Cancer Immunol Immunother (2012) 61:2067–2077
DOI 10.1007/s00262-012-1268-7

ORIGINAL ARTICLE

Toll-like receptor 4 gene polymorphism influences dendritic cell in vitro function and clinical outcomes in vaccinated melanoma patients

Andrés Tittarelli · Fermín E. González · Cristián Pereda · Gabriela Mora · Leonel Muñoz · Carlos Saffie · Tamara García · David Díaz · Cristián Falcón · Marcela Hermoso · Mercedes N. López · Flavio Salazar-Onfray

Received: 31 January 2012 / Accepted: 11 April 2012 / Published online: 3 May 2012

Abstract Toll-like receptor 4 (TLR4) is expressed on dendritic cells (DCs), sensing environmental danger molecules that induce their activation and maturation. Recently, we reported a method for the production of therapeutic DCs against melanoma, called tumor antigen-presenting cells (TAPCells), using a heat-shocked allogeneic melanoma cell lysate (TRIMEL) as an activation factor and antigen provider. Since TRIMEL contains endogenous TLR4 ligands, we evaluated the role of TLR4 in TAPCells differentiation by antibody neutralization and the association of a *Tlr4* polymorphism (896A/G) (Asp299Gly), determined by PCR–RFLP, with the in vitro activation capacity and the clinical outcome of TAPCells-vaccinated patients. Antibody blocking of monocyte TLR4 inhibited surface expression, determined by flow cytometry, of the major histocompatibility complex class I, CCR7, CD80, CD83 and CD86 on TAPCells, reduced interleukin (IL)-6 and tumor necrosis factor -α gene expression evaluated by qRT-PCR, and also inhibited the TAPCells-mediated interferon-γ (IFN-γ) secretion of melanoma-specific $CD8^+$ T cells determined by ELISpot ($p < 0.01$). Moreover, $CD8^+$ T-cell activation capacity was significantly reduced in TAPCells bearing the TLR4 Asp299Gly receptor ($p < 0.05$). Finally, TAPCells-vaccinated stage-IV melanoma patients bearing the *Tlr4* 896G allele showed a shortened post-therapy median survival rate compared with those carrying the *Tlr4* 896A allele ($p < 0.05$; log-rank test). Our results indicate that TLR4 is a key receptor for the tumor lysate-mediated in vitro generation of clinically efficient antigen-presenting cells. Further analysis of patients included in different vaccine protocols is necessary for definitively establishing a role for TLR4 polymorphism in clinical responses.

Keywords TLR4 · Dendritic cells · Immunotherapy · Melanoma

Electronic supplementary material The online version of this article (doi:10.1007/s00262-012-1268-7) contains supplementary material, which is available to authorized users.

A. Tittarelli · F. E. González · C. Pereda · G. Mora · L. Muñoz · C. Saffie · T. García · D. Díaz · C. Falcón · M. Hermoso · M. N. López · F. Salazar-Onfray (✉)
Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, 8380453 Santiago Chile, Chile
e-mail: fsalazar@med.uchile.cl

A. Tittarelli
e-mail: tittarelli@gmail.com

A. Tittarelli · F. E. González · C. Pereda · G. Mora · T. García · C. Falcón · M. N. López · F. Salazar-Onfray
Millennium Institute on Immunology and Immunotherapy, University of Chile, 8380453 Santiago, Chile

F. E. González
Department of Conservative Dentistry, Faculty of Dentistry, University of Chile, 8380492 Santiago, Chile

M. N. López
Research Support Office, University of Chile Clinical Hospital, 8380453 Santiago, Chile

Introduction

Dendritic cells (DCs) are professional antigen (Ag)-presenting cells (APCs) with an exceptional ability to internalize different proteins that are subsequently processed and presented to T cells within major histocompatibility complex (MHC) class I and class II molecules during their maturation process [1]. The events associated with DCs maturation and migration are partly the result of toll-like receptors (TLRs) activation, whose synthesis is regulated

by the environment in which these cells reside [2, 3]. To date, ten different kinds of TLRs have been described in humans capable of specifically recognizing different pathogen-associated molecular patterns (PAMPs) or endogenous danger-/damage-associated molecular patterns (DAMPs) [4]. These receptors mediate the interaction between the immune system cells and pathogens or stressed endogenous cells, and have thus a central role in the innate immune response [2]. Also, the signals mediated by different TLRs have a crucial impact on the induction and regulation of effective adaptive immune responses against pathogens and tumor cells [5].

With the purpose of improving immunotherapy against advanced cancer, emphasis has been placed on an efficient vaccine design to induce optimal DC maturation/activation, better Ag presentation and increased immunogenicity [6]. Recent progress in achieving the above involves the clinical development of TLR agonists [7]. For example, TLR7 agonist imiquimod and the attenuated *Mycobacterium bovis* preparation of bacillus of the Calmette-Guerin strain (that stimulates TLR2, TLR4 and TLR9) are approved for clinical use for the treatment of superficial basal cell carcinoma and bladder cancer, respectively [8, 9].

Human monocyte-derived DCs predominantly express TLR2 and TLR4 at the cell surface [10]. Moreover, it has been shown that the expression of TLR4 by DCs is very important for efficient tumor-associated Ag presentation from dying/stressed cancer cells. At this respect, heat shock proteins (HSPs) exposed by stressed tumor cells are recognized by TLR4 expressed on DCs, which facilitates intracellular Ag processing and presentation [11]. In addition, the pro-inflammatory factor high mobility group box 1 (HMGB1), released during tumor cell death, interacts with TLR4 on DCs promoting the tumor-derived Ags processing and presentation [12]. Moreover, ligation of TLR4 by HMGB1 inhibits the fusion of phagosomes with lysosomes, preventing the degradation of tumor Ags and facilitating their trafficking to the dedicated Ag cross-presenting compartment [12].

The *Tlr4* gene is highly polymorphic, and to date, 15 polymorphisms in their coding sequence have been described [13]. *Tlr4* has two main co-segregating non-synonymous single nucleotide polymorphisms (SNPs), A896G (Asp299Gly) and C1196T (Thr399Ile), which are found in 8–10 % of Caucasians populations [14]. Genetic studies showed that in Chilean population the Asp299Gly polymorphism is present in similar frequency than in Caucasian ones, with an allele frequency of 4.6 % [15]. The Asp299Gly SNP is located at the TLR4 extracellular domain and was found to alter the ability of the host to respond to environmental stresses like inhaled lipopolysaccharide (LPS) [16] and respiratory syncytial virus infection [17]. Importantly, it has been reported that patients with breast cancer, who carry at least one TLR4 loss-of-function allele, relapse more quickly after radiotherapy and chemotherapy than those carrying two wild-type TLR4 alleles [12]. Same authors demonstrated that TLR4 Asp299Gly SNP reduces the interaction between TLR4 and the endogenous danger signal HMGB1, resulting in reduced capacity of DCs to cross-present melanoma cell Ags to specific cytotoxic T cells [12]. Moreover, TLR4 polymorphisms have been linked with an increased susceptibility for gastric cancer and gallbladder cancer [18, 19]. Notably, patients with head and neck squamous cell carcinomas carrying the TLR4 Asp299 wild-type genotype showed significantly better disease-free survival than patients with the TLR4 Gly299 allele treated with adjuvant systemic therapies, including agents such as cisplatin and 5-fluoruracil [20].

