M2 macrophages on tumour growth and metastasis in hepatocellular carcinoma

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KEY MESSAGES

1. M2 macrophages contributed to poor survival outcome in liver cancer patients. An increase in the number of immune cells within the tumour correlated with reduction in the overall and relapse-free survival period.
2. An increase in M2 macrophages correlated with larger tumour size, increased venous infiltration, and higher tumour stage.
3. M2 macrophages secreted a chemokine CCL22 that promoted invasiveness of liver cancer cells and increased the incidence of metastasis.
4. Targeting the tumour-promoting macrophages is a potential therapeutic strategy against liver cancer.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most commonly diagnosed malignancy and the second most common cause of death from cancer worldwide. Over 500 000 new cases and deaths are estimated to occur every year. HCC is characterised by rapid disease progression and high postsurgical recurrence and high metastatic rate (50% to 70% over 5 years) and often results in poor clinical outcomes.[1] One major risk factor of recurrence and metastasis is sustained inflammation, which is found in up to 80% of cancer patients. Therefore, understanding the roles of inflammatory-related immune cells, particularly macrophages, in cancers is important. It remains controversial whether macrophages contribute to better or poorer clinical outcomes in HCC.

Macrophages can be sub-classified into classically (M1) and alternatively (M2) activated phenotypes based on surface receptors and functional characteristics. With a distinct secretory profile consisting of cytokines and growth factors, M2 macrophages are responsible for mediating wound healing processes via extracellular matrix remodelling, angiogenesis, and immunosuppression. In human cancers, these wound-healing features are exploited to facilitate tumour growth and dissemination. Collective evidence demonstrates that intratumoural macrophages known as tumour-associated macrophages exhibit M2 phenotypes and are correlated with poor prognosis in numerous malignancies.[2]

Methods

The present study comprised a clinical study, an in vivo study, and an in vitro study (Fig 1). The study was approved by the Institutional Review Board of the University of Hong Kong. Informed written consent was obtained from all patients. Animals (Control of Experiments) Ordinance (Hong Kong) and the institute's guidance on animal experimentation were strictly followed.

For clinical study, tumorous liver tissues (intra-tumour) and peritumoural liver tissues within 2 cm proximal to the tumour margin (peri-tumour) were collected from 95 patients (aged 18-83 years, 78% male) who underwent curative surgery for HCC in Queen Mary Hospital from 2004 to 2008.

For in vivo study, male athymic nude mice (BALB/c nu/nu, 4-6 weeks old) were used. Surgical procedures have been described in previous reports by our group. Briefly, 3 $\times 10^5$ MHCC97L cells suspended in 0.2 mL DMEM were injected subcutaneously into the flanks of mice. After 4 weeks, the subcutaneous tumours were resected and diced into 1 mm^3 cubes, which were then implanted in the left lobes of the livers of another group of nude mice. Simultaneously, 5 $\times 10^5$ M1 or M2 polarised THP-1 macrophages suspended in 0.2 mL DMEM were injected into the portal vein of the same group of mice. Nine mice were used for both the negative control and the M2 macrophage treatment group, whereas six mice were used for the M1 macrophage treatment group. Mice injected with pure DMEM were served as negative control. Tumour size and metastasis of MHCC97L xenograft were monitored weekly by Xenogen IVIS (Xenogen IVIS 100, Caliper Life Sciences). All mice were sacrificed at week 5 and the size of liver tumour was measured.

For in vitro study, to examine the direct effect on cell growth and migration of HCC, macrophages

