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Analysis of YKL-39 and stabilin-1 as indicators for alternative activation of macrophages in tumor microenvironment

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CONTENTS

Abbreviations

acLDL: acetylated low density lipoprotein

ALOX5AP: Arachidonate-5-lipoxygenase-activating protein

AMAC-1: Alternative macrophage activation-associated CC chemokine

AP-1: Activating protein 1

bHLH: basic helix-loop-helix

BMPs: Bone morphogenetic proteins

CCL: C-C motif chemokine ligand

CD163: Cluster of Differentiation 163

CD68: Cluster of Differentiation 68

CDK: Cell cycle protein-dependent kinase

CLPs: Chitinase-like proteins

COX-2: Cyclooxygenase-2

CSF1: Colony stimulating factor 1

DCs: Dendritic cells

ECM: Extracellular matrix

ECs: Endothelial cells

EGF: Epidermal growth factor

EMT: Epithelial-mesenchymal transition

FAK: Focal adhesion kinase

GAPDH: Glutaldehyde phosphate dehydrogenase

GCs: Glucocorticoids

GDFs: Growth differentiation factors

GDNF: Glial cell line-derived neurotrophic factor

HA: Hyaluronan

HAMP: Hepcidin antimicrobial peptide

Hb-Hp: Hemoglobin-haptoglobin complex

HLA-DR: Human leukocyte antigen DR

Id: Inhibitors of DNA binding

IFN-γ: Interferon-γ

IL-8: Interleukin-8

iNOS: inducible nitric oxide synthase

IRAK-M: Interleukin-1 receptor-associated kinase

IRF: Interferon regulatory factor

KLF: Krüppel-like factor

LAMPs: Lysosomal-associated, mucin-like membrane proteins

LNs: Lymph nodes

LPS: Lipopolysaccharide

LYVE-1: Lymphatic Vessel Endothelial Receptor 1

MafB: Maf transcription factor B

MAPK: Mitogen-activated protein kinase

MCP-1: Chemotactic protein-1

M-CSF: Macrophage colony stimulating factor

MDP: Monocyte-dendritic cell progenitor

MHC: Major histocompatibility complex

MMP-2: Metalloprotease 2

MPS: Mononuclear phagocyte system

MR: Mannose receptor

NFAT5: Nuclear factor of activated T cells 5

NO: Nitric oxide

PBMC: Peripheral blood mononuclear cell

PDGF: Platelet-derived growth factor

PGE_2: Prostaglandin E2

PLAUR: Plasmin urokinase activator

RELMa: Resistin like molecule alpha

RGS1: Regulator of G-protein signaling 1

ROS: Reactive oxygen species

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SI-CLP: Stabilin-1 interacting chitinase-like protein

SIRT1: Sirtuin 1

SPARC: Secreted protein, acid and rich in cysteine

STAT1: Signal transducer and activator of transcription 1

TAMs: Tumor-associated macrophages

TFEB: Transcription factor EB

TFs: Transcription factors

TGF-β: Transforming growth factor-beta

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor α

TREM1: Triggering receptor expressed on myeloid cells 1

uPA: urokinase plasminogen activator

VEGF: Vascular endothelial growth factor

YKL-39: cartilage chitinase-3-like protein 2

YKL-40: human cartilage glycoprotein-39

1 Introduction

1.1 Monocytes-derived macrophages: overview

1.1.1 Origin and tissue distribution of macrophages

Macrophages were initially discovered in the 19th century by a Russian bacteriologist, Ilya Mechnikov. Macrophages were found in all tissues and originate from blood monocytes[1]. In response to various stimuli, monocytes are recruited into tissues. Their life span is considerably longer than that of short-lived granulocytes[1]. Tissue macrophages originate from monocytes that differentiate from the bone marrow precursors and participate in forming the mononuclear phagocyte system (MPS)[2, 3].

Based on ontogeny and phagocytic activity, the whole MPS was described (Fig.1), including monocyte-derived dendritic cells (DCs), different networks of tissue macrophages and blood monocytes[4].

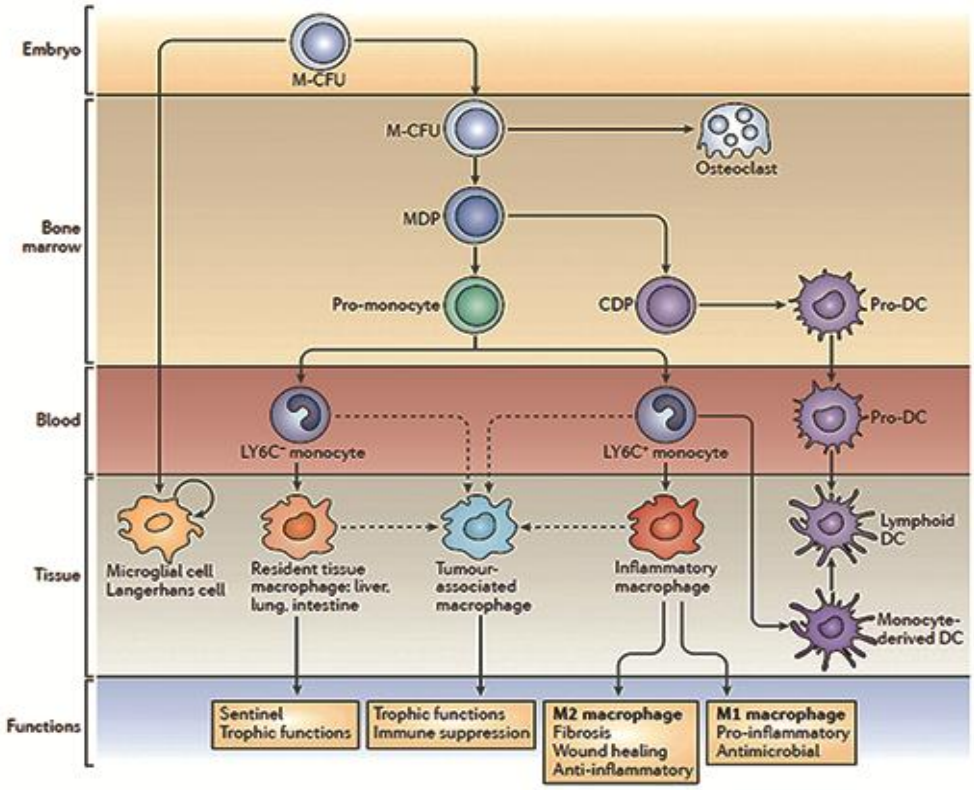


Figure 1. The mononuclear phagocyte system[4]. Permission from copyright © 2011, Springer Nature.

In the bone marrow, the key differentiation factor driving cascade of steps resulting in monocytes differentiation is colony stimulating factor 1 (CSF1). Monocytes develop from monocyte-dendritic cell progenitor (MDP)-derived pro-monocyte

precursor. In the homeostatic conditions, CSF1R/CSF1/IL-34 axis is important for the development of tissue-resident macrophage populations at embryonic stage and maintenance of certain tissue-resident macrophage population in adult organism[5, 6]. In adult organism, circulating monocytes can be recruited into tissues and give rise to inflammatory macrophages or replenish tissue-resident macrophages[3]. These macrophages can be polarized into specific phenotypes or activation states, that are historically described as M1 or M2 polarization vectors[4,7,8]. Tumor-associated macrophages (TAMs) can originate both from circulating monocytes and resident macrophages[9, 10].

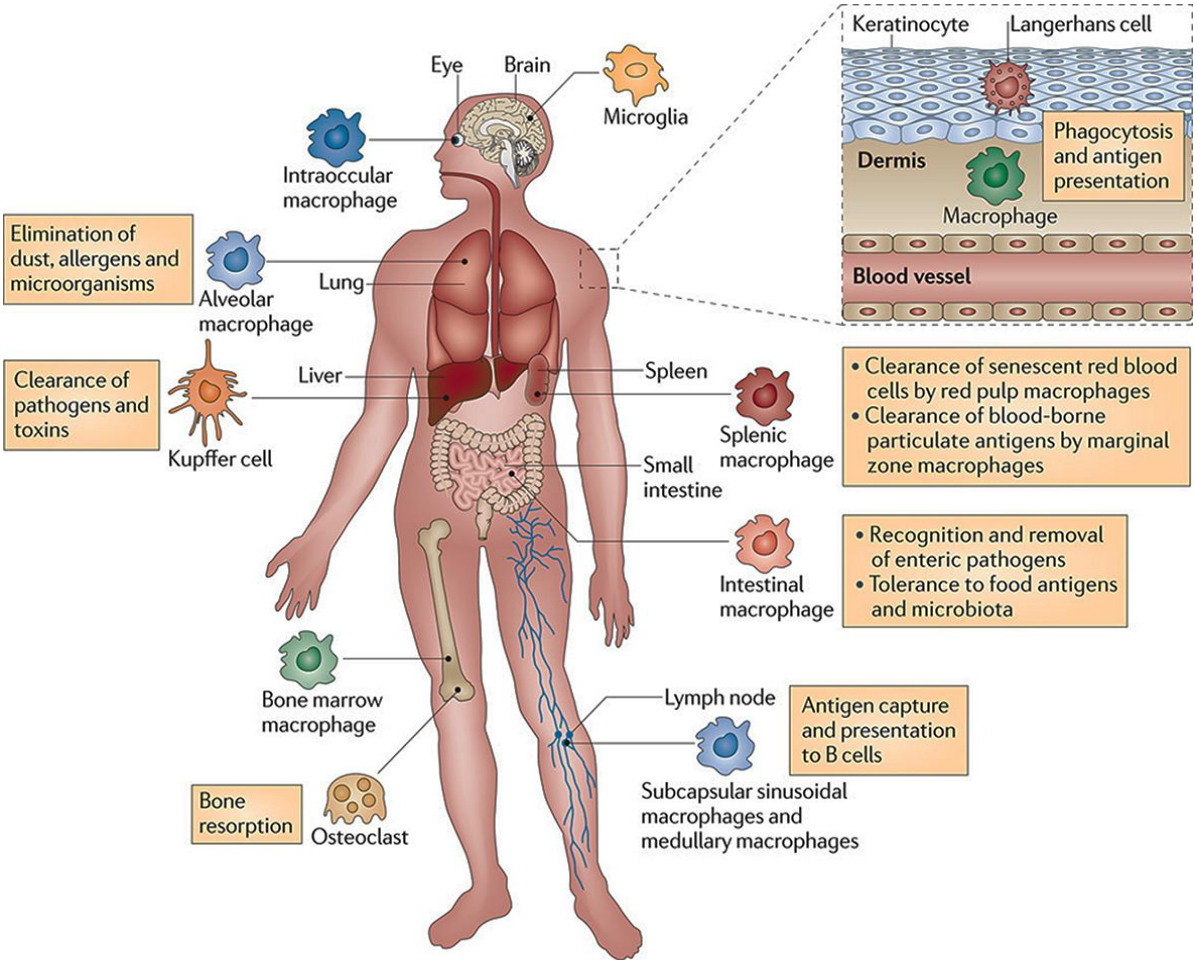


Figure 2. The distribution and function of macrophage in tissues[11]. Permission from copyright © 2011, Springer Nature

On the basis of location and functions, macrophages are classified into different subpopulations. According to different tissue localization, tissue-specific macrophages have been historically given specific names, for example Kupffer cells (liver), microglia, histiocytes (interstitial connective tissue), alveolar macrophages (lung), osteoclasts (bone) and others (brain) (Figure 2)[11]. Secondary lymphoid organs also contain different subpopulations of macrophages, such as marginal

zone macrophages which are proved to be distributed in the spleen, and macrophages present in the subcapsular sinus of lymph nodes[11]. These macrophages play roles in various physiological processes. For example, subcapsular sinusoidal macrophages in lymph nodes (LNs) eliminate foreign microorganisms out of the lymph and can initiate anti-viral humoral immune response[11].

1.1.2 Differentiation and plasticity of macrophages

The are two directions of macrophage functional polarization: M1, classically activated macrophages and M2, alternatively activated macrophage[12,13]. M1-macrophages are responsible for the acute inflammatory reactions and secrete pro-inflammatory cytokines, including reactive oxygen species (ROS), tumor necrosis factor (TNF-α), interleukin-8(IL-8), IL-1β, IL-6, nitric oxide (NO) and inducible nitric oxide synthase (iNOS) to eliminate bacteria and other foreign substance[14]. M2-macrophages can be divided into three subgroups (M2a, M2b and M2c), which participate in various processes, such as tissue repair/ wound healing, maintenance of tolerance and regulation of immunity. Under stimulation with corticoids, IL-10, TGF-β and IL-4/IL-13, macrophages acquire a distinct M2 phenotype[15-17]. Macrophages are characterized by high level of functional plasticity that allows mature M1 macrophages to change to M2 phenotype, and M2 to change to M1[18]. Stimulation of M1 macrophages with IL-4 and IL-10 caused secretion of M2 factors alternative macrophage activation-associated CC chemokine (AMAC-1) and IL-1Ra, while M2 macrophages stimulated with tumour necrosis factor gamma(IFN-γ) or lipopolysaccharide (LPS) start to produce M1 markers TNF-α and IL-1β[19]. Macrophage plasticity was suggested to have limitations after long-term residence in the tissues[20].

1.1.3 Biological functions of macrophages

Phagocytosis and endocytosis

Through fluid-phase or receptor-mediated processe，mammalian cells are capable of transporting macromolecules and particles from the extracellular environment[21]. Macrophages function specifically to internalize larger particles rapidly and to degrade the internalized bacteria and molecular complexes[21]. Phagocytosis is generally the process of uptake of particles around 1μm or greater in diameter (including bacteria and apoptotic cells). Phagocytosis is an evolutionally conserved

process. For example, in model organisms such as *C. elegans* and *D. melanogaster*, it was identified that phagocytosis plays key roles in the processes of embryonic development and elimination of apoptotic cells[22-24].

Two factors affect the process of phagocytosis: the nature and size of the particle. Firstly, macrophage recognizes the particle with specific receptors, then cell membrane is extended, and actin cytoskeleton is polymerized to support engulfment of a particle. The internalized material is localized in the early phagosome that is maturated by the dynamic interaction with endosomal/lysosomal system. As the end station, the materials is degraded in phagolysosomes formed by fusion with lysosomes to enable the internalized material digestion by lysosomal enzymes[23].

Phagocytosis functions as a front-line defense mechanism during pathogen attack. But, some pathogens can escape from the defense mechanism of macrophage by manufacturing anti-phagocytic capsules, or secreting toxins that are specifically harmful to macrophages[23, 25]. The most important role of phagocytosis is to identify foreign substances, and to distinguish dead and transformed cells[23]. Macrophage can also internalize and digest substances which are packed with opsonins such as specific antibodies or complement components[23].

In addition to phagocytosis, macrophages are able to mediate specific uptake of molecular factors from extracellular space or circulation by receptor-mediated endocytosis[26]. In this case, extracellular ligands are recognized by the endocytic receptors (e.g., LDL receptor, stabilin-1, CD163, transferrin) on the surface of macrophage, then the plasma membrane is invaginated, and the receptor-ligand complex is internalized[26]. In well studied clathrin-dependent receptor-mediated endocytosis, clathrin initiates formation of pits on the inner surface of plasma membrane, which facilitates engulfment of the receptor/ligand complex into clathrin-coated vesicles[27]. These vesicles are subsequently fused with early endosomes to initiate dissociation of receptor-ligand complex, endocytic receptors are retrieved to the cell surface and ligands are transported within the cell. Ligands can be delivered from early endosomes into recycling endosomal compartment or to the late endosomes, consequently, to lysosomes for degradation[27]. Endocytosis is an important part of a number of physiological and pathological processes, including nutrient uptake, antigen presentation, intracellular signaling and lipid processing[27,

[28].In pathological conditions, dysregulated endocytosis of specific extracellular compounds is involved in atherosclerosis and cancer progression[28-30].

Macrophages and adaptive immunity

Macrophages play an important role to prevent microbial invasion[31]. After ingesting a microbe, macrophages can present the antigen to T cells. After recognition of the antigen, T cells can be activated to kill the infected cell. T helper cells also induce B cells to produce antibodies. Each antigen may induce production of antibodies with multiple specificities, and also induce formation of immunological memory which allows rapid clearance of pathogens upon secondary infection[31, 32].

Macrophage recruitment and chemotaxis

One of the most important roles of macrophages in the adult organism is the maintenance of tissue homeostasis and healthy turn-over[33].In different physiological and pathological conditions, monocytes are induced to migrate into tissues and to differentiate into tissue-specific macrophages. In physiological healthy conditions, monocytes are recruited to the gut, dermis and heart to continuously replenish the resident macrophage population[34]. In pathological conditions, such as inflammation and infection, monocytes and resident macrophages migrate to the injured tissue site[35]. Following injury, chemoattractants, such as C-C motif chemokine ligand 2(CCL2), CCL3, CCL5, CCL7, CCL12 and CCL20 are released in the surrounding tissue to attract monocytes and macrophages[36-40,41]. Monocytes from peripheral blood firstly adhere to the vessel wall, next migrate through the endothelial cell barrier into tissue, and then migrate towards the stimulus. The resident tissue macrophages also release factors that attract neutrophils and monocytes. Local stimuli can trigger the resident macrophages to secrete cytokines which stimulate expression of cell adhesion factors such as P-selectin to promote the permeability of endothelial layer and to enable migration of inflammatory cells[42-46]. During the activation of complement or clotting cascades, pathogen-derived molecules and factors are produced, increased concentrations of pathogen-derived molecules and factors can also induce recruitment of monocytes into tissue[47].

Chemokines constitute a family of short proteins classified into different types on basis of core cysteine motifs responsible for the formation of disulphide bonds[43,48]. Chemokine orchestrates immune cell infiltration in pathology. For example, during

liver injury, hepatic macrophage produce several chemokines, like CCL3 (MIP-1α), CCL2 (MCP-1), CCL20 (MIP-3α) and other pro-migratory mediators, to recruit leukocytes that create conditions for chronic inflammation and fibrosis[48,49]. For glioblastoma, it was observed that the chemokine CCL2 produced by cancer cells and by immune cells in tumor microenvironment is required for the efficient angiogenesis and tumor cell proliferation[50].

1.1.4 Types of macrophages activation

Classically activated macrophages (M1)

Macrophages undergo M1 type of activation in response to microbial compounds, such as lipopolysaccharide (LPS) or interferon gamma (IFN-γ)[51]. In general, stimulation of IFN-γ and LPS drives macrophages toward the M1 phenotype. LPS induces Toll-like receptor (TLR)-mediated macrophage activation and production of TNF-α, which is a major acute stage inflammatory cytokine. M1 macrophages also are induced to increase expression of B-7 (CD86) and major histocompatibility complex (MHC) class II that is essential for the presenting antigen and for the elimination of pathogens[52] (Table 1).

Table 1. Expression profiles of classically (M1) and alternatively (M2) activated macrophages[52-57].

	Classical (M1) activation	**Alternative (M2) activation**
Cytokines	TNF-α IL-1β IL-6 IL-12 IL-15 IL-18	IL-10 TGF-β IL-1ra
Cytokine receptors	IL-1R type I	Type II IL1 decoy receptor
Chemokines	MIP-1α CCL2, 3, 4, 5 CXCL8, 9, 10, 11	AMAC-1 CCL13, 14, 17, 18, 22, 24
Chemokine receptors	CCR7	CCR2 CXCR1, 2
Immune/scavenger receptors	FcγRI (CD64) FcγRII (CD32) FcγRIII CD16) CD80 CD86	FcεRII (CD23s) MMR (CD206) SR-A I b-glucan R MARCO CD163 Stabilin-1
Enzymes of arginine metabolism	iNOS	Arginase
Others		Fibronectin

	b-I-H3 15-lipoxygenase FoxQ1[55] SI-CLP[56] YKL-39[57]

During inflammatory reaction, M1 phagocytose foreign microorganism and microorganism components, and transport microorganism to lysosomes. With the action of a variety of degradation enzymes including several cathepsins cysteine proteases, foreign microorganism and microorganism components are degraded. Processed bacterial antigens can be presented to T-cells to activate adaptive immune responses[58]. By production of various pro-inflammatory cytokines, macrophages can execute the pro-apoptotic activity[59]. By up-regulating production of reactive oxygen species and NO induced by iNOS, classically activated macrophages eliminate and kill intracellular microorganisms[60].

Alternatively activated macrophages (M2)

Alternatively activated macrophages (M2) were identified as a specific type of macrophage. Stimulation with IL-4 was first identified to induce expression of mannose receptor (CD206) in macrophages[61,62]. During next decades, several other inducers of M2 phenotype have been identified, including IL-13, IL-10, glucocorticoids, TGF-β and others[63-66]. It was identified that each of these M2 stimuli drives development of specific variants of M2 phenotype characterized by unique molecular profile and clear functional polarization[67].Alternatively activated macrophage also produce cytokines such as resistin like molecule alpha (RELMa) and TGF-β, counteracting inflammation[68-70]. M2 have pronounced healing and clearance functions needed for the wound repair and homeostatic tissue turn-over. However,pro-healing and immunosuppressing activities are detrimental if macrophages act within the tumor mass, and start to support tumor growth[71].

1.1.5 Macrophages and chronic inflammation

Inflammatory processes have multiple effects in carcinogenesis. Chronic inflammation is characterized by the absence of a balance between production of pro-inflammatory and anti-inflammatory/healing mediators[72].

Inability of M1 macrophages to switch to the healing M2 phenotypes can result in chronic inflammatory status, where matrix remodeling function is to be efficiently executed. M2 macrophages can produce and secrete mediators, growth factors and

anti-inflammatory cytokines to function on extracellular matrix metabolism and tissue repair, which was defined as the feature on counteracting the activity of M1 macrophages[73]. The two macrophage subpopulations induce different mediator and cytokine expression, and the changes in the levels of mediator and cytokine expression and their interaction ultimately affect the tissue response to chemical toxicants[73] (Figure 3). The inability to inhibit the inflammatory cytokine production prompts macrophages to compensate excess of inflammatory mediators by producing extracellular matrix components, which leads to fibrosis. Chronic inflammation is detrimenta，it increases the rate of somatic mutations in local tissue microenvironment and creates favorable conditions for the cell transformation[73].

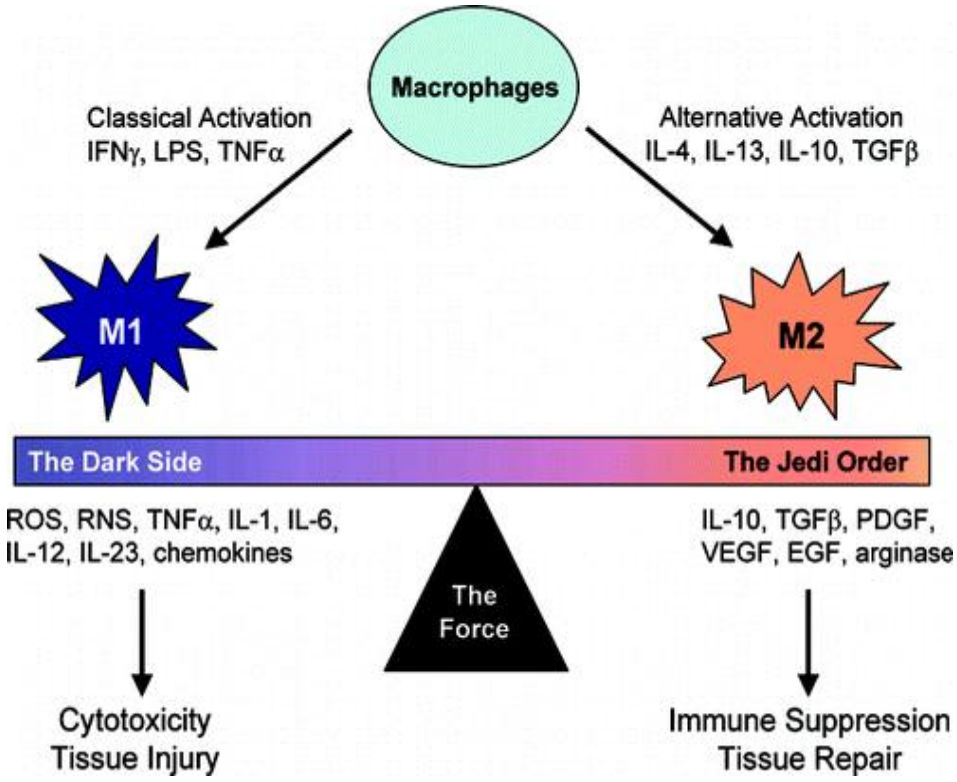


Figure 3. The role of macrophage in inflammation[73]. Permission from copyright © 2009, American Chemical Society

1.1.6 Macrophages and cancer

The development and progression of tumor goes through several stages including cell transformation, primary tumor growth, angiogenesis, cancer cell invasion and metastasis. Level of tumor invasion and metastasis define the levels of the aggressiveness of cancers[74]. Macrophages play crucial roles in tumor growth, tumor vascularization and in transition to the metastatic stage[75].