In the last 10 years, we have developed a DC-based immunization therapeutic approach that improves the long-term survival in patients with advanced melanoma [22–24]. In our approach, a lysate derived from heat-conditioned allogeneic melanoma cells, named TRIMEL, has been used as a tumor-associated Ags source, providing a unique strategy to obtain efficient tumor Ag-presenting cells with a mature DC-like phenotype (named TAPCells) [24]. Particularly, previously we proved that TRIMEL contain some DAMPs, such as the endogenous TLR4-ligand HMGB1, that mediate an optimal APC maturation and Ag cross-presentation [24]. For that reason, we investigated whether TLR4 Asp299Gly SNP would be important for the individual risk assessment of TAPCells-vaccinated melanoma patients.

In our current study, we demonstrated that TLR4 at the monocyte surface mediates some of TRIMEL effect on TAPCells maturation process and that the *Tlr4* polymorphism Asp299Gly negatively affects the outcome of TAPCells-based immunotherapy in metastatic melanoma patients. Our results indicate that TLR4 is a key receptor for the tumor lysate-mediated in vitro generation of clinically efficient DCs.

Materials and methods

Patients

This work is a retrospective clinical study, using clinical data from 72 melanoma patients vaccinated with TAPCells and followed up from November 2000 until October 2011, in accordance with the described protocols [23, 24]. The study was performed in agreement with the Helsinki Declaration and approved by the Bioethical Committee for Human Research of the Faculty of Medicine, University of Chile. All patients signed a letter of informed consent.

Cell lines and TAPCells generation

THP-1 monocytic/macrophagic cells were purchased from the American Type Culture Collection (ATCC); Mel1, Mel2 and Mel3 melanoma cell lines were established from samples of metastatic lymph nodes at the Institute of Biomedical Sciences, University of Chile. The cells were cultured at 37 °C under 5 % CO_2 in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin (all from Invitrogen). TRIMEL is a cell lysate derived from the allogeneic melanoma cell lines (Mel1, Mel2 and Mel3), prepared as described [22–24]. Briefly, each cell line was heat shock (HS)-treated at 42 °C for 1 h and then incubated for 2 h at 37 °C. Cells obtained were mixed in equal amounts and lysed through repeated freeze–thaw cycles in liquid nitrogen. Thereafter, the cell lysate was sonicated and irradiated with a 60-Gy dose. The protein concentration was estimated by Bradford's method using a biophotometer (Eppendorf). We demonstrated before that TRIMEL allows the optimal delivery of a wide-ranging pool of Ags (Table S1), coupled with the presence of factors promoting TAPCells differentiation, maturation and Ag cross-presentation to CTLs [24]. In particular, HS triggers the induction of calreticulin (CRT) and HMGB1, both of which act as danger signals, mediating an optimal Ag-presenting cell maturation and Ag cross-presentation [24].

Adherent monocytes isolated from peripheral blood mononuclear cells (PBMC) of melanoma patients or healthy donors were cultured in serum-free AIM-V medium (Invitrogen), with recombinant human interleukin 4 (rhIL-4: 500 U/mL; Miltenyi), and recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF: 800 U/mL; Shering Plough) for 22 h (activated monocytes, AM) and then stimulated or not for 24 h with TRIMEL (100 μg/mL). In some experiments, AM were additionally incubated with anti-human TLR4-IgA2 neutralizing monoclonal antibody (mAb) or an isotype control (2 μg/mL; both from Invivogen; clone numbers W7C11 and T9C6, respectively) 1 h before TRIMEL stimulation.

Immunofluorescence and confocal microscopy

THP-1 cells were incubated with TRIMEL for 2 h, washed twice in PBS and fixed in 3 % paraformaldehyde for 30 min. Cells were then incubated for 90 min at room temperature with the primary antibodies, washed 5 times with PBS-3 % bovine serum albumin and exposed to secondary Abs for 90 min. The primary Abs were mouse mAb anti-TLR4 (IgA2), clone W7C11 (Invivogen) and polyclonal-Ab anti-HMGB1 (Sigma-Aldrich, catalog number H9539). Secondary antibodies were Alexa Fluor® 488-conjugated goat anti-mouse and Alexa Fluor® 546-conjugated donkey anti-rabbit (Invitrogen, catalog numbers A-10667 and A-11010, respectively). Cells were mounted in cover slips using FluorSave reagent (Calbiochem).

Confocal images were collected using a Carl Zeiss LSM700 laser-scanning confocal microscope with a 63× (numerical aperture 1.4) oil immersion objective (Zeiss Plan-Apochromat). Single confocal sections of 0.7 μm were taken parallel to the cover slip (xy sections). Images were acquired and processed with the Zeiss Zen 2009 image software. Final image was compiled with Adobe Photoshop 8.0.1 software.

Co-localization analysis

Manders's co-localization coefficients [25] were calculated for HMGB1/TLR4 using NIH ImageJ software with the co-localization analysis plug-in JACoP. Manders's co-localization coefficient calculates the spatial overlap of two proteins, with M1 representing the percentage of HMGB1 pixels (red channel) that overlaps pixels in the green channel (TLR4), and conversely for M2. M1 and M2 values range from 0 to 1, with 0 corresponding to non-overlapping images and the latter reflecting 100 % co-localization between both images. Manders's coefficients are not influenced by differences in absolute signal intensities in each channel because pixel intensity in a particular channel is normalized to total pixel intensity across the image for that label. Values were reported as mean ± SD.

Tlr4 genotype determination

Total DNA was isolated from PBMC obtained from melanoma patients using the Chomczynski method [26]. *Tlr4* Asp299Gly (896 A/G) polymorphism was genotyped using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis as described [27]. The 249-bp amplicon was digested with *NcoI* restriction enzyme (1U; New England BioLabs) overnight at 37 °C. After digestion, the A allele consisted of one fragment of 249 bp and the G allele consisted of 223 and 26 bp fragments.

Flow cytometry

The following mAbs were used for cell staining: anti-HLA-ABC (clone W6.32), HLA-DR (clone L243), CD80 (clone 2D10.4), CD83 (clone HB15e), CD11c (clone 3.9), CD86 (clone IT2.2), CD40 (clone 5C3) and CCR7 (clone 3D12; all from eBioscience). Samples were acquired on a FACSCalibur (BD Biosciences) and analyzed using WinMDI 2.8 software.