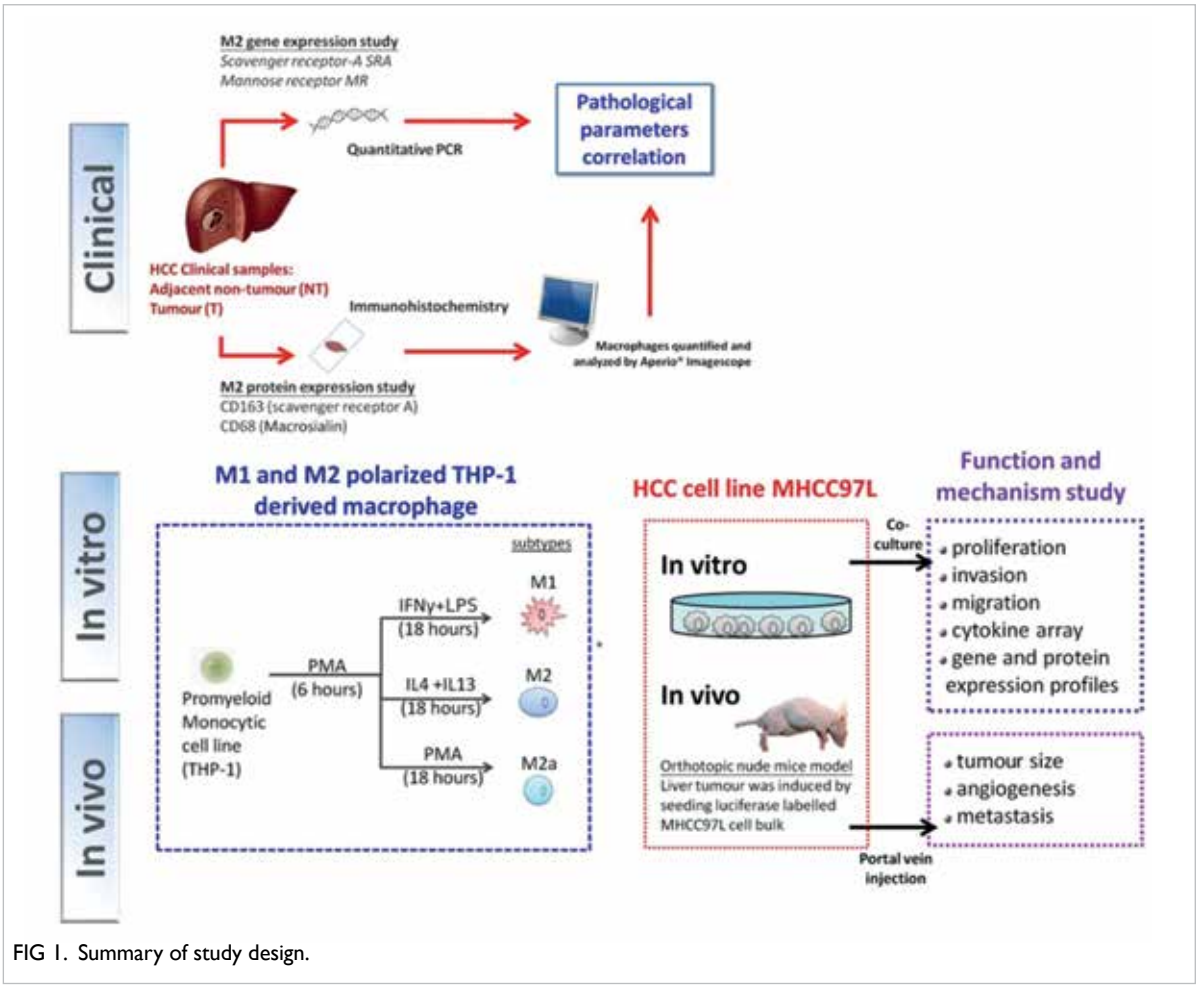

FIG 1. Summary of study design.

and MHCC97L cells cocultivation was conducted using the non-contact coculture transwell system (Corning). For cell growth, 1 ×10^5, 3 ×10^5, or 5 ×10^5 THP-1 cells were seeded in 0.4-μm sized pores inserts and polarised into M1 and M2 macrophages. Culture medium was then replaced by FBS-free RPMI 1640 medium for further 24 hours. Inserts containing M1 or M2 polarised THP-1 macrophages were transferred to 6-well cell culture plate seeded with MHCC97L cells (1 ×10^5 cells per well) in advance and cocultured for 72 hours. Inserts were discarded and the MHCC97L cells were washed with PBS three times and trypsinised with 0.05% Trypsin-EDTA (Invitrogen). The total number of MHCC97L cells after coculture with macrophages in each well was measured by the FC500 flow cytometer (Beckman Coulter). To evaluate the status of MHCC97L cells after co-culture, 1 mg/mL of D-luciferin was added to each well and visualised by In Vivo Imaging System (IVIS; Xenogen IVIS 100).

Statistical analysis was performed using GraphPad Prism 5.0 and PASW Statistics 18.0 (SPSS Inc.). A P value of ≤0.05 was considered statistically significant. Unpaired Student's *t* test and Fisher's exact test for dual comparison and log-rank test for comparison of survival in Kaplan-Meier survival plot were used. Prognostic value of each clinicopathologic parameter and expression level of macrophage marker was tested by univariate Cox proportional hazards regression analysis. The prognostic power of significant predictors in the univariate analysis was then evaluated by a multivariate survival model.