Tumor-associated macrophages (TAMs) are cells of the innate immune that infiltrate rapidly growing tumor and control biology of transformed cells, endothelial cells and other cells in tumor microenvironment. TAM control recruitment of different types of immune cells in tumor site and direct adaptive immune responses[71]. It is believed that TAMs can develop their functional polarization in the M1 and M2 direction. It was suggested that M1 exhibits mainly antitumor properties in the tumor microenvironment, where it induces the activation of the adaptive immune response and the inflammatory response of the body. In contrast, M2-like macrophages exhibit mainly suppressive immune function in the tumor microenvironment, which promote angiogenesis within tumor tissue and promote tumor growth and metastasis[71, 76]. In the majority of cancers types, TAM have pronounced M2-like phenotypes, however, TAM phenotypes are specific for each cancer type[71].

Tumor-associated macrophages: origin and markers

TAMs are derived from two types of cells: first, resident macrophages present in the tissues, second, form the circulating monocytes which are recruited into tumor site by tumor-produced growth factors and chemokines (such as CCL5, CCL2 and macrophage colony stimulating factor (M-CSF))[76-78]. CD68 is a general macrophage marker of TAMs. The main markers of classically activated M1 include HLA-DR, CD80/86 and iNOS. The typical markers of alternatively activated M2 mainly include surface receptors, such as macrophage mannose receptor 1 (CD206) and cluster of differentiation 163 (CD163). Stabilin-1 is also marker of M2 polarization of TAM[71]. Stabilin-1 is a scavenger receptor for tumor-related ligand cysteine-rich acidic secreted protein (SPARC) and an intracellular sorting receptor for the newly synthetized chitinase-like proteins SI-CLP and cartilage chitinase-3-like protein 2 (YKL-39)[71, 79-81] (Table 2).

Table 2. Biomarkers of TAMs (modified from[71])

Macrophage marker	Function	TAM polarization	Type of cancer
CD68	Transmembrane glycoprotein	General macrophage marker	Breast, colorectal, lung, ovarian, prostate
CD80	Immunoglobulin superfamily	M1	Colorectal, lung

CD163	Scavenger receptor for the hemoglobin–haptoglobin complex	M2	Breast, colorectal, lung
CD204 (MSR1)	Macrophage scavenger receptor	M2	Breast, colorectal, lung, prostate
CD206	Mannose receptor and C-type lectin	M2	Breast, colorectal, ovarian, prostate
HLA-DR	MHC class II cell surface receptor	M1	Lung, ovarian
iNOS	Enzymes catalyzing the production of NO from L-arginine	M1	Lung, ovarian
Stabilin-1	Scavenger and intracellular sorting receptor	M2	Breast, colorectal
YKL-39 (CHI3L2)	Chitinase-like protein, pro-angiogenic and monocyte chemoattractant	M2	Breast
YKL-40 (CHI3L1)	Chitinase-like protein, pro-angiogenic	M1	Breast, lung, prostate

The recruitment and polarization of macrophages in the tumor mass

During tumor development, macrophages are primary immune cells which infiltrate tumor lesions[82]. Cancer is frequently initiated at sites of chronic inflammation, and bone marrow derived monocytes are attracted to the growing tumor mass[83]. Chemotaxis of monocytes into the tumor is most frequently induced by colony stimulating factor 1 (CSF-1) and monocyte chemotactic protein-1 (MCP-1, CCL2)[84]. After monocytes migrate into tumor site, M-CSF and tumor cell-derived factors control their differentiation into TAMs. Tumor-derived factors include IL-10, prostaglandin E2 (PGE_2), IL-6, transforming growth factor β (TGF-β1), M-CSF and IL-4[84-86]. Most of tumor-derived mediators program M2-like TAMs. However, the analysis of M1-markers of TAM reveals that TAMs can have tumor-specific mixed phenotype[85, 87].

The phenotype and function of macrophages are mainly determined by transcription factors (TFs), epigenetic modifications and metabolic pathways[88]. In M1, the transcription factors that play a major role include interferon regulatory factor

1(IRF1), IRF3, IRF5, IRF8, Krüppel-like factor 4(KLF4), KLF6, nuclear factor of activated T cells 5 (NFAT5), signal transducer and activator of transcription 1 (STAT1) and transcription factor EB (TFEB)[89-97]. In M2, transcription factors include IRF4, IRF7, Maf transcription factor B (MafB), signal transducer and activator of transcription 3 (STAT3) and STAT6[98-102]. TFs are activated by different signals from the microenvironment, and cause functional programming of macrophages. Programming of TAMs is also controlled on the epigenetic levels by DNA methylation, histone modifications and microRNAs[103-106].

Pro-inflammatory and growth promoting properties of TAMs

It was found that macrophage express several soluble inflammatory cytokines and growth factors like platelet-derived growth factor (PDGF), TGF-β, epidermal growth factor (EGF), IL-1β and IL-6[107-109]. TAMs are major producers of EGF which stimulates tumor cells growth[110]. EGF produced by TAMs interacts with EGFR expressed by tumor cells, which promote their proliferation. Alternatively, activation of STAT3 pathway in TAMs results in the production of IL-6. As a pro-inflammatory cytokine, IL-6 can cause proliferation of malignant cells[111].TGF-β has controversial function in carcinogenesis[112]. Most carcinoma cells respond to TGF-β by proliferation and epithelial plasticity. TGF-β provides beneficial conditions for malignant tumour invasion and dissemination, stem cell properties and resistance to therapy[113]. TGF-β might inhibit tumor growth by reducing IL-6 secretion. Upregulated expression level of TGF-β enhance invasiveness of epithelial cancer cells and metastasis[114]. By producing of TGF-β, tumor cells also induce the production of interleukin-1 receptor-associated kinase (IRAK-M), a negative modulator of Toll-like receptors (TLR) signaling in TAM[115]. IL-1β also contributes to many physiological and pathological processes in the human body. It has both promoting and inhibiting effect cancer development and progression. IL-1β contributes to metastasis by regulation of epithelial-mesenchymal transition (EMT), cancer cell stemness, and migration/invasion[116]. During cancer chemotherapy, the role of IL-1β is uncertain and may promote or inhibit the anti-tumor immune response, therefore, for individuals undergoing cancer chemotherapy, the role of IL-1β should be analyzed specifically depending on the drug used[116].

Immunosuppressive properties of TAMs

TAMs can secrete IL-10 and TGF-β that inhibit T-cell responses[117]. STAT3 is involved in the TAM-mediated suppression of adaptive immunity. STAT3 can suppress STAT1-mediated Th1 antitumor immune responses, and STAT3 can also enhance the derivatization of immature myeloid cells with tumor immunosuppressive activity[118]. So, in different human tumors, activated STAT3 not only supports tumor growth, angiogenesis and metastasis, but also suppresses anti-tumor immunity as well[118]. In addition, TAMs secrete CCL17 and CCL22 that preferentially attract T lymphocytes without cytotoxic functions towards cancer cells (Th2 and Treg cells)[119-121]. And, in ovarian cancer, TAMs are major producers of CCL18. CCL18 is capable of recruiting naive T cells[122]. Therefore, TAMs suppresses the antitumor adaptive immune response.

Angiogenesis promoting properties of TAMs

Macrophages are essential for the induction of the angiogenic switch, as it has been first identified in murine breast cancer model[123]. Several macrophage derived molecules, such as TNF-α, IL-8 and vascular endothelial growth factor (VEGF), function as pro-angiogenic factors. Other molecules, including macrophage metalloprotease 2(MMP-2), MMP-7, MMP12, MMP-9 and cyclooxygenase-2 (COX-2) adjust the process of angiogenesis[124-126]. VEGF was found to be secreted by a specialized subpopulation of TAMs in hypoxic regions of breast cancer[127]. In murine tumor models, cells which express VEGF can be identified by Tie2 expression[127]. It was found in experimental mouse models that macrophage depletion can dramatically reduce vessel density and tumor growth[127].

The chitinase-like protein family comprises new type of regulators of angiogenesis. YKL-40 belongs to this family that also includes other members, such as YKL-39 (3-like chitinase 3-like 2, CHI3L2) and SI-CLP. In the study of breast and colon cancer, the upregulated expression level of YKL-40 induced angiogenesis and development of tumor[128,129]. With the involvement of integrin $\alpha_v\beta_3$ and membrane receptor syndecan-1 (S1), YKL-40 functions through activating downstream focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK)[129]. In addition, upregulated expression level of YKL-40 is related to vasculogenesis in human breast cancer. All of these evidences suggest that YKL-40 promotes angiogenesis in cancer[129, 130].

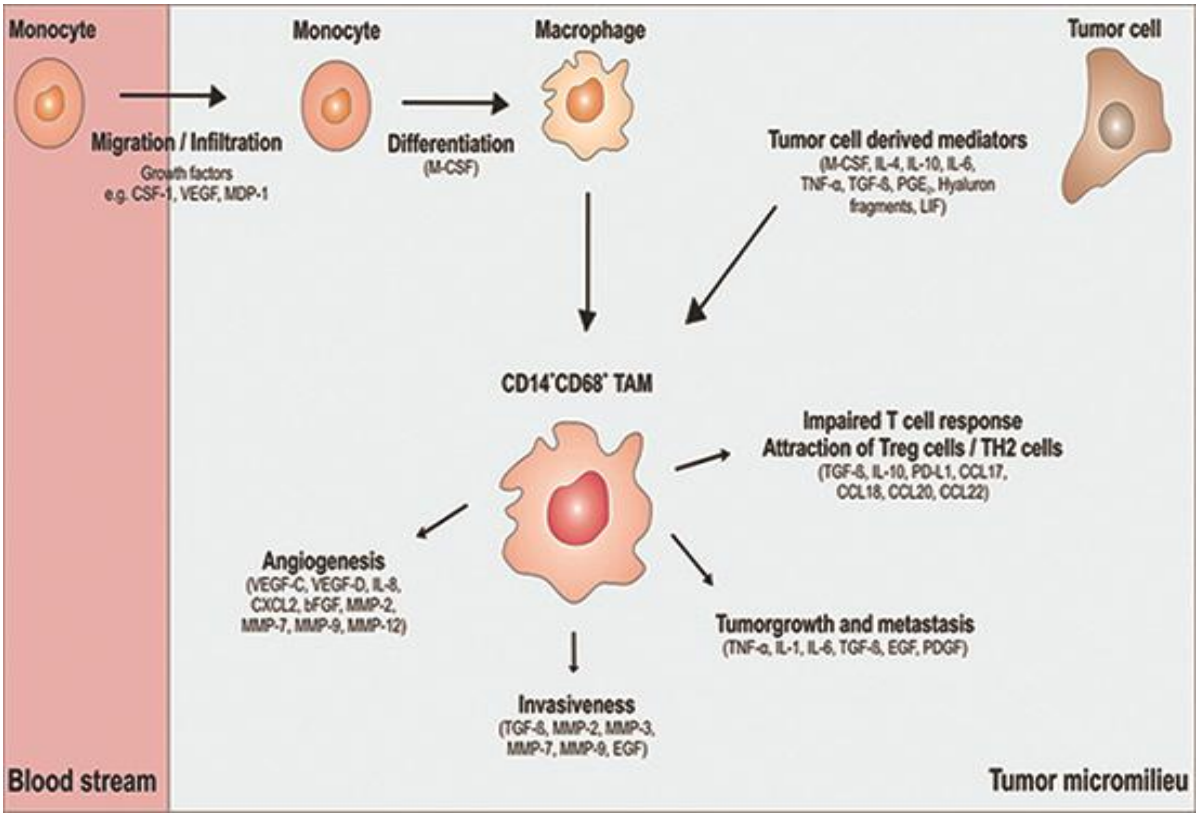


Figure 4. Differentiation and activity of TAMs in tumor microenvironment[131]. In the presence of growth factors and chemokine, circulating monocytes are recruited into the tumor tissue. Monocytes differentiated to macrophages under influence of local M-CSF. Soluble tumor derived factors trigger the differentiation process of macrophages into TAMs, which promote tumor angiogenesis, tumor growth and metastasis. TAMs can promote Treg cell aggregation, exert local immunosuppression or directly suppress T cell responses. Permission from copyright American Physiological Society.

Cancer cell invasion and metastasis promoting properties of TAMs

Invasion is a main feature of malignant tumor cells, and is also a necessary condition for tumor metastasis. It was found that tumor cells recruit macrophages by producing CSF-1 and TNF-α, which in turn produce matrix metalloproteinases, cathepsins and other proteolytic enzymes which cleave extracellular matrix[132]. In the tumor microenvironment, the extracellular matrix (ECM) is present around the tumor cells and inflammatory cells[133]. The ability of tumor cell to invade and to metastasize depends on how they interact with ECM[133]. TAMs reprogrammed by cancer cells are able to degrade ECM by producing several enzymes, including MMP-2 and MMP-9, which support tumor invasion[134]. Intravital imaging has indicated that macrophages present in the perivascular area of the breast are involved in the invasion of tumor cells[135]. Secreted tumor-derived chemotactic factors induce macrophages to move to the invasive front, where they enhance the ability of tumor

cells to invade surrounding tissues by the production of chemotactic factors including epidermal growth factor (EGF)[135]. When EGF signaling is inhibited or macrophages are depleted, the process of tumor cell entry into the circulation is inhibited[135, 136]. In the original tumor microenvironment, TAMs are able to promote tumor metastasis with two mechanisms. The first mechanism, TAMs generate proteases, such as urokinase plasminogen activator (uPA) and MMP2 and 9, which dissolve the tumor peripheral barrier and enhance escape of tumor cell[137, 138]. The second mechanism, TAMs secrete the Wnt ligand Wnt5a, which stimulates the non-classical planar cell polarity in cancer cells to contribute to invasion[139]. Investigation of TAMs subpopulations which are distributed around tumor vessels and are involved in tumor cell infiltration of surrounding tissues, it was further demonstrated that TAMs are able to express Wnt7b and increased expression of Wnt7b significantly correlates with the occurrence of lymph node metastasis[140]. It has been concluded that Wnt-signaling pathway are critical for the TAMs-mediated angiogenesis and tumor invasion[140].

1.2 TGF-β

1.2.1 TGF-β: overview

Transforming growth factor beta (TGF-β) participate in the regulation of proliferation and differentiation in many cell types. As an important cytokine, TGF-β functions in a wide range of diseases[141].

TGF-β superfamily

TGF-β superfamily comprises a range of proteins, including the TGF-β subfamily, growth differentiation factors (GDFs), glial cell line-derived neurotrophic factor (GDNF) family, the activin/inhibin family, and bone morphogenetic proteins (BMPs)[142]. TGF-β superfamily members are involved in the regulations of various physiological and pathological processes, including growth, development, tissue homeostasis and regulation of the immune system functions. TGF-β signaling acts in diverse cellular responses, such as cell extracellular matrix remodeling, differentiation, and proliferation[142].

Bone morphogenic proteins (BMPs) play a role in cytoskeletal structural alterations and also participate in the induction of movement of various cell types to areas of inflammation[143]. TGF-β/BMP pathway was found to be the most important signal system which includes SMAD and non-SMAD pathways[144, 145]. Dysfunctions of

TGF-β/BMP signaling pathway may contribute to a variety of diseases[146]. Previous studies have confirmed that the TGF-β signaling system play a dual role in the organism. In some environments, the TGF-β signaling pathway promotes cancer cell proliferation, however, in other environments, it is involved in regulating cell death, and the balance of these signaling pathways is of great importance. The TGF-β signaling system also contributes to the regulation of cancer cell metastasis, which can be considered by the design of metastasis inhibition therapy[147].

The role in tumor cell proliferation

The cell cycle includes four phases: G1 (gap 1) phase, S phase, G2 (gap 2) phase and M phase. TGF-β promotes the production of cell cycle protein-dependent kinase (CDK) inhibitors (CDK-IS). In neurons, epithelial cells and hematopoietic cells, TGF-β promotes the expression of three inhibitors, including P15INK4B, P21CIP1 and P27KIP1, which inactivate the cyclin-CDK complex. Consequently, the cell cycle process is interrupted, the cell cycle stays in the G1 phase, and the cells are unable to enter the S phase[148-151]. C-MYC induces cell division and leads to a significant increase in cell proliferation. TGF-β down-regulates expression of the C-MYC oncogene, and therefore suppresses cancer cell proliferation. Inhibitors of DNA binding (Id) family proteins, especially Id1, Id2 and Id3, are involved in the G1 to S phase transition of the cell cycle. TGF-β reduces the production of the Id protein family, that negatively affects cell proliferation. In addition, by limiting the function of the kinase of P70 S6, TGF-β contributes to cell cycle arrest in the G1 phase[152-154].

The role on tumor metastasis

Epithelial-mesenchymal transition (EMT) is a fundamental component of many pathological processes, and in case of cancer, EMT is a key process enhancing migratory and metastatic potential of tumor cells[155, 156]. Through the EMT process, the polarity of epithelial cells is lost, tight junctions and adhesions between cells are lost, allowing the cells to gain the ability to migrate. This transformation enhances the migrating and invading ability of cancer cells, promoting cancer metastasis[157]. TGF-β participates in the EMT process in tumor cells and TGF-β enhances tumor invasion through the SMAD signaling pathway[158].

The role in immunosuppression

Cytotoxic CD8+ T cells release many cytokines that cause cancer cell apoptosis. However, TGF-β inhibits the expression of these cytotoxic genes. TGF-β promotes tumor cells to evade the body's immune surveillance, leading to pro-cancer effects[159]. Dendritic cells (DCs) are antigen-presenting cells in tumors, they are involved in antitumor immunity by inducing T-cell responses[160]. TGF-β promotes the production of the differentiation inhibitor Id1 which inhibits the differentiation of DCs, leading to immunosuppression[161]. NK cell is another important anti-tumor immune cell type[88], which directly discriminates tumor cells, at the same time, mediates cytotoxic responses[162]. By downregulating the expression of IL-15, TGF-β prevents the activation of NK cells[163]. In addition, TGF-β promotes differentiation of M2-type of macrophages[164, 165]. M2 macrophages produce cytokines to promote tumor development[166].

The role in angiogenesis

Growing tumor require enhanced energy and oxygen supply that is provided by angiogenesis[167]. TGF-β signaling system is closely related to the activity and function of EC[168]. TGF-β promotes the production of connective tissue growth factor and vascular endothelial factor, regulates formation of capillaries, while the enhanced blood supply leads to a more rapid tumor growth[169, 170]. In non-small cell lung cancer, prostate cancer, hepatocellular carcinoma and renal cell carcinoma, increased level of TGF-β expression correlates with poor prognosis，that is related to increased angiogenesis and increased tumor invasiveness due to TGF-β[171-173].

1.2.2 TGF-β and macrophages

Macrophages are key regulatory cells in the pathogenesis of many diseases where TGF-β is involved. TGF-β helps monocytes gather into tumors[174] and induces monocytes to differentiate into macrophages[175]. Alternative activation macrophages (M2) is induced by Th2 cytokines, like IL-10, IL-13, IL-4, and by anti-inflammatory factor, like glucocorticoids (GCs)[176-178]. Stimulation of macrophages by a combination of IL-4 and TGF-β1 results in the expression of IL-17 receptor B (IL17RB) which functions as a receptor of Th2-related cytokine IL17E/IL-25[179].

Induced by IL-4 and TGF-β, macrophages differentiate into specific phenotypes which are involved in the formation of atherosclerotic lesions in disease[180, 181]. Tumor cells secrete large amounts of IL-4 and TGF-β to regulate function of monocytes/macrophages supporting tumor growth and metastatic spread[182]. TGF-β

activates signaling cascade through cell surface receptors (TGF-RII and TGF-RI). When TGF-β interacts with the complex of TGF-βRII and TGF-βRI, the TGF-βRII phosphorylation is induced, and then TGF-βRII activates TGF-RI[183]. By the process of phosphorylation of Smad2 and Smad3 (R-Smads), TGF-RI transmits biological signals. By binding to Smad4 to form a heterodimeric complex, R-Smad is transported into the nucleus[184]. More recently it was found that TGF-β1 can activate not only Smad2/3, but also Smad1/5 pathway in macrophage[185]. Activation of Smad1/5 signaling pathway promotes the expression of the hepcidin antimicrobial peptide (HAMP) gene which is involved in atherosclerosis. In addition, hepcidin has also been shown to participate in the regulation of iron metabolism and the expression of the receptor of plasmin urokinase activator (PLAUR) which is related to plaque instability in macrophages[185]. In M2 macrophages, upregulated expression of TGF-1 gene is involved in transcriptional and signaling regulation, this process may initiate subsequent effects of TGF-β and (Th2) immunomodulatory functions, affecting lipid metabolism and can promote atherosclerosis formation[186].

1.3 YKL-39

YKL-39 belongs to human Glyco_18 domain protein family[187].YKL-39 functions as an important factor on disease progression by initiation of autoimmune response and tissue remodeling[188].

Combination of IL-4 and TGF-β in contrast to single stimulations was shown to induce expression of YKL-39[189]. Increased mount of YKL-39 are also detected in diseases, such as atherosclerosis, neurodegenerative diseases and tumors[190-192]. It is a key process to gather monocytes into tumor tissue on all stages of tumor progression[193]. In vitro analysis of biological activities of YKL-39 revealed that YKL-39 can act as chemotactic factors for monocytes and can also induce angiogenesis[194]. These two activities can support tumor development[194]. In breast cancer, elevated levels of YKL-39 gene expression positively correlated with the metastatic relapse after neoadjuvant chemotherapy. YKL-39 has been proposed as a biomarker for metastasis relapse in breast cancer after chemotherapy[194, 195].

1.4 Stabilin-1

Stabilin-1 is multifunctional scavenger receptor and homeostatic receptor[196]. It was first discovered as an MS-1 antigen expressed on the sinusoidal endothelial cells in human spleen[197]. Due to the difference in protein structures, scavenger receptors

are categorized into 8 types, which are labeled with A-H[198] (Fig 5). Scavenger receptors are responsible for the removal of “unwanted self" macromolecules, pathogens and dying cells[26]. Most scavenger receptors on macrophages possess the function of scavenger receptor-mediated endocytosis of lipoproteins, and some family members have also been proven to be involved in the phagocytosis of bacteria and apoptotic cells by macrophages[199]. The structure of a scavenger receptor determines its ability to bind a wide range of ligands, and this property of the scavenger receptor plays a critical role in the elimination of inflammatory substances and tissue turn-over[199]. Scavenger receptors can have both tumor-promoting and tumor-inhibiting activities, depending on the tumor context[200].

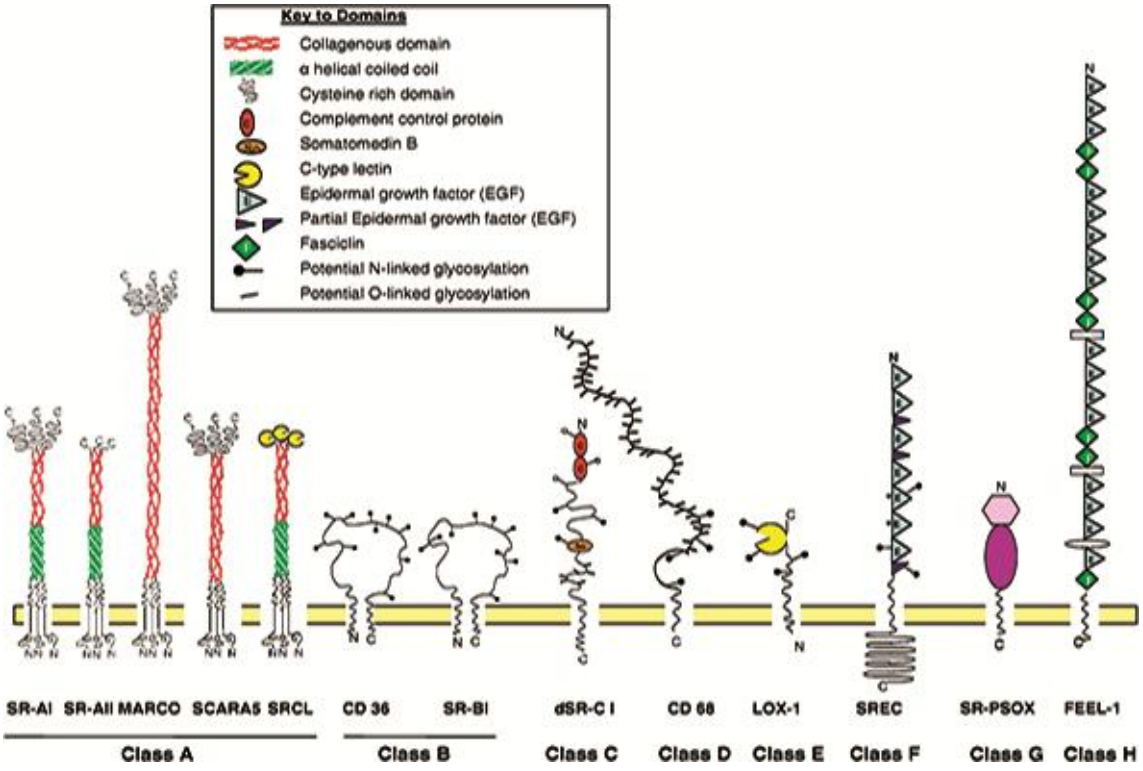


Figure 5. Different classes of scavenger receptors. Specific domains are highlighted by the codes indicated within the figure[198]. Permission from copyright © 2007 Elsevier Inc.

