAMNR-2 (Fig. 4E-H). In contrast, MR1+ cells were double-labeled with antibodies detecting markers specific for B-cell lineage, including CD19 (Fig. 4K) and HLA-DR (Fig. 4N). MR1+ cells were also co-stained with markers of plasma cell or plasmablasts, CD38 and CD138 (Fig. 5). Most MR1+ cells were co-stained with anti CD138 monoclonal antibodies (Fig. 5C) and all of the 26.5 + cells were co-stained with the anti CD38 antibody (Fig. 5F) suggesting that the MR1 positive cells were plasma cells or plasmablasts. CD38 can also be expressed by other cell types, including lamina propria T lymphocytes. When we performed staining with anti-CD3 and 26.5 monoclonal antibodies we did not detect double positive cells, which indicates that mucosal T cells do not express MR1. Interestingly, we found some instances of images suggesting interactions between some T cells and MR1 positive cells, as can be observed in figure 5L. Our results indicate that MR1 expression is restricted to plasma cells or plasmablasts. In order to analyze the Ig isotype produced by these MR1 positive cells we performed double staining with anti MR1 and anti IgA antibodies, since most of the plasma cells present in the mucosa are IgA positive. As shown in figure 5 some IgA+ cells co-express MR1 (Fig. 5I), whereas all the MR1 positive cells were also IgA+ (Fig. 5I).

In order to analyze whether MR1 is expressed on the cells surface or intracellulary we performed confocal analysis. Most of the MR1 positive cells showed an intracellular and vesiculated staining pattern (Fig. 6A), however, some cells display a cell-membrane pattern (Fig. 6B-C). Double staining with anti CD38 and anti MR1 was also performed (Fig. 6D-F).

In summary, these results indicate that protein MR1 expression "in vivo" is restricted to plasma cells or B-cell blasts which produce immunoglobulins of the IgA isotype in the human intestinal mucosa.

Discussion

MR1 molecules display a high degree of similarity to HLA class Ia molecules, especially in the α1 and α2 domains (Bjorkman and Parham, 1990). Furthermore, MR1 is highly conserved between species and the regions most conserved are also the α1 and α2 domains (Yamaguchi et al., 1997). The conservation between species also applies to the MR1-restricted MAIT lymphocytes (Tilloy et al., 1999; Treiner et al., 2003), suggesting that these molecules and cells are important in the immune response. However, the function of MR1 molecules and MAIT cells are currently unknown. Two major points need to be determined regarding the MR1/MAIT function: what type of cells express MR1 "in vivo", and the nature of the antigens presented by MR1 to MAIT lymphocytes.

Our primary goal was to study MR1 expression in human tissues and cells. Previous results suggested that MR1 expression on the surface of transfected cells was limited. However, a high expression level of transfected MR1 was observed using recently developed anti MR1 monoclonal antibodies (Huang et al., 2005). Also, the antisera developed in our laboratory, as well as the

