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The Repulsive Guidance Molecule – A attenuates chemotaxis induced neutrophil migration and dampens inflammation.

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INDEX

1 Introduction

1.1 Migration of leukocytes in response to acute inflammation.

Intensive care medicine is often challenged by acute inflammation as it is primarily seen in pathologies like peritonitis or respiratory distress syndrome. Despite all progress in modern medicine both diseases still have a lethality that might increase to more than 50% depending on the patients condition. In this context a better understanding of the pathology of acute inflammation is fundamental to improve existing therapies. Acute inflammation is characterized by hyperemia, increased vascular permeability and invasion of immune cells(1). Since Mechnikov's discovery in 1901, white blood cells or leukocytes are known to be the key hematopoietic cells to mediate the initial acute inflammation(2). As already known by Mechnikov, leukocytes have to be able to migrate to sites of the inflammatory injury to eliminate pathogens. Such migration of leukocytes is seen in all forms of inflammation, as mentioned above to a particularly high extent in intensive care medicine dealing with peritonitis or respiratory distress(3,4). Here, inappropriate migration of immune cells into the region of inflammation can result in tissue destruction, organ dysfunction or even organ failure(5). Therefore studying a better understanding of the mechanisms involved into leukocyte migration is clinically of major interest and can improve therapies by pointing out new drug targets.

Leukocyte migration is regulated in order to ensure adequate host defense and prevent an exaggerated immune response(6). To give a rough overview of how leukocyte migration is regulated a three-step model of extravasation can be used. It describes the fundamental precondition of leukocytes crossing the endothelium, the process of adhesion (Figure 1): In brief, leukocytes trafficking through postcapillary venules roll on the surface of endothelial cells. During this time period leukocytes can receive signaling from endothelial cells. This is facilitated by hemoconcentration following vascular leakage in inflammation, as well as through cell adhesion molecules known as selectins, transmembrane-molecules expressed on the surface of the endothelium. In a second step, tethered leukocytes are triggered by proteins bound to the endothelial surface. These proteins belong to a group of messenger proteins (cytokines) known as chemokines (chemotactic cytokines), which are

produced by either tissue cells or immune cells and selectively activate leukocytes(7). At last, activated leukocytes induce integrins, proteins that bind to cell adhesion molecules, and the process of transmigration, also known as diapedesis, is initiated (Figure 2). Diapedesis is based on active migration facilitated by an accurately defined reorganization of the cytoskeleton(8).

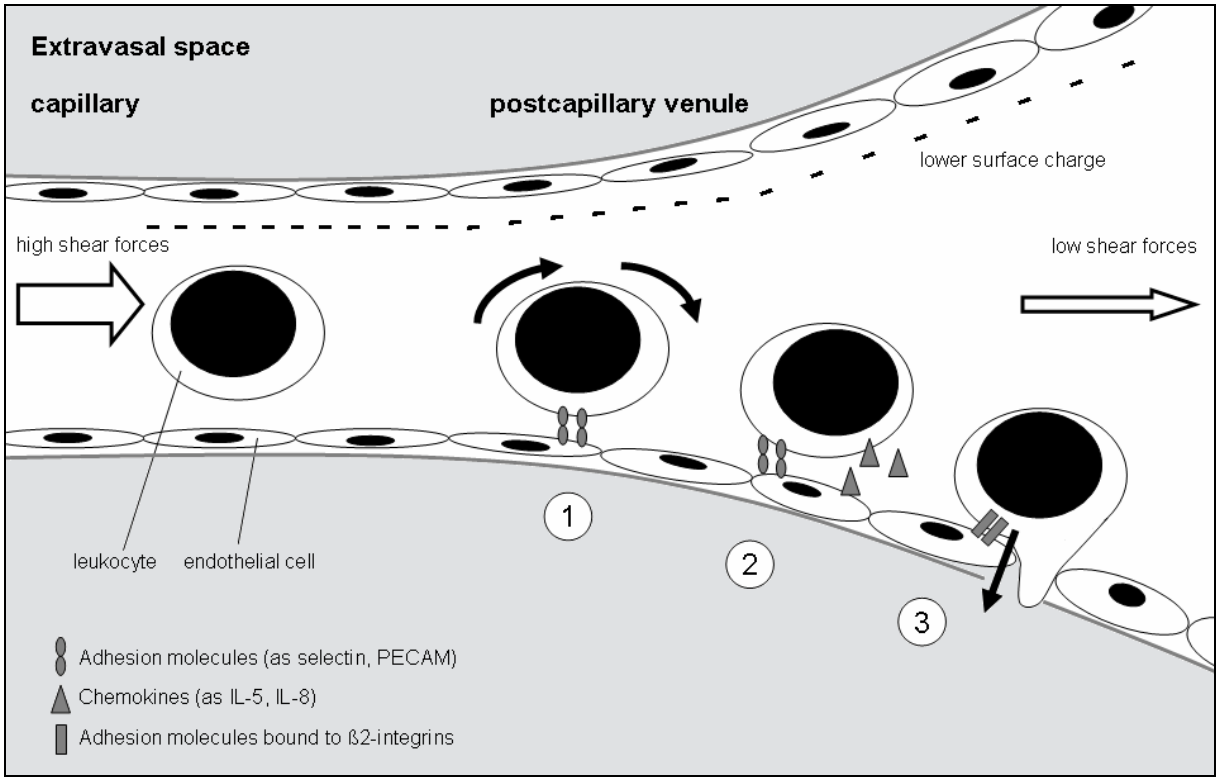

Figure 1. Adhesion as a precondition of leukocyte migration, described in a three-step model(7). (1) Tethering: Slowing down of circulating leukocytes, rolling along endothelial surface. Adhesion molecules initiate cell-cell interaction. (2) Triggering: Leukocytes receive signals via chemokines. This leads to (3) Latching: Cell adhesion molecules on endothelial cells bind to integrins on leukocytes. Cell adhesion molecules are induced by chemokines. Physical properties that influence cell adhesion are shear forces and surface charge on the endothelium. High shear forces and higher surface charge in capillaries compared to postcapillary venules prevent adhesion.

