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The common heat shock protein receptor CD91 is up-regulated on monocytes of advanced melanoma slow progressors

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SUMMARY

Despite advances in our understanding of tumour immunology there is no therapy of proven survival benefit for advanced melanoma. Nevertheless, disease progression is slow in a small proportion of patients with metastatic melanoma, suggesting a contribution to outcome from host factors. Recent data have indicated the importance of the heat shock protein receptor CD91 in immune responses to, and progression of, infectious disease. Here we investigate the relationship between CD91 expression and outcome in malignancy. Rare melanoma patients were recruited with advanced disease that was progressing unusually slowly. CD91 expression on their monocytes was compared with control patients with more typical rapidly advancing metastatic disease. Th1 and Th2 cytokines, as well as innate and adaptive immune subsets, were also measured in the two groups. A significant increase in median CD91 expression levels was observed in slow progressors ($P = 0{\cdot}006$). There were no differences in other immune subset markers or inflammatory cytokines. The ability of CD91 to internalize and cross-present tumour antigens through the major histocompatibility complex class I pathway may maintain CD8-positive cytotoxic T cell responses and contribute to slow progression of advanced melanoma.

Keywords CD91 skin cancer/melanoma

INTRODUCTION

Advances in tumour immunology have led to an improved understanding of the immune responses to malignancy [1]. No interindividual differences in host immunity have been identified so far that might account for the observed variation in host melanocyte-specific immunity. In an earlier study, the large cell surface dimer CD91 was found to be up-regulated on monocytes of human immunodeficiency virus (HIV) type-1 infected individuals that do not develop AIDS (termed long-term non-progessors, LTNP), while other immune markers and cytokine levels remain generally unchanged [2]. Furthermore we have shown recently that functionally impaired dendritic cells from immunosuppressed patients with cancer can respond successfully to tumour antigens via CD91 [3].

The high efficiency stimulation of cytotoxic T cells has been attributed in many cases to direct binding to, and internalization of, heat shock protein (HSP)–peptide complexes via the CD91 molecule (also called α_2-macroglobulin receptor, or the low-density lipoprotein-related protein). CD91 has been identified as a common receptor for the HSPs hsp70, hsp90, gp96, hsp110 and calreticulin [4,5]. Alternatively, CD91 function unrelated to HSPs may be important; CD91 has been identified as the receptor for α-defensins [6], molecules known to be secreted from activated T cells of LTNP [7]. Such CD91-mediated pathways may provide a mechanism for the maintenance of cytotoxic T cell responses in LTNP [8,9].

There is currently no therapy of proven survival benefit in metastatic malignant melanoma [10], and yet a small proportion of patients with AJCC stage IV disease live very significantly longer than the median survival of 12 months. In order to investigate possible mechanisms for this variable outcome we applied 'lessons learnt from HIV' [11] to patients with metastatic melanoma by measuring innate and adaptive immune subsets, Th1 and Th2 cytokines and levels of CD91 on monocytes.

PATIENTS AND METHODS

Patients and samples

Consenting individuals with non-progressive metastatic melanoma ($n = 8$) were recruited and defined as slow progressors

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based on survival for (i) 6 years or more following diagnosis of cutaneous metastases; (ii) 6 years or more following diagnosis of lymph node involvement that was unresectable or beyond the regional nodes; or (iii) 2 years or more following diagnosis of visceral metastases (lung, liver or bowel). The next eight consecutive patients with cutaneous metastases or unresectable primary melanoma, but who did not meet the criteria for slow progression, were recruited as controls. All individuals were enrolled between May and October 2003. The study received appropriate ethical approval and all volunteers were from St George's Hospital NHS Trust, London.

Each patient provided 20 ml of blood which was collected into heparinized vacutainers and processed within 2 h of drawing. Plasma was collected and peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll-Histopaque® gradient (Sigma, Poole, UK).

Flow cytometry

Total lymphocyte and subset analysis was performed using whole blood stained with murine antihuman monoclonal antibodies (TetraOne, Beckman Coulter, High Wycombe, UK) to CD3 (total lymphocyte), CD4 (T helper subset), CD8 (cytotoxic T subset), CD16/56 (natural killer) and CD19 (B cell) and were evaluated on an Epics XL-MCL (Beckman Coulter) flow cytometer.