Quantitative real-time PCR

Total RNA was isolated using TRIzol® reagent as recommended by the supplier (Invitrogen) and quantified in a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies). cDNAs were synthesized from 1 μg of total RNA with the Transcriptor First Strand cDNA kit and random primers according to the manufacturer's instructions (Roche). Quantitative real-time PCR was performed using the FastStart Taqman Probe Master (Roche) with 1 μg of cDNA template in the ABI PRISM 7900 Sequence Detector System (Applied Biosystems), according to the manufacturer's instructions. The primers used are shown in Table S2. Gene expression levels were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$) normalized to the mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

ELISpot assay

Tumor infiltrating lymphocytes (TILs), mainly $CD8^+$ $CD45RO^+$ T cells and highly responsive to a HLA-A2-restricted Mart-$1_{27–35}$ peptide (Fig. S1), were obtained as previously described [28]. TRIMEL or a non-HS melanoma cell lysate, with or without a conditioned supernatant (derived from HS-treated melanoma cells), were used to stimulate for 24 h AM from HLA-$A2^+$ healthy donors or melanoma patients carrying a TLR4 Asp299Asp or Gly allele. HMGB1 from the conditioned supernatants was blocked adding 10 μg of a polyclonal-Ab anti-HMGB1 (Sigma-Aldrich) 1 h before use. Specific Abs against TLR4 (2 μg/mL; Invivogen) were used to block TLR4 on AM 1 h before TRIMEL/Melanoma lysate stimulation. TILs (5×10^4) were co-cultured for 16 h with the different HLA-$A2^+$ APCs or AM at 5:1 TIL/APC (or AM) ratio. Interferon-gamma (IFN-γ) release was tested by the enzyme-linked immunosorbent spot (ELISpot) assay according to the manufacturer's instructions (Mabtech).

Statistics

Statistical analysis was achieved using GraphPad PRISM 5.03 and Stata 7.0 (Stata Corp, College Station, TX, USA) software. Differences between treatments were tested by applying Student's *t* test and also by Dunn's multiple comparison tests. Results are presented as mean ± SD. Survival curves estimations were calculated by applying the Kaplan–Meier method and log-rank test. For subgroup analysis, the chi-square test was used to compare proportions of categorical variables, unless the frequency of expected events was fewer than 5, in which case, Fisher's exact test was used.

Results

TLR4 is involved in TRIMEL-mediated differentiation of activated monocytes into DC-like cells with a mature phenotype

Recently, we demonstrated the effectiveness of TAPCells immunization for improving long-term survival in patients with metastatic melanoma [22–24]. Furthermore, we demonstrated that HMGB1, a tumor-associated DAMP, present in the heat-conditioned melanoma cell lysate TRIMEL, mediates the optimal APC activation [24]. Here we observed that TLR4 from human monocyte cells (THP-1 cell line) co-localizes with HMGB1 present in the TRIMEL (Fig. 1a). TLR4 displayed 78.4 ± 13 % co-localization with HMGB1 in TRIMEL-incubated THP-1 cells and only 30 ± 17 % in control condition (Fig. 1b). To determine whether TLR4 is involved in TRIMEL-mediated differentiation/activation of AM into TAPCells, we stimulated AM, pre-incubated with neutralizing anti-TLR4 mAbs or an isotype control, with TRIMEL lysate. TLR4 blockade attenuated TRIMEL-mediated induction of MHC-I, CD83, CD80, CD86 and CCR7 on TAPCells (Fig. 1c, d). As expected, the anti-TLR4 neutralizing mAbs used specifically inhibited LPS-mediated maturation markers induction in AM, whereas it did not have any effect on the induction of maturation markers in AM stimulated with the TLR2 agonist Pam3Cys (Fig. S2). Moreover, TLR4 blockade significantly inhibited the TRIMEL-induced gene expression of the pro-inflammatory cytokines IL-6 and TNF-α on TAPCells (Fig. 1e). Together, these results suggest that some DAMPs present in TRIMEL, such as HMGB1, can induce the activation and differentiation of AM into mature DC-like APCs through the ligation of TLR4.

Tlr4 Asp299Gly polymorphism decreases the TAPCells' capacity to activate melanoma-specific T cells in vitro

A SNP in *Tlr4* (896A/G, Asp299Gly, rs4986790) affecting the extracellular domain of TLR4 is associated with a reduction in endotoxin response [17], deficient LPS signaling [29] and reduced HMGB1 binding [12]. The defective binding of HMGB1 to the mutated TLR4 was associated with an impaired capacity of monocyte-derived DCs to cross-present melanoma Ags to cytotoxic T lymphocytes (CTL) [12], a fundamental requirement for clinically effective DC-based tumor vaccines [6]. We addressed the possibility that the TLR4 Asp299Gly polymorphic protein at the AM surface could affect the TRIMEL-mediated TAPCells differentiation and activation. For that purpose, 72 melanoma patients included in a phase