Chemokines play a crucial role in this process since the orchestration of chemokines determines whether or not a leukocyte passes the vascular barrier(9). Approximately 50 chemokines have been identified, starting with interleukin 8 (IL-8) in 1987(10). Chemokines are small proteins characterized by four cysteins forming two disulphide bounds, a loop region that recognizes the chemokine receptor and an N-terminal motif that triggers the receptor after binding took place. Chemokine

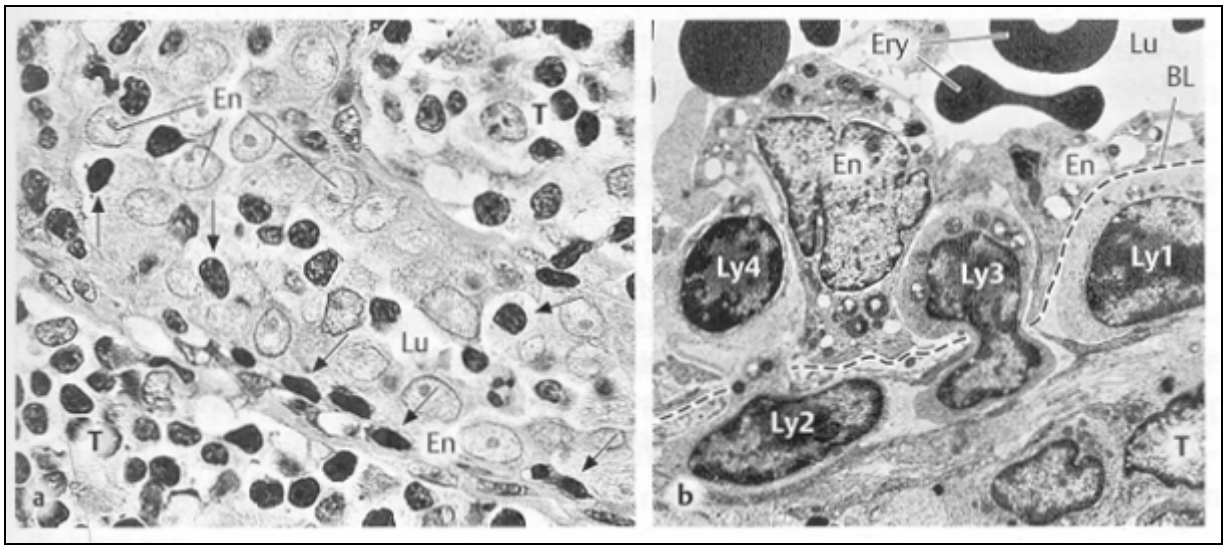

Figure 2. Leukocyte migration in a postcapillary venule (a. human lymph node, b. rat lymph node, from Lüllmann-Rauch(11), 2003). a. Lymphocytes (marked by arrows) migrating through endothelium (En) and basal lamina (BL) into the T-zone (T) of the extravascular space. b. Lymphocytes (Ly1-4) at different stages of diapedesis. Erythrocytes (Ery) in the lumen of the vessel (Lu). (Giemsa stain, magnification 640x, electron-microscopical image, magnification 2000x)

receptors are heptahelical, coupled to guanosine triphosphate (GTP) binding proteins and classified into CC and CXC chemokines depending on the position of their disulphide bounds. 8 receptors for CC chemokines are known and 4 CXC receptors, termed CCR1-8 and CXCR1-4. Besides the activation and adhesion mentioned above binding of chemokines and chemokine receptors on leukocytes results in an increase in intracellular free calcium, a release of granules and the initiation of cytoskeletal reorganization that enables active migration. Chemokines induce a polarization of leukocytes through the intracellular distribution of actin, a major component of the cytoskeleton, so that leukocytes acquire a polarized shape that defines the direction of movement(8). Chemokines can be specific for subtypes of leukocytes - interleukin 5, as an example, recruits eosinophils while IL-8 attracts neutrophils and macrophages. In the past, therapeutical approaches tried to interfere with chemokine signaling to influence inflammation at an early stage. For example, an anti IL-8 antibody proved to be successful in repressing acute inflammation in animal experiments(12,13). This is plausible as neutrophil granulocytes are among the first cells to invade inflamed tissue. An IL-8 antibody even proved therapeutically beneficial in humans(14): a recent study pointed out that palmoplantar pustolosis, a chronic skin disease, is partly mediated by IL-8 and can be dampened by a

monoclonal IL-8 antibody. Chemokines can further be separated into two major groups, one present under pathological conditions, and a second group that is expressed constitutionally to regulate physiological leukocyte traffic, as homing of lymphocytes. Interestingly, previous work has also suggested that chemokines could be involved in migratory processes of organ development(15,16).

1.2 Neuronal guidance molecules as immune modulating proteins.

Cellular migration guided through messenger proteins is not only found in the immune system but has been well characterized during neuronal development(17,18). During development of the central nervous system (CNS) axonal endings have to migrate remarkable distances to form neuronal projections. To guide axon growth cones proteins are expressed that either have an attracting or repulsing effect, acting as cell-bound or soluble protein. So far five families of such guidance molecules have been described: netrin, slit, ephrin, semaphorin and repulsive guidance molecule. Concentration gradients of these guidance molecules and the balance of their attractive and repulsive signals determine the precise direction of axonal growth. Interestingly, several neuronal guidance molecules were reported to also guide leukocyte migration (19-25). This led to the speculation that guiding mechanisms of the central nervous system were conserved in the immune system(26). This concept is comprehensible as both migratory processes are based on the reorganization of parts of the cytoskeleton. Nevertheless, the fact that neuronal guidance molecules could influence leukocyte migration was not obvious since migration in the nervous system differs from migration of leukocytes in speed, cellular environment or morphology(18).

Slit-2 was the first protein to be characterized in this regard(19), 14 years after the discovery of the first known chemokine IL-8. The major difference is the fact that slit-2 showed an inhibitory and repelling effect on leukocytes in contrast to chemokines, which, without exception, promote migration. These findings led to a new understanding of leukocyte migration: Immune cells can probably both be attracted and repelled by endogenous factors. Antagonists of chemokines were described previously, but only of viral origin(27,28). Slit-2 is an extracellular matrix protein involved in the development of commissural axon pathways. The inhibitory effect of slit-2 on leukocytes was assessed by in-vitro transmigration assays. After

suggesting that other neuronal guidance proteins could show similar influence netrin-1, a protein also guiding commissural axon growth, was studied(20). Netrin-1 is known to act as a repulsive and attractive cue at the same time depending on the receptors it binds to. Similarly to slit-2, netrin-1 revealed an inhibitory effect on leukocyte migration, demonstrated in-vitro and in-vivo. This work also demonstrated an expression of netrin-1 in human tissue and a down-regulation after treatment with tumor necrosis factor α (TNF α), an important inflammatory cytokine. Finally, Uncoordinated (UNC) 5b, a receptor mediating netrin's repulsive effect in the CNS, might be involved in reducing leukocyte migration. Further studies corroborated the findings concerning slit-2 and netrin-1. As examples, slit-2 was reported to regulate T-cell migration(23), netrin-1 was shown to attenuate leukocyte traffic in inflammation due to hypoxia(21). Interpreting these results it can be presumed that proteins such as slit-2 and netrin-1 fulfill the task to prevent inappropriate leukocyte migration. In contrast, other neuronal guidance molecules support the activation of immune cells. Semaphorin 7A was shown to enhance inflammatory responses(24,25). Semaphorins build a large group of either repelling or attracting guidance cues. Semaphorin 7A, that is known to promote axonal outgrowth, is involved in the formation of the olfactory tract. In an immunological context it was reported to be expressed on activated T-cells and to stimulate cytokine production. The growing evidence about the immunological properties of neuronal guidance proteins prompts to focus on similar, not yet characterized proteins.