CD91 receptor levels were performed on 5×10^5 washed PBMCs stored previously in liquid nitrogen. These were labelled for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-α2-macroglobulin α-chain (BioMac, Leipzig, Germany) and phycoerythrin (PE)-conjugated anti-CD14 (Pharmingen, Oxford, UK). A minimum of 100 000 cells were acquired in the live gate and analysed on a Becton Dickinson FacsCaliber using CellQuest® software. Positive staining for each marker was determined by comparison to appropriate isotype-matched controls and identical settings were used on each sample.

Cytokines

Plasma samples were stored at –80°C and analysed together on single plates for each cytokine to reduce variability in results, using quantitative sandwich immunoassay kits. Enzyme-linked immunosorbent assays (ELISAs) were performed for tumour necrosis factor (TNF)-α (Roche, Mannheim, Germany), interferon (IFN)-γ, monocyte chemotactic protein-1 (MCP-1) and interleukin (IL)-2, IL-4, IL-6 and IL-12 (Diaclone, Besançon, France).

Between-group comparisons were made using Mann–Whitney *U*-tests on the VASSARSTATS program.

RESULTS

Patients and treatment

Tables 1 and 2 demonstrate the characteristics of patients in both the slow progressor and control groups. All patients were from the same treatment centre and so had access to the same standard of medical intervention, surgery and supportive care during the course of their illness. Of note, at enrolment five of eight slow progressors, but none of the controls, had survived for 2 years or more following a diagnosis of visceral metastases (Table 2).

Cytokines

Results were confounded by heterogeneity in cytokine levels (Table 3), a situation observed previously in other studies [12]. There were no significant differences between the slow progressors and controls in levels of TNF-α, IFN-γ, MCP-1, IL-2, IL-4, IL-6 or IL-12. One slow progressor (patient 2) had levels at the upper limit of detection for IL-6 and MCP-1 (IL-6 measured 2000 pg/ml, MCP-1 was 20 000 pg/ml), while IL-4 levels were measurable in two of eight controls but were undetectable (<1 pg/ml) in plasma from all slow progressors.

Surface staining

There were no statistically significant differences in innate and adaptive absolute numbers or percentages of lymphocyte subsets including CD3 (all lymphocytes), CD4 (T helper cells), CD8 (T suppressor cells), CD19 (B cells) and CD16/56 (natural killer cells) (Table 4).

The percentage of cells staining for CD91, including monocytes, showed no statistical differences. The fluorescence intensity of CD91 staining was, however, significantly higher on the monocytes of slow progressors *versus* controls (median ± 95% confidence interval = 70·3 ± 22·9 *versus* 39 ± 9·4; $P = 0{\cdot}006$; Fig. 1). Although there was considerable variation in the CD91 median fluorescence intensity on the monocytes of slow progressors, all but one had a CD91 median fluorescence intensity of greater than 60, whereas all controls had an intensity of less than 55 (Fig. 1d). CD91 levels did not correlate with cytokines or lymphocyte subset values.

DISCUSSION

A number of tumour and host factors have been implicated in the pathogenesis and progression of melanoma. Host factors include two major susceptibility genes for melanoma, *CDKN2A* and *CDK4*; *GST* and *MC1R* contribute to only a minority of cases [13–15]. Studies have not, however, previously examined host factors that may protect against rapid progression. Here we demonstrate that CD91 expression is up-regulated on $CD14^+$

Table 1. Clinical characteristics of patients: comparison of slow progressors with controls

	Slow progressors	Controls
n	8	8
Median age (range)	52 (30–69)	72 (36–91)
Median survival (range) since diagnosis of primary melanoma, in months	102 (52–156)	37 (16–228)
Median survival (range) since diagnosis of first metastatic disease, in months	72 (24–120)	15 (0–48)

Table 2. Clinical characteristics of patients: survival and sites of metastatic disease

		Survival (months) since diagnosis of:		
	Patient	Primary melanoma	Cutaneous recurrence or unresectable regional lymph node metastasis	Visceral or distant nodal metastasis
Slow progressors	1	79	71	
	2	52		24
	3	156	81	47
	4	156	120	94
	5	82	58	47
	6	98	74	
	7	107	107	
	8	126	67	63
Controls	9	41	41	
	10	228	48	
	11	34	4	
	12	22	5	
	13	16	12	
	14	17	17	
	15	46	18	
	16*	40		

*This elderly patient was receiving medical therapy for an unresected lentigo maligna melanoma of the face.