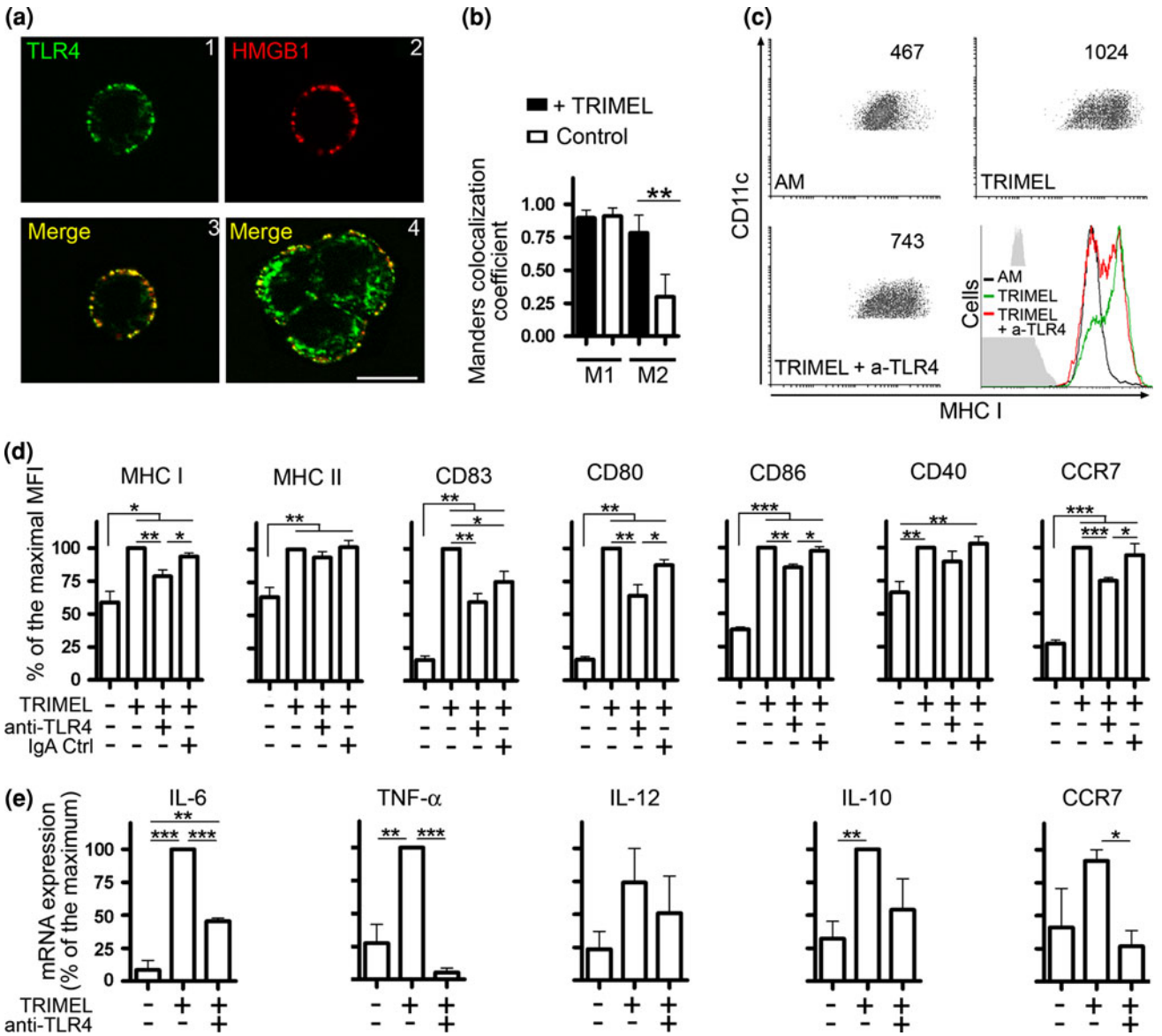

Fig. 1 Role of TLR4 in the TRIMEL-mediated differentiation of TAPCells. **a** HMGB1 from TRIMEL co-localizes with monocyte TLR4. THP-1 cells were incubated (1–3) or not (4) with TRIMEL for 2 h, washed, fixed and then incubated with a rabbit polyclonal antibody (pAb) anti-HMGB1 and a mouse monoclonal Ab (mAb) anti-TLR4. The TLR4 (1) and HMGB1 (2) signals were detected using the Alexa Fluor® 488-conjugated goat anti-mouse and the Alexa Fluor® 546-conjugated donkey anti-rabbit secondary Abs, respectively. Scale bar, 10 μm. **b** The co-localization coefficients for HMGB1 (M1 channel) and for TLR4 (M2 channel) were calculated using Manders's automatic threshold determination. Manders's coefficients range from 0 (no overlap) to 1 (complete overlap). *Values* appear indicated as mean ± SD of at least 20 different cells per condition. **c**, **d** Surface markers expression was evaluated by flow cytometry in activated monocytes (AM) stimulated or not with TRIMEL for 24 h. Specific Abs against TLR4 were used to block TLR4 on AM 1 h before TRIMEL stimulation. **c** Representative *dot-plots* and the histogram show the analysis for MHC-I expression. Numbers in each *dot-plot* are mean fluorescence intensities (MFI) values. The *gray* histogram corresponds to unstained cells. **d** The data show the average percentage of the MFI from $CD11c^+$ gated cells relative to TRIMEL-stimulated AM. **e** Expression levels of mRNA of IL-6, TNF-α, IL-12, IL-10 and CCR7 were evaluated by qRT-PCR in AM (blocked or not for TLR4) stimulated or not with TRIMEL for 24 h. The mRNA levels were normalized to the endogenous level of GAPDH, and the data are showed as percentage of the maximum (TRIMEL-stimulated AM). **d**, **e** The data represent the average ± SD of at least 3 independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

II clinical trial [23, 24] were genotyped for the 896A/G polymorphism by PCR–RFLP analysis (Fig. 2a). At this respect, the *Tlr4* 896G allele was found in 9 out of 72 melanoma patients (12.5 %)—eight of them were heterozygotes and one homozygote—corresponding to an allelic frequency of 7.46 %. The analyzed cohort included 57

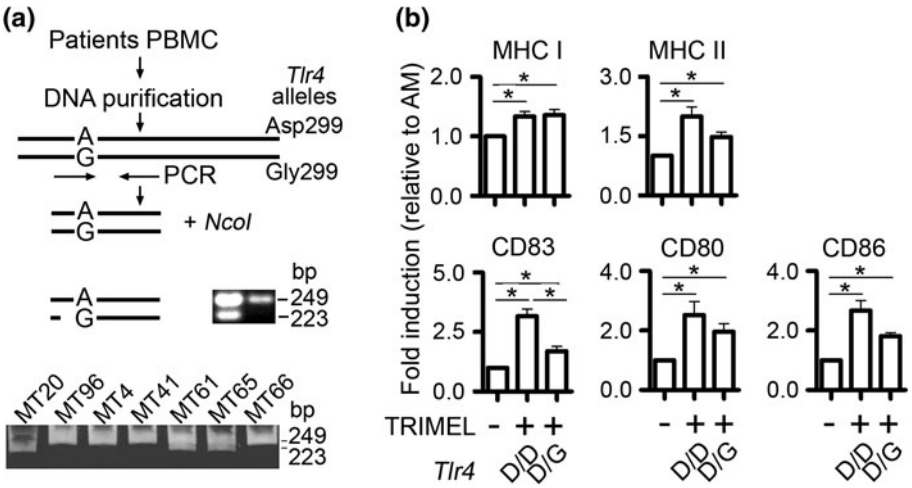

Fig. 2 Association of phenotype of melanoma patient's TAPCells with the Tlr4 Asp299Gly polymorphism. **a** Strategy for the detection of the *Tlr4* polymorphism Asp299Gly (896A/G) by PCR–RFLP in melanoma patients treated with TAPCells. Representative results are shown for 7 tested melanoma patients. Patients MT61 and MT65 show heterozygosis and patient MT20 homozygosis for the 896G allele. **b** Surface markers expression was evaluated by flow cytometry in activated monocytes (AM) stimulated or not with TRIMEL for 24 h. AM were differentiated from monocytes of melanoma patients with or without the *Tlr4* Asp299Gly polymorphism (D/G and D/D, respectively). The data represent the average increase in the mean fluorescence intensities (MFI) from $CD11c^+$ gated cells relative to AM. Data represent at least 3 patients' samples for each genotype. $^*p < 0.05$

stage-IV, 13 stage-III and 2 stage-IB high-risk melanoma patients (Table 1).

AM derived from patients bearing the different *Tlr4* genotypes were ex vivo treated with TRIMEL for inducing differentiation into TAPCells. Our results showed that CD83 expression was significantly lower on TAPCells carrying the mutated TLR4 Asp299Gly compared with TAPCells bearing the normal TLR4 Asp299Asp (Fig. 2b).