1.3 The Repulsive Guidance Molecule A.

The Repulsive Guidance Molecule (RGM) is one of the previously described guidance proteins involved in neuronal development. It was initially described in 1990 in the chick visual system based on the observation that growing temporal retinal axons could be repelled by membranes of the posterior tectum(29). Led by these findings, a novel glycosylphosphatidylinositol(GPI)-anchored protein of 33 kDa size was isolated and later named RGM according to its action.

Monnier and Sierra could further biochemically characterize it(30). A detailed analysis confirmed that RGM is GPI-anchored and partly contains molecular structures known from different human proteins (Figure 3): Two hydrophobic domains at the N- and C-terminus, a partial von Willebrand factor domain and a cell adhesion

sequence, the amino acid motif arginine-glycine-aspartic acid (RGD). Von Willebrand factor is a glycoprotein that is constitutively produced by endothelial cells as well as megacaryocytes, precursor cells of platelets. It plays multiple roles in blood hemostasis. By binding to platelet receptors and to collagen it connects platelets and connective tissue after damage to blood vessels(31). Binding and stabilizing factor VIII is another function of von Willebrand factor and requires a module called D domain(32). Besides RGM, the D domain can be found in various other proteins including integrins and complement factors. Another main structural feature of RGM is its RGD site. The sequence arginine-glycine-aspartic acid is a cell attachment site recognized by integrins and is of great importance for cell adhesion and migration. Previous work has demonstrated that short peptides containing an RGD site influence cell adhesion. It can be enhanced if a surface is coated with those peptides, whereas RGD containing peptides prevent cell adhesion if previously presented to cells(33). Interestingly, RGM does not share sequence homologies to one of the four already known families of guidance proteins. Furthermore, Monnier and Sierra described an expression of RGM in the embryonic chicken tectum, gradually increasing from the anterior to the posterior pole. Its repulsive function could be demonstrated in a stripe assay in-vitro: temporal retinal axons specifically avoided growing towards stripes containing membranes of RGM-transfected cells.

In mammals three subtypes of RGM exist, termed RGM-A, -B, -C, with RGM-A, gene locus on chromosome 15q26.1(34), having the greatest homology to chick RGM(35), which is 80%. Their expression was examined in mouse embryos: RGM-A was expressed in various central nervous areas, predominantly in the hippocampus, midbrain, parts of the brainstem and spinal cord(36,37). More recent studies pointed out that RGM-A is also expressed beyond the central nervous system(37-39), as its mRNA was detected in rat heart, lung, liver, skin, kidney and testis. RGM-B expression shows a similar distribution, while RGM-C, that is expressed in muscle tissue, differs in some aspects. An influence of RGM on the retinotectal map formation in mammals could not be reported, instead, the repulsive effect of RGM-A was described to be involved in the formation of the dentate gyrus(40), in neuronal tube closure(35) and in the development of the enteric nerve system(41). In rats as well as humans RGM-A was induced following central nervous system injury(42,43). This fact is clinically particularly interesting since it was possible to improve the regeneration of spinal cord injury by an anti RGM-A antibody in rats (44).

The molecular mechanisms of RGM-A signaling need to be further explored. Mainly, one receptor of RGM-A, neogenin, has been identified and examined(45). The fact that RGM-A exerts its function through the neogenin receptor, which is also a known receptor for netrin-1, was demonstrated in binding studies. Neogenin was first described in 1994 as a protein involved in neural development and is a 160 kDa size transmembrane protein consisting of 4 immunoglobulin G domains and 6 fibronectin type III repeats (Figure 3). RGM-A is supposed to bind within the extracellular fibronectin repeats(46). It is unlikely that RGM-A interacts with the same binding site as netrin-1, as both proteins do not share a significant sequence homology. The affinity of RGM-A binding neogenin was described to be about 10-fold higher than netrin binding neogenin, having a dissociation constant of 230 pM(47). The conclusion that RGM-A signaling in the neuronal system depends on neogenin was confirmed as repulsive effects of RGM-A can be blocked by an anti neogenin antibody. No binding of RGM-A to other known netrin receptors has been described to date, although a close relation to neogenin can be found in a receptor named Deleted in Colorectal Cancer (DCC). Still, it is remarkable that a recent study showed an indirect interaction of RGM-A with the previously mentioned netrin receptor UNC5b(48).

Generally speaking, inhibitors of axonal growth are likely to be associated with RhoA GTPases(49). Rho GTPases are small messenger proteins binding guanosyltriphosphate (GTP) and functioning as signal transducer. They are known to link membrane bound receptors and changes of the assembly of the actin cytoskeleton(50). They are reported to be activated after spinal cord injury(51), which, as mentioned above, also leads to an increased expression of RGM-A. The resulting assumption that RGM-A signaling involves RhoA could be confirmed(44), and a following study pointed out that RGM-A signaling via neogenin is supposed to be besides RhoA Rock and protein kinase C dependent(52). Additionally it has to be mentioned that RGM-A functions as a coreceptor for bone morphogenetic proteins (BMP). A study revealed that the extracellular part of RGM-A forms a complex with BMP and initiates its classical pathway involving proteins of the smad family and Inhibitor of Differentiation (Id1) protein(38).