Results

We analysed the level of M2 macrophages in tumour tissue collected from 80 HCC patients. Three antibody targeting surface receptors: monocyte/macrophage (CD14), macrophage (CD68), and M2 macrophage (CD163) were used to identify distinct subpopulation residing in the intratumoural and peritumoural regions of HCC tissues via immunohistochemistry. The ratio of positively stained macrophages to total cell population in each sample was determined. All macrophages were highly expressed in the tumoural region, and most of the populations demonstrated M2 subtype. Consistent results were observed in the mRNA expression study involving two M2

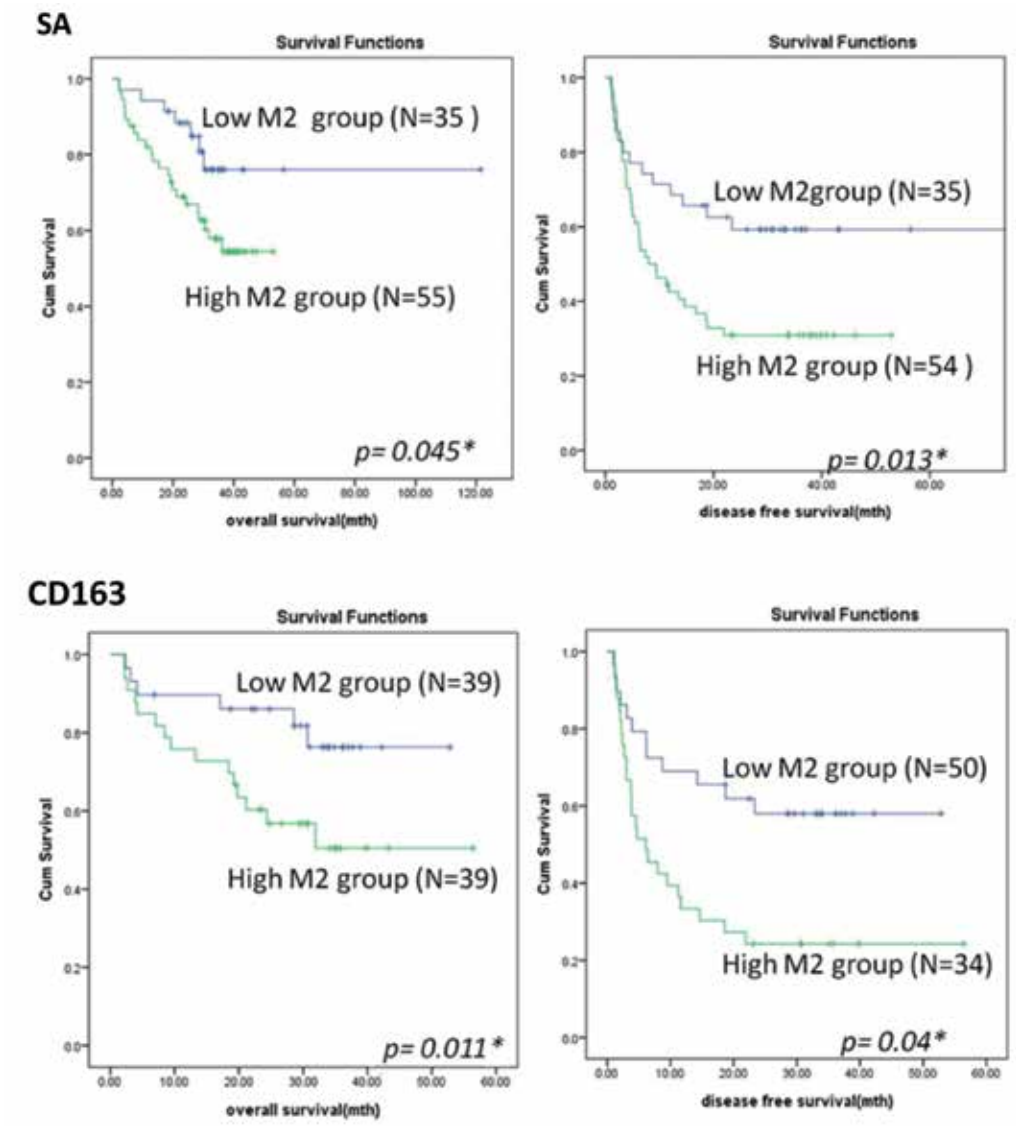

FIG 2. Overall and disease-free survival analysis of M2 macrophages in patients with hepatocellular carcinoma

macrophage-specific transcripts: class A scavenger receptor (SA) and mannose receptor (MR).