Furthermore, the involvement of TLR4 in TRIMEL-mediated increased APC capacity to functionally activate melanoma-specific T cells was assessed. Firstly, we observed that TAPCells from normal (D/D) melanoma patients induced the activation of a HLA-A2-restricted Mart-$1_{27–35}$ specific TIL line in a TLR4-dependent manner, inducing the IFN-γ release by the CTL line (Fig. 3a). In contrast, TAPCells from melanoma patients bearing the *Tlr4* D/G genotype induced a significantly lower amount of IFN-γ release by TILs (Fig. 3a). Secondly, we confirmed that a supernatant from HS-treated melanoma cells (Mel1 + Mel2 + Mel3) improves the capacity of TAP-Cells to activate the CTL line when added to non-HS-treated melanoma lysate, as previously described [24]. This effect was blocked when supernatants were pretreated with anti-HMGB1 Abs or, to the same extent, when AM were pre-incubated with anti-TLR4 neutralizing Abs (Fig. 3b). These results suggest that TAPCells expressing the TLR4 Asp299Gly show a reduced capacity to be activated by TRIMEL and consequently show a limited capacity to activate melanoma-specific T cells response.

Tlr4 Asp299Gly polymorphism is associated with a reduced TAPCells clinical efficacy in stage-IV melanoma patients

To evaluate the impact of the *Tlr4* 896G allele presence in the clinical efficacy of TAPCells, we analyzed the clinical outcome of 53 stage-IV patients that completed the immunotherapy protocol (Table S3). In this regard, 8 out of 53 stage-IV melanoma patients (15.1 %) carried the mutated *Tlr4* 896G allele. The median follow-up period was 48 months (range, 18–132 months), and the overall median survival of the stage-IV patients was 16 months. In line with our previous reports, the majority of treated patients (54.7 %; 29 out of 53) showed a TRIMEL-specific DTH response after the vaccination protocol. A similar response proportion was detected in the *Tlr4* 896A group of patients (57.8 %; 26 out of 45). Only 3 out of 8 patients (37.5 %) bearing the *Tlr4* 896G allele developed a DTH response (Table S3). No statistically significant differences between the groups were detected when additional demographic parameters, such as gender, mean age, disease stage or previous treatment, were analyzed (Table 1 and S4).

Finally, we separately analyzed the overall survival of *Tlr4* 896A/G patients. Our results showed that stage-IV melanoma patients bearing the *Tlr4* 896G allele showed a tendency to shortened post-therapy median survival than those carrying the *Tlr4* 896A allele (12 and 21 months, respectively), although this finding was not statistically

Table 1 Demographic characteristics of patients included in the study

	D/D patients 63 (100 %)	D/G, G/G patients 9 (100 %)	*p* value (D/D vs. D/G)
Gender-no. (%)			
Male	31 (49.2 %)	4 (44.4 %)	nd
Female	32 (50.8 %)	5 (55.6 %)	
Mean age at enter			
Years ± SD (range)	50.4 ± 14.2 (19–83)	47.3 ± 20.3 (20–81)	
Stage[e]			
IB	2 (3.2 %)	0	
IIIA	3 (4.7 %)	1 (11.1 %)	
IIIB	3 (4.7 %)	0	
IIIC	6 (9.5 %)	0	
IV	49 (77.9 %)	8 (88.9 %)	
Immunological response (DTH)-no. (%)			
+	37 (58.7 %)	4 (44.4 %)	
–	20 (31.7 %)	5 (55.6 %)	
nd	6 (9.6 %)	0	
Stage-IV patients (completed treatment)	**D/D patients 45 (100 %)**	**D/G or G/G patients 8 (100 %)**	
Gender-no. (%)			
Male	23 (51.1 %)	4 (50 %)	0.581[a]
Female	22 (48.9 %)	4 (50 %)	
Mean age			
Years ± SD (range)	51.1 ± 14.9 (19–83)	50 ± 20 (20–81)	0.421[b]
M stage-no. (%)			
M1a	14 (31.1 %)	2 (25 %)	0.796[c]
M1b	12 (26.7 %)	3 (37.5 %)	
M1c	19 (42.2 %)	3 (37.5 %)	
Median follow-up			
Months	46	57	0.332[d]
Median post-therapy survival			
Months (range)	21 (3–88)	12 (6–58)	0.175[d]
+	26 (57.8 %)	3 (37.5 %)	
Immunological response (DTH)-no. (%)			
–	17 (37.8 %)	5 (62.5 %)	0.207[a]
nd	2 (4.4 %)	0	

SD standard deviation, *M* Metastasis, *DTH* delayed type IV hypersensitivity, M1a, transit skin; M1b, lung; M1c, other organs; *no* number, *nd* not determined, *yr* year

[a] Fischer test

[b] *t* test

[c] Chi-square test

[d] log rank

[e] According to the American Joint Committee on Cancer (AJCC) criteria

significant ($p = 0.175$) (Fig. 4a; Table 1). Then, we excluded patients with short post-therapy survival (less than 6 months) from the analysis, because they were probably outside the therapeutic range of the treatment. Our results show that patients bearing the *Tlr4* 896G allele had a significantly lower post-therapy median survival than those with the normal *Tlr4* 896A allele (12 *vs.* 28 months, $p = 0.026$; Fig. 4b and Table S4), suggesting that TLR4 SNP affects the immunological responses in vaccinated melanoma patients.