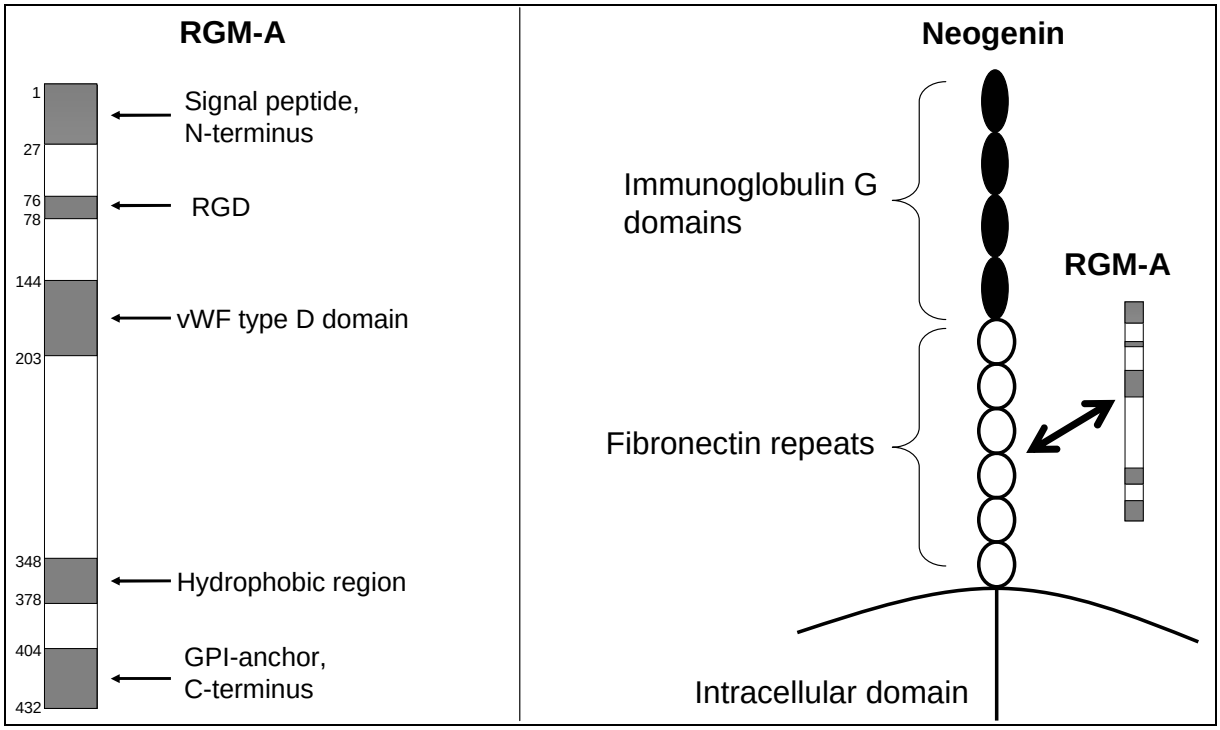

Figure 3. Main structural features of the Repulsive Guidance Molecule A and its receptor neogenin. a. Signal peptide, arginine-glycine-aspartic acid (RGD), a partial von Willebrand factor (vWF) type D domain, a hydrophobic region and a glycosylphosphatidylinositol(GPI)-anchor. b. Neogenin: 4 immunoglobulin G domains and 6 fibronectin type III repeats form the extracellular part. RGM-A probably binds within the fibronectin repeats.

1.4 Hypothesis and goal of this study.

The mechanism of leukocyte migration is a complex process and important signal molecules for leukocyte trafficking have been revealed over recent years(9). In addition to chemokines, neuronal guidance proteins like netrin-1 have been discovered as guidance cues in inflammatory processes(19-25). Therefore, it is promising and sensible to focus on other neuronal guidance molecules like RGM-A. In addition, the molecular structure of RGM-A including a VWF domain and an RGD site suggests a possible immunological function.

This study investigates if RGM-A has an immune modulating effect by functioning as a signal in leukocyte migration. Here, the first question to be pursued is whether RGM-A is according to previous studies expressed in relevant amounts outside the central nervous system. Next, RGM-A should be evaluated in its ability to influence leukocyte migration in-vitro. Transmigration assays should be used as a

tool to investigate this. As adhesion is a precondition of leukocyte migration the possible impact of RGM-A on leukocyte adhesion should be examined in an adhesion assay. To translate the possible results in-vitro RGM-A should further be tested in-vivo in a murine model of zymosan-A induced peritonitis. If RGM-A shows an influence on inflammation, it should further be an aim of this study to gain insight into the mechanisms involved. Therefore the role of possible receptors of RGM-A need to be looked at. Their expression on leukocytes is supposed to be examined and antibodies against possible target receptors should be tested in their capacity to neutralize effects. To investigate the importance of a signal pathway in-vivo knockout mice lacking certain receptors are valuable. Additionally, knowing that certain cytokines play an important role in leukocyte migration this study tries to investigate a possible influence of RGM-A on their level both in-vitro and in-vivo. The selected cytokines are tumor necrosis factor (TNF), interleukin (IL)-1ß, -6 and macrophage inflammatory protein (MIP) 1α.

In summary, this study tries to get insight into the following:

1. Extraneuronal expression of the neuronal guidance molecule RGM-A in adult murine tissue.
2. Impact of RGM-A on leukocyte migration and adhesion in-vitro.
3. Immunemodulatory effect of RGM-A in a mouse model of induced peritonitis.
4. Dependence of RGM-A mediated effects on possible target receptors both in-vitro and in-vivo.
5. Effect of RGM-A on pro-inflammatory cytokine levels in-vitro and in-vivo.

1.5 Support and cooperation concerning this study.

The experiments of this work are part of team work and - at the present point of time – partly submitted to be published beyond this doctoral thesis. In this context some experiments have to be mentioned as they belong to this study, yet were not exclusively produced by the author but partly by members of the same or cooperating research groups (names of scientists in brackets): Immunohistochemical staining (Jan Schwab, figure 5), transmigrational assays (Valbona Mirakaj, figure 10), adhesion assays (research group of Gerd Klein, figure 11) and mouse model of zymosan-A induced peritonitis (Valbona Mirakaj, Marthe Schmit, figure 17, 18, 19).

2 Materials and Methods

2.1 Materials

2.1.1 Transcriptional studies

Name of product	Product number	Company	Registered office
Ultra PURE Distilled Water, RNAse, DNAse free	10977	GIBCO	Gaithersburg, MA, USA
Ethanol absolut	A3678	AppliChem	Darmstadt, Germany
β-Mercaptoethanol	M-6250	Sigma	Taufkirchen, Germany
TRIzol® Reagent	15596-018	Invitrogen	Carlsbad, CA, USA
Chloroform 99%	C2432	Sigma	Taufkirchen, Germany
2-Propanol 99%	I9516	Sigma	Taufkirchen, Germany
iScript cDNA Synthesis Kit	170-8891	Bio-Rad Laboratories, Inc.	Munich, Germany
iQ™ SYBR® Green Supermix	170-8880	Bio-Rad Laboratories, Inc.	Munich, Germany
Primer : Murine RGM-A Sense: 5'- CAG CAA GCT CAC CAT CAT CT- 3' Antisense: 5'- CCC GAC ACC TTC TCT GTG AT -3' Murine ß-actin Sense: 5'-GGC TCC TAG CAC CAT GAA GA -3' Antisense: 5'-TCT GCT GGA AGG TGG ACA G -3'		Biomers	Ulm, Germany