High expression levels of M2 macrophage markers SA and CD163 in tumour area significantly correlated with shortened overall and disease-free survival in HCC patients (Fig 2). Using multivariate Cox proportional hazards analyses, only the M2 macrophage markers $CD163^+$ and SA were significant independent prognosis factors in overall survival ($CD163^+$: hazard ratio [HR]=3.14, 95% confidence interval [CI]=1.2-8.2, P<0.05) and disease-free survival (CD163+: HR=3.64, 95% CI=1.0-7.0, P=0.043; SA: HR=3.56, 95% CI=1.0-12.2, P=0.044).

Increased levels of CD163 and SA were significantly associated with late tumour stages, venous infiltration, and multiple tumour nodules (P<0.05) in HCC patients. High levels of M2 macrophages induced invasiveness in tumour cells.

To validate the tumour-promoting effects of M2 macrophages, we tested M2 macrophages on an orthotopic nude mice model of liver cancer. 5 $\times 10^5$ THP-1-derived M1 or M2 macrophages were introduced into the liver of nude mice via portal vein injection immediately after orthotopic liver transplantation. Tumour growth and metastasis were monitored using Xenogen IVIS100, and the mice were sacrificed at week 5 after transplantation. The tumour volume in the mice injected with M2 macrophages increased 3.26-fold compared with the control group (1.27±0.36 cm^3 vs 0.39±0.05 cm^3, P=0.032, Fig 3a). By contrast, the mice injected with M1 macrophages exhibited a significant 2.79-fold reduction in tumour volume (0.14±0.02 cm^3,