Table 3. Plasma cytokine values (pg/ml) in slow progressors and controls

Cytokine measured	Slow progressors	Controls	*P*-value (Mann–Whitney)
TNF-α	93·15 ± 106	25 ± 89	0·23
IFN-γ	<3·125	<3·125	1
MCP-1	481·2 ± 6868	467 ± 162	0·79
IL-2	<31·25	<31·25	1
IL-4	<1	1 ± 1·46	0·40
IL-6	39·1 ± 691	6·25 ± 463	0·34
IL-12	<6·25	<6·25	1

Table 4. Immune cell subsets in slow progressors and controls with melanoma (median ± standard deviation)

Subset measured (normal range)	Slow progressors	Controls	*P*-value (Mann–Whitney)
CD3% (65–90)	73·2 ± 7·3	67·3 ± 4·7	0·17
CD3 (800–2660)	1576 ± 344	1191 ± 276	0·17
CD19% (5–25)	11·8 ± 3·7	10·9 ± 1·7	0·56
CD19 (100–600)	231 ± 106	204 ± 30	0·10
CD4% (30–65)	40 ± 8·8	41 ± 7	0·75
CD4 (450–1660)	805 ± 281	720 ± 82	0·09
CD8% (15–50)	27·8 ± 5·4	25·4 ± 1·7	0·29
CD8 (190–1210)	551 ± 155	441 ± 275	0·24
CD16/56% (3–30)	11·6 ± 9·1	16·6 ± 2·5	0·09
CD16/56 (50–620)	264 ± 249	214 ± 220	0·91
ISUM (950–3880)	2230 ± 488	1651 ± 536	0·34
CD4 : CD8 ratio	1·3 ± 0·7	1·6 ± 0·6	0·75

The percentage of each cell type as well as absolute values (cell counts are measured in cells/mm^3) were recorded. ISUM = immunological sum of all subsets.

monocytes of melanoma slow progressors, a finding with parallels in HIV disease. Increased CD91 levels may not prevent tumours (or viral infections) occurring, but may modulate the course of the disease once it has been established by maintaining cytotoxic adaptive immunity.

Pathways regulating expression of CD91 are poorly understood, although correlative evidence exists for down-regulation of CD91-mediated immune activation as a mechanism of immune escape. Thus levels of a CD91 ligand, α2-macroglobulin, are elevated in animal models of cancer [16], and during recovery of animals from autoimmune encephalitis [17]. Indeed, infusion of animals with α2-macroglobulin prevents experimental autoimmune pathology. It is conceivable that antimelanoma therapy in the slow progressor group (particularly immunotherapies) led to higher CD91 levels on monocytes, which these data suggest may be protective against rapid progression. However, no immunotherapy or other treatment modality has yet been associated with survival benefit in metastatic melanoma.

The binding of CD91 either to antigenic peptides and HSPs, or to T cell-derived α-defensins, could represent a potential mechanism of slow progression in melanoma. CD91 is the only independently confirmed HSP receptor [4,5,18]; previous work has shown that HSP–peptide complexes are taken up by antigen-presenting cells, and that the peptides are re-presented by the major histocompatibility complex (MHC) class I on these cells to CD8$^+$ cytotoxic T cells [3,19–21]. The CD91-mediated internalization of HSPs represents an exogenous portal by which chaperoned antigen may enter the classical proteosome/TAP-dependent