2.1.2 Immunoblotting experiments

Name of product	Product number	Company	Registered office
Dulbecco's Phosphate-Buffered Saline (DPBS) 1x without Ca^{2+} and Mg^{2+}	14190	GIBCO	Gaithersburg, MA, USA
DPBS 1x with Ca^{2+} and Mg^{2+}	14040	GIBCO	Gaithersburg, MA, USA
BCA™ Protein Assay Kit	23225	Pierce	Bonn, Germany
Aprotinin	A2132	AppliChem	Darmstadt, Germany
Leupeptin	A2183	AppliChem	Darmstadt, Germany
Pepstatin A	77170	Sigma	Taufkirchen, Germany
Orthovanadate	S6508	Sigma	Taufkirchen, Germany
Phenylmethyl-sulfonyl fluoride (PMSF) 200mM	P7626	Sigma	Taufkirchen, Germany
Radioimmunoprecipitation assay buffer (RIPA) ♦ Ethylenediamin-tetra acetic acid (EDTA) ♦ NaCl ♦ Tris (hydroxy methyl) aminomethane (Tris)-base (pH 7.4) ♦ Octoxynol-9 (Triton® X-	A3145 A4857 CA-630	AppliChem Merck AppliChem Sigma	Darmstadt, Germany Darmstadt, Germany Darmstadt, Germany Taufkirchen, Germany

Name of product	Product number	Company	Registered office
100) 2% ♦ NP-40 Igepal	234729 17771	Sigma Sigma	Taufkirchen, Germany Taufkirchen, Germany
Laemmli loading buffer (4x), non-reducing ♦ 1M Tris-HCl pH 6.8 ♦ Glycerol ♦ Sodium dodecyl sulphate ♦ Bromophenol blue ♦ Aqua dest.	 G-6279 71729 A512.1 A3540	 Sigma Fluka Biochemika Roth Applichem	 Taufkirchen, Germany Buchs, Switzerland Karlsruhe, Germany Darmstadt, Germany
Rotiphorese® Gel 30	3029	Roth	Karlsruhe, Germany
Methanol	1.06009.2511	Merck	Darmstadt, Germany
N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	T9281	Sigma	Taufkirchen, Germany
Ammoniumpersulfate (APS) 10% in A.d.	A-9164	Sigma	Taufkirchen, Germany
Upper Buffer pH 6.8: ♦ 0.5M Trisbase ♦ 0.4% Sodium dodecyl sulphate	 T1503 71729	 Sigma Fluka Biochemika	 Taufkirchen, Germany Buchs, Switzerland

Name of product	**Product number**	**Company**	**Registered office**
Lower Buffer pH 8.8: ♦ 1.5M Trisbase ♦ 0.4% Sodium dodecyl sulphate	 T1503 71729	 Sigma Fluka Biochemika	 Taufkirchen, Germany Buchs, Switzerland
MagicMark™ XP Western Standard	LC5602	Invitrogen	Carlsbad, CA, USA
SeeBlue® Plus 2, Prestained Standard	LC5925	Invitrogen	Carlsbad, CA, USA
10x Tris/Glycine Buffer	161-0771	Bio-Rad Laboratories, Inc.	Munich, Germany
10x Tris/Glycine/ sodium dodecyl sulfate (SDS) Buffer	161-0772	Bio-Rad Laboratories, Inc.	Munich, Germany
Tris buffered saline (TBS): ♦ Trisbase ♦ NaCl	T1503 1.06404 A4857	Sigma Merck AppliChem	Taufkirchen, Germany Darmstadt, Germany Darmstadt, Germany
TWEEN® 20	A4974	AppliChem	Darmstadt, Germany
Nonfat dried milk powder	A0830	AppliChem	Darmstadt, Germany
Bovine Serum Albumin (BSA)	A7906	Sigma	Taufkirchen, Germany
Dimethylsulfoxide (DMSO)	D5879	Sigma	Taufkirchen, Germany
Luminol	09253	Fluka Biochemika	Buchs, Switzerland

Name of product	Product number	Company	Registered office
p-coumeric acid	C9008	Sigma	Taufkirchen, Germany
Hydrogen peroxide solution 30%	1.08597	Merck	Darmstadt, Germany

2.1.3 Immunohistochemistry

Name of product	Product number	Company	Registered office
Formaldehyde 37%	F1268	Sigma	Taufkirchen, Germany
Paraffin wax	A6330	Sigma	Taufkirchen, Germany
Xylol	28975.325	VWR Prolabo Chemikalien; VWR International GmbH	Darmstadt, Germany
Ethanol absolut	A3678	AppliChem	Darmstadt, Germany
Sodium citrate	A0548.0500	AppliChem	Darmstadt, Germany
Methanol	1.06009.2511	Merck	Darmstadt, Germany
Hydrogen peroxide solution 30%	1.08597	Merck	Darmstadt, Germany
Porcine serum	26250-084	GIBCO	Gaithersburg, MA, USA
Mayer’s hemalum Solution	1.09249	Merck	Darmstadt, Germany
Streptavidin, peroxidase conjugated	S-911	Invitrogen	Carlsbad, CA, USA

2.1.4 Histopathology

Name of product	Product number	Company	Registered office
Xylol	28975.325	VWR Prolabo Chemikalien; VWR International GmbH	Darmstadt, Germany
Ethanol absolut	A3678	AppliChem	Darmstadt, Germany
Acetone	24201	Honeywell Riedel-de-Haën	Morris Township, New Jersey
Hydrochloric acid	1.09057	Merck	Darmstadt, Germany
Mayer's hemalum Solution	1.09249	Merck	Darmstadt, Germany
Eosin	1.01424	Merck	Darmstadt, Germany
Eukitt® quick-hardening mounting medium	03989	Fluka Biochemika	Buchs, Switzerland

2.1.5 Cytology and immunofluorescence

Name of product	Product number	Company	Registered office
DPBS 1x without Ca^{2+} and Mg^{2+}	14190	GIBCO	Gaithersburg, MA, USA
Cytofix™ fixation buffer	554655	BD biosciences	Erembodegem, Belgium
Prolong® Gold anti-fade reagent with DAPI	P36931	Molecular Probes, Inc.	Eugene, OR, USA