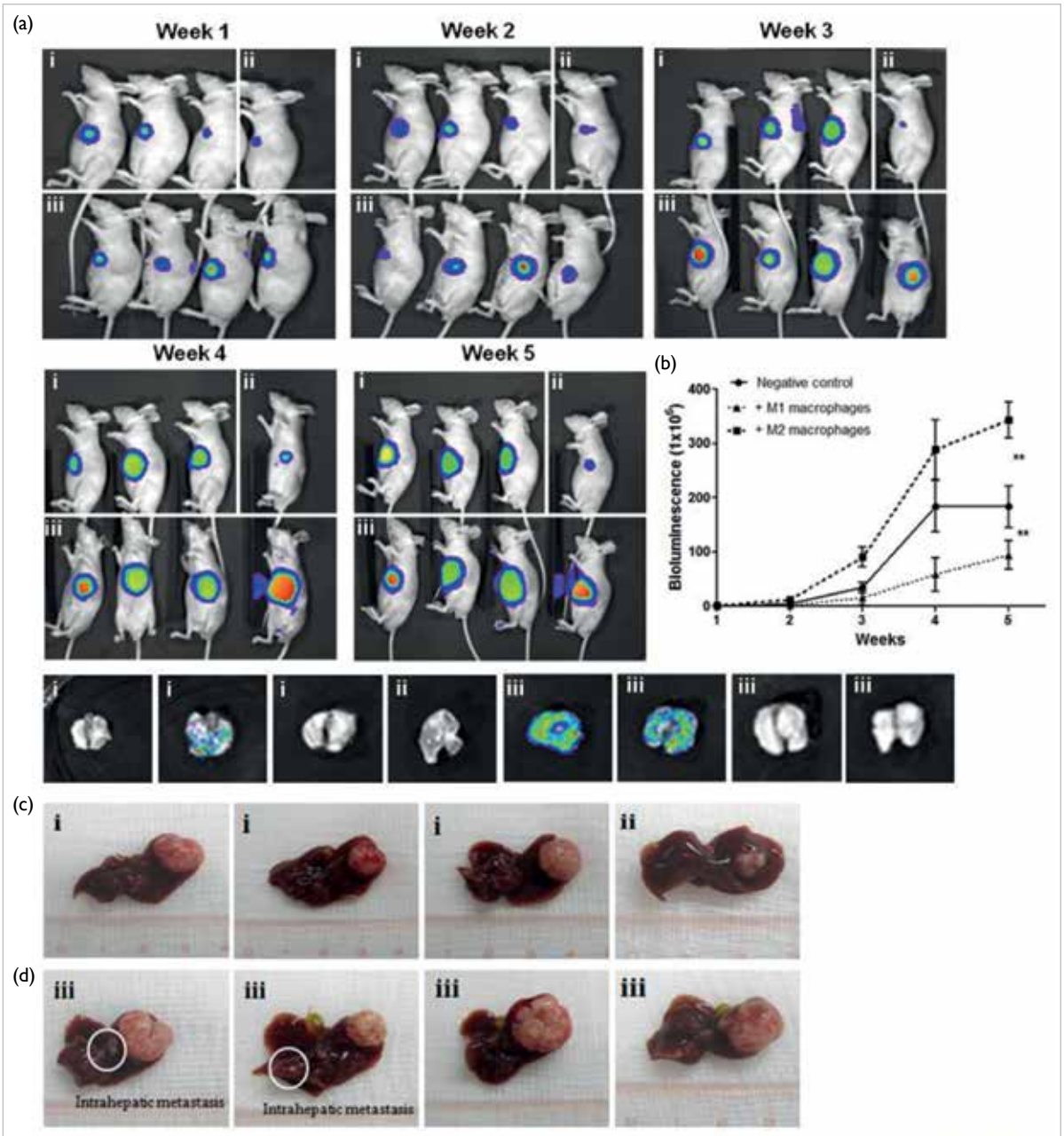

FIG 3. Orthotopic liver tumour nude mice model: male athymic mice bearing orthotopically grafted MHCC97L-Luciferase tumours were injected with (i) DMEM (negative control), (ii) M1 macrophages, or (iii) M2 macrophages into the portal vein. (a) Monitoring of tumour growth by Xenogen IVIS at week 1 to 5. (b) Measurements of mean in vivo liver tumour bioluminescence of each group over time. Bioluminescent signals were quantified in photons/s at each imaging time point. (c) Lung metastasis and (d) tumour volume was examined.

P=0.044). An increased rate of lung metastases was observed in the M2-treated group compared with the control (57% vs 25%, P<0.05, Fig 3b and c). To identify the location of the injected M2 macrophages, immunohistochemistry with anti-human CD163 antibody was applied, and positively stained cells were observed in the peritumoural region (Fig 3d). M2 macrophages stimulated the growth of tumour as well as increased the metastasis incidents in liver cancer.

To further verify whether M2 macrophages directly induce the growth and invasiveness of HCC cells, in vitro studies were conducted by co-culturing HCC cell line MHCC97L with M2 macrophages. After 72 hours, the cell number of MHCC97L significantly increased 1.3-fold compared with the single MHCC97L culture (P<0.05). M2 macrophages were found to enhance the migration of HCC cells. Applying a similar co-cultivation methodology but with 8 μm inserts and 24 hours of incubation to allow

cell migration across the membrane, the number of migrated MHCC97L increased by 3.2-fold upon co-cultivation with M2 macrophages compared with the control ($P<0.01$).

Given that cytokines and chemokines represent the major functional responses of macrophages, a signalling mechanism between M2 macrophages and HCC cells may account for the pro-tumoural activities. The expression level of 42 cytokines in M1 and M2 monoculture supernatants as well as M1/MHCC97L and M2/MHCC97L co-culture supernatants were examined using an antibody cytokine array. Only the cytokine C-C motif chemokine 22 (CCL22) known as macrophage-derived chemoattractant was significantly up-regulated in M2–MHCC97L, in contrast to the other three configurations (Fig 3d). To exclude the possibility that CCL22 may derive from MHCC97L before or after co-cultivation with M2 macrophages, transcript and protein analysis were performed; no CCL22 mRNA and protein were detected in both cases (data not shown).

Discussion

M2 macrophages in tumour area significantly contributed to the disease progression. The level of peritumoural M2 macrophages was a predictor of the survival in HCC. In vivo and in vitro experimental evidences showed a significant increase in tumour growth and migration in the presence of M2 macrophages confirming their pro-tumour functions. One underlying mechanism identified was CCL22/CCR4, which enhanced HCC invasiveness through epithelial-mesenchymal transition activation.

Acknowledgements

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Results from this study have been published in: Yeung OW, Lo CM, Ling CC, et al. Alternatively activated (m2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. J Hepatol 2015;62:607-16.

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