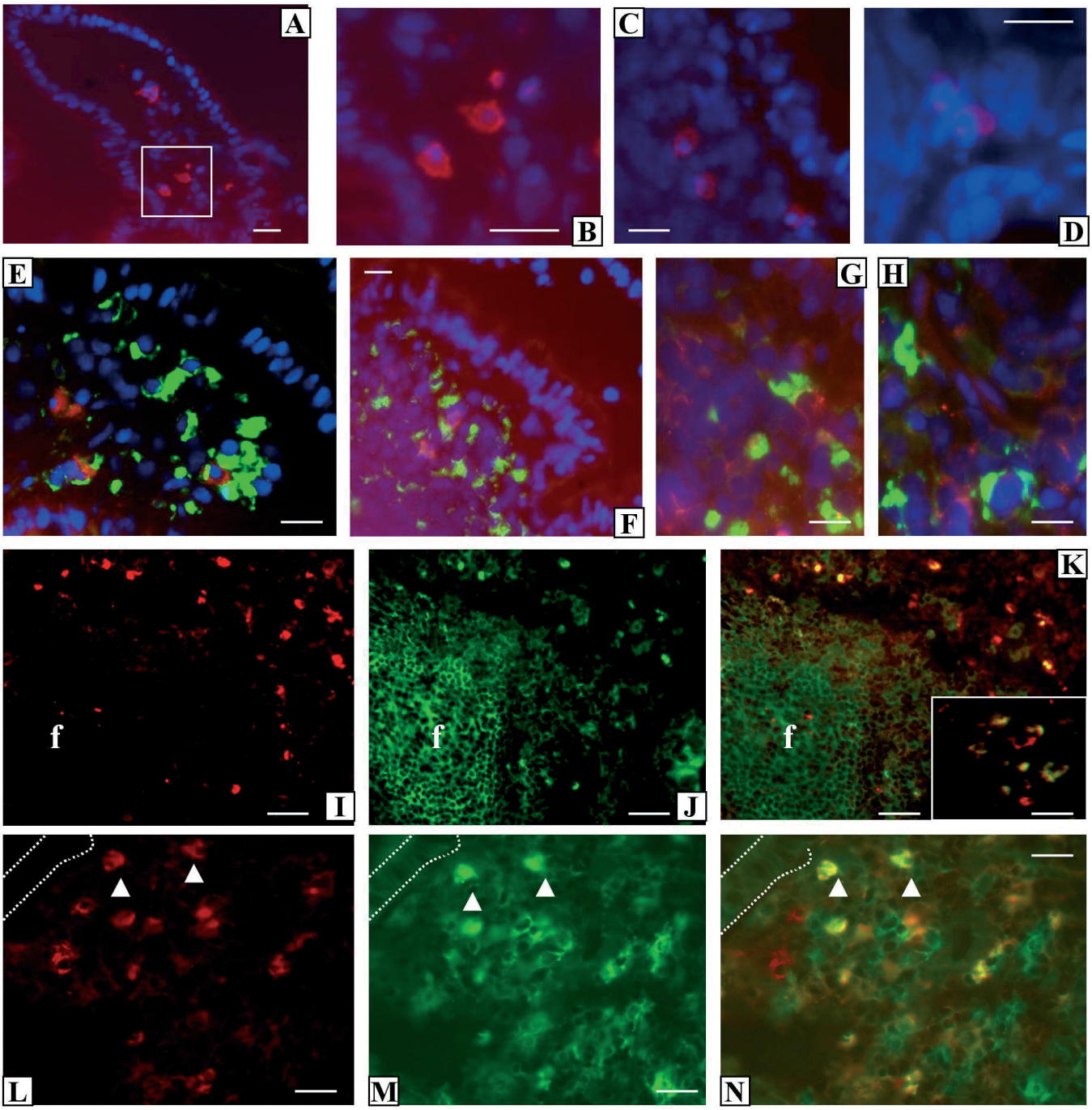

Fig. 4. Immunohistological detection of MR1 in human tissues. The epithelium limit is indicated by dotted lines. 26.5+ cells (red) can be located in lamina propria beneath epithelium villi in ileum (**A, B** is a higher magnification detail of **A)**, colon **(C)** and appendix **(D)**. Double immunofluorescence staining was performed for CD11c (green; **E, G**) or CD68 (green; **F, H**) with moAb 26.5 (red; **E, F**) or anti RAMRN-2 antisera (red; **G ,H**), in Ileum samples. We did not observe double staining for these markers. On the same ileum image, 26.5+ cells (red, **I**) are located outside lymphoid follicle **(F)**, CD19 k(green, **J**) is expressed in follicle and lamina propria, and coexpression (yellow) of 26.5 (red) and CD19 (green) can be observed outside of follicular (f) areas in lamina propria (**K**, insert is a magnification). Most of the 26.5+ cells (**L**, red) in lamina propria are positive also for HLA-DR (**M**, green; **N**, merge in yellow). Nuclei of cells were stained with DAPI (blue). Arrows point to examples of what is indicated. Scale bars: A-H, L-N, insert K, 25 μm; I-K, 100 μm.