Stabilin-1 is a protein encoded by the STAB1 gene which is located on the chromosome 3p21.1. Stabilin-1 consists of 7 fasciclin, a C-type lectin-like hyaluronan-binding Link module, 16 epidermal growth factor (EGF)-like and 2 laminin-type EGF-like domains[196].

Fasciclin domain (FAS1 domain) was defined as an extracellular domain, which consists out of about 140 amino acid residues, and it is one of ancient cell adhesion domains of plants and animals[201]. The domains of FAS1 are distributed in various

secreted and membrane-anchored proteins, which are usually anchored by GPI and include several domains, such as a single FAS1 domain, a tandem array of FAS1 domains, or FAS1 domain(s) interspersed with other domains[201]. Growth factor (EGF)-like domain is present in many animal proteins and consists out of 30 to 40 amino-acid residues, and most typically EGF-like domain are included in the extracellular domain of membrane-bound proteins or are present in secreted proteins(Fig 6)[196].

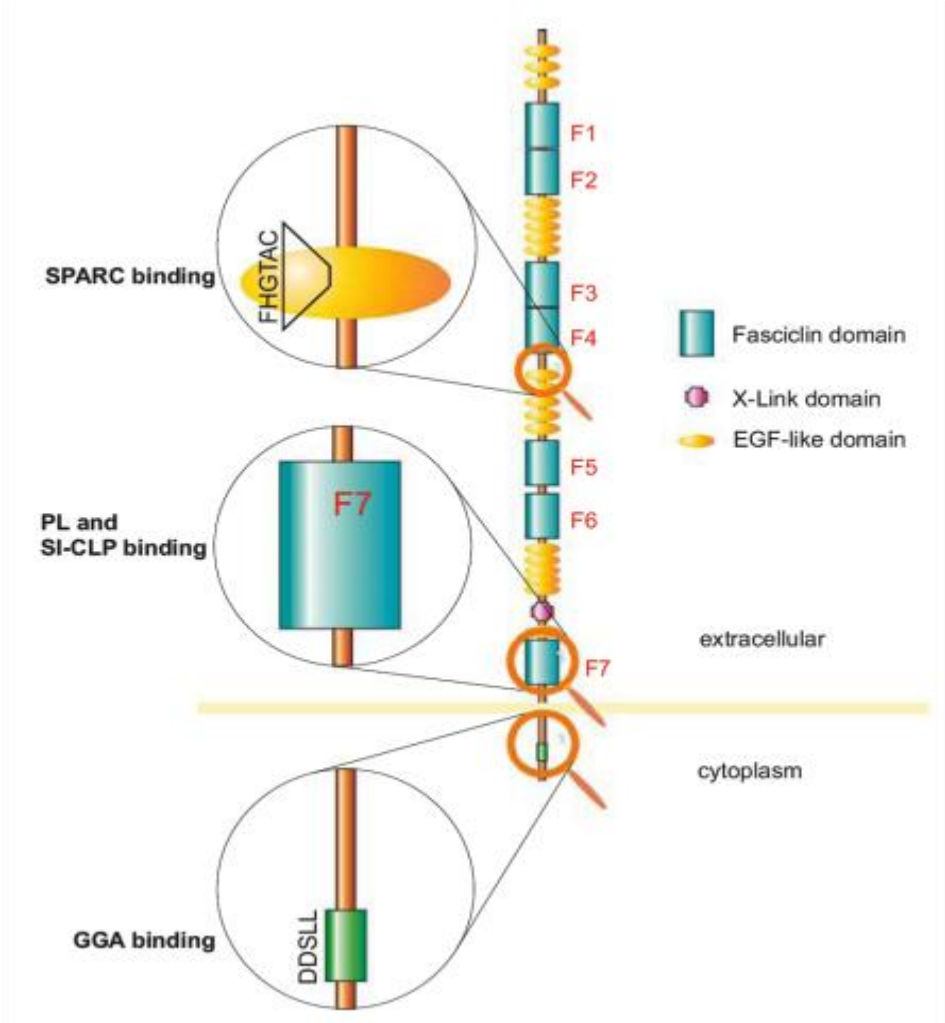


Figure 6. Schematic representation of stabilin-1 structure and ligand-binding sites[196]. Large extracellular domain contains seven fasciclin domains, F1-F7, multiple EGF-like domains and X-Link domain. FHGTAC is a SPARC binding site located within the EGF domain between F4 and F5. PL and SI-CLP bind to F7. The DDSLL motif is the binding site for GGA adaptors. Permission from copyright American Physiological Society.

In the healthy organs and tissues, expression of stabilin-1 has been predominantly found on tissue macrophages and sinusoidal endothelial cells. During chronic inflammation and tumorigenesis, stabilin-1 expression can be elevated both on macrophages as well as on various types of endothelial cells[196]. Stabilin-1 plays a

role in two main intracellular trafficking pathways: in the receptor-mediated endocytosis and in the intracellular sorting. In alternatively activated macrophages, acetylated low density lipoprotein (acLDL) and secreted protein, acid and rich in cysteine (SPARC) fractions are internalized and transported to the lysosomal degradation pathway by stabilin-1[202, 203]. SPARC is a soluble component of extracellular matrix that affects the level of cancer cell adhesion and motility[204, 205]. Moreover, stabilin-1 participates in the intracellular sorting and lysosomal transport of the novel stabilin-1-interacting chitinase-like protein (SI-CLP)[206]. Stabilin-1 was also found to be involved in cell adhesion and transmigration, such as tumor cells, leukocytes and lymphocytes[196, 207-209]. In general, stabilin-1 regulates the context of extracellular cytokine and growth factor composition by the internalization of extracellular ligands and transport to the secretory pathway of intracellular ligands[210]. It was demonstrated that expression of stabilin-1 was significantly increased in macrophages cultured in vitro under stimulation with the combination of IL-4 and dexamethasone. When IFN-γ was used as an inducer to stimulate macrophages cultured in vitro, expression of stabilin-1 was significantly inhibited. Therefore, the combination of IL-4 and dexamethasone is used for in vitro induction of stabilin-1 expressing alternatively activated macrophage phenotypes[211].

In previous study, stabilin-1 was found to be expressed on CD163 positive macrophages in patients with bacterial infection caused by *Tropheryma whipplei* (Whipple's disease)[212]. Alternative activation of macrophages during this pathology may prevent effective clearance of *Tropheryma whipplei* from organism[212]. Stabilin-1 was also expressed on subpopulation of LYVE-1 positive tissue macrophages in murine tumor models and in the excision wound healing model[213]. Sstabilin-1 positive macrophages were found on the periphery of the tumor in the murine cell line B16F1 melanoma, whereas only single stabilin-1 positive macrophages were localized inside the tumor mass[214].

1.5 CD68

Cluster of Differentiation 68 (CD68) is a 110-kDa membrane protein[215]. It is closely associated with the family of lysosomal-associated, mucin-like membrane proteins (LAMPs)[215]. It is involved in the processes of endocytosis and lysosomal transport, that belong to the most important biological activities of macrophages[216]. By binding to lectins or selectins with tissue and organ specificity, CD68 promotes the aggregation of macrophage subpopulations to specific tissues or cells. Moreover,

CD68 enhances the ability of macrophages to interact with the surfaces of other cell types; for example, macrophages have been observed to move on vascular endothelium under the induction of CD68 and selectins[216]. CD68 is continuously recycled between plasma membrane and intracellular vesicles, including endosomes and lysosomes, and the ability of macrophages to interact with selectin-possessing stroma or other cells is enhanced through this process. In addition, CD68 can be proteolytically cleaved to form soluble CD68, which is released into the blood and urine[217]. CD68 is used as a basic biomarker to label majority of resident and monocytes-derived macrophages in the tissues. Among all CD68 monoclonal antibodies identifying the CD68 antigen, KP1 and PG-M1 are the most useful antibodies in the field of diagnostic pathology, and both KP1 and PG-M1 have consistent immunoreactivity when applied to paraffin-embedded sections[218].

1.6 CD163

As a cell-surface glycoprotein receptor, CD163 has been identified on the surface of most of macrophage subpopulations existing in tissues[219]. Functionally, CD163 mediates endocytosis of the hemoglobin-haptoglobin complex (Hb-Hp)[219]. It has been suggested that CD163 prevents Hb-induced oxidative tissue damage under the conditions of excessive hemolysis, and it induces elimination of free Hb from the circulation[219]. CD163 is expressed on macrophages in the bone marrow and on other subpopulations of macrophages involved in erythroid island formation. CD163 may be involved in modulating erythroid formation as an erythrocyte adhesion receptor[219]. Apart from these homeostatic functions, CD163 can be involved in regulation of inflammation and host defense, and the combination of CD163 and CD163 monoclonal antibodies can induce the expression of inflammatory factors, including nitric oxide, interleukins IL-1β, IL-6, and IL-10[220-222].

CD163 on macrophage can be involved in phagocytosis, and mediates the binding of macrophages to Gram-negative or Gram-positive bacteria[223]. It has been shown that the peptide motif present in the structural domain of CD163 facilitates the early process of erythroblast binding and regulates the capability of bacterial binding[223].

Significantly upregulated expression levels of CD163 have been mainly found in mature tissue macrophages, such as those in the liver, spleen, and thymus. In addition, large numbers of CD163-positive macrophages are also found in various inflammatory tissues[223]. In response to stimuli, such as LPS or IL-4 and IL-13, the expression levels of CD163 are significantly elevated on M2 macrophages;

therefore, CD163 is considered by some researchers to be a marker of M2 macrophages[224].

1.7 CD206

Cluster of Differentiation 206 (CD206) is defined as a mannose receptor. It is mainly distributed on the surface of dendritic cells, macrophages, and skin cells, including human dermal fibroblasts and keratinocytes[225]. The receptor plays an important role in both the innate and adaptive immune systems[225]. CD206 is an endocytic and phagocytic receptor, it can recognize various microorganisms, such as parasites, fungi, viruses, and bacteria[226, 227].

During the development of an adaptive immune response, the mannose receptor (MR) can present mycobacterial antigens to T cells[228]. By the MR-mediated endocytic pathway, T cells can also be successfully activated, indicating that MR can be applied in vaccine therapy for tuberculosis and other infectious diseases[228].

CD206 is important for the maintenance of homeostasis. It can bind with many endogenous ligands, including extracellular matrix components, normal as well as tumoral mucins, enzymes, cell membranes, and hormones. These endogenous ligands can trigger an anti-inflammatory and tolerogenic process in dendritic cells and macrophages[227]. CD68, CD163, and CD206 biomarkers are used largely for immunohistological characterization of macrophages, although flow cytometry analysis with these markers is also possible. Additionally, an increasing number of experimental results have confirmed that CD163 and CD206 can be used as markers of M2 macrophages in tumors[71].

1.8 LYVE-1

Lymphatic Vessel Endothelial Receptor 1 (LYVE-1) is a receptor homologous to CD44, which is primarily distributed on the lymphatic endothelial cells[229]. The lymphatic system is a part of the circulatory system that includes a network of lymphatic vessels. The primary function of the lymphatic system is to collect leaked plasma and interstitial fluid and return them back to the blood[229]. This process involves two aspects, i.e., fluid is transported through lymph vessels, and dissolved macromolecules in lymph nodes and spleen are taken up and degraded. Among these macromolecules, hyaluronan (HA) is a large mucopolysaccharide (10^5–10^7 Da) co-polymer of N-acetyl D-glucosamine and D-glucuronic acid, and it is distributed mainly in the extracellular matrix of tissues throughout the body[229, 230].

LYVE-1 is a type of HA transporter, and it functions as a co-receptor rather than as a primary receptor in the process of HA uptake in the liver and spleen sinusoidal endothelium[230]. LYVE-1 is co-expressed with HARE (stabilin-2) in liver and spleen sinusoids, suggesting that LYVE-1 is involved in the metabolism process of HA in those particular organs[230].

On the lymphoid surface, the process of LYVE-1-induced HA aggregation may contribute to the development of immune response or cancer cell migration. In the presence of LYVE-1 in vitro, HA can interaract with CD44 to form a substrate, which directs the process of transmigrating CD44+ leukocytes or tumor cells[231]. Apart from the liver and lymphatic endothelium, various types of cells, including Kupffer cells, renal epithelium, cortical neurons, and the islets of Langerhans, can express LYVE-1[232].

The process of initial metastatic spread via the lymphatics has been identified in various types of carcinomas. Therefore, for cancer models and naturally occurring tumors, the application of specific LYVE-1 antibodies to the lymphatics as a marker for lymphangiogenesis has been proven useful and effective[233].

1.9 The aims of the study

- Establishment of test-system for the analysis of specificity of newly generated anti-YKL-39 antibodies.
- Analysis of the specificity of newly generated polyclonal and monoclonal a-YKL-39 antibodies.
- Analysis of TGF-β effect on the expression of YKL-39 in different subpopulations of human macrophages in the presence and absence of Th1 and Th2 cytokines and glucocorticoids.
- Analysis of intracellular localization of YKL-39 in M2 macrophages and its co localization with stabilin-1.
- Examination of expression of YKL39 in TAM subpopulation in glioblastoma samples.

2 Materials and methods

2.1 Chemicals, materials and reagents

Table 3. Chemicals, materials and reagents.

Products	Company
Acrylamide / bis acrylamide 30%	Sigma
AEC chromogen	DAKO
Ammonium persulfate (APS)	Cell Biolabs, Inc.
Biocoll separating solution	Fermentas
Bovine Serum Albumin (BSA)	Roche
CD14 Micro Beads	Fermentas
Citrate buffer pH 6.0	ZYTOMED
DAKO Fluorescent Mounting Medium	DAKO
Dexamethasone	Invitrogen
4′.6-diamidino-2-phenylindole (DAPI)	Bio-Rad
(DMEM) + GlutaMAX	Invitrogen
DRAQ 5	Biochrom AG
10x EDTA buffer pH 8.0	ZYTOMED
Ethanol	J.T.Baker
Faramount mounting medium	DAKO Cytomation
Filter paper, 3 mm	Merk
Fetal calf serum (FCS)	Biochrom AG
FuGENE HD transfection reagent	Roche
Gel Code Blue stain reagent	Pierce
IL-4	Peprotech
Isopropanol	Merck
Laemmli sample buffer	Bio-Rad
Loading dye, 6x	Fermentas
MACS separation columns	Miltenyi Biotec
ß-Mercaptoethanol	Sigma
Methanol	Merk
$MgCl_2$ 100 mM	BIORON
Nitrocellulose transfer membrane	Whatman

Page Ruler Plus Prestained Protein Ladder (10-250 kD)	Fermentas
Parafilm	American National Can
Paraformaldehyde (PFA)	Fluka
PBS Dulbecco, w/o Ca^{2+}, Mg^{2+}	Biochrom AG
Percoll	Biochrom AG
Phosphate buffered saline **(**D-PBS), sterile 1x	Sigma
Primers	Eurofins Genomics
Sodium dodecyl sulphate (SDS) 10%	Bio-Rad
TaqMan probes	MWG Biotech
Taq polymerase 5 u/µl	BIORON
TEMED	Genaxxon
TGF-β	Peprotech
TGS (Tris/Glycine/SDS) buffer 10x	Bio-Rad
Tri-EDTA buffer pH 9.0, 10x	ZYTOMED
Tris-Acetate EDTA (TAE) buffer, 10x	Eppendorf
Triton X-100	Sigma
Trypan blue solution	Sigma
Trypsin/EDTA solution, 0.05%	Biochrom AG
Tween 20	Sigma
X-VIVO 10 medium	Invitrogen
96 well white plates with flat bottom	Biowhittaker

2.2 Kits

Table 4. Kits.

Kits	Company
E.Z.N.A. total RNA Kit I	Omega bio-tek
Fermentas Revert Aid cDNA synthesis Kit	Thermo

2.3 Equipments

Table 5. Equipments.

Equipment	Company
Agarose electrophoresis unit i-Mupid	Erogentec
Autoclave VX-95	Systec

Blot chambers	Bio-Rad
Cell counter	Beckmann
Cell culture flasks	Greiner
Centrifuge 5415 D	Eppendorf
Centrifuge 5804 R	Eppendorf
Centrifuge 6K15	Sigma
Centrifuge RC 5C Plus	Sorvall
Confocal laser scanning microscope	Leica
Deep freezer (-80 C)	Sanyo
Eppendorf concentrator 5301	Eppendorf
Freezer (-20 C)	Liebherr
Ice machine	Scotsman AF100
Incubator 37°C	Edmund Bühler GmbH
Laminar flow hood	Nuaire
Magnetic stirrer MR3000	Heidolph
Monocyte Isolation Kit II	MACS Miltenyi Biotec
Petri 6-Well Cell Culture Plates	Nunc
Pipets	Gilson, Eppendorf
Pipet Controller	Accu Jet Pro, Brand
Real-time PCR instrument Mx3005	Stratagene
SDS-PAGE power unit Power-Pac 200	Bio-Rad
SDS-PAGE unit	Biometra
Shaker KS 260 basic	IKA
Thermomixer comfort	Eppendorf
Tubes	Falcon
Vortex Genie 2	Scientific Industries
Water bath	Memmert
Water bath SW-21	Julabo
X-ray film processor X-OMAT 480RA	Kodak

2.4 Buffers and solutions

2.4.1 Buffers for immunohistochemical and immunofluorescent staining

Citrate buffer (pH 6.0, 1x) Citrate buffer concentrate (pH 6.0, 10x) was diluted

	1:10 with deionized water, and pH value was measured with pH meter.
Tri-EDTA buffer (pH 9.0, 1x)	Tri-EDTA buffer concentrate (pH 9.0, 10x) was diluted 1:10 with deionized water, and pH value was measured with pH meter.
EDTA buffer (pH 8.0, 1x)	EDTA buffer concentrate (pH 8.0, 10x) was diluted 1:10 with deionized water, and pH value was measured with pH meter.
0.1% Tween 20/PBS	1 ml Tween 20 was filled up to 1000 ml with PBS

2.4.2 Buffers for electrophoresis and Western blotting

Running buffers for agarose gel electrophoresis

1x TAE buffer (1 liter)	2 M TRIS-Acetate, 0.05 M EDTA, pH = 8.3
	20 ml 50x TAE buffer
	20 µl Ethidium bromide (EtBr)
	Filled up to 1000 ml with deionized water

Transferring buffer for Western blotting

Transferring buffer	100 ml 10x TGS
	200 ml Methanol
	700 ml distilled water

Running buffer for Western blotting

	100 ml 10x TGS
	900 ml distilled water

Loading buffer for Western blotting

1x Laemmli sample buffer (10 ml)	9.5 ml Laemmli buffer
	0.5 ml β-Mercaptoethanol

2.4.3 Solutions for immunological methods

Blocking solutions

For Western blotting	6% non-fat milk powder in PBS
For immunofluorescence	3% BSA in PBS
For immunohistochemistry	3% BSA in PBS

Washing solutions

For Western blotting	0.1% Tween 20/PBS

For immunohistochemistry	PBS
	0.1% Tween 20/PBS
For immunofluorescence	PBS
	0.1% Tween 20/PBS
Staining substances	
For immunohistochemistry	AEC-chromogen
For immunofluorescence	DAPI, DRAQ 5

2.4.4 Cell fixation solutions

Fixation solution (4% PFA)

40 g PFA was weighted, then added to 700 ml PBS in a 1 L beaker (it was prepared under a fume hood); pH value was 11.4 (adjusted by 1 N NaOH); the solution was mixed with a magnetic stirrer for 1 hour to dissolve PFA completely; appropriate amount of 1 N HCl was added until the pH values reached 7.4. It was filled up to 1 L with PBS; the pH value was checked again; the solution was filtered with 0.22 µm filter and stored at -20°C.

Fixation solution (2% PFA)	4% PFA was diluted 1:1 with PBS.
Fixation solution (Acetone)	100% Acetone
Permeabilization solution	0.5% Triton X-100 in PBS

2.4.5 Buffers for Western blotting

"Towbin" buffer	700 ml distilled H_2O
	200 ml methanol
	100 ml 10x TGS

2.5 Cell culture related methods

2.5.1 Cells applied for the study

Cell lines and primary cells used in the study are shown in Table 7. DMEM complete medium used in the study contained 10% FCS and 100 µg/ml Penicillin/Streptomycin.

Table 6. List of cells used in the study.

Name of the cell line or primary cells	Growth medium	Growth conditions	Cell type
TS/A	RPMI complete medium with 10% FCS and 100 µg/ml	5% CO_2	Mouse mammary adenocarcinoma cell

	penicillin/streptomycin		line
Primary human macrophages	X-Vivo 10 serum free medium	7.5% CO_2	Primary human cells

2.5.2 Antibiotics

For cell culture:

Penicillin/Streptomycin solution (10000 U/ml penicillin, 10000 µg/ml streptomycin) was applied at 1:100 dilution.

2.5.3 Thawing of cell lines

- RPMI complete medium (10% FCS/1% penicillin/streptomycin) was prepared and warmed up at 37ºC water bath.
- 5 ml of complete medium was added in 50 ml falcon tube.
- Complete medium was prepared in flasks (12 ml in T75 flask).
- Cells were picked up from liquid nitrogen tank.
- Cells were thawed at 37°C in water bath for several seconds.
- Cells were pipetted in falcon tube, and 10 ml PBS was added to wash DMSO away.
- The falcon tube was centrifuged 1200 rpm at room temperature for 8 min.
- Supernatant was discarded.
- Pellet was resuspended in 1-2 ml medium and transferred into the new flask.
- Cells were cultivated at 5% CO_2 at 37°C until they reached proper confluence.
- If there were too many dead cells on next day, just change medium.

2.5.4 Splitting of adherent cells

- RPMI complete medium and trypsin were prepared and warmed up at 37°C (water bath).
- 15 ml falcon tubes were labeled and 3 ml of complete medium was added.
- The flasks with cultured cell were checked under microscope, if confluence of cells reached 70%-90%, the cells were split. If not, medium should be changed and kept in incubator until cell reached proper confluence.
- The old medium was aspirated from flasks.
- The cells were washed 1-2 times with 10 ml PBS (w/o Mg^{2+} and Ca^{2+}).
- 3 ml trypsin was added into flask.
    - Trypsin destroys the contact between cells.

    - Trypsin should be warmed up at 37°C before it was applied to the cells.
- The flasks were incubated at 37°C for 3 min, then checked under microscope to ensure all cells had been detached from bottom, or they should be kept in incubator for two more minutes.
- 5 ml PBS was added into each flask to resuspend the cells.
- Cells were centrifuged at 1200 rpm, in 20°C for 8 min.
- New T75 (or T25, T175) flasks were prepared and 12-14 ml medium was added into flasks.
- The supernatant was aspirated without touching pellet.
- The pellet was resuspended with 1 ml complete medium.
- When necessary, cells were counted using hemacytometer or coulter counter.
- Pelleted cells were resuspended in an appropriate volume of growth medium and seeded into sterile flasks.
- Cells were cultured at 5% CO_2 and 37°C.

2.5.5 Counting cells with hemacytometer

- RPMI complete medium and trypsin were prepared and warmed up at 37°C water bath.
- 15 ml falcon tubes were labeled and 3 ml complete medium was added.
- The flasks with cultured cell were checked under microscope, if confluence of cells reached 70%-90%, the cells were split; if not, medium should be changed and kept in incubator until cell reached proper confluence.
- The old medium was aspirated away from flasks, and ensured that it won't detach cells from bottom.
- The cells were washed 1-2 times with 10 ml PBS (w/o Mg^{2+} and Ca^{2+}).
- 3 ml trypsin was added into flask.
    - Trypsin destroys the contact between cells.
    - Trypsin should be warmed up at 37°C before it was added to the cells.
- The flasks were incubated at 37°C incubator for 3 min, then checked under microscope to ensure all cells had detached from bottom, or they should be kept in incubator for two more min.
- 5 ml PBS was added to wash cells away from bottom, suspension was taken up and then everything was pipetted into falcon tube which was prepared with complete medium.