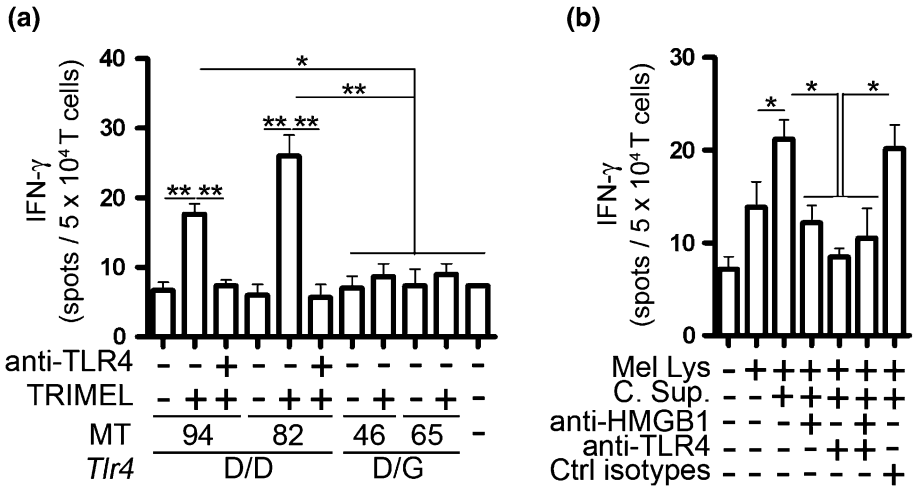

Fig. 3 Association of T-cell activation capacity of melanoma patients' TAPCells with the *Tlr4* Asp299Gly polymorphism. **a** The HLA-A2-restricted Mart-$1_{27–35}$-tumor infiltrating lymphocyte line was co-cultured with activated monocytes (AM) stimulated or not with TRIMEL, derived from monocytes from HLA-$A2^+$ melanoma patients with the D/D or D/G *Tlr4* genotype (MT94, MT82 or MT46, MT65, respectively). The AM were pre-incubated or not with anti-TLR4 Abs 1 h before TRIMEL stimulation. IFN-γ release was measured by ELISpot. **b** HLA-$A2^+$ AM were incubated for 24 h with a non-heat shock (HS)–treated melanoma cell lysate (Mel Lys) in the presence or absence of a conditioned supernatant derived from HS-treated melanoma cells (C. Sup.), pre-incubated or not with an anti-HMGB1 polyclonal antibody. Specific Abs against TLR4 were used to block TLR4 on AM 1 h before TRIMEL stimulation. Treated antigen presenting cells were co-cultured for 16 h with the HLA-A2-restricted Mart-$1_{27–35}$-tumor infiltrating lymphocyte line, and IFN-γ release was measured by ELISpot. Data represent the average ± SD of 3 different healthy donors. $^{**}p < 0.01$; $^{*}p < 0.05$

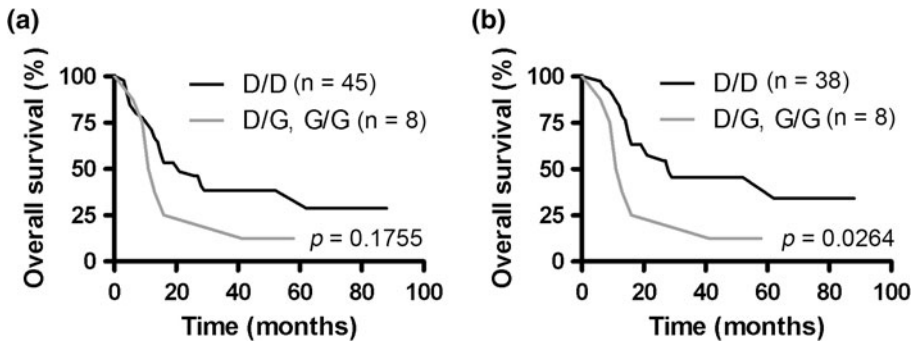

Fig. 4 Kaplan–Meier post-immunotherapy survival estimation of stage-IV melanoma patients by Tlr4 genotype. **a** Overall survival of stage-IV melanoma patients after immunotherapy with autologous TAPCells (inclusion criteria: complete treatment; $n = 53$), grouped according to their *Tlr4* genotype. **b** Overall survival of stage-IV melanoma patients after immunotherapy with autologous TAPCells (inclusion criteria: complete treatment + post-therapy survival time ≥6 months; $n = 46$)

Discussion

Despite the high expectations for DC-based vaccination trials, until now response rates to immunotherapy vary among cancer patients, leading to low clinical objective responses that have caused disappointment in the medical and scientific community [6, 30]. Inter-patient variations in the efficacy of antitumor immunotherapy can be explained by tumor escape mechanisms, such as Ag loss and defects in MHC expression, as well as differences in the tumor and the immune system-associated genes [31–33]. In our present study, we analyzed the role of TLR4 in the phenotypic and functional modulation of therapeutic APCs and we also evaluated its impact on the immune and clinical outcome of melanoma patients vaccinated with TAPCells.

Recently, we demonstrated that TRIMEL, which is derived from three HS-allogeneic melanoma cell lines, has the capacity to differentiate AM to APCs with a committed mature DC-like phenotype and a high-capability to activate $CD8^+$ and $CD4^+$ melanoma-specific T cells [24]. Moreover, we demonstrated that TRIMEL activates the NF-κB pathway inducing the surface expression of molecules associated with Ag presentation, co-stimulation of *naïve* T cells and natural killer cells, and lymph node migration activity. Additionally, we proved that TRIMEL induces the release of Th1-polarizing and pro-inflammatory cytokines

such as IL-6, TNF-α and IL-12 [24]. In the present study, we showed that TLR4 is involved in TRIMEL-mediated phenotypic and functional monocyte differentiation to mature DC-like cells associated with increased surface marker expression and cytokine gene expression (Fig. 1). The ability of TLR4 to induce gene expression and/or protein secretion of pro-inflammatory cytokines was previously reported in response to different PAMPs/DAMPs. Such examples include murine DCs in response to heparin-binding hemagglutinin from *Mycobacterium tuberculosis* [34], murine macrophages and DCs in response to peroxiredoxin 1 [35], and in human and murine macrophages in response to HMGB1 [36, 37].

Genetic polymorphisms (mainly SNPs) are common variants within a population that are found at a frequency of over 1 %. SNPs may alter the amino acid sequence (non-synonymous SNPs), the promoter function, or can be completely "silent." Within the genes involved in the immune recognition, several distinct SNPs have been described, including pattern recognition molecules and cytokines [38, 39]. Besides increasing the susceptibility to infectious disease [13], SNPs of immune genes can affect the antitumor immune responses induced by chemotherapy [12] or immunotherapy [40, 41]. For example, lymphoma patients with the FcγRIIIa V/F and F/F genotypes have lower progression-free survival rate after idiotype vaccination than patients with the wild-type genotype [40]; the presence of a deletion mutation in the chemokine receptor 5 gene (CCR5Δ32) in patients with stage-IV melanoma results in a decreased survival following different types of immunotherapies [41].

TLR4 is a signal-transducing receptor for structurally diverse microbial and endogenous molecules, such as the Gram-negative enterobacterial LPS, fungal mannans, heat shock proteins and HMGB1 [38]. Importantly, Apetoh and co-workers demonstrated that the TLR4 SNP Asp299Gly affects the binding of HMGB1 to the extracellular domain of the receptor, compromising the DC-mediated cross-presentation and decreasing the response to anthracyclines in breast cancer patients bearing the mutant allele [12].

Due to the biological impact of TRIMEL-HMGB1 on the functional phenotype of our therapeutic DCs [24], we speculate that the identification of TLR4 Asp299Gly SNP can be important for the individual risk assessment of TAPCells-vaccinated melanoma patients [23, 24]. *Tlr4* 896A/G genotypes were detected by PCR–RFLP in a cohort of 72 melanoma patients treated with TAPCells (Table 1 and S3). The *Tlr4* 896G allele was found in 12.5 % of the melanoma patients included in the analysis, 8 heterozygotes and one homozygote, corresponding to an allelic frequency of 7.46 %, slightly higher than the one observed in other study involving Chilean population [15]. We then analyzed the impact of the TLR4 Asp299Gly polymorphic protein expression in the capacity of TRIMEL to induce maturation markers on TAPCells in vitro. Our results show that the presence of the mutant allele generates TAPCells with lower levels of maturation markers than cells with the normal allele (Fig. 2b), particularly for the molecule CD83. Recently, it has been elucidated that the TLR4-signaling induces CD83 mRNA and a post-translational pathway mediated by CD83 that promotes MHC-II and CD86 expression on DCs [42]. The impact of the TLR4 and the Asp299Gly SNP in the TRIMEL-mediated phenotypic maturation of TAPCells was perfectly correlated with a null capability to activate melanoma-specific TILs (Fig. 3a and b). These results that suggest a role for TLR4 in the mediation of cross-presentation agree fairly well with those of Apetoh and co-workers, who observed that monocyte-derived DCs from individuals bearing the *Tlr4* 896G allele did not cross-present oxaliplatin dying melanoma cells-derived Mart1 Ag to CTL clones in an HMGB1-dependent manner [12].