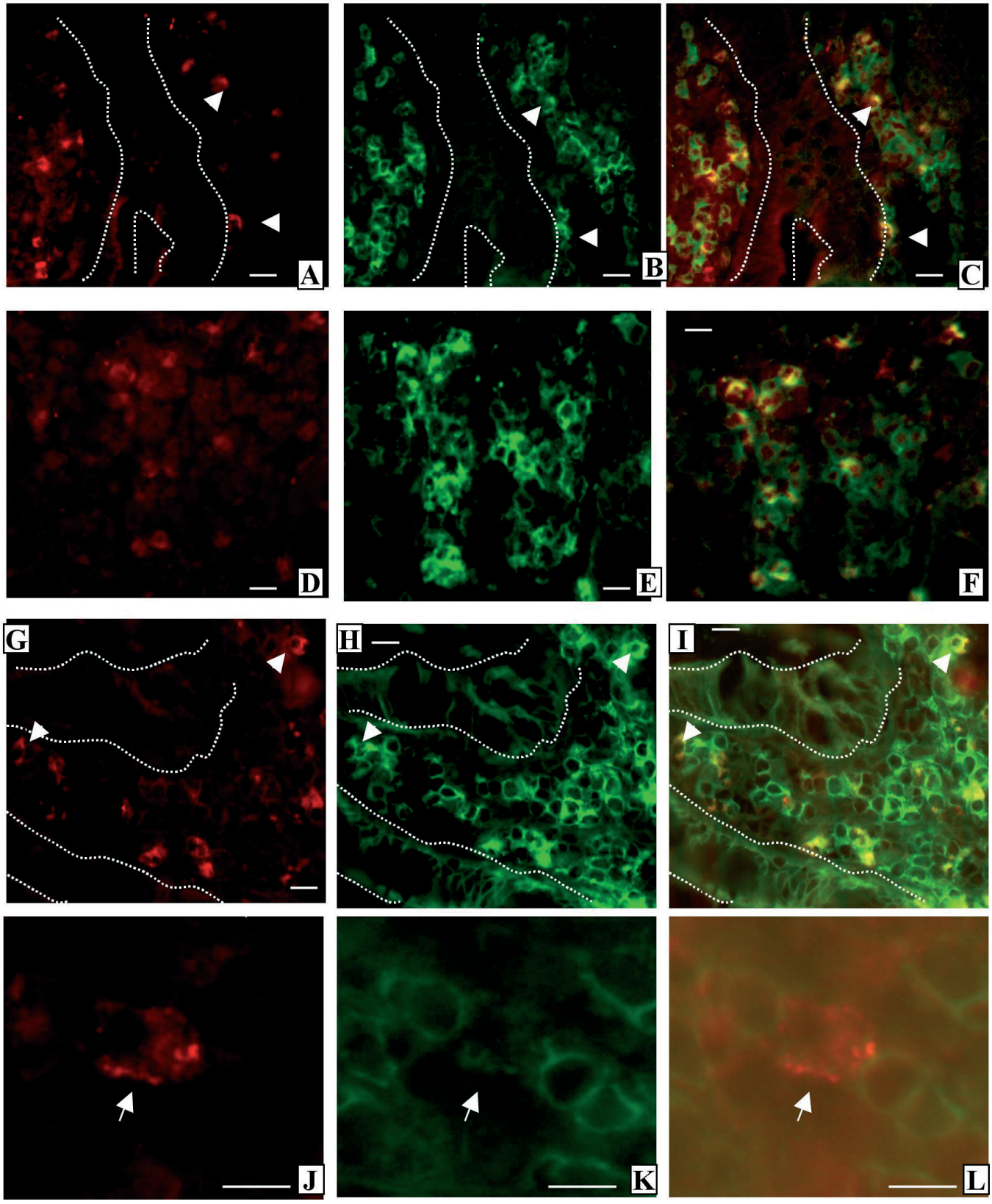

Fig. 5. Double immunofluorescence of human tissue sections from ileum. The epithelium limit is indicated by dotted lines. Most of the 26.5+ cells located in lamina propria (**A**, red) are also positive in the same tissue section for CD138 (**B**, green; **C**, merge in yellow). All of 26.5+ cells (**D, G**; red) represent a population of CD38+ cells (**E**, green; **F**, merge in yellow) and IgA+ cells (**H**, green; **I**, merge in yellow) inside lamina propria of villi. Typical 26.5+ cells presenting a large size and irregular shape, and sometimes 26.5+ membrane staining (**J**, red) may present synapse images with CD3+ lymphocytes (**K**, green; **L**, merge). Arrows point to examples of what is indicated. Scale bars: A-I, 25 μm; J-L, 10 μm.

monoclonal antibodies (Huang et al., 2005), showed that our MR1.221 and MR1.CIR transfectant cell lines are able to express high levels of MR1 on the cell surface. These transfectants were selected only with anti-ß2m or anti-MR1 antibodies in order to obtain high levels of MR1 on the cell surface without adding exogenous antigens. Thus, if MR1 needs ligands to reach the cell surface these ligands can be endogenous and/or they can be provided by the FCS added to the cell culture. In this respect, experiments adding *E. coli* extracts to our cell cultures did not increase the expression of MR1 on the cell surface (B. Gozalbo, unpublished results). It must be taken into account that the antibodies 12.2 and 26.5 detect only the "folded" form of MR1. Whether this ligand is of a peptidic nature or is different is not known, and our efforts to extract and identify peptides binding to MR1 have been unsuccessful thus far (B. Gozalbo unpublished results), which can be explained if the natural ligand of MR1 is of lipidic nature, as the experiments with exogenous ligands suggest (Okamoto et al., 2005; Shimamura et al., 2007a,b).

Regarding cell lines, we found a very low expression of MR1 in human cell lines of lymphoid origin. In addition, we did not detect the presence of MR1 in primary peripheral blood leucocytes or in monocyte-derived dendritic cells or macrophages. This prompted us to investigate expression in cells from a different source. The most probable location for MR1-expressing cells is thymus and mucosa, because MAIT cells are thymus-dependent and are located in high numbers in the mucosa. In preliminary analysis we did not detect MR1 expression in samples of human thymus (Gomez del Moral, unpublished results). In contrast, staining of intestinal mucosa samples with anti MR1 antibodies revealed the presence of positive cells. These cells were of non-lymphocyte morphology and to characterize them we performed double stainings with different markers. The MR1+ cells were negative for markers specific for T cells (CD3), dendritic cells (CD11c) or macrophages (CD14, CD68). We decided to include CD68 in our stainings because in several previous reports intestinal macrophages have been described as CD14 negative (Smith et al., 1997, 2001).