- Cells were centrifuged at 1200 rpm, in 20°C for 8 min.
- New T75 (or T25, T175) flasks were prepared and 12-14 ml medium was added into flasks.
- The supernatant was aspirated away without touching pellet.
- The pellet left was resuspended with 1 ml complete medium.
- 50 µl of cells) were diluted with 50 µl of Trypan blue working stock.
- Hemacytometer was filled by capillary action. Pipette filled with cells/Trypan blue was placed at the edge of the hemacytometer to pipette the cells out allowing the chamber to fill itself.
- The number of cells in 5 of the outer “large” squares was counted.
- The cell concentration was then calculated as follows:
  Cell concentration (in cells/ml) = average count x 2 x 10.000 x dilution factor.

2.5.6 Cryopreservation of cells

- Cells were collected as described above.
- After centrifugation, cell pellet was resuspended in 10 ml of medium for cryopreservation (FCS, 10% DMSO).
- Cells were counted and defined amount of the cells ($5x10^6$ cells/1.5 ml) was transferred into 2 ml cryotubes.
- The cryotubes were placed into the isopropanol freezing box and frozen at -80°C.
- On the next day the cryotubes were transferred into liquid nitrogen tank (-180°C).

2.5.7 Transfection with FuGENE HD reagent

- 6-well plate was prepared for transfection and sterile glass coverslips were inserted into wells.
- RPMI complete medium and trypsin were prepared and warmed up at 37°C water bath.
- 15 ml falcon tubes were labeled and 3 ml complete medium was added.
- The flasks with cultured cell were checked under microscope, if confluence of cells reached 70%-90%, it was time to split it; if not, medium should be changed and kept in incubator until cell reached proper confluence.
- The old medium was aspirated away from flasks, and ensured that it won’t detach cells from bottom.
- The cells were washed 1-2 times with 10 ml PBS (w/o Mg^{2+} und Ca^{2+}) every time.
- 3 ml trypsin was added into flask.

- Trypsin destroys the contact between cells.
- Trypsin should be warmed up at 37°C before it was added to the cells.

- The flasks were incubated at 37°C incubator for 3 min, then checked under microscope to ensure all cells had detached from bottom, or they should be kept in incubator for two more min.
- 5 ml PBS was added to wash cells away from bottom, suspension was taken up and then everything was pipetted into falcon tube which was prepared with complete medium.
- They were centrifuged 1200 rpm, in 20°C for 8 min.
- New T75 (or T25, T175) flasks were prepared and 12-14 ml medium was added into flasks.
- The supernatant was aspirated away without touching pellet.
- The pellet left was resuspended with 1 ml complete medium.
- 50 µl of cells (at an unknown concentration) were diluted with 50 µl of Trypan blue working stock.
- Pipette filled with cells/Trypan blue was placed at the edge of the hemacytometer to fill hemacytometer.
- The number of cells in 5 of the outer “large” squares was counted.
- These counts were added together and divided by 5 to get an average.
- The cell concentration was calculated as follows:
  Cell concentration (in cells/ml) = average count x 2 x 10,000 x dilution factor of original cells.
- One day before transfection, proper number ($3x10^5$ cell/ml) of cells was seeded in a 6-well plate to ensure cell confluence will reach 50%-70% on the day of transfection, cells were cultured in incubator with 5% CO_2 and 37°C for one day.
- On the day of transfection, plasmid DNA was diluted to 2 µg per 100 µl of serum-free medium.
- 2–10 µl of FuGENE®HD transfection reagent was added to achieve the proper ratio of reagent to DNA, and incubated for 30 min.
- Then mixture of transfection reagent and DNA was added to each well, and mixed gently.
- The plate was incubated at 37°C for 48 hours or an appropriate time for further experiments.

2.5.8 Isolation of CD14+ monocytes from buffy coats

- Human monocytes were isolated from buffy coats of healthy donors as described with modifications[179]. Blood used for the isolation was not depleted of platelets. A unique donor number was given for each isolation.
- 1st gradient solution was prepared before experiment: 15 ml of biocoll separating solution was added in 50 ml falcon tube.
- 2nd gradient solution was prepared: every 30 ml percoll gradient solution was prepared with 13.5 ml percoll (Amersham), 15 ml MEM Spinner modification (Sigma) and 1.5 ml 10x Earle's salt solution (Biochrom).
- MACS buffer was prepared in advance: 2.5 g of BSA was dissolved in 500 ml PBS, 2 ml of 0.5 M EDTA was added and the mixture was filtered to a sterile flask.
- PBS was prepared in T-75 flask, blood was transferred from the plastic bag to the T-75 flask, then mixed by pipetting up and down for 3 times.
- 30 ml of diluted blood were added to biocoll tube, 2 tubes for each donor. The tubes were centrifuged at 1600 rpm, 20°C for 30 min.
- Meanwhile, percoll-gradient solution was mixed well, distributed 30 ml to each 50 ml tube. Desired amount of X-VIVO 10 solution was poured into a sterile flask and kept at room temperature.
- The upper yellow phase (serum) was carefully removed and discarded. The next phase (white blood cells) was collected with a 10 ml pipet and transferred into a new 50 ml tube filled with 10 ml PBS.
- The mixture was added up to 50 ml with PBS and the leukocytes were harvested by centrifugation at 1400 rpm, 20°C for 10 min with break. The supernatant was discarded.
- The cells were washed with 50 ml PBS and centrifuged at 1200 rpm, 20°C for 10 min with brake.
- The cell pellet from each donor was resuspended with 3 ml PBS using 5 ml pipet and collected in a new tube (optional). Tubes were washed twice with 2 ml PBS and the solution was added together to harvest as many cells as possible. The new tubes for each donor were filled up to 50 ml with PBS. The cells were counted and centrifuged again. The supernatant was discarded.
- Percoll-gradient solution was handled very carefully.

- The cells were resuspended with 3 ml PBS and transferred into the percoll-gradient solution against the tube wall very slowly.
- Cells were centrifuged at 1600 rpm, 20^{o}C, for 30 min.
- The upper phase (PBS) and second phase (monocytes) were collected into a 50 ml tube with 10 ml PBS inside.
- The solution was mixed well, filled up to 50 ml with PBS and centrifuged at 1400 rpm, 10^{o}C for 15 min with break. The supernatant was discarded.
- The cell pellet was resuspended with 3 ml PBS and transferred into a 15 ml tube. The 50 ml tube was washed with 4 ml PBS. The solution was added into 15 ml tube and filled up to 10 ml.
- Cells were counted and rest of them were centrifuged at 1100 rpm, 10°C for 10 min with brake.
- The cell pellet was re-suspended in CD14 micro beads and pre-chilled MACS buffer (5 µl CD14 and 95 µl MACS buffer per 1x10^{7} cells).
- The cells were incubated for 20 min on a rotator at 4^{o}C.
- The tubes were filled up to 10 ml with MACS buffer and centrifuged at 1400 rpm，10^{o}C for 10 min.
- During this time, the columns were attached to the magnetic stand (maximal cells for one column is 1x10^{8} cells) and 15 ml tubes were placed under columns. The columns were washed with 3 ml of MACS buffer.
- Cell pellet was re-suspended in 1 ml cold MACS buffer and500 µl of cell suspension was added to each column.
- The column was washed 3 times with 3 ml MACS buffer each time.
- The column was removed from the magnetic separation unit and placed on the top of a fresh 15 ml tube.
- CD14^{+} monocytes were eluted from the column with 5 ml MACS buffer. Cells were counted and centrifuged at 1400 rpm, 10^{o}C for 10 min.
- Cell pellet was resuspended in X-VIVO medium at a concentration of 1x10^{6} cells/ml.

2.5.9 Cytokines stimulation of CD14+ monocytes from buffy coats

- Monocytes were counted.
- 50 ml falcon tube was prepared, monocytes were resuspended to 1x10^{6} cells/ml in X-VIVO medium.

- X-VIVO medium with monocytes was distributed into 6-well plate, with 3 ml medium in each well.
- Cytokines were added in 6-well plate: M-CSF at 1 ng/ml, IL-4 at 10 ng/ml, TGF-β at 10 ng/ml and dexamethasone $1x10^{-7}$ M.
- Cells were incubated at 37°C with 7.5% CO_2 for 6 days. Further, cells were harvested for RNA isolation, protein analysis or used for functional endocytosis assays.

2.5.10 Harvesting cells

- Eppendorf tubes were prepared and labeled in advance.
- The 6-well plates with stimulated macrophages were taken out of incubator.
- The top layer of supernatant was aspirated and collected.
- The plates were put on the ice for 30 min.
- The medium in wells was mixed. Afterwards, 1.5 ml medium was transferred into an Eppendorf tube, and centrifuged at 2000 rpm for 10 min. The medium was discarded and only pellet was left.
- Attached cells were scraped carefully, transferred into centrifuged tubes, and centrifuged again at 2000 rpm for 10 min.
- The tubes were taken out of centrifuge, the medium was aspirated carefully using 1000 µl tip until about 80-100 µl medium left. Small volume tips with 20 µl were used to remove leftover medium.
- 80 µl PBS was added to wash pellet, centrifuged again, and discarded, and only pellet was left.
- The tubes with pellet were stored at -80°C overnight.
- Next day, the pellets were lysed with 200 µl Lammili buffer, incubated at 95°C for 5 min, mixed by vortex and stored at -80°C.

2.6 Human tissue material

Frozen sections of human neuroblastoma were provided by Prof. Dr. rer. nat. Christel Herold-Mende, Neurological Clinic, Medical Faculty Heidelberg, University of Heidelberg.

2.7 Immunological methods

2.7.1 Antibodies

2.7.1.1 Primary antibodies

Table 7. List of primary antibodies (wd: working dilution).

Antibody	Species	Company	Catalogue no.	Concentration	IHC (wd)	IF (wd)	WB (wd)
Anti-human CD68	Mouse	ZYTOMED	556028	500 µg/ml	1:100	1:100	
Anti-human CD163	Mouse	Invitrogen	R960-25	1 mg/ml	1:200	1:200	
Anti-human CD206	Mouse	BD Bioscience	555953	0.5 mg/ml	1:200	1:200	
Anti-human stabilin-1	Rabbit	Non commercial			1:1000	1:1000	
Anti-human YKL-39	Mouse	Non commercial			1:5	1:5	1:5
Anti-human YKL-39	Rat	Non commercial			1:5	1:5	
Control antibodies							
Anti-human GAPDH	Rabbit	Abcam	ab37168	1 mg/ml			1:2000

2.7.1.2 Secondary antibodies

Table 8. List of secondary antibodies and staining substances (wd: working dilution).

Antibody	Species	Company	Catalogue no.	Concentration	IHC (wd)	IF (wd)	WB (wd)
Anti-mouse IgG, Alexa488-conjugated	Donkey	Dianova	715-164-153	1.5 mg/ml	1:400	1:400	
Anti-mouse IgG, Cy3-conjugated	Donkey	Dianova	715-165-150	1.5 mg/ml	1:400	1:400	
Anti-mouse IgG, HRP conjugated ECL	Sheep	GE Healthcare	NA931-1ML	1 mg/ml	1:400	1:400	1:4000
Anti-rabbit IgG, Alexa488-conjugated	Donkey	Dianova	711-165-156	1.5 mg/ml	1:400	1:400	
Anti-rabbit	Donkey	Dianova	711-165-	1.5 mg/ml		1:400	

IgG, Cy3-conjugated			152		1:400		
Anti-rat IgG, Alexa488-conjugated	Donkey	Dianova	712-545-150	1.5 mg/ml	1:400	1:400	
DAPI		Sigma	D9542	5 ug/ml	1:1000	1:1000	
DRAQ 5		Cell Signaling	4084	50 ul	1:1000	1:1000	

2.7.2 Western blot analysis

2.7.2.1 Gel preparation

- Plates were washed with soap, tap water, deionized water and 100% ethanol, then dried with tissue paper.
- Rubber seals were put around glass plate with spacer (plain side up).
- Notched glass plates were put on top and fixed with clips, two clips were put on each side separately, two clips were put on top and bottom separately.
- 25 ml of separation gel were prepared for one glass sandwich.
- 10% APS and TEMED were added at the end and mixed.
- Gel solution was pipetted in between the glass plates.
- 1-2 cm space was left for stack gel, 500 µl of methanol were pipetted immediately on top of the separation gel.
- Methanol was poured out and deionized H_2O was added to wash for 2 times after polymerization.
- 6 ml stack gel were prepared for each separation gel and pipetted on top of separating gel.
- Comb was inserted into stack gel.
- After about 20 min, gel polymerization was completed.

2.7.2.2 Sample preparation

- Sample buffer were prepared: 950 µl Laemmli sample buffer + 50 µl ß-mercaptoethanol.
- Target proteins were diluted in Laemmli sample buffer with 1:1, then heated for 2-5 min (3 min is optimal) at 95℃ in Thermo mixer (Eppendorf).
- If cells were lysed directly in Laemmli sample buffer, they were thawed and heated for 2-5 min (3 min is optimal) at 95℃ in Thermo mixer (Eppendorf) then loaded directly on the gel without dilution.

2.7.2.3 Marker preparation

- 5 µl protein-standard were pipetted in first well. Prestained protein ladder was used (protein size range 10-180 kDa).

2.7.2.4 Electrophoresis

- 20-25 µl of sample was pipetted using Hamilton glass syringe into the wells.
- SDS-chamber was connected to power-supply, gel was run at 20 mA for 20 min. After that amperage was increased to 40 mA.
- The gel was run for 6-8 hours.
- Before samples were pipetted, chamber was filled with 1x TGS firstly, the existence of 'Gel-wall' between different lanes should be confirmed and it was not allowed to insert the tip of syringe into stacking gel.

2.7.2.5 1000 ml "Towbin" buffer was prepared to transfer membrane

- 700 ml distilled H_2O
- 200 ml methanol
- 100 ml 10x TGS

2.7.2.6 Preparation of blotting cassette in plastic chamber with Towbin buffer

- 1 x blottingsponge
- 2 x Whatmanpaper
- 1 x blottingmembrane (nitrocellulosetransfermembrane)
- 1 x or 2 x gel
- 2 x Whatmanpaper
- 1 x blotting sponge
- The transfer was performed at 0.25 A for 3 hours (2.15 h) or overnight in cold room at 0.15 A.

2.7.2.7 Checking membrane with Ponceau S

- Membranes were immersed in Ponceau S solution and stained for 20 min with gentle shaking.
- Membrane was washed with distilled water until bands were visible.

2.7.2.8 Blocking membrane with 6% milk

- Membrane was blocked with 6% milk/PBS (Milk: blotting grade blocker non-fat dry milk).
- Membrane was incubated for 30 min to 1 h in a plastic chamber at room temperature on shaker, or incubated in cold room for 24 h.

2.7.2.9 Primary antibody incubation

- First antibody was diluted in 1% milk/PBS.
- First antibody was incubated in cold room overnight, or incubated at room temperature for 2-3 hours.

  (Note: antibody could be stored in 4^{o}C with sodium azide 0.02% or 0.05% (V/V))

2.7.2.10 Washing steps

- The membrane was shortly pre-washed with PBS/0.1% Tween 20.
- The membrane was washed with PBS/0.1% Tween 20 for 3 times (5 min every time).

2.7.2.11 Secondary antibody incubation

- Secondary antibody (donkey a-rabbit-IgG-HRP or donkey a-mouse-IgG-HRP) was diluted in 1% milk/PBS and incubated for 45 min to 1 hour.

2.7.2.12 Washing steps

- The membrane was shortly pre-washed with PBS/0.1% Tween 20.
- The membrane was washed 3x10 min with PBS/0.1% Tween 20.

2.7.2.13 Development

- The membrane was placed with protein-side up in a container or clear plastic sheet protector.
- 10-12 ml HRP substrate were added to the membrane and incubated in plastic chamber at room temperature for 2-5 min, membranes should be completely covered.
- After development, two pieces of plastic foils were prepared, then membrane was taken out from plastic chamber and put in between plastic foils and membrane should be kept humid.
- Then a development cassette was prepared, covered membrane was put inside and brought to darkroom.

- In darkroom, film was developed for 1, 5, 60 min or overnight.
- Film was labeled with date, sample loading volume, marker size, running and blotting date, primary and secondary antibodies used.

2.7.3 Protein visualization using Page Blue™ solution

- Plates were washed with soap, tap water, deionized water and 100% ethanol, then dried with tissue.
- Rubber seals were put around glass plate with spacer (plane side up).
- Clean glass plates were put on top and fixed with clips, two clips were put on each sides separately, two clips were put on top and bottom separately.
- 25 ml separation gel was prepared for one set of plates.
- 10% APS and TEMED were added at the end and mixed (because it polymerize very quickly).
- Gel solution was pipetted in between the glass plates.
- 1-2 cm space were left for stack gel, 500 µl of methanol were pipetted immediately on top of the separation gel.
- Methanol was poured out and deionized H_2O were added to wash for 2 times after polymerization.
- 6 ml stack gel were prepared for each separation gel and pipetted on top of separating gel.
- Comb was inserted into stack and air bubbles were prevented.
- After about 20 min, gel polymerization was completed.
- Sample buffer was prepared: 950 µl Laemmli sample buffer + 50 µl ß-mercaptoethanol.
- Target proteins were diluted in Laemmli sample buffer with 1:1, then heated for 2-5 min (3 min is optimal) at 95℃ in Thermo mixer (Eppendorf).
- If lysis of cells was already prepared with Laemmli sample buffer, it should be taken from -80ºC fridge directly and then heated for 2-5 min (3 min is optimal) at 95℃ in Thermo mixer (Eppendorf) then loaded directly in gel without dilution.
- 5 µl protein-standard were pipetted in no.1 well. Prestained protein ladder ca. 10-180 kDa (for smaller size proteins).
- 20-25 µl samples were pipetted with Hamilton glass syringe in wells.

- SDS-chamber was connected to power-supply, gel was run at 20 mA for 20 min, then it was possible to run at 40 mA by which target protein moved faster.
- The gel was run until target protein arrived in the middle of the gel, usually the time was about 6-8 hours.
- After electrophoresis the gel was washed 3 times for 10 min each time in deionized water with gentle agitation.
- Page blue protein staining solution was added and incubated at room temperature for 60 min with gentle agitation.
- The staining solution was discarded and the gel was rinsed for 2 times with deionized water.
- The gel was washed with 100-200 ml of deionized water for 5 min.

Note: Washing the gel for a longer period or frequently replacing the water will enhance sensitivity. Additionally, placing a folded Kimwipes™ Tissue in the container to absorb excess dye will accelerate the detaining process.

2.7.4 Re-blotting with stripping solution

- The blots or individual strips that are to be re-used were prepared for stripping immediately after their first usage. If stripping could be done right away, membranes were wrapped in plastic wrap and stored in PBS at 4°C.
- Blots were placed in plastic tray with appropriate amount of 1x antibody stripping solution.
- Blots were submerged in stripping solution and incubated with gentle mixing for 15 min at room temperature.

  Note: It is necessary to increase the stripping incubation time when blots that have been stripped previously are used. The stripping time can be increased by 5 to 10 min, if needed.
- A clean plastic tray was filled with an equal amount of blocking buffer (5% milk). The blots were washed two times for 5 min with blocking buffer and re-used for staining.

2.7.5 Drying gel

- Gel was immersed in distilled water overnight.
- The gel was taken out, recognized up side and down side, then it was unfolded on a clean glass plate with up-side.

- One piece of big thick filter paper was cut into two pieces of small papers according the shape of gel.
- Two pieces of small papers were unfolded on the down side of gel, which should exactly match with edge of gel.
- The plate with gel and paper was reversed, the gel was detached from plate and combined with paper.
- The gel was covered with Saran wrap which should exactly match with edge of gel, preventing air bubbles.
- The combination of gel and filter paper was put in the middle of the bottom of platform.
- The mains power was turned on.
- The vacuum pump was turned on, the vacuum pump timer was set to 2 h.
- A seal should be created between the rubber overlay and platen.
- The temperature was set 80°C , and temperature timer was set 2 h.
- After 2 h, it should be confirmed heating was stopped, and temperature was going down.
- The gel was kept in machine for overnight.
- The gel should be removed after drying was completed.
- Vacuum pump was turned off.
- Vacuum pump timer and heating element timer were turned off.
- The silicone rubber overlay was removed, or vacuum would be broken.
- The stacking layers were removed to reach the dried gel.

2.8 Preparation of cell and tissue sections for staining

2.8.1 Paraffin section

- Water bath was heated for at least 10 min.
- Blade and cutting surface were cleaned with a wipe and a little bit of ethanol, and they should be kept dry completely.
- Excess wax from embedded sample and embedding ring was removed.
- Microtome blade was locked in place and it must be confirmed that blade guards were closed, microtome handle was locked and embedded sample was clamped onto microtome.
- Sample position was adjusted to site straight and even right above the blade, and it was locked into place.

- The handle was unlocked and quickly turned until sample was cutted a little. The first full section was captured using brush.
- Sections of 1 µm thickness were prepared using Leica rm 2155. Sections were unfolded on the water bath, then dried 30 min, and stored in incubator at 60°C overnight.

Deparaffinisation

- Sections were deparaffinized:

Xylol:	2 x 10 min, under hood
100% ethanol:	2 x 5 min
96% ethanol:	1 x 5 min
90% ethanol:	1 x 5 min
80% ethanol:	1 x 5 min
70% ethanol:	1 x 5 min
Distilled water:	1 x 5 min

Antigen retrieval

- Water bath was set at 100°C, coplin jar with buffer was put in water bath before water got boiled.
- After water bath was boiled, sections were inserted into jar and kept in water bath for 20 min.
- Coplin jar was taken out of water bath, and cooled down for 15-20 min at room temperature.
- Circles were made around sections with DAKO pen.
- Sections were washed shortly in PBS/0.1% Tween 20.
- Sections were blocked with 3% BSA for 30 min-1 h.
- Sections were blocked with 0.3% peroxide/PBS for 5 min.
- Sections were washed with 0.1% Tween 20/PBS for 30 sec.

2.8.2 Acetone fixation of cells and frozen tissue sections

Acetone fixation of cells grown in 6-well plate

- Supernatant was removed.
- Cells were washed gently for 2 times with 2 ml of PBS, cells were fixed for 10 min with acetone.

- Coverslips were replaced into new plate and dried for 30 min
- Wells were made circle with the DAKO pen.
- Cells were washed with PBS/0.1% Tween 20 for 30 sec.
- 100 µl 0.03% peroxidase with PBS were applied, and incubated in room temperature for 5 min.
- Cells were washed with PBS/0.1% Tween 20 for 30 sec.

Acetone fixation of frozen section

- The slides were moved out of -80°C, then slides were dried in room temperature for 15 min.
- Sections were fixed in 100% acetone for 10 min in room temperature, then were dried for 30 min.
- Sections were made circle with the DAKO pen.
- Sections were washed with PBS/0.1% Tween 20 for 30 sec.
- Sections were blocked with 3% BSA/PBS for 1 h in room temperature, and sections were incubated in humid chamber.
- Sections were washed with PBS/0.1% Tween 20 for 30 sec.

2.8.3 PFA fixation of cells and tissue sections

PFA-fixation of cells grown in 6-well plate

- Cells were washed gently 2 times with 2 ml of PBS Cells were fixed with 1 ml of 2% PFA for 10 min.
- Cells were incubated with 1 ml of 0.5% Triton X-100/PBS at room temperature for 15 min.
- Cells were fixed with 1 ml 4% PFA for 10 min.
- Cells were washed with 2-3 ml of PBS on shaker for 4-5 times (10 min each time).
- 3 ml of PBS were added into the wells and plates were stored at 4ºC for 1 week.
- Alternatively, plates were dried completely, sealed with parafilm and stored at -80ºC. If plates would be stored for a long time, plates should be dried very well under the hood,
  then packed with parafilm and frozen at -80ºC.

PFA-fixation of tissue (frozen sections)

All procedure were performed at RT.

- 4% PFA was thawed, 2% PFA was prepared by dilution 1:1 with PBS.

- 0.5% Triton X-100/PBS was prepared in advance and stored in 4ºC. Pre-warmed and used at RT.
- Sections were washed 2 times with 2 ml PBS very carefully with pipetting on the edge of well.
- Sections were fixed with 1 ml 2% PFA for 10 min.
- Sections were washed with 1 ml 0.5% Triton X-100/PBS for 15 min.
- Sections were fixed for 10 min with 1 ml 4% PFA.
- Sections were washed with 2-3 ml PBS on shaker for 4-5 times with 10 min every time.