The induction of a specific CTL response is crucial for tumor rejection, the control of metastatic dissemination and an effective clinical response. Thus, we analyzed the clinical outcome of stage-IV melanoma patients bearing or not the mutant allele of TLR4. Of the 53 stage-IV patients who completed the TAPCells vaccination protocol [23], 8 (15.1 %) carried the mutated *Tlr4* 869G allele and the group did not differ from patients displaying the normal *Tlr4* allele for gender, mean age, metastasis stage or previous treatment (Table 1, S3 and S4). However, we observed a non-significant, but clear trend toward lower post-immunotherapy survival in patients bearing the *Tlr4* 869G allele compared to those with the more frequent gene. Interestingly, we observed that patients bearing the *Tlr4* 896G allele showed a significantly lower post-therapy median survival than those with the normal *Tlr4* 896A allele, when we excluded patients with short post-therapy survival (less than 6 months) from the analysis, assuming that they were probably outside the therapeutic range of the treatment (Fig. 4). The mechanism of cancer vaccines, based on inducing CTLs, infiltrating tumors, and exerting T cell-mediated cytotoxic effects, is quite different from that of cytotoxic chemotherapy. In fact, cancer vaccines do not work as quickly as the direct cytotoxic effect of chemotherapy and can take up to several months to exert an immunological effect and impact on the clinical outcome [43], so a very short post-therapy survival probably indicates a lack of clinical effect, which is independent of the patient's genetic background.

The reduced median of survival observed in patients bearing the *Tlr4* 896G allele suggests that TLR4 SNP can affect the immunological responses induced by tumor lysate-loaded DC (TAPCells). Previously, we demonstrated that TAPCells immunotherapy induces an immunological

response against TRIMEL Ags, measured by DTH reaction, in approximately 60 % of the patients [23, 24]. TRIMEL-specific DTH is associated with an in vivo $CD4^+/CD8^+$ memory T-cell accumulation at the reaction site, together with reduced rates of progression and prolonged patient survival, thus linking ex vivo events with clinical antitumor responses [23, 24]. According to the results obtained in this work, short post-therapy survival in some patients may be related to the presence of the *Tlr4* 869G allele. This can be a consequence of a poor ex vivo differentiation of TAPCells, or a deficient specific in vivo immune response induction by the vaccine, rather than a generalized immune dysfunction, since 4 out of 9 patients bearing the *Tlr4* 896G allele developed a DTH response (Table 1).

Our results were in line with those obtained in a German study that includes a population with head and neck squamous cell carcinomas, there patients carrying the *Tlr4* Asp299 wild-type genotype showed significantly better disease-free survival than patients with the *Tlr4* Gly299 allele treated with adjuvant systemic therapies [20]. Moreover, it is also demonstrated that colon cancer patients carrying the minor allele of *Tlr4* (Gly299) exhibited worse progression-free survival and overall survival after treatment with chemotherapy [44]. Despite those observations, another study involving 763 melanoma patients showed that patients carrying the *Tlr4* Gly299 allele have a prolonged survival after first metastasis regionally spread, than patients carrying the *Tlr4* Asp299 wild-type genotype [21]. However, this study have some critical differences with our present work, as almost all included patients were in early stages of the disease (81.4 % in stages-0, I and II; 14.7 % in stage III), and only the minority were treated with immunotherapy.

Here, we demonstrated that TLR4 polymorphisms might impact the therapy outcome; however, our study has clear limitations due to the small sample size. Further analysis of patients included under different vaccine protocols and the inclusion of genotype information in prospectively planned randomized controlled trials in various populations are necessary in order to definitively establish a role of TLR4 polymorphism in clinical response. Other factors such as the genetic background and activation status of the immune system, the progression stage of the disease, tumor characteristics and micro-environmental conditions are possibly important elements that may impact patient post-therapy survival.

Finally, our observations may result in benefit for the melanoma patient when the immune defect induced by deficient TLR4 signaling can be clinically overcome by combining immunotherapy with another treatment regimen, such as chemotherapy, radiation or gene therapy. Preclinical models indicate the importance of a complex integrated immune response and more standard strategies in eliminating established tumors validating the exploration of combinatorial treatments, which are anticipated to be far more effective than monotherapies [45].

Acknowledgments This study was supported by grants from the National Fund for Scientific and Technological Development (FONDECYT 1090238, and 1090243), the Fund for the Promotion of Scientific and Technological Development (FONDEF DO5I10366), and the Millennium Science Initiative from the Ministry for the Economy, Development and Tourism (P09/016-F).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Steinman RM, Banchereau J (2007) Taking dendritic cells into medicine. Nature 449:419–426
2. Pasare C, Medzhitov R (2005) Toll-like receptors: linking innate and adaptive immunity. Adv Exp Med Biol 560:11–18
3. Nembrini C, Abel B, Kopf M, Marsland BJ (2006) Strong TCR signaling, TLR ligands, and cytokines redundancies ensure robust development of type 1 effector T cells. J Immunol 176:7180–7188
4. Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune response. Nat Immunol 5:987–995
5. Schreibelt G, Tel J, Sliepen KH, Benitez-Ribas D, Figdor CG, Adema GJ, de Vries IJ (2010) Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy. Cancer Immunol Immunother 59:1573–1582
6. Frankenberger B, Schendel DJ (2012) Third generation dendritic cell vaccines for tumor immunotherapy. Eur J Cell Biol 91:53–58
7. Kanzler H, Barrat FJ, Hessel EM, Coffman RL (2007) Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med 13:552–559
8. Geisse J, Caro I, Lindholm J, Golitz L, Stampone P, Owens M (2004) Imiquimod 5 % cream for the treatment of superficial basal cell carcinoma: results from two phase III, randomized, vehicle-controlled studies. J Am Acad Dermatol 50:722–733
9. Adams S (2009) Toll-like receptor agonists in cancer therapy. Immunother 1:949–964
10. Re F, Strominger JL (2001) Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem 276:37692–37699
11. Srivastava P (2002) Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol 2:185–194
12. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A et al (2007) Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 13:1050–1059
13. Schröder NWJ, Schumann RR (2005) Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis 5:156–164
14. Ferwerda B, McCall MB, Alonso S, Giamarellos-Bourboulis EJ, Mouktaroudi M, Izagirre N et al (2007) TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. Proc Natl Acad Sci USA 104:16645–16650
15. Santos JL, Lera L, Pérez-Bravo F, Albala C (2006) Adiposity and bone mineral density of Chilean elderly women in relation to toll-like receptor 4 gene polymorphisms. Ann Hum Biol 33:585–592

16. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M et al (2000) *TLR4* mutations are associated with endotoxin hyporesponsiveness in humans. Nat Gen 25:187–191
17. Tal G, Mandelberg A, Dalal I, Cesar K, Somekh E, Tal A et al (2004) Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. J Infect Dis 189: 2057–2063
18. Santini D, Angeletti S, Ruzzo A, Dicuonzo G, Galluzzo S, Vincenzi B et al (2008) Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in gastric cancer of intestinal and diffuse histotypes. Clin Exp Immunol 154:360–364
19. Kutikhin AG (2011) Impact of Toll-like receptor 4 polymorphisms on risk of cancer. Hum Immunol 72:193–206
20. Bergmann C, Bachmann HS, Bankfalvi A, Lotfi R, Pütter C, Wild CA et al (2011) Toll-like receptor 4 single-nucleotide polymorphisms Asp299Gly and Thr399Ile in head and neck squamous cell carcinomas. J Transl Med 9:139
21. Gast A, Bermejo JL, Claus R, Brandt A, Weires M, Weber A et al (2011) Association of inherited variation in Toll-like receptor genes with malignant melanoma susceptibility and survival. PLoS ONE 6:e24370
22. Escobar A, López MN, Serrano A, Ramírez M, Pérez C, Aguirre A et al (2005) Dendritic cell immunizations alone or combined with low doses of interleukin-2 induce specific immune responses in melanoma patients. Clin Exp Immunol 142:555–568
23. López MN, Pereda C, Segal G, Muñoz L, Aguilera R, González FE et al (2009) Prolonged survival of dendritic cell–vaccinated melanoma patients correlates with tumor-specific delayed type IV hypersensitivity response and reduction of tumor growth factor β-expressing T cells. J Clin Oncol 27:945–952
24. Aguilera R, Saffie C, Tittarelli A, González FE, Ramírez M, Reyes D et al (2011) Heat-shock induction of tumor-derived danger signals mediate rapid monocyte differentiation into clinically effective dendritic cells. Clin Cancer Res 17:2474–2483
25. Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, Lockett S (2004) Automatic and quantitative measurement of protein–protein co localization in live cells. Biophys J 86: 3993–4003
26. Chomczynski P, Mackey K, Drews R, Wilfinger W (1997) DNAzol: a reagent for the rapid isolation of genomic DNA. Biotechniques 22:550–553
27. Figueroa C, Peralta A, Herrera L, Castro P, Gutiérrez A, Valenzuela J et al (2006) NOD2/CARD15 and Toll-like 4 receptor gene polymorphism in Chilean patients with inflammatory bowel disease. Eur Cytokine Netw 17:125–130
28. Mendoza-Naranjo A, Sáez PJ, Johansson CC, Ramírez M, Mandakovic D, Pereda C et al (2007) Functional gap junctions facilitate melanoma antigen transfer and cross-presentation between human dendritic cells. J Immunol 178:6949–6957
29. van der Graaf C, Kullberg BJ, Joosten L, Verver-Jansen T, Jacobs L, Van der Meer JW, Netea MG (2005) Functional consequences of the Asp299Gly Toll-like receptor-4 polymorphism. Cytokine 30:264–268
30. Andrews DM, Maraskovsky E, Smyth MJ (2008) Cancer vaccines for established cancer: how to make them better? Immunol Rev 222:242–255
31. Zaloudik J, Vagunda V, Drahokoupilova M, Janakova L, Talac R, Kalabis J, Sheard M (1997) Biomarkers for predicting response to regional chemo-immunotherapy in liver metastases from colorectal carcinoma. Int J Immunopharmacol 19:481–485
32. Hicklin DJ, Marincola FM, Ferrone S (1999) HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. Mol Med Today 5:178–186
33. Ahirwar D, Kesarwani P, Manchanda PK, Mandhani A, Mittal RD (2008) Anti- and proinflammatory cytokine gene polymorphism and genetic predisposition: association with smoking, tumor stage and grade, and bacillus Calmette-Guérin immunotherapy in bladder cancer. Cancer Genet Cytogenet 184:1–8
34. Jung ID, Jeong SK, Lee CM, Noh KT, Heo DR, Shin YK et al (2011) Enhanced efficacy of therapeutic cancer vaccines produced by co-treatment with *Mycobacterium tuberculosis* heparin-binding hemagglutinin, a novel TLR4 agonist. Cancer Res 71:2858–2870
35. Riddell JR, Wang XY, Minderman H, Gollnick SO (2010) Peroxiredoxin 1 stimulates secretion of proinflammatory cytokines by binding to TLR4. J Immunol 184:1022–1030
36. Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ et al (2006) HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. Shock 26:174–179
37. El Mezayen R, El Gazzar M, Seeds MC, McCall CE, Dreskin SC, Nicolls MR (2007) Endogenous signals released from necrotic cells augment inflammatory responses to bacterial endotoxin. Immunol Lett 111:36–44
38. Casanova JL, Abel L, Quintana-Murci L (2011) Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. Annu Rev Immunol 29: 447–491
39. Loeffler J, Ok M, Morton OC, Mezger M, Einsele H (2010) Genetic polymorphisms in the cytokine and chemokine system: their possible importance in allogeneic stem cell transplantation. Curr Top Microbiol Immunol 341:83–96
40. Weng WK, Czerwinski D, Timmerman J, Hsu FJ, Levy R (2004) Clinical outcome of lymphoma patients after idiotype vaccination is correlated with humoral immune response and immunoglobulin G Fc receptor genotype. J Clin Oncol 22:4717–4724
41. Ugurel S, Schrama D, Keller G, Schadendorf D, Bröcker EB, Houben R et al (2008) Impact of the CCR5 gene polymorphism on the survival of metastatic melanoma patients receiving immunotherapy. Cancer Immunol Immunother 57:685–691
42. Tze LE, Horikawa K, Domaschenz H, Howard DR, Roots CM, Rigby RJ et al (2011) CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10–driven MARCH1-mediated ubiquitination and degradation. J Exp Med 208:149–165
43. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbé C et al (2009) Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clin Cancer Res 15:7412–7420
44. Tesniere A, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F (2010) Immunogenic death of colon cancer cells treated with oxaliplatin. Oncogene 29:482–491
45. Baxevanis CN, Perez SA, Papamichail M (2009) Combinatorial treatments including vaccines, chemotherapy and monoclonal antibodies for cancer therapy. Cancer Immunol Immunother 58: 317–324