MR1+ cells were stained with markers of the B-cell

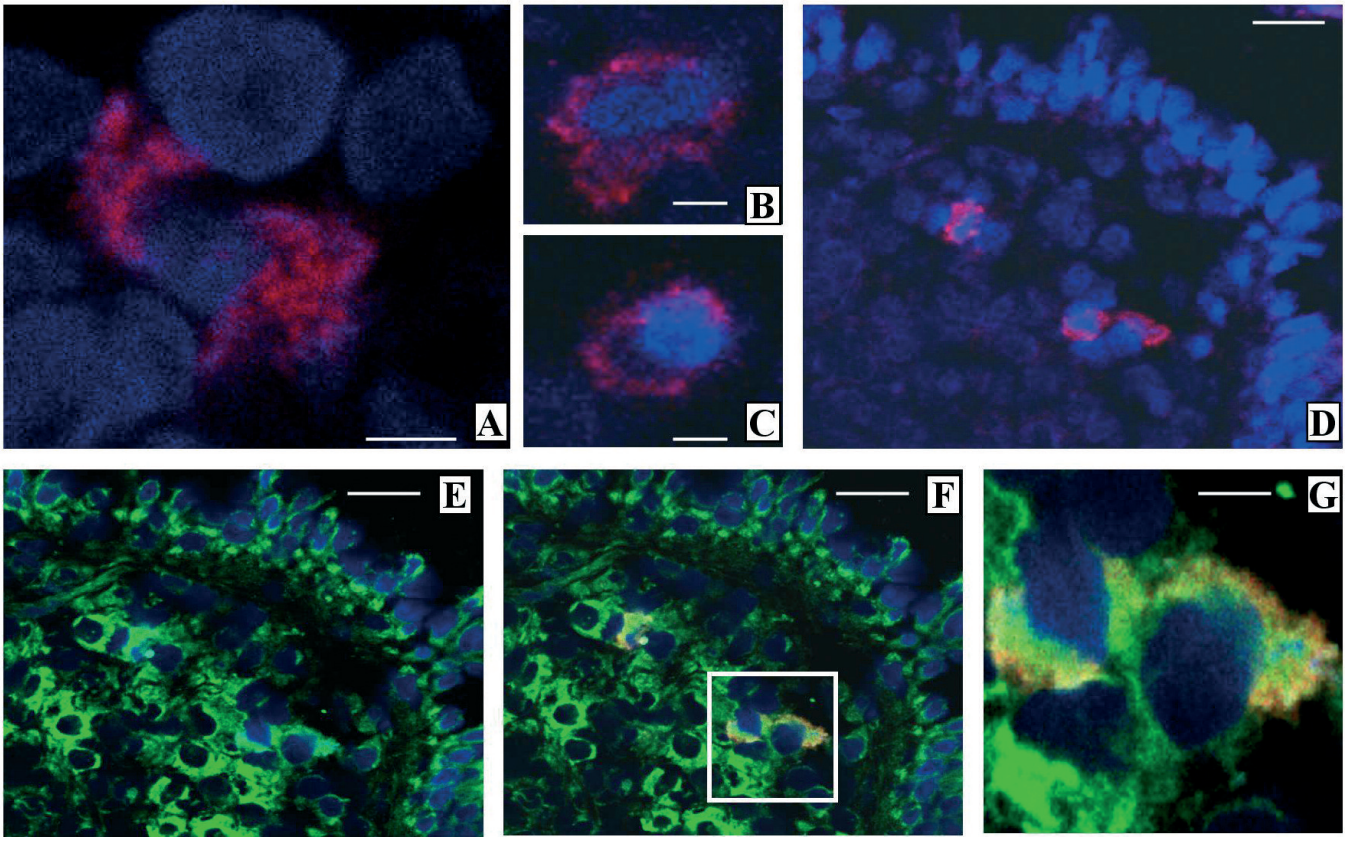

Fig. 6. Confocal images of MR1 positive cells in human tissue sections from Ileum. Most of the 26.5+ cells, located in lamina propria showed an intracellular vesiculated pattern (**A**, red). Some 26.5+ positive cells mainly showed a membrane pattern (**B-C**, red). Double immunostaining for 26.5 (**D**; red) and CD38 (**E**; green; **F**, merge in yellow; **G**, is a magnification of **F** box). Nuclei of cells were stained with DAPI (blue). Scale bars: A-C, insert G, 5 μm; D-F, 25 μm.

lineage like CD19, HLA-DR, CD38 or CD138, although their expression by the MR1 positive cells was not homogeneous. Taking into account these phenotypes, it is plausible to think that MR1 is expressed by a population of cells present in a developing stage between mature B cells and fully developed plasma cells. This would explain the presence of CD19 or HLA-DR positive cells at an early stage, or the CD138 staining corresponding to fully developed plasma cells (Jego et al., 1999). It is interesting to note that all MR1+ cells were positive for CD38; this marker can be present in plasmablasts or fully developed plasma cells. Taking this into account, it is tempting to speculate that MR1 could be involved in the transition between B cells and plasma cells through the interaction with MAIT cells. Also interesting is that every MR1 positive cell was also stained with anti IgA antibodies, although they were a relatively minor fraction of the whole IgA+ population. This fact is in agreement with the hypothesis that MR1 would be present mainly during the B blast stage (CD38+) and would be lost in a late stage (CD138+, IgA producing) of plasma cell development. For MR1 function, expression on the cell surface seems to be a prerequisite. In some previous reports expression of MR1 on the cell surface of transfected cells seem to be low (Miley et al., 2003), especially in non B-cells (Aldemir, 2008). In our L721.221 and C1R cells transfectants expression of MR1 on the cell surface was high. In the lamina propria, MR1 positive cells show an intracellular vesiculated pattern. However, in some cells we observed a cell membrane pattern which could suggest a possibility to present antigen to MAIT cells