2.1.6 ELISA analysis of cytokine levels

Name of product	Product number	Company	Registered office
Heparin	Liquemin®	Roche	Grenzach-Wyhlen, Germany
Zymosan A	Z4250	Sigma	Taufkirchen, Germany
Recombinant human RGM-A	2459-RM	R&D Systems	Minneapolis, MN, USA
Human TNFα DuoSet®	DY210	R&D Systems	Minneapolis, MN, USA
Mouse TNFα DuoSet®	DY410	R&D Systems	Minneapolis, MN, USA
Human IL-1ß DuoSet®	DY201	R&D Systems	Minneapolis, MN, USA
Mouse IL1-ß DuoSet®	DY401	R&D Systems	Minneapolis, MN, USA
Human IL-6 DuoSet®	DY206	R&D Systems	Minneapolis, MN, USA
Mouse IL-6 DuoSet®	DY406	R&D Systems	Minneapolis, MN, USA
Human MIP1-α DuoSet®	DY270	R&D Systems	Minneapolis, MN, USA
Mouse MIP1-α DuoSet®	DY450	R&D Systems	Minneapolis, MN, USA

2.1.7 CaCo-2 cell culture

Name of product	Product number	Company	Registered office
Minimal Essential Medium (MEM)	M-4655	Sigma	Taufkirchen, Germany

Name of product	Product number	Company	Registered office
Fetal calf serum (FCS) Gold	A15-641	PAA	Pasching, Austria
Sodium pyruvate	S-8636	Sigma	Taufkirchen, Germany
MEM amino acid solution	M-7145	Sigma	Taufkirchen, Germany
Antibiotic-Antimycotic Solution	A5955	Sigma	Taufkirchen, Germany
DPBS 1x with Ca^{2+} and Mg^{2+}	14040	PAA	Pasching, Austria
Accutase	L11007	PAA	Pasching, Austria
Dimethylsulfoxide (DMSO)	D5879	Sigma	Taufkirchen, Germany

2.1.8 Isolation of human polymorphonuclear leukocytes and transepithelial migration assays

Name of product	Product number	Company	Registered office
Percoll ™ sterile ♦ 63% (63% Percoll, 30% DPBS and 7% DPBS 10x, not containing $MgCl_2$ or $CaCl_2$) ♦ 72% (72% Percoll, 20% DPBS and 8% DPBS 10x, not containing $MgCl_2$ or $CaCl_2$)	17-0891	GE Healthcare Bio-Science AB	Uppsala, Sweden
Coulter® Isotone® II Diluent	8448011	Beckman Coulter GmbH	Krefeld, Germany
Coulter Clenz™ Cleaning agent	8417-222	Beckman Coulter GmbH	Krefeld, Germany
Lysis Buffer ♦ Ethylenediamin-	A3145	AppliChem	Darmstadt,

Name of product	Product number	Company	Registered office
tetra acetic acid (EDTA) ♦ Sodium Bicarbonate ♦ Ammonium chloride	 S-8875 A0988	 Sigma AppliChem	Germany Taufkirchen, Germany Darmstadt, Germany
DPBS 1x without Ca^{2+} and Mg^{2+}	14190	GIBCO	Gaithersburg, MA, USA
Hank's buffered salt solution (HBSS) +/- $CaCl_2$ +/- $MgCl_2$	14175	GIBCO	Gaithersburg, MA, USA
N-formyl-methyl-leucyl-phenylalanine (fMLP)	F-3506	Sigma	Taufkirchen, Germany
Recombinant human RGM-A	2459-RM	R&D Systems	Minneapolis, MN, USA
Bovine Serum Albumin (BSA)	A7906	Sigma	Taufkirchen, Germany
Citric Buffer: ♦ 200 mM Sodium Citrate ♦ 200 mM Citric Acid	 3580 8.18707	 Roth Merck	 Karlsruhe, Germany Darmstadt, Germany
Triton 10% ♦ Triton® X-100	 X100	 Sigma	 Taufkirchen, Germany
2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	A1888	Sigma	Taufkirchen, Germany
Hydrogen peroxide solution 30%	1.08597	Merck	Darmstadt, Germany

2.1.9 Cell adhesion assay

Name of product	Product number	Company	Registered office
RPMI 1640 medium	22400-121	Invitrogen	Carlsbad, CA, USA
Human serum albumin	A9511	Sigma	Taufkirchen, Germany
DPBS 1x without Ca^{2+} and Mg^{2+}	14190	GIBCO	Gaithersburg, MA, USA
Recombinant human RGM-A	2459-RM	R&D Systems	Minneapolis, MN, USA
Bovine Serum Albumin (BSA)	A7906	Sigma	Taufkirchen, Germany

2.1.10 Antibodies

Name of product	Product number	Company	Registered office
Immunoblot			
Anti RGM-A	Ab26287	Abcam	Cambridge, UK
β-actin rabbit monoclonal antibody (13E5)	4970	Cell Signaling Technology, Inc.	Danvers, MA, USA
Goat anti rabbit IgG-HRP	Sc-2004	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
Donkey anti goat IgG-HRP	Sc-3851	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
Immunohistochemistry			
Anti RGM-A	Raised against the sequence: RMPEE VVNAVEDRDSQGL	Provided(43)	

Name of product	Product number	Company	Registered office
Biotinylated swine-anti rabbit IgG $F(ab)_2$	E0431	DAKO	Hamburg, Germany
Goat anti Neogenin antibody	Sc-6537	Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Rabbit anti CD45 antibody	Sc-25590	Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Alexa Fluor® 488 donkey anti goat	A11055	Invitrogen	Carlsbad, CA, USA
Alexa Fluor® 594 goat anti rabbit	A-11012	Invitrogen	Carlsbad, CA, USA
Goat IgG	Sc-2028	Santa Cruz Biotechnology, Inc.	Heidelberg, Germany
Rabbit IgG	Sc-2027	Santa Cruz Biotechnology, Inc.	Heidelberg, Germany
Cytology and immunofluorescence			
Anti CD45 antibody	Caltag	MHCD4506	Burlingame, CA, USA
Rabbit anti neogenin antibody	Sc-15337	Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Mouse $IgG_{2\alpha}$	349051	Becton Dickinson	Heidelberg, Germany
Goat anti rabbit IgG FITC conjugate	816111	ZyMax	San Francisco, USA
Transmigration assay			
Anti human RGM-A antibody	AF2459	R&D Systems	Minneapolis, MN, USA
Goat anti neogenin antibody	Sc-6537	Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Goat IgG	Sc-2028	Santa Cruz Biotechnology, Inc.	Heidelberg, Germany
Cell adhesion assay			

Name of product	Product number	Company	Registered office
Goat anti neogenin antibody	Sc-6537	Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Mouse anti HLA-1	ab23755	Abcam	Cambridge, UK