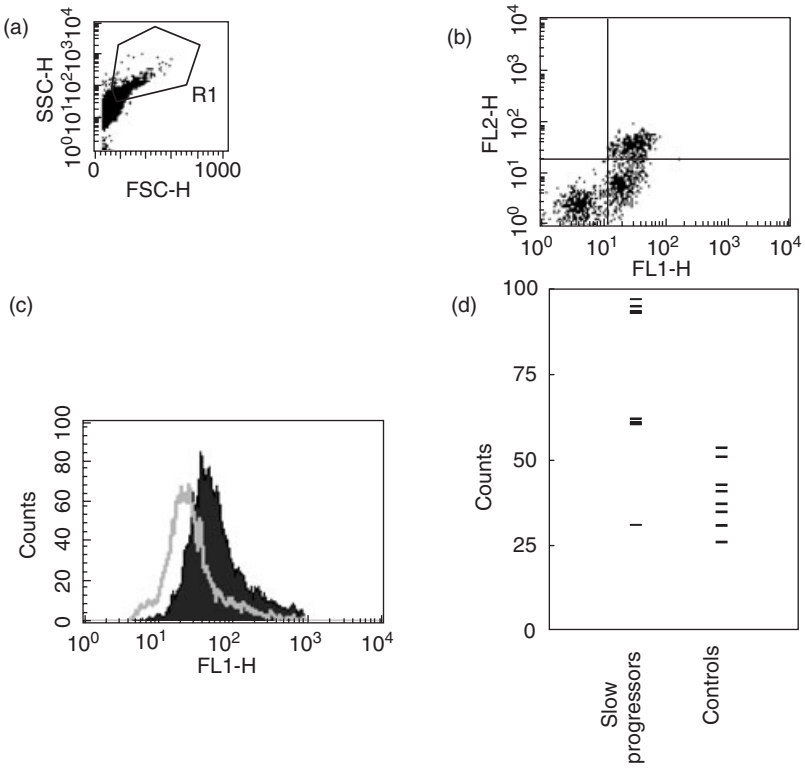

Fig. 1. CD91 levels are increased on monocytes of melanoma slow progressors. Cells in the live gate (a) were analysed for CD91 (FL1 *x*-axis) and CD14 staining (b, FL2 *y* axis). The $CD14^+$ cells were gated and the CD91 median fluorescence intensity on these was plotted on a histogram (c). CD91 fluorescence intensity is shown for a representative slow progressor (solid area) and control (open area). Fluorescence intensity for each patient is plotted in (d); the median CD91 fluorescence intensity was significantly higher in slow progressors than controls (see text). Isotype control staining was less than 2·5%.

MHC class I pathway, so-called cross-presentation [22,23]. Thus relatively efficient cross-presentation of HSP-chaperoned antigen, via CD91, may represent a mechanism explaining the association between CD91 expression and outcome in both melanoma and HIV disease.

This small study demonstrates no differences in lymphocyte, natural killer subsets and levels of plasma cytokines between slow progressors and controls. It is of interest that TNF-α levels were detectable in all the patients with slow progression and the role of TNF-α in the disruption of tumour endothelium is becoming established [24,25]. Intriguingly, TNF-α is thought to lead to the production of both IL-6 and MCP-1 and hence may contribute to the regulation of tumour progression [26].

We and others [27] have been unable to detect significant quantities of CD91 on peripheral blood myeloid or plasmacytoid dendritic cells, and the limited CD91 expression that we are able to detect disappears after a few days in culture. The lack of CD91 on blood dendritic cells may reflect the fact that they are immature cells *en route* to the tissues where they become fully competent in antigen capture. We have found no evidence of CD91 expression on $CD3^+$ T cells but consistently observe expression on $CD14^+$ monocytes, the majority of which stain for CD91. There are also high levels of expression on monocyte-derived dendritic cells [2,28]; unlike peripheral blood dendritic cells these can be obtained in significant quantities and have been shown to uptake, process and present antigen efficiently [29]. Pulsing of monocyte-derived DCs represents a potential avenue for use in therapy.

Up-regulation of CD91-mediated processes, possibly including endocytosis of HSP–peptide complexes and cross-presentation of tumour antigens, may represent a useful antitumour therapeutic strategy. Alternatively, CD91 up-regulation may serve as a surrogate marker for response to therapy.

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