The expression of MR1 in the B-cell compartment is also in agreement with the fact that development of MAIT cells appears to be dependent on B cells in mice as well as in humans (Treiner et al., 2003). Interstingly, K.O. mice without transmembrane region of the immunoglobulin- μ chain (μMT-/-) lack most B cells, although they have normal levels of MAIT cells (Treiner et al., 2003); this was explained by the fact that μMT-/- mice do have B cells producing IgA (Macpherson et al., 2001). The development of MAIT cells is also dependent on the thymus. Using the monoclonal antibody 26.5 Huang et al. (2008) detected the expression of MR1 by immunoprecipitation and subsequent western blot in the thymus and other tissues from mice, although they did not describe in what kind of cells the protein was expressed within the tissues. In contrast, we did not detect MR1 positive cells in a sample of human thymus. Interestingly, Treiner et al. did not detect MAIT cells in the mouse thymus either (Treiner et al., 2003). One explanation for this could be that thymic MR1 expression and MAIT selection are fixed in a time window during development, e.g. before birth. Another explanation could be that MAIT cells develop extrathymically but the precursors (or a population of selecting B cells) come from the thymus.

In summary, we describe for the first time the identification of a cell population expressing the protein MR1 "in vivo", which are B-cell blasts or plasma cells that produce immunoglobulins of the IgA isotype, suggesting a function for MR1 involved in the development of IgA producing plasma cells.

Acknowledgements. We thank Dr. T.H. Hansen for the anti MR1 monoclonal antibodies 12.2 and 26.5 and Lonza for providing the plasmids for the GS system. This work was supported by grants from Spanish Ministerio de Sanidad (Ref. FIS -PI050512), Fundación Mutua Madrileña and Dirección General de Universidades e Investigación (CAM)/ Universidad Complutense de Madrid (Ref. 920631) to E. Martínez-Naves. B. Gozalbo-López was supported by a pre-doctoral fellowship from Spanish Ministerio de Educación y Ciencia. Yolanda Campos-Martín was supported by a pre-doctoral fellowship from Universidad Complutense. The fluorescence and confocal images were performed in microscopies from the Servicio Común de Investigación, Facultad de Biología, Universidad Complutense de Madrid.

References

Aldemir H. (2008). Novel MHC class I-related molecule MR1 affects MHC class I expression in 293T cells. Biochem. Biophys. Res. Commun. 366, 328-324.

Bjorkman P.J and Parham P. (1990). Structure, function, and diversity of class I major histocompatibility complex molecules. Annu. Re. Biochem. 59, 253-288.

Campos-Martin Y., Gomez del Moral M., Gozalbo-Lopez B., Suela J. and Martinez-Naves E. (2004). Expression of human CD1d molecules protects target cells from NK cell-mediated cytolysis. J. Immunol. 172, 7297-7305.

Collins S.J., Gallo R.C. and Gallagher R.E. (1977). Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature 270, 347-349.

Croxford J.L., Miyake S., Huang Y.Y., Shimamura M. and Yamamura T. (2006). Invariant V(alpha)19i T cells regulate autoimmune inflammation. Nat. Immunol. 7, 987-994.

Edwards P.A., Smith C.M., Neville A.M. and O'Hare M.J. (1982). A human-hybridoma system based on a fast-growing mutant of the ARH-77 plasma cell leukemia-derived line. Eur. J. Immunol. 12, 641-648.

Fogh J., Wright W.C. and Loveless J.D. (1977). Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J. Natl. Cancer Inst. 58, 209-214.