2.9 IHC staining

- Primary antibodies were diluted with 1% BSA/PBS, 100 µl of diluted antibodies were added per each section and incubated at room temperature for 45 min.
- Sections were washed with PBS/0.1% Tween 20 for 3 times (5 min each time).
- Secondary antibodies were diluted with 1% BSA, 100 µl of diluted antibodies were added per each section and incubated at room temperature for 30 min.
- Sections were washed with PBS/0.1% Tween 20 for 3 times (5 min each time).
- AEC-substrate-chromogen solution was added on sections. Sections were incubated for 5 -15 min. Color development was observed under a microscope to prevent sections to be overstained.
- Sections were rinsed with PBS/0.1% Tween 20
- The slides were counterstained with Mayer's Haemalaun at room temperature for 1 min.
- The sections were washed with tap water for 3 times (5 min each time).
- Sections were mounted using DAKO aqueous mounting medium, dried and stored at RT: 1 drop of mounting medium was used for 1 cycle and a coverslip with the size 24 x 30 mm was put on; if it was 2 sections on one slide: 1 drop was used for per section and a coverslip with the size 24 x 60 mm was put on.
- After mounting medium was dried, sections could be put in special slide holder and kept in room temperature.

2.10 IF staining

- The primary antibody was diluted with 1% BSA/PBS, 200 µl diluted antibody were applied for per section, then incubated for 1.0 h in humid chamber.
- Sections were washed 3 times and 5 min for every time with PBS on shaker.

- Sections were washed with PBS/0.1% Tween 20 for 30 sec.
- The secondary antibody was diluted with 1% BSA/PBS in red light protected 1.5 ml Eppendorf tube, DAPI (1:1000) and DRAQ 5 (1:1000) were added to each tube.
- 200 µl diluted antibody were applied for per section, then incubated for 45 min in humid chamber.
- Sections were washed 4 times and 5 min for every time with PBS on shaker.
- DAKO Fluorescent mounting medium was applied and 24 x 30 mm coverslip was put on it.
- Slides were kept in the dark at 4°C, and aluminum foil was put around the map.

2.10.1 Confocal microscopy analysis

Confocal microscopy was performed using Leica TCS SP2 microscope. Three emitting wavelengths were used, including: an argon laser emitting at 488 nm, a krypton laser emitting at 568 nm and a helium/neon laser emitting at 633 nm, and 63 x 1.32 objectives.

2.11 Molecular biology methods

2.11.1 Isolation of total RNA

E.Z.N.A. total RNA Kit I was used for the RNA isolation. The lysis buffer was prepared by adding 10 µl of mercaptoethanol to each 1 ml of the TRK buffer (guanidine isothiocyanate containing buffer which suppresses RNase activity) and mixed well.

- Cells (3-5 x 10^6 cells) were harvested and lysed in 350 µl of lysis buffer and completely disrupted by passing the lysate 20 times through a needle fitted to a syringe.
- 350 µl of 70% ethanol were added to the lysate. The sample was vortexed and applied to HiBind RNA spin column placed into a 2 ml collection tube.
- Column was centrifuged at 10000 x g for 1 min. After centrifugation, the flow-through was discarded and column was washed 1 time with 500 µl of RNA washing buffer I and 2 times with 500 µl of RNA washing buffer II.
- After last centrifugation, the column was placed in fresh RNase free microcentrifuge tube. RNA was eluted 2 times with 30 µl of RNase free water which was preheated at 70°C.

- The concentration of isolated RNA was determined photometrically and the quality of the obtained RNA samples was analyzed on 1% agarose gel. Isolated samples were stored at –20°C for experiments.

2.11.2 cDNA synthesis

For cDNA synthesis, RevertAid H Minus First Strand Synthesis Kit from Fermentas was used. Prior to cDNA synthesis, all RNA samples were digested with DNase I to remove possible contamination of isolated RNA with fragments of genomic DNA. For this procedure, following components were mixed:

Total RNA (2 µg)	6 µl
10x DNase I buffer with $MgCl_2$ (Fermentas)	1 µl
RNase free DNase I (Fermentas)	1 µl
Distilled water (RNase free)	2 µl

Digestion was done at 37°C for 40 min in thermo block followed by enzyme inactivation at 70°C for 10 min. After this procedure, cDNA was generated according to the following protocol:

- 1 µl of Oligo $(dT)_{18}$ primer was added to DNase I digested RNA sample.
- Volume was adjusted to 12 µl with water and primer annealing was done at 70°C for 5 min.
- Samples were placed on ice for 1 min.
- After that, following components were added and mixed well:

5x buffer for polymerase	4 µl
Ribolock RNase inhibitor	1 µl
10 mM dNTP mix	2 µl

- Mixture was incubated at 37°C for 5 min followed by addition of 1 µl of RevertAid H minus reverse transcriptase.
- Reaction was incubated at 42°C for 1 h at thermo block and enzymatic activity was stopped by additional incubation at 70°C for 10 min.
- cDNA sample was diluted 10 times with distilled water.

2.11.3 Real-time PCR with TaqMan probe

All primers and dual-labelled probes were ordered from MWG Biotech. Dual-labelled probes contained FAM at 5’ end and BHQ1 quencher at 3’ end of sequence.

List of used primers was shown in Table 11. All primer sequences were shown from 5’ end to 3’ end direction. For analysis of gene expression, Real-time PCR reaction was set up from the following components:

TaqMan Gene Expression Master Mix	5.0 µl
TaqMan Assay	0.5 µl
cDNA	1.0 µl
Distilled water	3.5 µl

Table 9. List of primers used for PT-PCR.

Internal No.		Target gene	Sequence (5'-3' direction)
2065	F	YKL-39	CCTCCTGTCCTTTGACTTCCAT
2066	R	YKL-39	CCTTGCTCAGAGGGCTGTTG
2067	T	YKL-39	TTGGGAAAAGCCCCTTATCACTG
2012	F	GAPDH	CGCTCTCTGCTCCTCCTGTT
2013	R	GAPDH	CCATGGTGTCTGAGCGATGT

Each cDNA sample was analyzed in quadruplicates. For normalization, the expression of GAPDH mRNA was analyzed for each probe. Amplification was performed using LightCycler480 instrument. The following program was used:

- 95°C for 3 min
- 95°C for 10 sec
- 60°C for 30 sec
- go to step 2, repeat 50 times

Samples were stored in -20^{o}C.

2.12 Statistical analysis

To determine significance of difference between two groups, t-test was performed using GraphPad Prism 5 software. For data of more than two groups, P-value was analyzed with ANOVA, P-value less than 0.01 was considered statistically significant.

3 Results

3.1 Selection of antibodies for detection of YKL-39 expression in human monocyte-derived macrophage and tissues

To analyze the localization and expression of YKL-39 in human monocyte-derived macrophages and various tissues, highly specific anti-YKL-39 antibodies are needed. The production of murine, rat and guinea pig antibodies recognizing peptide fragments of YKL-39 (YKL-39-N1, YKL-39-N2, YKL-39-HNSP) was performed by our collaborator, Dr. Elisabeth Kremmer (Helmholtz Zentrum München). After immunization with YKL-39 peptides, 43 hybridoma supernatants of independent clones were obtained from mice, 73 hybridoma supernatants of independent clones were obtained from rat, and 3 serums were obtained from immunized guinea pig. Secondly, in order to select the best specific antibody, murine mammary adenocarcinoma cell line (TS/A) were transiently transfected with pcDNA3-YKL-39 (P825) expression construct for the recombinant human YKL-39 fused with FLAG tag. The reactivity of murine and rat monoclonal antibodies, and guinea pig polyclonal antibodies towards recombinant YKL39 expressed in TS/A was examined by double fluorescence immunostaining and confocal microscopy techniques to select most specific. The most specific antibodies were further used to analyze the expression and distribution of YKL-39 in various tissues and cells.

3.1.1 Analysis of mouse anti-YKL-39 monoclonal antibodies

The results of immunofluorescent (IF) staining revealed that most of mouse anti-YKL-39 antibody clones (including YKL-39.1 raised towards immunogenic peptide YKL-39-N1, YKL-39.2 towards peptide YKL-39-N2, and YKL-39H towards peptide YKL-39-HNSP) specifically recognized recombinant human YKL-39 in transfected TS/A cells (Figures 7, 8 and 9). Based on the results of IF staining, TS/A-YKL-39 clones were sub-divided into negative, weakly, moderately positive, and strongly positive (Table 12). Strongly positive clones including H6G4, H3E4, and H5A2, 14E4, 14C12, 14E10, 11H 3, 15D12 and 24A6 were further sub-cloned to increase expression of YKL-39 (Table 13), the clone 14E10 was selected for further experiments.

Table 10. Analysis of specificity of mouse monoclonal antibodies with murine TS/A. Specificity of mouse monoclonal antibodies was analyzed by immunofluorescent staining and confocal microscopy.

Number	Clone number of hybridoma supernatant	Expression level
1	YKL-39-H 3A8 (M-2B)	Moderate
2	YKL-39-H 3A11 (M-2B)	Moderate
3	YKL-39-H 3E4 (M-2B)	Strong
4	YKL-39-H 3G10 (M-G1)	Moderate
5	YKL-39-H4E1 (M-2)	Moderate
6	YKL-39-H 5A2 (M-2A)	Moderate
7	YKL-39-H 5G1 (M-G3)	Moderate
8	YKL-39-H 6G4 (M-2A)	Strong
9	YKL-39.2 2B2 (M-G1)	Weak
10	YKL-39.2 4A6 (M-2a)	Moderate
11	YKL-39.2 4A7 (M-2a)	Moderate
12	YKL-39.2 6G2 (M-2a)	Moderate
13	YKL-39.2 7A12 (M-G3)	Moderate
14	YKL-39.2 7C10 (M-G3)	Weak
15	YKL-39.1 1F7 (M-2)	Negative
16	YKL-39.1 1H3 (M-2B)	Strong
17	YKL-39.1 1H5 (M-2B)	Moderate
18	YKL-39.1 2E1 (M-2)	Weak
19	YKL-39.1 2F3 (M-2B)	Moderate
20	YKL-39.1 3A1 (M-2A)	Weak
21	YKL-39.1 3A2 (M-2A)	Weak
22	YKL-39.1 3B5 (M-2A, M2B)	Weak
23	YKL-39.1 3C9 (M-2B)	Negative
24	YKL-39.1 3D10 (M-2B)	Strong
25	YKL-39.1 3G10 (M-2B)	Negative
26	YKL-39.1 4C5 (M-G1)	Strong
27	YKL-39.1 4C12 (M-2A)	Strong
28	YKL-39.1 4D9 (M-2B)	Negative
29	YKL-39.1 4E4 (M-G1)	Strong
30	YKL-39.1 4E8 (M-2B)	Negative
31	YKL-39.1 4E10 (M-G1)	Strong
32	YKL-39.1 4E12 (M-2A)	Moderate
33	YKL-39.1 4F6 (M-G1, M-2A)	Negative
34	YKL-39.1 4G7 (M-2A)	Moderate
35	YKL-39.1 4H2 (M-2)	Negative
36	YKL-39.1 5D12 (M-2A)	Moderate
37	YKL-39.1 5E1 (M-2A)	Moderate
38	YKL-39.1 5F8 (M-G1)	Strong
39	YKL-39.1 5F10 (M-2B)	Strong
40	YKL-39.1 6A3 (M-2B)	Strong
41	YKL-39.1 6A9 (M-2A)	Moderate
42	YKL-39.1 8A4 (M-2)	Strong
43	YKL-39.1 8E11 (M-2B)	Strong

Table 11. Final selection of mouse monoclonal anti-YKL-39 antibodies for further experiments.

YKL-39 H 6G4 (M-2A)	YKL-39 H 3E4 (M-2B)	YKL-39 H 5A2 (M-2A)	YKL-39.1 4E4 (M-G1)
YKL-39.1 4C12 (M-2A)	YKL-39.1 4E10 (M-G1)	YKL-39.11H3 (M-2B)	YKL-39.1 5D12 (M-2A)
YKL-39.2 4A6 (M-2a)			

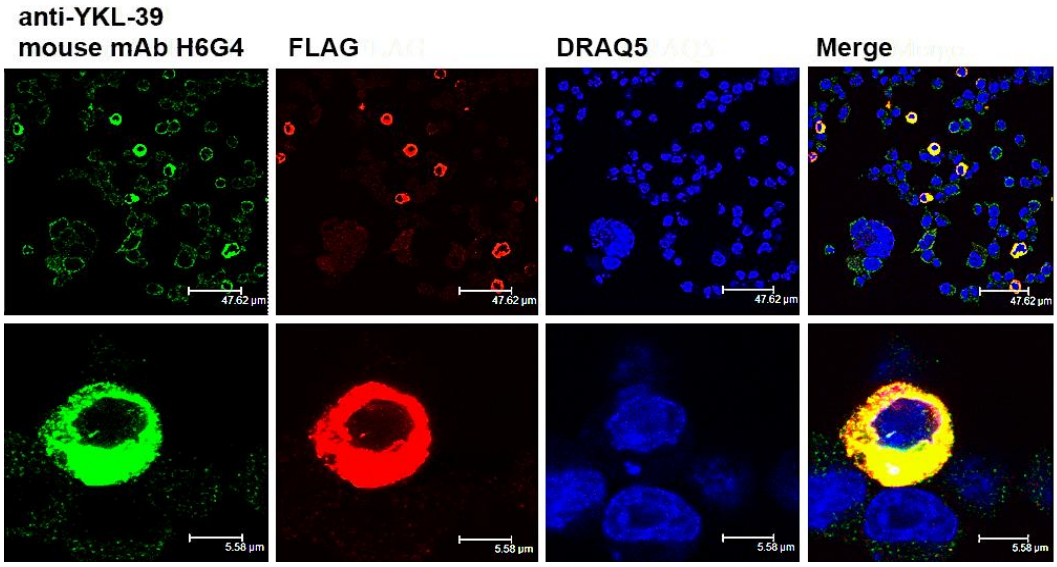


Figure 7. Confocal microscopy analysis of TS/A cells transiently transfected with YKL-39 expression construct with mouse anti-YKL-39 monoclonal antibody (immunogenic peptide YKL-39-HNSP). YKL-39 cells were detected with mouse anti-YKL-39 mAb (H6G4) and secondary Alexa488-conjugated anti-mouse IgG Ab. FLAG tag was detected with rabbit anti-FLAG Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Yellow color indicated co-localization of YKL-39 and FLAG. Scale bars: 47.62 µm and 5.56 µm.

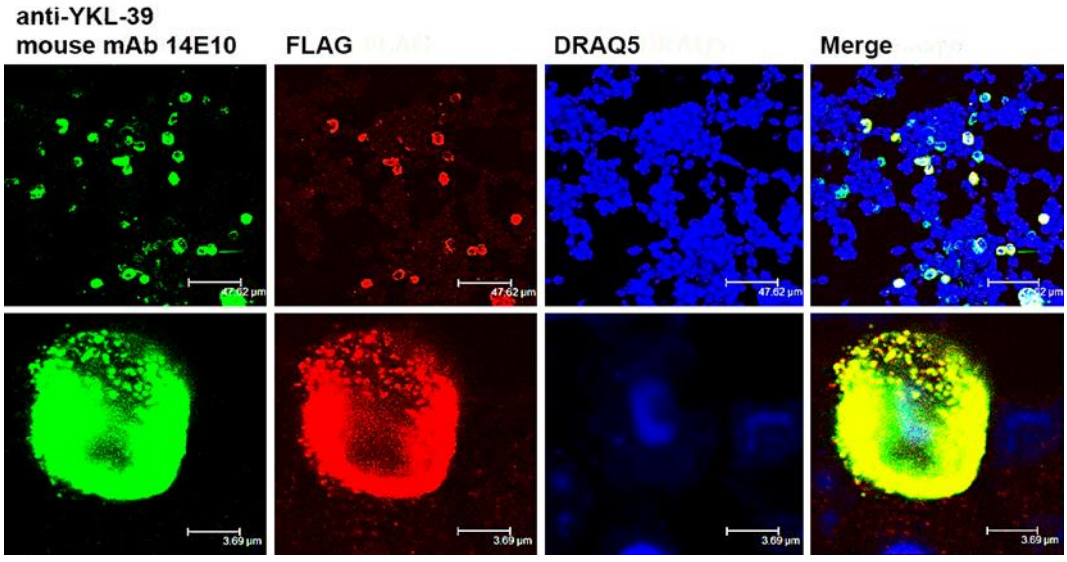


Figure 8. Confocal microscopy analysis of TS/A cells transiently transfected with YKL-39 expression construct with mouse anti-YKL-39 monoclonal antibody

(immunogenic peptide YKL-39-N1). YKL-39 cells were detected with mouse anti-YKL-39 mAb (14E10) and secondary Alexa488-conjugated anti-mouse IgG Ab. FLAG tag was detected with rabbit anti-FLAG Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ 5. Yellow color indicated co-localization of YKL-39 and FLAG. Scale bars: 47.62 μm and 3.69 μm.

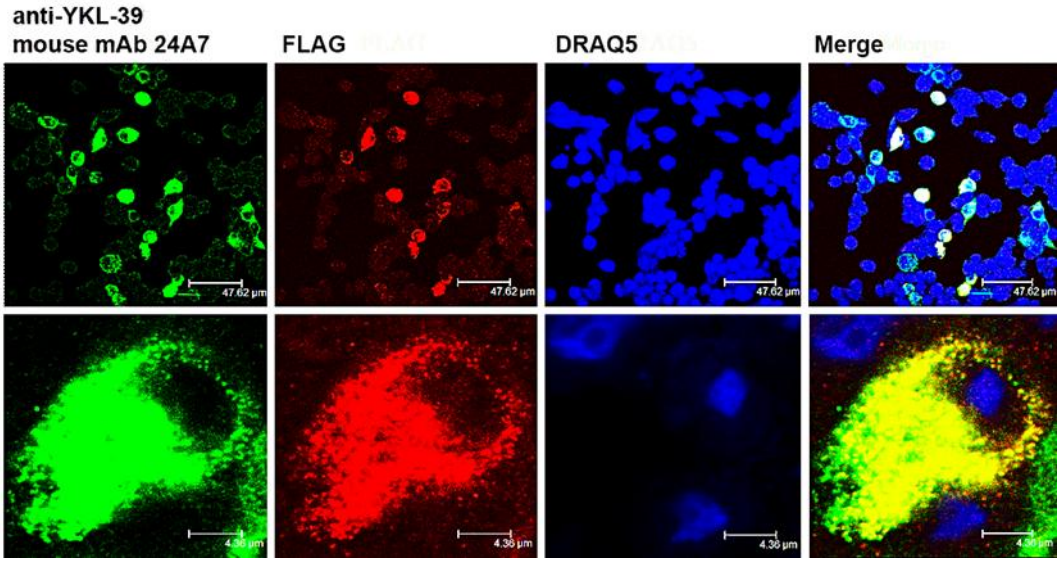


Figure 9. Confocal microscopy analysis of TS/A cells transiently transfected with YKL-39 expression construct with mouse anti-YKL-39 monoclonal antibody (immunogenic peptide YKL-39-N2). YKL-39 cells were detected with mouse anti-YKL-39 mAb (24A7) and secondary Alexa488-conjugated anti-mouse IgG Ab. FLAG tag was detected with rabbit anti-FLAG Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ 5. Yellow color indicated co-localization of YKL-39 and FLAG. Scale bars: 47.62 μm and 4.36 μm.

3.1.2 Analysis of rat anti-YKL-39 monoclonal antibodies

Table 14 summarizes the analysis of rat monoclonal anti-YKL39 antibodies, where YKL-39.1 corresponds to the immunogenic peptide YKL-39-N1, YKL-39.2 corresponds to the immunogenic peptide YKL-39-N2, YKL-39H corresponds to the immunogenic peptide YKL-39-HNSP. The results showed that most of the generated antibodies clones specifically recognized YKL-39, the clones which were detected strongly positive were picked out for sub-cloning, including: 213D7, 218H10, 114D7, H9A7 and H10G8. The strongest clone 218H10 was selected for further experiments (Table 14, Table 15, Figures 10-12).

Table 12. Results of first round of rat monoclonal anti-YKL-39 antibodies analysis. Expression of YKL-39 was analyzed by immunofluorescent staining using rat anti-YKL-39 monoclonal antibody.

Number	Clone number of hybridoma supernatant	Percentage of positive cells	Expression level
1	YKL-39.1 3A8 (R-2c)		Negative
2	YKL-39.1 4D4 (R-2a)		Negative
3	YKL-39.1 5H3 (R-G1)	> 60%	Strong
4	YKL-39.1 6E7 (R-2b)	> 70%	Strong
5	YKL-39.1 7E2 (R-2b, R-2c)		Negative
6	YKL-39.1 10B8 (R-G1)	> 40%	Moderate
7	YKL-39.1 10C6 (R-2b, R-2c)		Negative
8	YKL-39.1 10H6 (R-G1)	> 30%	Moderate
9	YKL-39.1 14A6 (R-2a)		Negative
10	YKL-39.1 14D7 (R-2a)	> 40%	Strong
11	YKL-39.1 16A2 (R-G1, R-2c)	> 50%	Strong
12	YKL-39.1 16G12 (R-G1)		Negative
13	YKL-39.1 17E12 (R-2c)		Negative
14	YKL-39.1 17H3 (R-G1)	> 60%	Strong
15	YKL-39.1 18F9 (R-2c)		Negative
16	YKL-39.1 18G4 (R-2a, R-2c)	> 40%	Strong
17	YKL-39.1 20B8 (R-2a)		Negative
18	YKL-39.1 20C4 (R-G1)	> 50%	Strong
19	YKL-39.1 21B5 (R-2c)		Negative
20	YKL-39.1 21E11 (R-2a)	> 60%	Strong
21	YKL-39.1 22B11 (R-2c)		Negative
22	YKL-39.1 23B8 (R-2c)		Negative
23	YKL-39.1 26B (R-G1)		Negative
24	YKL-39.2 4F9 (R-2A)		Negative
25	YKL-39.2 6F11 (R-2c)		Negative
26	YKL-39.2 7E11 (R-2c)		Negative
27	YKL-39.2 7F4 (R-2a)		Negative
28	YKL-39.2 7G7 (R-2A)		Negative
29	YKL-39.2 8A11 (R-2A)		Negative
30	YKL-39.2 9D2 (R-2A)		Negative
31	YKL-39.2 9E4 (R-2b)		Negative
32	YKL-39.2 9E7 (R-g1)		Negative
33	YKL-39.2 10H4 (R-2C)		Negative
34	YKL-39.2 11G6 (R-2C)		Negative
35	YKL-39.2 12B1 (R-G1, R-2C)	> 50%	Strong
36	YKL-39.2 12B3 (R-2C)		Negative
37	YKL-39.2 12B10 (R-2A, R-2C)		Negative
38	YKL-39.2 12E4 (R-G1)		Negative
39	YKL-39.2 13A1 (R-2C)		Negative
40	YKL-39.2 14B6 (R-2A)		Negative
41	YKL-39.2 13D7 (R-2C)	> 40%	Moderate
42	YKL-39.2 13E9 (R-2C)		Negative
43	YKL-39.2 13H2 (R-2A)		Negative
44	YKL-39.2 13H3 (R-G1)		Negative
45	YKL-39.2 14C6 (R-2C)		Negative
46	YKL-39.2 14E6 (R-2A)		Negative
47	YKL-39.2 14E9 (R-G1)		Negative

48	YKL-39.2 15B2 (R-2C)		Negative
49	YKL-39.2 15F8 (R-2C)		Negative
50	YKL-39.2 16B3 (R-2C)		Negative
51	YKL-39.2 16D10 (R-2C)		Negative
52	YKL-39.2 18D7 (R-2C)		Negative
53	YKL-39.2 18F4 (R-2A)		Negative
54	YKL-39.2 18H10 (R-2A)	> 30%	Moderate
55	YKL-39.2 19D10 (R-2C)	> 50%	Moderate
56	YKL-39.2 19G1 (R-2C)		Negative
57	YKL-39.2 22F3 (R-2A; R-2C)		Negative
58	YKL-39.2 20D10 (R-2C)		Negative
59	YKL-39.2 22C5 (R-2A)		Negative
60	YKL-39.2 23B5 (R-2C)		Negative
61	YKL-39.2 23C9 (R-2C)		Negative
62	YKL-39.2 24C11 (R-2A; R-2C)		Negative
63	YKL-39H 3B2 (R-2A)	> 50%	Strong
64	YKL-39H 5F11 (R-2A)	> 60%	Strong
65	YKL-39H 9A7 (R-2A)	> 50%	Strong
66	YKL-39H 10D12 (R-2A)		Negative
67	YKL-39H 10G8 (R-2A)	> 40%	Strong
68	YKL-39H 16B12 (R-G1)		Negative
69	YKL-39H 16E2 (R-2A)	> 60%	Strong
70	YKL-39H 18F7 (R-G1)	> 40%	Strong
71	YKL-39H 19H4 (R-2A)	> 30%	Moderate
72	YKL-39H 21G1 (R-G1)	> 30%	Moderate
73	YKL-39H 21H5 (R-2A)		Strong

Table 13. Final clones with strongest reactivity for YKL-39 selected for further applications.