2.1.11 Zymosan-A induced peritonitis model and genotyping

Name of product	Product number	Company	Registered office
Zymosan A (ZyA)	Z4250	Sigma	Taufkirchen, Germany
Recombinant murine RGM-A	2458-RG	R&D Systems	Minneapolis, MN, USA
DPBS 1x with Ca^{2+} and Mg^{2+}	14040	GIBCO	Gaithersburg, MA, USA
Bovine Serum Albumin (BSA)	A7906	Sigma	Taufkirchen, Germany
Pentobarbital	P3761	Sigma	Taufkirchen, Germany
Heparin	Liquemin®	Roche	Grenzach-Wyhlen, Germany
Ferricyanide	244023-100G	Sigma	Taufkirchen, Germany
Ferrocyanide	455989-25G	Sigma	Taufkirchen, Germany
Magnesium chloride	M8266-100G	Sigma	Taufkirchen, Germany
Bromo-chloro-indolyl-galacto-pyranoside (X-gal)	B4252-100MG	Sigma	Taufkirchen, Germany
Sodium deoxycholate	D6750-10G	Sigma	Taufkirchen, Germany
NONIDET P 40	17771-25ML	Sigma	Taufkirchen, Germany

2.1.12 Technical equipment

Name of product	Product number	Company	Registered office
iCycler	170-8740	Bio-Rad Laboratories, Inc.	Munich, Germany
Gene expression macro		Bio-Rad Laboratories, Inc.	Munich, Germany
Novex Mini-Cell	1167482-625	Invitrogen	Carlsbad, CA, USA
Mini Trans-Blot® Electrophoretic Transfer Cell	170-3930	Bio-Rad Laboratories, Inc.	Munich, Germany
Trans-Blot® Cell Electrophoretic Transfer Cell	369/2832	Bio-Rad Laboratories, Inc.	Munich, Germany
Powerpac 3000		Bio-Rad Laboratories, Inc.	Munich, Germany
HERAEUS Cytoperm 2	51011660	Kendro Laboratory Products GmbH	Hanau, Germany
Sterile workbench Lamin Air HB 2472	50033854	Kendro Laboratory Products GmbH	Hanau, Germany
Photometer Ultrospec 3000 pro		Biochrom Ltd	Berlin, Germany
Cyclone 25 FTGENE2L	119539-4	peQLab Biotechnologie GmbH	Erlangen, Germany
GENios microplate reader	F129004	Tecan Group Ltd	Männedorf, Austria
Magellan software		Tecan Group Ltd	Männedorf, Austria
Prism software		Graphpad Software, Inc.	San Diego, CA, USA
TD-20/20	TD 2020	Turner Designs	Sunnyvale, CA,

Name of product	Product number	Company	Registered office
Luminometer	2-2118-CE	BioSystems, Inc.	USA
Disposable Cuvettes	E2371	Promega	Madison, WI, USA
Hettich Zentrifuge	EBA 12 R	Hettich Zentrifugen	Tuttlingen, Germany
Centrifuge 5417 R	0021875	Eppendorf	Hamburg, Germany
HERAEUS Megafuge 1.0R	75003060	Kendro Laboratory Products GmbH	Hanau, Germany
Cellstar® TC-Tube, sterile	163 160	Greiner bio-one	Frickenhausen, Germany
Coulter® Z2	AB094 157	Beckman Coulter GmbH	Krefeld, Germany
Vortex Genie	G-560E	Scientific Industries, Inc.	Bohemia, NY, USA
Mastercycler gradient	533100082	Eppendorf	Hamburg, Germany
Mikroskop DM-IL	520802	Leica	Bensheim, Germany
Rotary microtome	RM 2235	Leica	Bensheim, Germany
Flattening bath HI 1210	0415 21466	Leica	Bensheim, Germany
Flattening table HI 1220	0423 21474	Leica	Bensheim, Germany
Cell Lifter 3008		Corning-Costar	Mexico, USA
Eppendorf Cups		Eppendorf	Hamburg, Germany
15 ml tubes 50 ml tubes	181171 352070	Greiner bio-one BD Falcon	Frickenhausen, Germany Franklin Lakes, NJ, USA
Microplates F-	655001	Greiner bio-one	Frickenhausen,

Name of product	Product number	Company	Registered office
Bottom 96 well			Germany
Immuno-Blot™ Polyvinylidene Fluoride (PVDF) Membrane 0.2 µm	162-0177	Bio-Rad Laboratories, Inc.	Munich, Germany
Tissue Homogenizing System MICCRA D8		Art Labortechnik	Müllheim, Germany
MicroAIR (U22)	070 501 015	OMRON	Mannheim, Germany
Fully-enclosed Tissue Processor TP1050	04612711R001	Leica Microsystems GmbH	Wetzlar, Germany
Embedding center, dispenser and hot plate EG1160	14038630527	Leica Microsystems GmbH	Wetzlar, Germany
Super Frost® Plus Object Plate	03-0060	R. Langenbrink Labor- und Medizintechnik	Teningen, Germany
Konfocal Laser Scanning Microscope LSM 510^{MK4}		Carl Zeiss Advanced Imaging Microscopy	Jena, Germany
96 well 0.2 ml Thin-Wall PCR Plates	223-9441	Bio-Rad Laboratories, Inc.	Munich, Germany
Analytic Balance AE240		Mettler-Toledo GmbH	Giessen, Germany
Thermomixer, Incubator/Shaker	5436	Eppendorf	Hamburg, Germany
Plastic Cassettes 1.5 mm	NC 2015	Invitrogen	Carlsbad, CA, USA
BD Insyte I.V.	REF 381223	Becton Dickinson	Madrid, Spain

Name of product	Product number	Company	Registered office
Catheter 22GA 0.9x25mm		S.A.	
Tissue culture dishes		Cellstar	Frickenhausen, Germany
Tubes 50ml/15ml		Becton Dickinson	Le Pont De Claix, France
Glass Slides		NalgeNuc International	Naperville, IL, USA
Permeable transwell inserts		Costar	Mexico, USA
Polystyrene permeable inserts (3 µm)		Costar	Mexiko, USA
Cytospin cytocentrifuge system		Thermo Scientific	Shandon, Pittsburgh
Diana Chemiluminiscence Camera		Raytest	Straubenhardt, Germany
Zeiss LSM 510		Carl Zeiss Micro-Imaging GmbH	Göttingen, Germany
FACSort Cell Sorter		Becton Dickinson	Heidelberg, Germany

2.2 Methods

2.2.1 Transcriptional studies

To assess the transcription of RGM-A in various murine tissues RNA was extracted from brain, lung, spleen, intestine, kidney and liver samples using an established method described below (Trizol® reagent RNA isolation protocol). Part of the freshly separated RNA was then rewritten into complementary DNA by reverse transcriptase and used for performing a reverse transcription polymerase chain reaction (RT PCR). A PCR is a method of amplifying DNA in-vitro, and, as a real time PCR was performed, it provides the possibility to quantify the rate of transcription.