Hansen T.H., Huang S., Arnold P.L. and Fremont D.H. (2007). Patterns of nonclassical MHC antigen presentation. Nat. Immunol. 8, 563-568.

Hashimoto K., Hirai M. and Kurosawa Y. (1995). A gene outside the human MHC related to classical HLA class I genes. Science 269, 693-695.

Huang S., Gilfillan S., Kim S., Thompson B., Wang X., Sant A.J., Fremont D.H., Lantz O. and Hansen T.H. (2008). MR1 uses an endocytic pathaway to activate mucosal-associated invariant T cells. J. Exp. Med. 205, 1201-1211.

Huang S., Gilfillan S., Cella M., Miley M.J., Lantz O., Lybarger L., Fremont D.H. and Hansen T. H. (2005). Evidence for MR1 antigen presentation to mucosal-associated invariant T cells. J. Biol. Chem. 280, 21183-21193.

Illes Z., Shimamura M., Newcombe J., Oka N. and Yamamura T. (2004).

Accumulation of Valpha7.2-Jalpha33 invariant T cells in human autoimmune inflammatory lesions in the nervous system. Int. Immunol. 16, 223-230.

Jego G., Robillard N., Puthier D., Amiot M., Accard F., Pineau D., Harousseau J.L., Bataille R. and Pellat-Deceunynck C. (1999). Reactive plasmacytoses are expansions of plasmablasts retaining the capacity to differentiate into plasma cells. Blood 94, 701-712.

Kawachi I., Maldonado J., Strader C. and Gilfillan S. (2006). MR1-restricted V alpha 19i mucosal-associated invariant T cells are innate T cells in the gut lamina propria that provide a rapid and diverse cytokine response. J. Immunol. 176, 1618-1627.

Klein E., Klein G., Nadkarni J.S., Nadkarni J.J., Wigzell H. and Clifford P. (1968). Surface IgM-kappa specificity on a burkitt lymphoma cell in vivo and in derived culture lines. Cancer Res. 28, 1300-1310.

Lanotte M., Martin-Thouvenin V., Najman S., Balerini P., Valensi F. and Berger R. (1991). NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood 77, 1080-1086.

Lozzio C.B. and Lozzio B.B. (1975). Human chronic myelogenous leukemia cell-line with positive philadelphia chromosome. Blood 45, 321-334.

Macpherson A., Lamarre A., McCoy K., Harriman G., Odermatt B., Dougan G., Hengartner H. and Zinkernagel R.M. (2001). IgA production without μ or δ chain expression in developing B cells. Nat. Immunol. 2, 625-631.

Miley M.J., Truscott S.M., Yu Y.Y., Gilfillan S., Fremont D.H., Hansen T.H. and Lybarger L. (2003). Biochemical features of the MHC-related protein 1 consistent with an immunological function. J. Immunol. 170, 6090-6098.

Minowada J., Onuma T. and Moore G.E. (1972). Rosette-forming human lymphoid cell lines. I. establishment and evidence for origin of thymus-derived lymphocytes. J. Natl. Cancer. Inst. 49, 891-895.

Murakami H. and Masui, H. (1980). Hormonal control of human colon carcinoma cell growth in serum-free medium. Proc. Natl. Acad. Sci. USA 77, 3464-3468.

Okamoto N., Kanie O., Huang Y.Y., Fujii R., Watanabe H. and Shimamura M. (2005). Synthetic alpha-mannosyl ceramide as a potent stimulant for an NKT cell repertoire bearing the invariant Valpha19-Jalpha26 TCR alpha chain. Chem. Biol. 12, 677-683.

Parra-Cuadrado F., Gomez del Moral M., Garcia-Pavia P., Setien F. and Martinez-Naves E. (2001). Characterization of the MHC class I-related MR1 locus in nonhuman primates. Immunogenetics 53, 643-648.

Parra-Cuadrado F., Navarro P., Mirones I., Setien F., Oteo M. and Martinez-Naves, E. (2000). A study on the polymorphism of human MHC class I-related MR1 gene and identification of an MR1-like pseudogene. Tissue Antigens 56, 170-172.