YKL-39-2 13D7 (R-2C)	YKL-39.2 18H10 (R-2A)	YKL-39-1 14D7 (R-2a)	YKL-39-H 9A7 (R-2A)
YKL-39-H 10G8 (R-2A)			

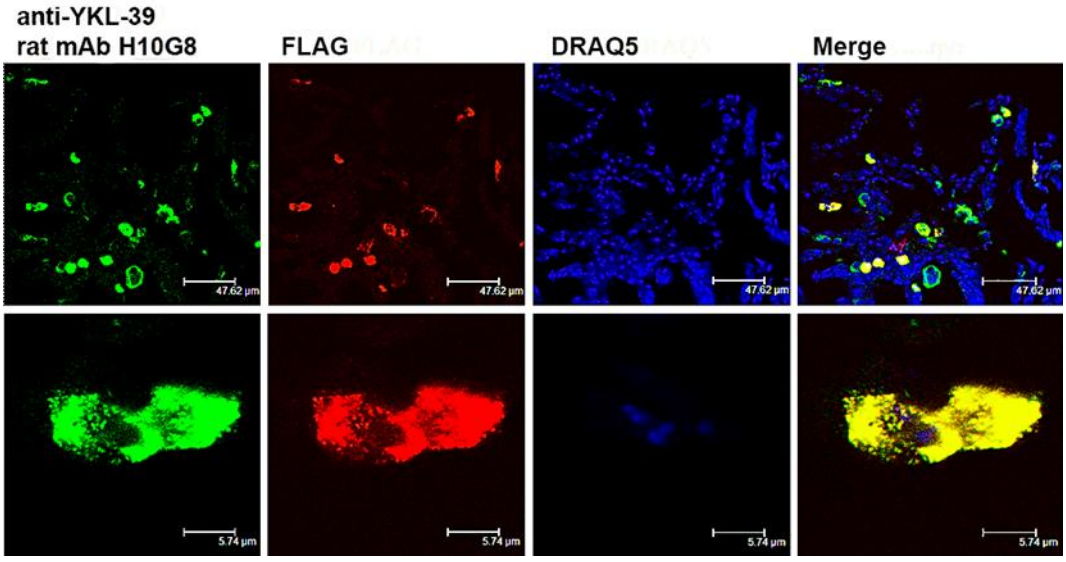


Figure 10. Analysis of the expression of YKL-39 in TS/A cells using immunofluorescent staining and confocal microscopy with rat anti-YKL-39

monoclonal antibodies towards immunogenic peptide YKL-39-HNSP. YKL-39 cells were detected with rat anti-YKL-39 mAb (clone H10G8) and secondary Alexa488-conjugated anti-rat IgG Ab. FLAG tag was detected with rabbit anti-FLAG Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Yellow color indicated co-localization of YKL-39 and FLAG. Scale bars: 47.62 μm and 5.74 μm.

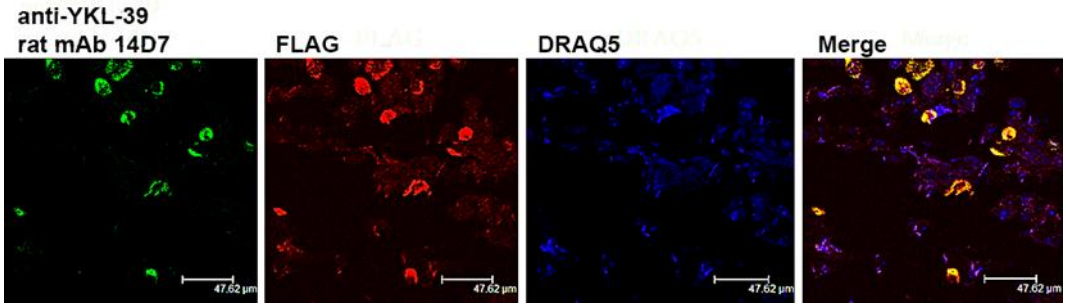


Figure 11. Analysis of the expression of YKL-39 in TS/A cells using immunofluorescent staining and confocal microscopy with rat anti-YKL-39 monoclonal antibodies towards immunogenic peptide YKL-39-N1. YKL-39 cells were detected with rat anti-YKL-39 mAb (clone 14D7) and secondary Alexa488-conjugated anti-rat IgG Ab. FLAG tag was detected with rabbit anti-FLAG Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Yellow color indicated co-localization of YKL-39 and FLAG. Scale bar: 47.62 μm.

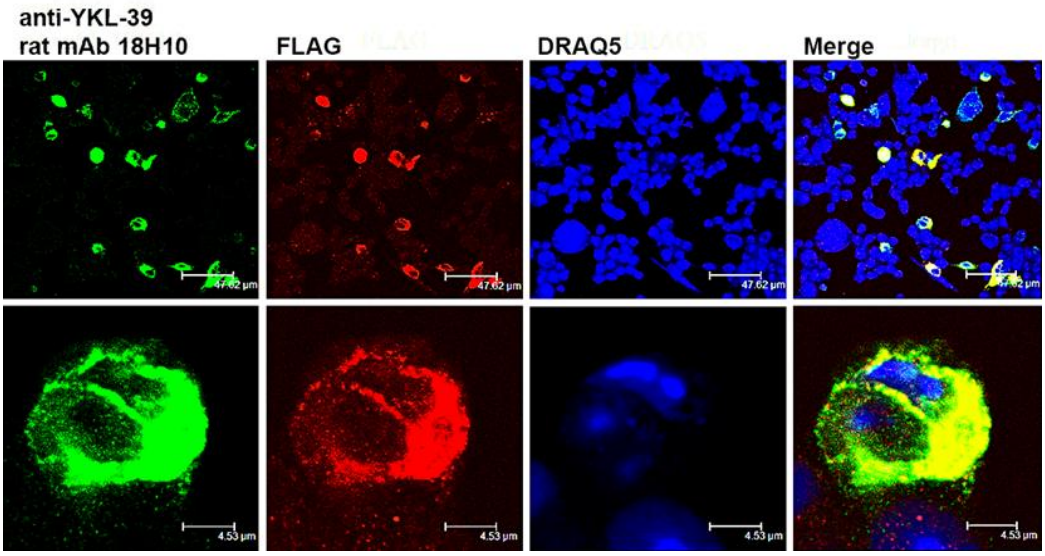


Figure 12. Analysis of the expression of YKL-39 in TS/A cells using immunofluorescent staining and confocal microscopy with rat anti-YKL-39 monoclonal antibodies towards immunogenic peptide YKL-39-N2. YKL-39 cells were detected with rat anti-YKL-39 mAb (clone 18H10) and secondary Alexa488-conjugated anti-rat IgG Ab. FLAG tag was detected with rabbit anti-FLAG Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Yellow color indicated co-localization of YKL-39 and FLAG. Scale bars: 47.62 μm and 4.36 μm.

3.1.3 Analysis of guinea pig anti-YKL-39 polyclonal antibodies

The results revealed that all tested samples of guinea pig anti-YKL-39 antibodies specifically recognized YKL-39 in transfected TS/A cells. The best clone (Immunized Serum I4) was selected for further experiments (Table 14, Figure 13, Figure 14).

Table 14. Results of guinea pig polyclonal anti-YKL-39 antibody analysis. Expression of YKL-39 was analyzed by immunofluorescent staining using guinea pig anti-YKL-39 polyclonal antibody.

Number	Names of clone	Percentage of positive cells	Expression level
1	Immunized Serum I2	> 20%	weak
2	Immunized Serum I3	> 20%	weak
3	Immunized Serum I4	> 20%	moderate

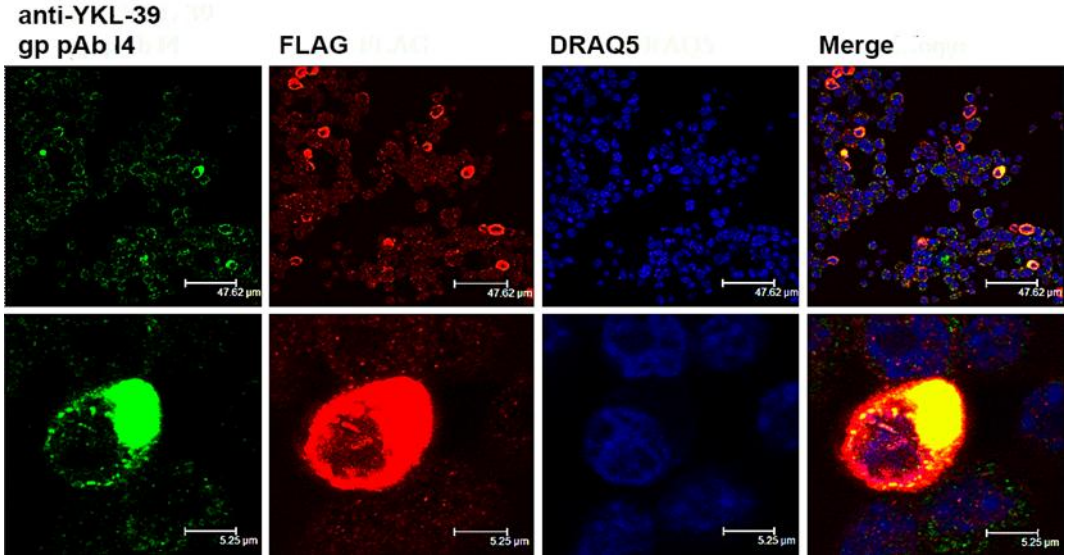


Figure 13. Analysis of the expression of YKL-39 in TS/A cells using immunofluorescent staining and confocal microscopy with guinea pig anti-YKL-39 polyclonal antibodies. YKL-39 cells were detected with gp anti-YKL-39 mAb (I4) and secondary Alexa488-conjugated anti-gp IgG Ab. FLAG tag was detected with rabbit anti-FLAG Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Yellow color indicated co-localization of YKL-39 and FLAG. Scale bars: 47.62 µm and 5.25 µm.

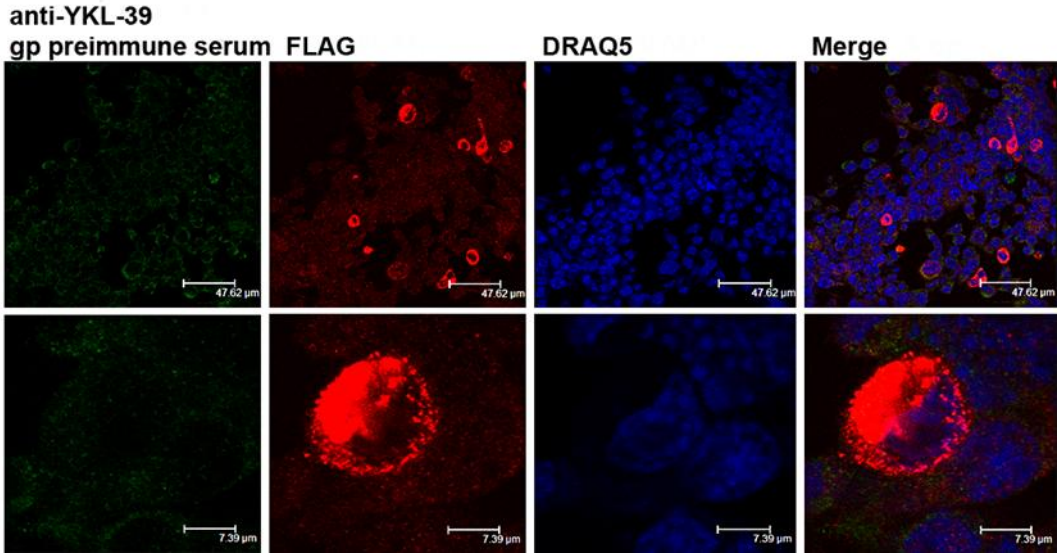


Figure 14. Analysis of the expression of YKL-39 in TS/A cells using immunofluorescence staining and confocal microscopy with guinea pig preimmune serum. YKL-39 expressing cells were detected with guinea pig pre-immune serum and secondary Alexa488-conjugated anti-gp IgG. FLAG tag was detected with rabbit anti-FLAG Ab and secondary Cy3-conjugated anti-rabbit IgG. Nuclei were visualized with DRAQ5. Scale bars: 47.62 µm and 7.39 µm.

3.2 Analysis of YKL-39 expression in monocyte-derived macrophages

3.2.1 Real-time PCR analysis of YKL-39 gene expression in monocyte-derived macrophages

YKL-39 belongs to human Glyco_18 domain containing protein family which comprises chitinases and chitinase-like proteins. Previously, YKL-39 was confirmed to be massively expressed in synovial fibroblasts in patients with osteoarthritis[188]. However, its expression in macrophages has not been carefully investigated. To analyze expression level of YKL-39 in macrophages, monocytes from human blood were stimulated with combinations of M2-driving factors: IL-4, IL-4+dexamethasone and IL-4+dexamethasone+TGF-β. Non-stimulated monocytes were used as a control. Monocytes were allowed to differentiate in X-VIVO medium at 37°C, 7.5% CO_2 for 6 days. Cells were harvested, mRNA was isolated and cDNA was synthesized to quantify the expression of YKL-39 by Real-time PCR.

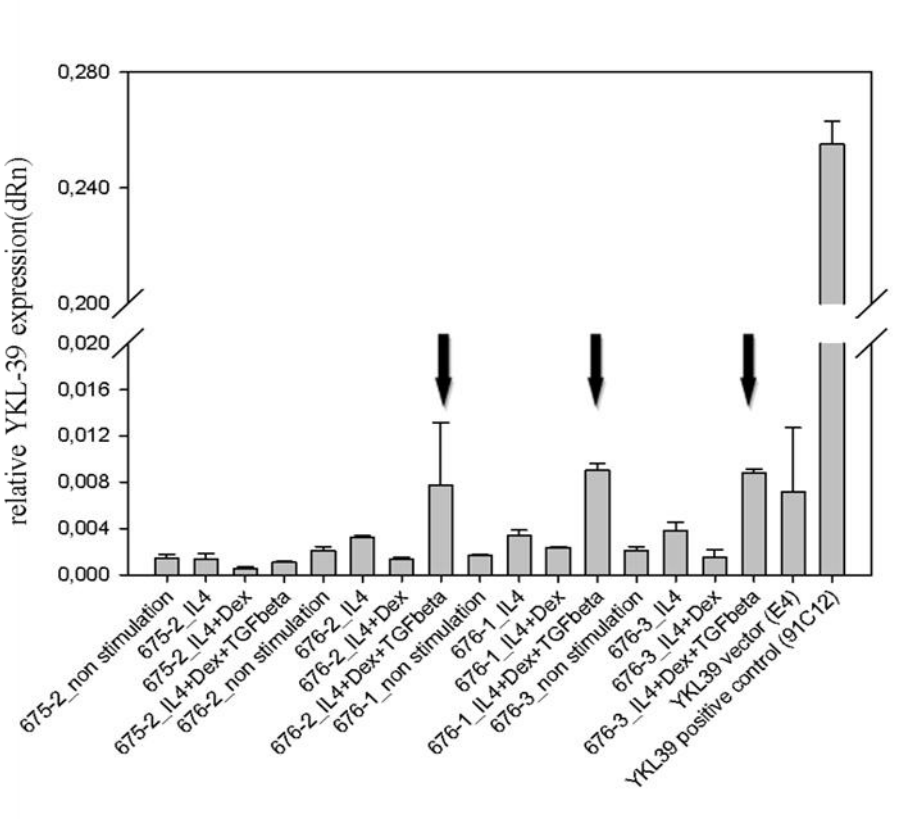


Figure 15. Real-time PCR analysis of YKL-39 expression in human monocyte-derived macrophages. Human peripheral blood monocytes were isolated and stimulated with different cytokines (as indicated on the diagram). YKL-39 stably transfected HEK293 clones (91C12) and vector clones (E4) were used as positive and negative controls. Number indicates individual macrophage culture derived from a single donors.

The results demonstrated that the expression of YKL-39 was upregulated in macrophages stimulated by combination of IL-4+dexamethasone+TGF-β in three out of four analyzed donors (Figure 15). Although the patterns of YKL-39 expression were similar in 4 donors, the levels of expression were donor-specific. For this reason, the data is shown for each donor separately. For three out of the four donors，analysis revealed that the expression of YKL-39 in macrophage showed a statistically significant increase under combined stimulation of IL-4 and TGF-β (P-value was obtained using ANOVA, P value < 0.01). Overall, both IL-4 and TGF-β could stimulate the expression of YKL-39 in monocyte-derived macrophages. But TGF-β was the strongest inducer of YKL-39 in human monocyte-derived macrophages.

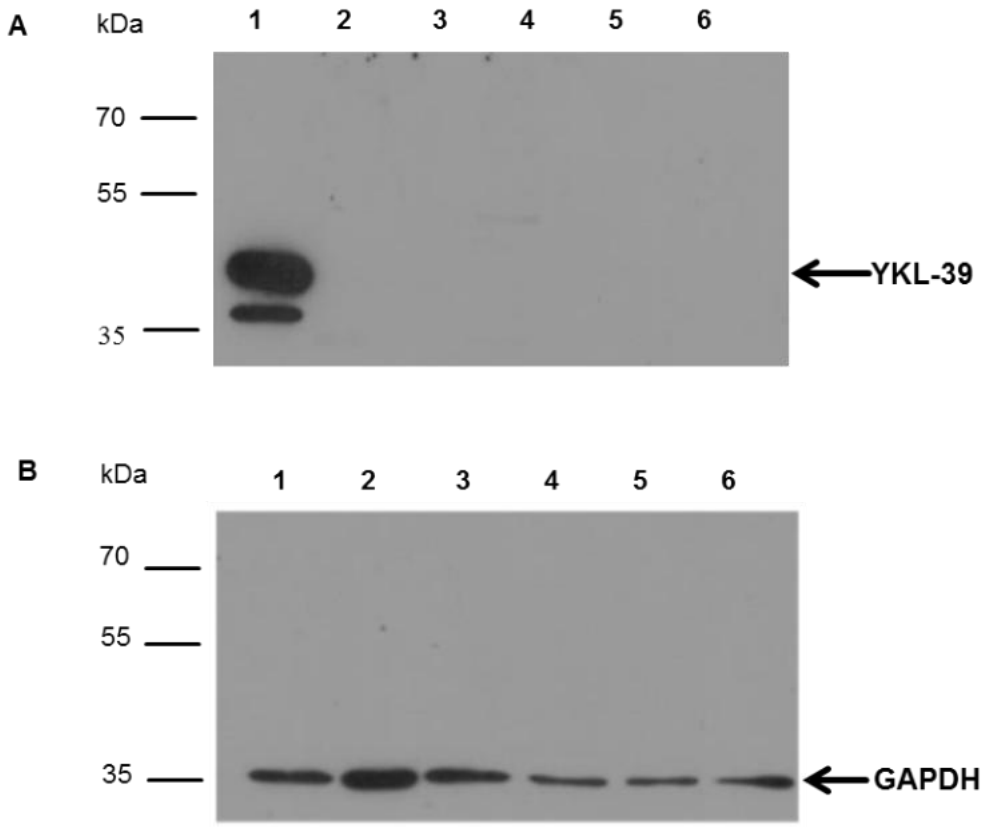
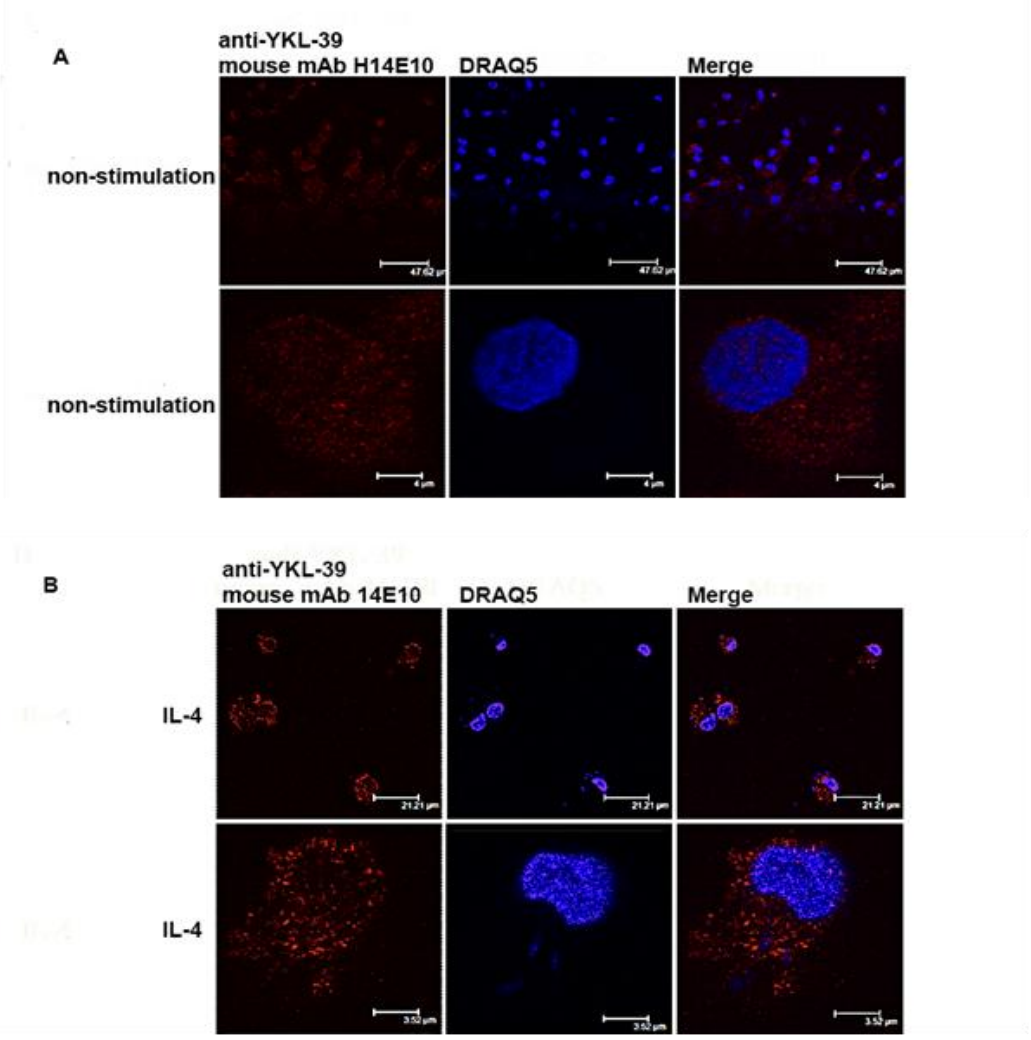


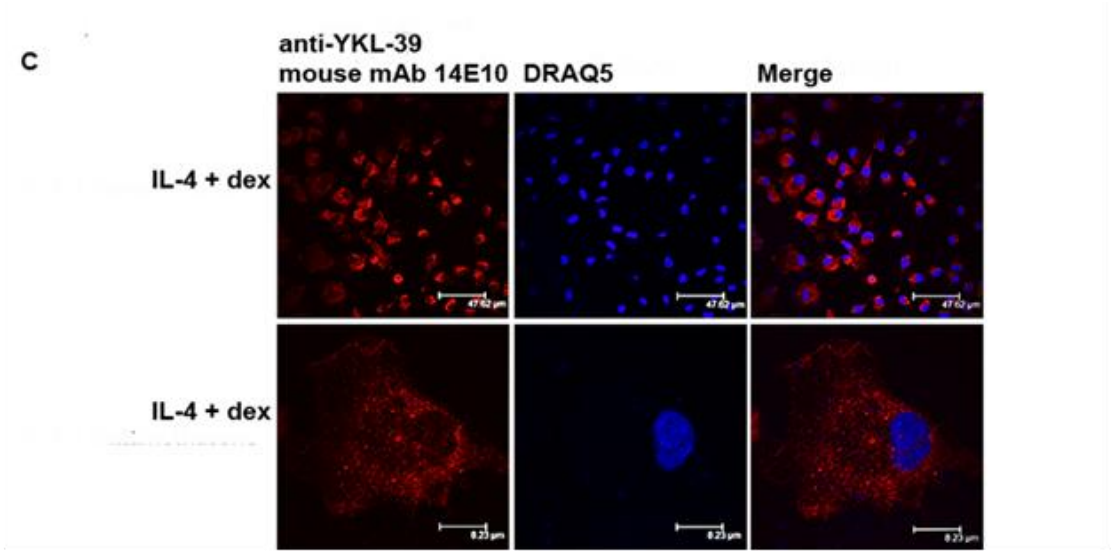
Figure 16. Analysis of YKL-39 expression in monocyte-derived macrophages by Western blotting. YKL-39 was detected with mouse anti-YKL-39 mAb (clone: 14E10) and secondary HRP-conjugated anti-mouse IgG. Lanes: (1). Positive control (clone 91C12) from stably transfected cell line HEK293; (2). Empty vector control (E4) from stably transfected cell line HEK293; (3). Non-stimulated macrophages; (4). Macrophages under IL-4 stimulation; (5). Macrophages under IL-4 + dexamethasone stimulation; (6). Macrophages under IL-4 + dexamethasone + TGF-β stimulation.