Parra-Cuadrado J.F. (2002). Análisis genético y bioquímico de la molécula MR1 (MHC related 1). Thesis/Dissertation.

Ravid Z., Goldblum N. and Zaizov R., Schlesinger M., Kertes T., Minowada J., Verbi W. and Greaves M (1980). Establishment and characterization of a new leukaemic T-cell line (peer) with an unusual phenotype. Int. J. Cancer 1980. 25, 705-710.

Riegert P., Wanner V. and Bahram S. (1998). Genomics, isoforms, expression, and phylogeny of the MHC class I-related MR1 gene. J. Immunol. 161, 4066-4077.

Rodgers J.R. and Cook R.G. (2005). MHC class 1b molecules bridge innate and acquired immunity. Nat. Rev. Immunol. 5, 459-471.

Schneider U., Schwenk H.U. and Bornkamm G. (1977). Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-hodgkin lymphoma. Int. J. Cancer 19, 621-626.

Shimamura M., Huang Y.Y., Okamoto N., Suzuki N., Yasuoka J., Morita K., Nishiyama A., Amano Y. and Mishina, T. (2007a). Modulation of Valpha19 NKT cell immune responses by alpha-mannosyl ceramide derivatives consisting of a series of modified sphingosines. Eur. J. Immunol. 37, 1836-1844.

Shimamura M., Huang Y.Y., Okamoto N., Watanabe Y., Murakami Y., Kinoshista T., Hirabayashi Y., Murakata C., Ito Y. and Ogawa T. (2007b). Glycolipids with nonreducing end alpha-mannosyl residues that have the potential to activate invariant Valpha NKT cells. FEBS J. 274, 2921-2932.

Shimizu Y. and DeMars R. (1982). Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. J. Immunol. 142, 3320-3328.

Smith S.D., Shatsky M., Cohen P.S., Warnke R., Link M.P. and Glader B.E. (1984). Monoclonal antibody and enzymatic profiles of human malignant T-lymphoid cells and derived cell lines. Cancer Res. 1984 44, 5657-5660.

Smith P.D., Janoff E.N., Mosteller-Barnum M., Merger M., Orenstein J.M., Kearney J.F. and Graham M.F. (1997). Isolation and purification of CD14-negative mucosal macrophages from normal human small intestine. J. Immunol. Methods. 202, 1-11.

Smith P.D., Smythies L.E., Mosteller-Barnum M., Sibley D.A., Russell M.W., Merger M., Sellers M.T., Orenstein J.M., Shimada T., Graham M.F. and Kubagawa H. (2001). Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities. J. Immunol. 167, 2651-2656.

Sundstrom C. and Nilsson K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer 17, 565-577

Terhorst C., Parham P., Mann D.L. and Strominger J.L. (1976). Structure of HLA antigens: Amino-acid and carbohydrate compositions and NH2-terminal sequences of four antigen preparations. Proc. Natl. Acad. Sci. USA 73, 910-914.

Tilloy F., Treiner E., Park S.H., Garcia C., Lemonnier F., de la Salle H., Bendelac A., Bonneville M. and Lantz O. (1999). An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class ib-restricted alpha/beta T cell subpopulation in mammals. J. Exp. Med. 189, 1907-1921.

Treiner E., Duban L., Bahram S., Radosavljevic M., Wanner V., Tilloy F., Affaticati P., Gilfillan S. and Lantz O. (2003). Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. Nature 422, 164-169.

Walter L.. and Gunther E. (1998). Isolation and molecular characterization of the rat MR1 homologue, a non-MHC-linked class I-related gene. Immunogenetics 47,477-482.

Yamaguchi H. and Hashimoto K. (2002). Association of MR1 protein, an MHC class I-related molecule, with beta(2)-microglobulin. Biochem. Biophys. Res. Commun. 290, 722-729.

Yamaguchi H., Hirai M., Kurosawa Y., Hashimoto K. (1997). A highly conserved major histocompatibility complex class I-related gene in mammals. Biochem. Biophys. Res. Commu. 238, 697-702.

Accepted June 5, 2009