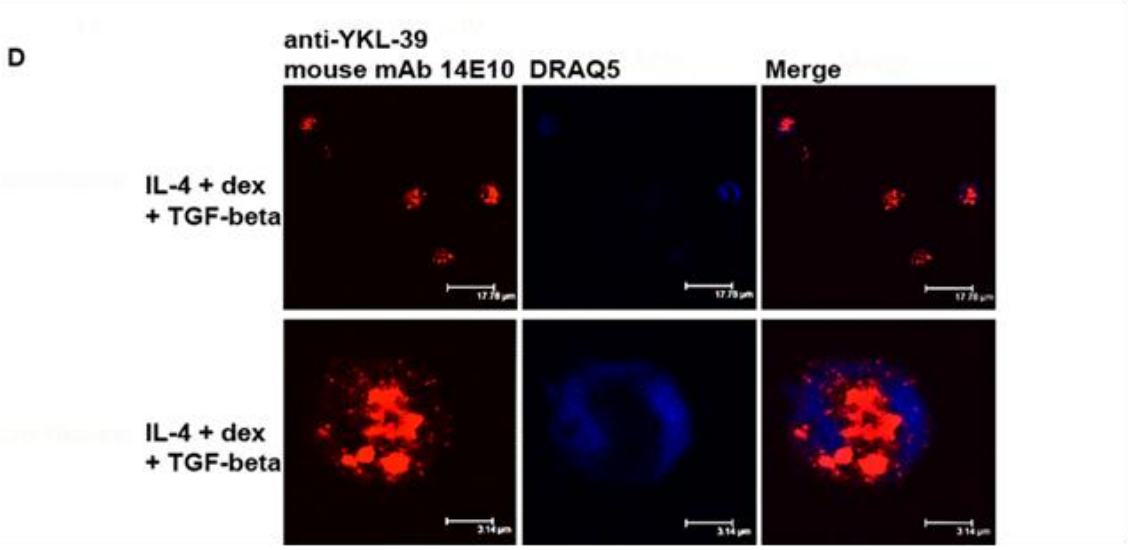
Western blotting demonstrated that recombinant YKL-39 protein was strongly expressed in HEK293 cells stably transfected with YKL-39 expression construct. However, its expression was not found in macrophages under any stimulation used (Figure 16). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein to demonstrate the protein loading. These data suggest that YKL-39 expression levels in human macrophages are insufficient to be detected by Western blotting. The decision was made to use immunofluorescent staining and confocal microscopy to detect endogenous YKL-39 protein in macrophages.

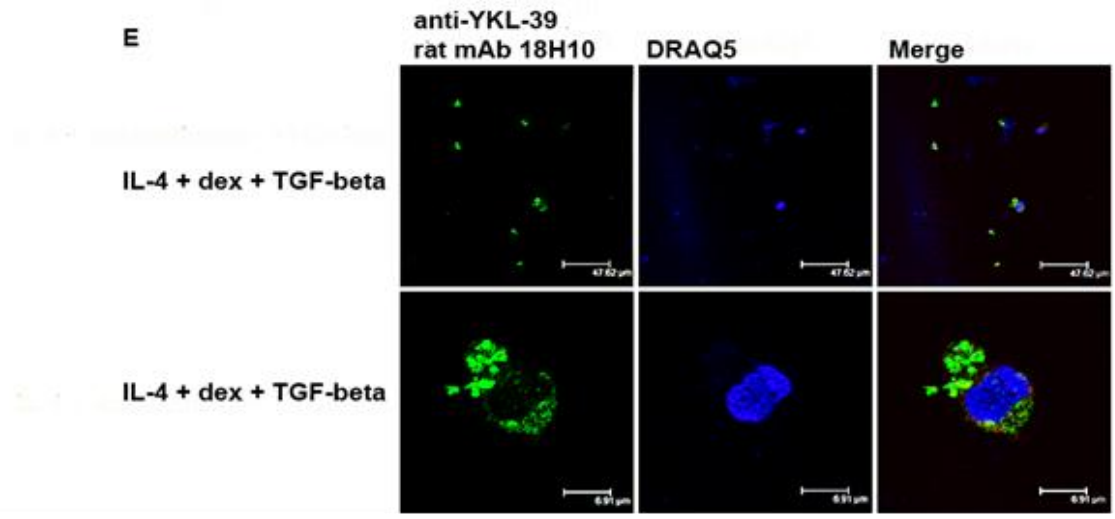
3.2.2 Immunofluorescent analysis of protein localization of YKL-39 in monocyte-derived macrophage

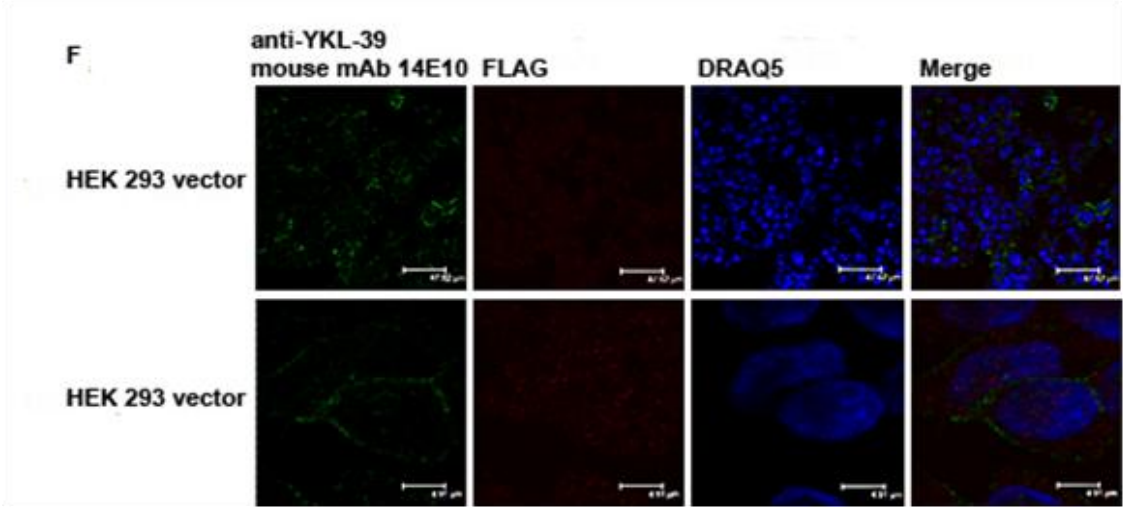
Monocyte-derived macrophages were stimulated with IL-4, IL-4+dexamethasone and IL-4+dexamethasone+TGF-β, and cultured for 6 days. Cells were fixed by 4% PFA and stained with mouse anti-YKL-39 monoclonal antibody (clone 14E10).


A
anti-YKL-39
mouse mAb H14E10
DRAQ5
Merge
non-stimulation
non-stimulation
B
anti-YKL-39
mouse mAb 14E10
DRAQ5
Merge
IL-4
IL-4


C
anti-YKL-39
mouse mAb 14E10
DRAQ5
Merge
IL-4 + dex
IL-4 + dex


D
anti-YKL-39
mouse mAb 14E10
DRAQ5
Merge
IL-4 + dex
+ TGF-beta
IL-4 + dex
+ TGF-beta


E
anti-YKL-39
rat mAb 18H10
DRAQ5
Merge
IL-4 + dex + TGF-beta
IL-4 + dex + TGF-beta


F
anti-YKL-39
mouse mAb 14E10
FLAG
DRAQ5
Merge
HEK 293 vector
HEK 293 vector

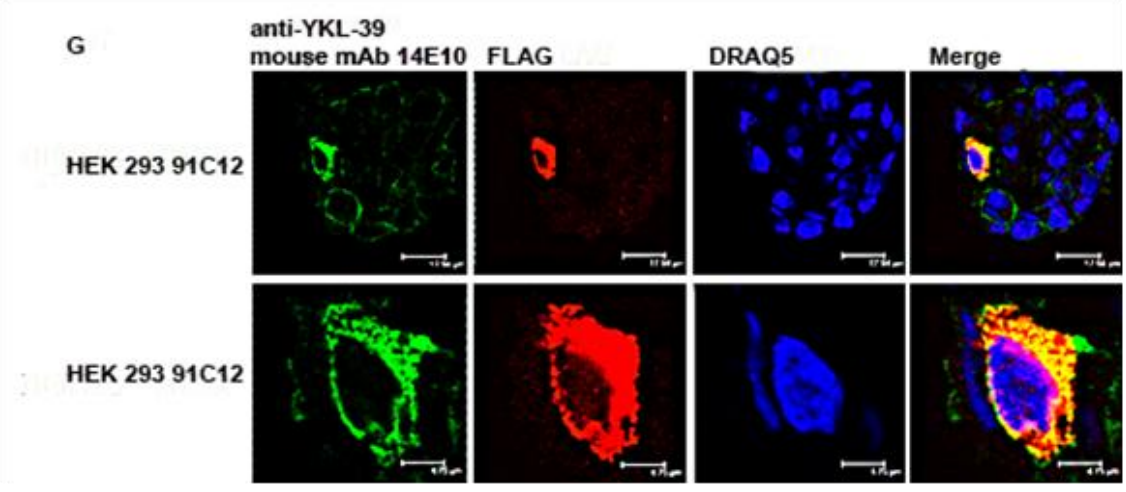

G
anti-YKL-39
mouse mAb 14E10
FLAG
DRAQ5
Merge
HEK 293 91C12
HEK 293 91C12

Figure 17. Immunofluorescent analysis of YKL-39 expression in monocyte-derived macrophages stimulated with different combinations of cytokines and stably transfected cell line using HEK239 clones 91C12 and E4. Monocytes were stimulated with IL-4, IL-4 + dexamethasone, IL-4 + dexamethasone + TGF-β. Cells were detected with mouse anti-YKL-39 mAb (clone 14E10 (A-D)) or rat anti-YKL-39 mAb (clone 18E10 (E)) and Cy3-conjugated anti-mouse IgG Ab (A-D) and Alexa488-conjugated anti-rat IgG Ab as secondary antibody. DRAQ5 was used for visualization of nuclei (A-G). YKL-39 stably transfected HEK293 clone 91C12, E4 was used as a positive control (F), and HEK293-vector was used as a negative control (G). Analysis was performed by confocal microscopy. Scale bars: 47.62 μm and 4.00 μm (A), 47.62 μm and 6.00 μm (B), 47.62 μm and 8.23 μm (C), 47.62 μm and 3.74 μm (D), 47.62 μm and 6.91 μm (E), 47.62 μm and 4.51 μm (F), 47.62 μm and 4.75 μm (G).

The results of immunofluorescent staining revealed that YKL-39 protein was strongly expressed in YKL-39 stably transfected HEK293 clone. Macrophages treated by IL-4 and IL-4 + dexamethasone did not express YKL-39, whereas only in macrophages stimulated by combination of IL-4 + dexamethasone + TGF-β, endogenous YKL-39 protein was detected using both mouse anti-YKL-39 and rat anti-YKL-39 monoclonal antibodies (Figure 17).

3.3 Analysis of YKL-39 expression as M2 marker in glioblastoma

Glioblastoma is a common, highly malignant tumor of the nervous system with a poor prognosis. In the past, it was mainly treated with a combination of surgical resection, chemotherapy and radiotherapy, but the data proved that these traditional treatment were not effective and the median survival was still less than one year[234]. Currently, research is focused on the possibility of cellular therapy as a new treatment. By intervening in the immune system, immune cells are used to directly remove tumor cells or to stop their growth and invasion[234].

During glioblastoma, a population of macrophages infiltrate tumor. These macrophages (TAMs) account for a large percentage of the tumor[235]. Although researchers usually considered this group of macrophages to be M2 subtype, there was no conclusive evidence to adequately define the phenotype of this group of TAMs[235, 236]. TAMs can promote tumor cell growth, proliferation and invasive development, furthermore, increased numbers of TAMs are linked to poorer prognosis of tumors[236]. In this study, we learned the characteristics and polarization status of TAMs subtypes in glioblastoma, and we suggest YKL-39 to be used as biomarker for TAMs in glioblastoma.

3.3.1 Analysis of YKL-39 expression in macrophages in glioblastoma

To identify the subpopulation of TAMs which produce YKL-39, we used of stabilin-1 as a marker of alternative activation of TAMs. In some regions of section, expression of YKL-39 was stronger than stabilin-1. YKL-39 was only partially co-expressed with stabilin-1 on macrophages (Figure 18, A). In other regions, stronger expression of stabilin-1 was detected, and YKL-39 was expressed by stabilin-1-negative cells (Figure 18, B).

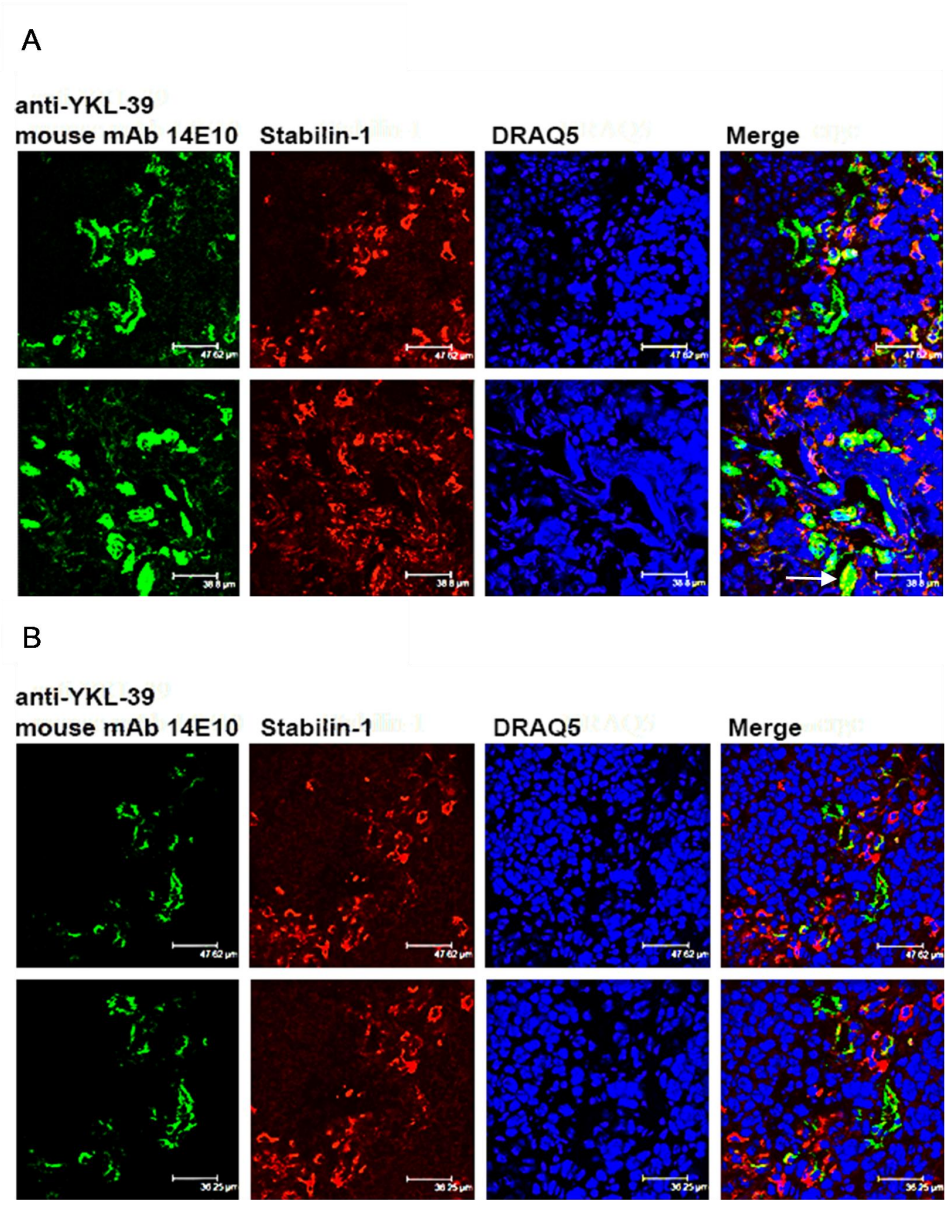


Figure 18. Analysis of YKL39 expression in glioblastoma in combination with stabilin-1. Glioblastoma samples were detected with mouse anti-YKL-39 mAb (clone 14E10) and secondary Alexa488-conjugated anti-mouse IgG Ab. And glioblastoma samples were detected with rabbit anti-stablilin-1 Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Yellow color indicated co-localization of YKL-39 and Stabilin-1. Scale bar: 47.62 μm and 38.8 μm (A), 47.62 μm and 36.25 μm (B).

3.3.2 Analysis of YKL-39 and LYVE-1 expression in macrophages in glioblastoma

LYVE-1 is a specific marker for lymphatic vessel endothelial cells[229]. The lymphatic system is one of the most important systems of the body and the body needs it to function in order to maintain proper functions. First, when the body is confronted with a foreign substance, the lymphatic system is capable of eliminating it by an effective and rapid immune response; second, the lymphatic system contributes to maintaining the balance of the body's tissue fluids[229]. It was found that TAMs can express LYVE-1 which is one of the established markers of lymphatic endothelium, and TAM can secrete pro-lymphangiogenic factors and differentiate into lymphatic endothelial cell to support tumor lymph angiogenesis[213, 229, 237]. As a core modulator affecting tumor progression, in vitro, TGF-ß significantly upregulated the synthesis of YKL-39 in macrophages[189], therefore, we hypothesized that YKL-39 might have the possibility to become a new biomarker for the subpopulation of TGF-ß-stimulated macrophages which are present in the tumor microenvironment and are involved in tumor progression.

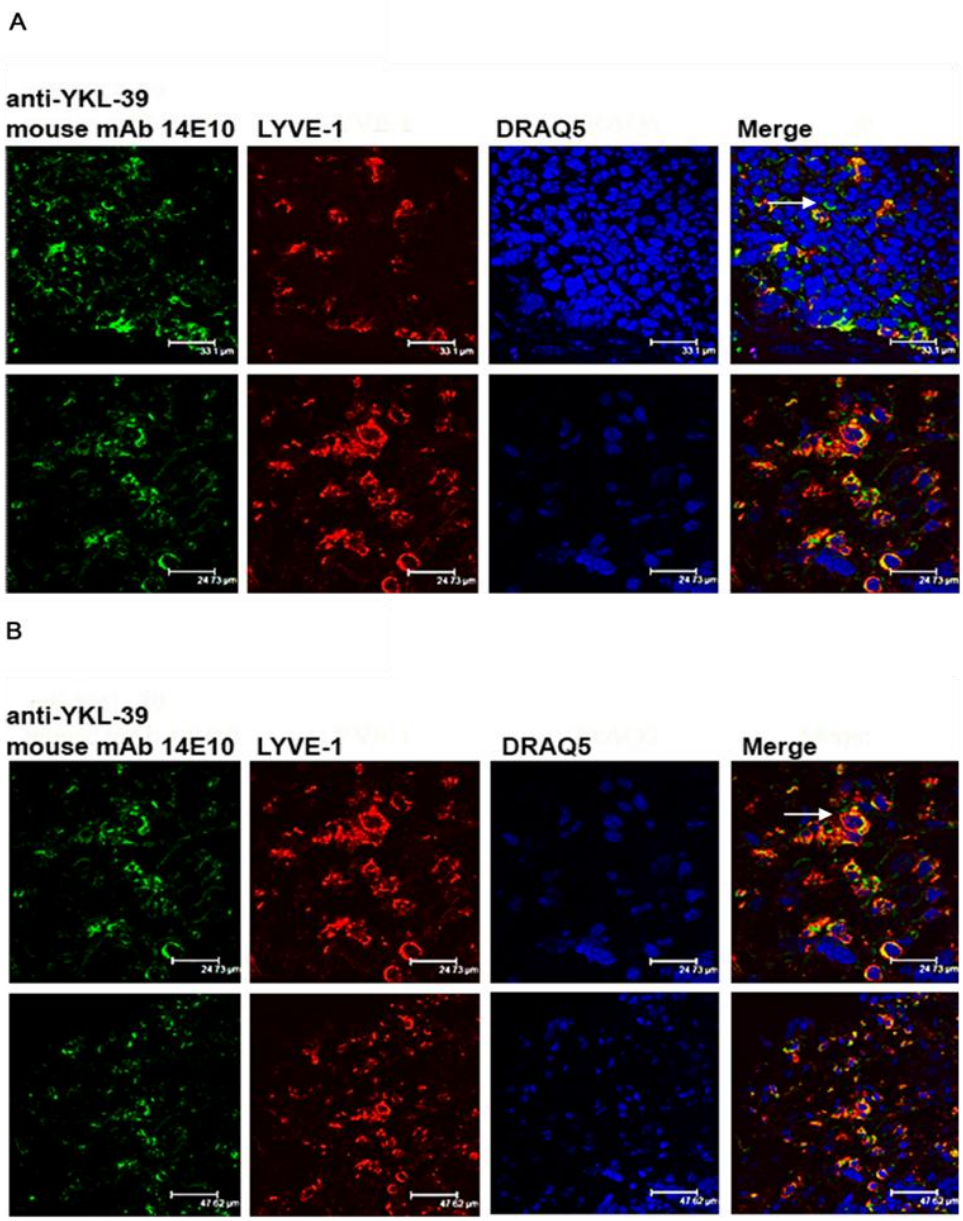


Figure 19. Analysis of YKL-39 protein localization in glioblastoma samples. Glioblastoma samples were analysed using mouse anti-YKL-39 mAb (clone 14E10) and secondary Alexa488-conjugated anti-mouse IgG Ab. And glioblastoma samples were detected with rabbit anti-LYVE-1 Ab and secondary Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Yellow color indicated co-localization of YKL-39 and LYVE-1. Scale bar: 28.74 μm and 33.1 μm (A), 24.73 μm and 47.62 μm (B).

The results of immunofluorescent staining revealed that YKL-39 was highly expressed in some tumor regions, however, the expression of LYVE-1 was low (Figure 19, A). In other regions, expression of LYVE-1 was stronger, and YKL-39 was partially co-localized with LYVE-1 positive macrophages (Figure 19, B).

3.3.3 Analysis of stabilin-1 expression in glioblastoma in combination with macrophage markers CD68, CD163 and CD206

CD68, CD163 and CD206 were widely applied for immunohistological characterization of macrophages[71]. Among them, CD68 is mainly used as a broad-spectrum biomarker of the monocyte-macrophage system to indicate the distribution of macrophages in human tissues and organs, while CD163 and CD206 are more frequently used as biomarkers of M2 macrophages[71]. The studies have demonstrated that an increase in the number of TAMs is strongly linked to a worse prognostic for patients with several major cancer types[235]. Studies in our laboratory demonstrated stabilin-1 is expressed in tissue-specific macrophages from different tissues, including: myocardium with myocardial infarction[238], breast[239] and monocyte-derived macrophage with stimulation of IL-4 + dexamethasone in vitro[240]. To study the expression of stabilin-1 in glioblastoma patients, immunofluorescent staining was performed in combination with antibodies recognizing CD68, CD163 and CD206 to determine whether stabilin-1 can be used as a marker of M2 macrophages in glioblastoma.

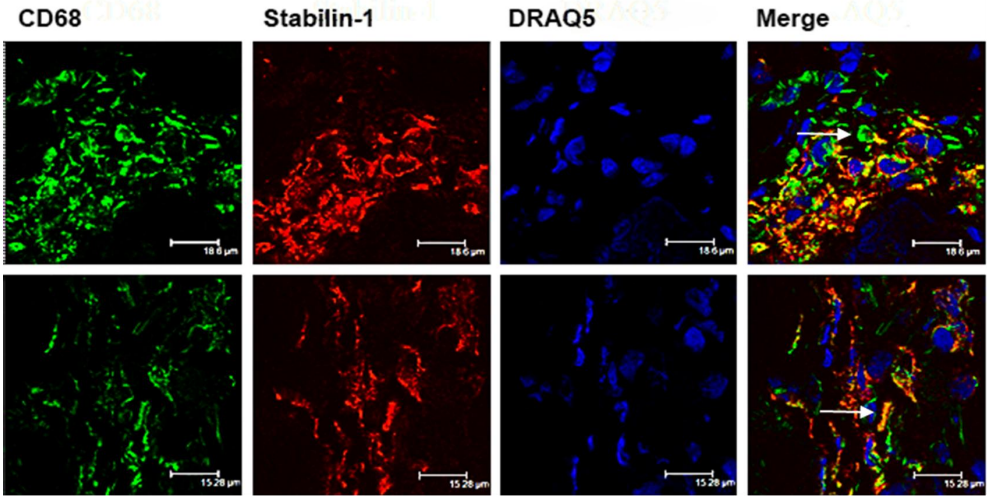


Figure 20. Analysis of stabilin-1 expression in glioblastoma in combination with CD68. Glioblastoma samples were detected with mouse anti-CD68 mAb and secondary Alexa488-conjugated anti-mouse IgG Ab. And glioblastoma samples were detected with rabbit anti-stabilin-1 Ab and Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Confocal microscopy was used to detect the expression and localization of stabilin-1 in combination with CD163 in glioblastoma. Scale bar: 18.6 μm and 15.28 μm.

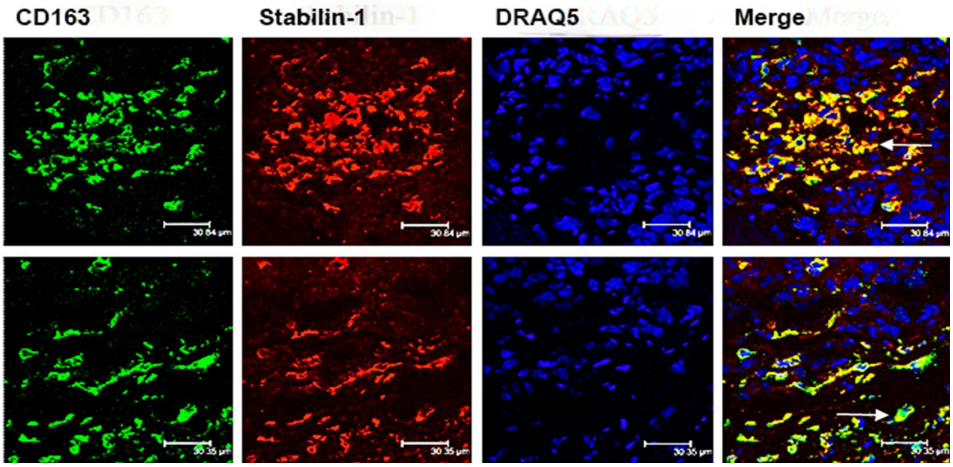
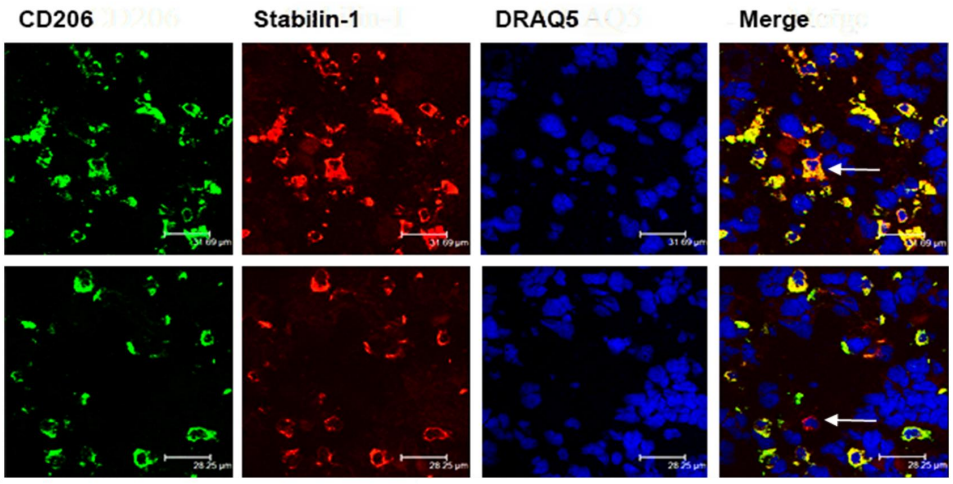


Figure 21. Analysis of stabilin-1 expression in glioblastoma in combination with CD163. Glioblastoma samples were analysed with mouse anti-CD163 mAb and secondary Alex488-conjugated anti-mouse Ab. Also, glioblastoma samples were analysed with rabbit anti-stabilin-1 Ab and secondary Cy3-conjugated anti-rabbit Ab. Nuclei were visualized with DRAQ5. Confocal microscopy was used to detect the expression and localization of stabilin-1 in combination with CD163 in glioblastoma. Scale bar: 30.84 μm and 30.35 μm.

A



B

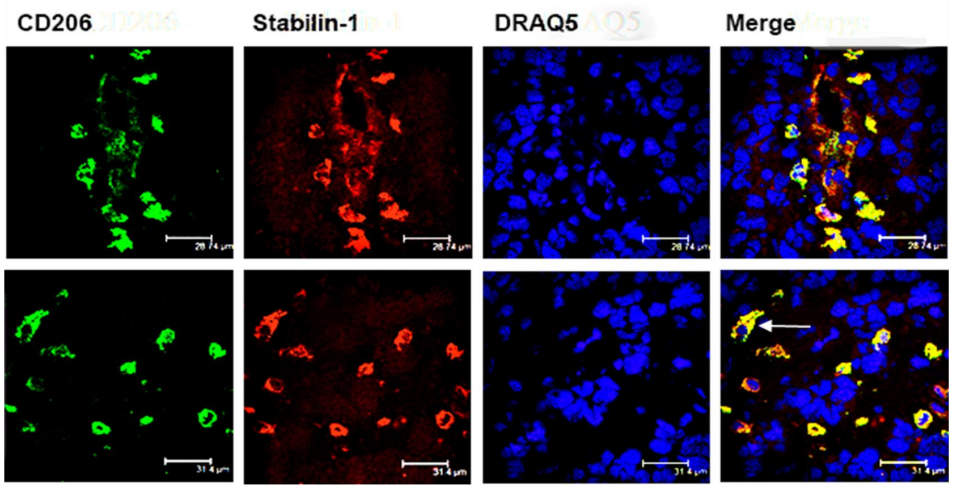


Figure 22. Analysis of stabilin-1 expression in glioblastoma in combination with CD206. Glioblastoma samples were analysed with mouse anti-CD206 mAb and secondary Alex488-conjugated anti-mouse IgG Ab. And glioblastoma samples were analysed with rabbit anti-stabilin-1 Ab and Cy3-conjugated anti-rabbit IgG Ab. Nuclei were visualized with DRAQ5. Confocal microscopy was used to detect the expression and localization of stabilin-1 in combination with CD206 in glioblastoma. Scale bar: 31.69 μm and 28.25 μm (A), 28.74 μm and 31.4 μm (B).

The results showed that stabilin-1 was partly expressed by $CD68^+$ cells and co-expressed by majority of $CD163^+$ or $CD206^+$ cells in different regions of tumor tissue. In some regions stabilin-1 was also expressed by $CD206^-$ macrophages (Figure 22, A). These results suggested that stabilin-1 can be used as M2 macrophage marker in glioblastoma. (Figures 20-22)

4 Discussion

4.1 Generation and selection of anti-YKL-39 antibodies

YKL-39 belongs to human Glyco_18 domain protein family. The family is composed of chitinases and chitinase-like proteins (CLPs). The family of human CLPs comprises YKL-40, YKL-39, and SI-CLP. Production of CLPs has been found in several cell types, including chondrocytes, neutrophils, macrophages, synoviocytes, cancer cells, and others[241, 242]. In a previous experiment, SI-CLP antibody was generated and selected by Shuiping Yin in our laboratory[81]. He established TS/A transfected cell lines using pcDNA3.1-SI-CLP-FLAG and pcDNA3.1-FLAG and validated expression of SI-CLP by an anti-SI-CLP monoclonal antibody (generated in collaboration with Dr. Elisabeth Kremmer, Helmholzzentrum, Münich)[81].

In the present study a similar test system was used to validate YKL-39 antibodies produced in the different animals aimed to increase their specificity and also to increase the number of antibodies combinations that are needed to investigate YKL-39 expression in different cell types. The fragment of human YKL-39 was used as the immunogen, a series of antibodies from guinea pig (polyclonal antibodies), mouse and rat (monoclonal antibodies) were generated. Their specificity was tested using a murine mammary adenocarcinoma cell line (TS/A) transiently transfected with pcDNA3-YKL-39 (P825) vector expressing recombinant human YKL-39 fused with the FLAG tag. After 36–48 hours of transfection, the expression of YKL-39 in the transfected cells was analyzed by immunofluorescent staining and confocal microscopy. Based on the results of immunofluorescent staining, anti-YKL-39 antibodies were sub-divided into negative, weakly positive, moderately positive, and strongly positive. Strongly positive antibodies were selected for further experiments. Through the screening process, we obtained several high-quality mouse and rat monoclonal antibodies. These antibodies provided specific signal and clearly recognized YKL-39, and were further used in this study. In our group, these high-quality antibodies have widely been used in subsequent experiments[194, 195]. However, the results also revealed that although all anti-YKL-39 antibodies from guinea pig specifically recognized YKL-39 in the transfected cells, the signal was relatively weak.

4.2 Assessment of YKL-39 expression in in vitro model of monocyte-derived macrophages under cytokine stimulations

In a previous study, YKL-39 was found to be secreted abundantly in the cultured primary human articular chondrocytes[189]. To examine expression of YKL-39 in human macrophages, the models of human blood monocyte-derived macrophages was used. In this system expression level of YKL-39 was significantly upregulated in response to IL-4 and TGF stimulation, however the data were limited to the quantification of mRNA levels[189]. Expression of YKL-39 protein in cells and tissues remained unknown due to the absence of specific antibodies. In our model $CD14^+$ monocytes were isolated from buffy coats and stimulated with combinations of M2-driving factors, namely IL-4, a combination of IL-4 + dexamethasone, and a combination of IL-4 + dexamethasone + TGF-β. RT-PCR analysis demonstrated that YKL-39 is strongly induced by TGF-β in the presence of IL-4 and dexamethasone, however, no signal was detected when Western blotting was performed while specific signal was detected in the control a TS/A-YKL39 cell line expressing recombinant YKL-39. However, when the same YKL-39 antibody was used for immunofluorescent staining and confocal microscopy, clear, strong and specific signal was detected in macrophages differentiated under stimulation with a combination of IL-4 + dexamethasone + TGF-β. These results are consistent with those obtained in the past by our laboratory. In this experiment, endogenous YKL-39 protein was detected by both mouse anti-YKL-39 and rat anti-YKL-39 monoclonal antibodies (which were produced in our lab) in macrophages stimulated by the combination of IL-4 + dexamethasone + TGF-β. TGF-β acted as the strongest inducer of YKL-39. These results suggested that YKL-39 as a promising macrophage biomarker that can reflect the activity of TGF-β in pathologies, including cancer.

4.3 TAMs diversity and markers used in humans

Tumor-associated macrophages (TAMs) are the dominant leukocyte population in tumor microenvironment. TAMs are involved in tumor growth, tumor angiogenesis, and suppression of adaptive immune responses and creation of optimal conditions for the metastatic spread of cancer cell[243-245]. However, depending on the cancer types, TAMs are presented in various subpopulations, where some subpopulations may retain anti-tumor abilities[71]. TAMs can be activated in 2 directions: classically activated M1 and alternatively activated M2. M1 macrophages can potentially have antitumor effects and stimulate the adaptive immunity and inflammatory response,

while M2 macrophages will rather inhibit the immune response and promote tumor growth and metastasis[71]. It has been shown that TAMs have specific phenotypes in different types of cancer, moreover, different subtypes of TAMs are present in different sites of the tumor[71]. To discriminate between different TAMs subtypes, different markers have been used to classify TAMs[71]. In previous studies, a number of markers corresponding to TAMs subtypes have been identified (Table 15).

Table 15. Biomarkers of subpopulations TAMs[71, 80, 194, 240, 246-250]

Macrophage marker	Function	TAM subpopulations	Type of cancer
CCL8 (MCP2)	Monocyte chemoattractant protein	M2	Breast
CD68	Transmembrane glycoprotein	General macrophage marker	Breast, colorectal, lung, ovarian, prostate
CD80	Immunoglobulin superfamily	M1	Colorectal, lung
CD163	Scavenger receptor for the hemoglobin–haptoglobin complex	M2	Breast, colorectal, lung
CD204 (MSR1)	Macrophage scavenger receptor	M2	Breast, colorectal, lung, prostate
CD206	Mannose receptor and C-type lectin	M2	Breast, colorectal, ovarian,prostate
YKL-39 (CHI3L2)	Chitinase-like protein, pro-angiogenic and monocyte chemoattractant	M2	Breast, gastric
YKL-40 (CHI3L1)	Chitinase-like protein, pro-angiogenic	M1	Breast, lung, prostate

For YKL-39, from studies on breast cancer in our lab, screened mouse monoclonal anti-YKL-39 (clone 4E10) antibody and anti-YKL-39 rat monoclonal antibody (clone 18H10) were applied in immunohistochemical staining. We found that significantly upregulated expression levels of YKL-39 were positively correlated with an increased risk of distant metastasis of tumor cells and poor response (stable or disease progression) after chemotherapy. It revealed that YKL-39 can be applied as a potential marker for determining tumor prognosis[130]. In addition, the data from the study of 68 female BC patients treated with anthracycline-containing NAC revealed that clinical response was associated with M2+ macrophage phenotype (YKL-39-

CCL18+ or YKL-39+CCL18-)[251].In gastric cancer, it has also been demonstrated that the significantly upregulated expression level of YKL-39 positively correlates with the degree of tumor-associated macrophage infiltration and angiogenesis, and YKL-39 has been suggested as a potential prognostic marker for gastric cancer[250].

4.4 TGF-β and human macrophages

Transforming growth factor beta (TGF-β) is an essential protein that is widely involved in the regulation of cell growth, differentiation and other critical physiological processes[252]. TGF-β is believed to be mostly anti-inflammatory cytokine, which induces the formation of heteromeric transmembrane serine/threonine kinase receptor complexes[252]. TGF-β can drive Smad-mediated signaling in number of cell types. In macrophages, TGF-β can drive both Smad2/3 and Smad1/5 signaling pathways, activating both pro-fibrotic and chronic inflammatory programs[146, 253].

In patients with traumatic brain injury, the combination of M-CSF, IL-6, and TGF-β1 has the ability to trigger the generation of tissue-repairing macrophages[254]. Moreover, TGF-β1 can function as a microglia differentiation factor, and the combination of M-CSF, IL-6, and TGF-β1 plays a role in the regulation of microglia function[254].

Earlier studies demonstrated that, with the stimulation of IL-4, macrophages differentiated from monocytes can express TGF-βRII on the cell surface, but the receptor was usually lost within 72 h[16]. However, a relatively low concentration of dexamethasone (1×10^{-8} M) was sufficient to support surface presence of TGF-βRII on M2 macrophages during their differentiation out of monocytes in vitro[16]. And under the stimulation of IL-4 + TGF-β, the gene expression of YKL-39 was dramatically upregulated[189]. The results of the study confirmed the previous results in our lab, and demonstrated that additional of dexamethasone, that enhances responsiveness of macrophages to TGF-β can also enhance YKL-39 expression.

4.5 YKL-39 and stabilin-1 are markers of M2 macrophages in glioblastoma

Glioblastoma is a common, highly malignant primary tumor of the brain. Patients with glioblastoma have a very low survival rate and exhibit resistance to anticancer treatment[255, 256]. There are two main types of cells in the microenvironment of glioblastoma, namely neoplastic and non-neoplastic cells. Among the non-neoplastic cells, the largest population are TAMs, which can constitute up to 40% of the tumor tissue[235]. In studies analyzing the significance of TAMs in tumors, there are usually two aspects. One aspect is the amount of TAMs, which is usually reflected by the

expression level of CD68. Another aspect is the phenotype of TAMs, which is reflected by specific M1 and M2 markers[71]. CD68 has been shown to be a broad-spectrum biomarker for macrophages, and CD163 and CD206 have been identified as M2 biomarkers[71]. LYVE-1 was believed be highly specific marker for lymphatic vascular endothelial cells[229]. However, it has been found that TAMs can express LYVE-1, and TAMs can secrete pro-lymphangiogenic factors and differentiate into lymphatic endothelial cells (ECs) to support tumor lymphangiogenesis[257].

In a previous studies, stabilin-1 has already been proven to be a marker of M2 macrophages under several physiological and pathological conditions[79]. However, there are no published reports on the expression of stabilin-1 in glioblastoma. In our study, we performed immunofluorescence staining using stabilin-1, CD68, CD163, CD206, and LYVE-1. The expression of stabilin-1 in M2 macrophages suggests that it may serve as M2 marker in glioblastoma. The results showed that stabilin-1 was expressed by $CD68^+$ cells and co-expressed in the majority of $CD163^+$ or $CD206^+$ cells in different regions of the tumor tissue. In some regions, stabilin-1 was also expressed by $CD206^-$ macrophages. These results suggest that stabilin-1 can be used as an M2 macrophage marker in glioblastoma.

In glioblastoma, the expression of YKL-39 (CHI3L2) and stabilin-1 was heterogeneous. The only previous study on the relationship between the expression of YKL-39 and cancer was performed by Kavsan et al.[258], who found that CHI3L2 gene expression was significantly upregulated in glioblastoma. Recently, a study conducted it our laboratory confirmed that YKL-39 is abundantly expressed on TAMs of breast cancer[130]. The aim of the present study was to clarify whether YKL-39 is strongly expressed in glioblastoma as it is in breast cancer. In addition, in previous studies conducted at our laboratory, stabilin-1 has been reported as a sorting receptor for SI-CLP in macrophages. However, further research is needed to confirm that stabilin-1 acts as a sorting receptor for YKL-39. YKL-39 was only partially co-expressed by stabilin-1^+ macrophages in some regions; in other regions, stronger expression of stabilin-1 was detected, and YKL-39 was expressed by stabilin-1^- macrophages. It may be suggested that YKL-39 can be a potential M2 macrophage marker but YKL-39 still can be found in other types of stabilin-1^- macrophages, and phenotypes of YKL-39^+ macrophages needs further investigation. And we have revealed that stabilin-1 acts as a sorting receptor for YKL-39 in breast cancer in our

lab[130], subsequent experiments are needed to confirm whether stabilin-1 can function as an intracellular sorting receptor for YKL-39 in glioblastoma.

LYVE-1 is a HA receptor that is predominantly located on the lymphatic vessel endothelium, which coincides with the area of distribution of HA on the luminal surface of lymphatic vessels[259]. According to the reports from our and other research groups, the expression levels of LYVE-1 in TAMs can be upregulated in human and mouse cancers[213, 259]. In addition, several studies have demonstrated that LYVE-1$^+$ macrophages may mainly play a role in lymphatic tract remodeling[260]. And a special subset of TAMs characterized by co-expression of stabilin-1$^+$ and LYVE-1$^+$ was suggested to contribute to tumor lymphangiogenesis[213, 261].The data generated by the current work demonstrated that macrophages can produce YKL-39 in vitro under stimulation of TGF-β. YKL-39 is thought to be a biomarker for TGF-β-programmed macrophage subsets in the tumor microenvironment[189]. However, only limited data were available for the expression of YKL-39 on macrophages in vivo. In the present study, YKL-39 was found to be highly expressed in some tumor regions, however, the expression of LYVE-1 was low. In other regions, the expression of LYVE-1 was stronger, and YKL-39 partially co-localized with LYVE-1$^+$ macrophages. This might suggest that a subpopulation of YKL-39$^+$ TAMs distribute and play roles in deep lymphatic vessels in tumors. It can be hypothesized that TAMs characterized by co-expression of YKL-39$^+$ and LYVE-1$^+$ may contribute to lymphatic tract remodeling and tumor lymphangiogenesis.

5 Summary

Macrophages are key cells of innate immune system. Pathological activation of macrophages results in chronic inflammation, which leads to serious human pathologies, including solid tumors. The analysis of macrophage biomarkers allows the classification and identification of macrophages subpopulations in inflammation and in tumors. TGF-β is an important driver of pathological macrophage programming in cancer. Previous studies have demonstrated that TGF-β can stimulate expression of YKL39 gene expression in macrophages. However the antibodies to examine YKL-39 expression in cells and tissues were not available. The aims of the current project included: 1) establishment of a test system for the analysis of specificity of newly generated anti-YKL-39 antibodies; 2) analysis of specificity of the newly generated anti-YKL-39 antibodies; 3) analysis of the TGF-β effect on the expression of YKL-39 in different subpopulations of human macrophages; 4) analysis of intracellular localization of YKL-39 in M2 macrophages and its co-localization with stabilin-1; 5) examination of expression of YKL-39 in TAMs subpopulations in human glioblastoma samples. The test system was designed the by transient transfection of pcDNA3-YKL-39-FLAG into mouse breast adenocarcinoma cell line TS/A. Immunization of mice, rat and guinea pigs with YKL39 peptides has been performed in cooperation with Dr. H-R. Rackwitz (Peptide Speciality Lab, Heidelberg) and Dr. E. Kremmer (Helmholzzentrum, München). 43 murine hybridomas, 73 rat hybridomas and 4 sera from immunized guinea pigs were tested in this work using TS/A-YKL-39-FLAG and TS/A-vector control cells for the specific recognition of recombinant YKL-39. Monocytes were isolated out of buffy coats by gradient centrifugation and CD14+ positive selection. M2 subpopulations were generated by stimulation with IL-4 alone, IL-4+dexamethasone, and IL-4+dexamethasone+TGF-β directly after monocyte isolation. The expression of YKL-39 protein was analyzed using quantitative RT-PCR, and by immunofluorescent staining/confocal microscopy using the newly generated antibodies. Using a-stabilin-1 RS1 antibody, co-localization of YKL-39 and stabilin-1 was studied in glioblastoma in combination with CD68, CD163, CD206, and LYVE-1 using triple immunofluorescent staining and confocal microscopy. Most of the anti-YKL-39 antibodies generated in mice specifically recognized YKL-39. Clones H6G4, H3E4, H5A2, 14E4, 14C12, 14E10, 11H3, 15D12, and 24A6, which were strongly positive, were selected for the sub-cloning. The strongest clone 14E10 was selected

for further experiments. A few of the anti-YKL-39 antibodies generated in rats specifically recognized YKL-39. Clones 13D7, 18H10, 14D7, H9A7, and H10G8 were strongly positive and were selected for the sub-cloning. The strongest rat mAb 18H10 was selected for further experiments. As for the YKL-39 antibodies generated in guinea pigs, the strongest serum (I4) was tested. The expression of YKL-39 mRNA was strongly upregulated in macrophages stimulated by the combination of IL-4+dexamethasone+TGF-β in three out of four donors. Only IL-4+dexamethason+ TGF-β–stimulated macrophages expressed the endogenous YKL-39 protein, as detected by immunofluorescent staining and confocal microscopy using mYKL-39 and rYKL-39 monoclonal antibodies. In glioblastoma sections, YKL-39 was partially co-expressed with stabilin-1+ and LYVE-1+ in intratumoral macrophages. Stabilin-1 was partially expressed by CD68+ cells and expressed in majority of CD163+ or CD206+ cells, suggesting that stabilin-1 can be used as an M2 macrophage marker in glioblastoma. YKL-39 can be used as a potential biomarker of M2 macrophages in glioblastoma. Nevertheless, since YKL-39 is only partially co-expressed by stabilin-1+ CD68+ macrophages, it should be used in combination with other M2 markers. YKL-39 can serve as a biomarker for the reaction of TAMs in TGF-β in tumor microenvironment. Newly generated murine mAb 14E10 and rat mAb 18H10 were tested in immunohistology and demonstrated high specificity and intensity of the reactions and can be further used for the analysis of YKL-39 expression in patients' samples and in in vitro experimentation.

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7 Figure Index

8 Table Index

9 Curriculum Vitae

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