Mouse organs were harvested, collected in cryotubes and immediately frozen in liquid nitrogen. Mice were killed by exposure to diethyl ether at a high concentration (2 minutes), and by injecting NaCl solution (0.9%) in the heart's left ventricle organs were rinsed to reduce the contain of blood. Approximately 100 mg of each tissue sample was transferred into test tubes containing 1 ml of Trizol® reagent, a solution of phenol and guanidine isothiocyanate, and homogenized. During an incubation of 5 minutes at room temperature Trizol® reagent is able to lyse cells as well as dissolve its components while maintaining the integrity of the RNA. Now 200 µl of chloroform were added to each tube, which was then intensively shaken by hand to assure a uniform mixture. After an additional incubation lasting 5 minutes at room temperature samples were centrifuged at 12000 g for 15 minutes at a temperature of 4℃. The mixture separates into a red phase containing phenol and chloroform, an interphase and an aqueous supernatant that contains the RNA. To separate RNA the aqueous phase was transferred into another tube and precipitated using isopropyl alcohol (500 µl). Samples were incubated for 10 minutes at room temperature, RNA was then spinned down at 12000 g for 10 minutes at 4 ℃ and now became visible as a gel-like pellet at the bottom of the tubes. The supernatant was carefully removed and the remaining pellet washed using 75% ethanol. Another centrifugation followed (7500 g, 5 minutes, 4 ℃). The supernatant was discarded and the RNA-pellet airdryed for 10 minutes before 40 µl RNAse free water was added to finally dissolve the RNA. Before the RNA was frozen at -80℃ an aliquot of 15 µl was taken for the experiment and transcribed into complementary DNA (cDNA).

Into every aliquot reverse transcriptase and nucleotides as substrate for the enzyme was added (4 µl 5x iScript Reaction Mix, 1 µl iScript Reverse Transcriptase). Transcription took place during 15 minutes at room temperature. From then on cDNA could be stored at -20℃ if necessary.

1 µl of the newly synthesized cDNA was diluted in 99 µl of nuclease free water to be measured so that afterwards an approximately equal amount of cDNA could be used for the PCR. Measurement was performed at a photometer to assess the absorption at a wavelength of 260 nm. The photometer further measured the purity of the sample by a ratio of the 260 nm and 280 nm absorption. The original sample of cDNA was diluted to achieve a dilution of 100 µg of cDNA per ml. A 96 well plate was used to perform the PCR. 4 µl of each diluted cDNA sample were pipetted in a well, every sample duplicate to find out eventual mistakes as imprecise pipetting. 21 µl of a reaction mixture were added that contained 12.5 µl SYBR® Green Supermix, 2 µl of a primer mix of two primers and 7.5 µl nuclease free water. The 96 well plate was briefly centrifuged (200 g, 1 minute) so that the whole volume of the sample was at the bottom of each well. The PCR was performed using iCycler (Bio-Rad Laboratories).

In brief, the principle of a PCR is an in-vitro amplification of a specific sequence of a DNA strand that is characteristic of the examined gene. By designing two primers, a sense and an antisense primer, this sequence can be defined and transcribed by a DNA dependent DNA polymerase. Primers mark the starting point for the polymerase after the cDNA double strand had been separated by heating the sample. After transcription the newly synthesized double strand is separated again by heat and cooled down until primers adsorb in an optimal way and another replication is initiated. Those cycles result in an exponential amplification of the sequence marked by the primers until there is a lack of enzyme, primer or nucleotides. To avoid a degeneration of the polymerase while the sample is heated a heat stable polymerase derived from Thermus aquaticus is used.

A further development of the conventional PCR is a real time PCR. After each cycle the amount of double stranded DNA is assessed using a fluorescent dye that only fluoresces being bind to double stranded DNA. By defining a threshold of fluorescence different samples that will reach this threshold after a different number of cycles can be compared. As one cycle represents a doubling of DNA this assessment yet is semi quantitative. At the end of a real time PCR all samples are

heated step by step while their fluorescence is measured. The purity of the specific amplified DNA strand is reflected in a sudden drop in fluorescence at a defined temperature (melting curve).

The RGM-A primer sets contained 10pM of the sense primer with following sequence: 5’ - CAG CAA GCT CAC CAT CAT CT - 3’ and the antisense primer: 5’ - CCC GAC ACC TTC TCT GTG AT - 3’. Samples were also controlled for murine ß-actin as housekeeping gene using following primers: sense 5' - GGC TCC TAG CAC CAT GAA GA - 3', antisense 5' - TCT GCT GGA AGG TGG ACA G - 3'. The primer set was amplified using cycles of 95℃ for 0:30 minutes, 66℃ for 0:45 minutes, 72℃ for 0:45 minutes, and a final extension of 72℃ for 3 minutes. DNA-Strands were separated at 95℃ for 3 minutes before the first cycle was started. Generated data was evaluated using gene expression macro software (Bio-Rad Laboratories).

2.2.2 Immunoblotting experiments

Immuno or western blotting experiments are targeted on assessing the expression of a specific protein. In addition to the measurement of transcription the RGM-A expression on the level of translation could be assessed.

Western blot analysis consists of extracting proteins, separating them by size using SDS gel electrophoresis, transferring them to a PVDF membrane (Western blot in its narrower sense) and detecting a specific protein. For this detection primary antibodies directed against an epitope of the protein to detect were used and a secondary antibody that detects the FC-region of the primary antibody and is itself linked to peroxidase so that it can indirectly be detected in an enzymatic reaction. Organ samples were harvested as described above, thawed, homogenized in 500-1000 µl radioimmunoprecipitation assay (RIPA)-buffer (depending on organ weight) and lysed for 15 minutes at 4℃ while slightly being shook, then transferred into microfuge tubes. After spinning at 12000 g to remove cell debris the pellet was discarded. From then on proteins in the supernatant were stored at -80℃ and rethawed no more than 3 times.

To measure the protein concentration of the supernatant two aliquots of each sample were diluted (1:10 and 1:50) and the concentration was measured using BCA Protein Assay Kit (Pierce). This colorimetric method allows measuring the amount of total protein using bovine serum albumin as standard (6 concentrations from 2 mg/ml

to 0.0625 mg/ml). The BCA Protein Assay Kit is based on a two-step reaction. First protein or oligopeptides react with cupric ions (reduction of Cu^{2+} in alkaline environment containing sodium potassium tartrate, known as Biuret reaction), then bicinchoninic acid (BCA) is forming a purple colored chelate complex with cuprous cation (Cu^{1+}) that provides a sensitive detection to be photometrically measured at 562 nm.

Aliquots of the protein samples were diluted using RIPA buffer and Laemmli sample buffer so that samples contained Laemmli buffer (4x) at a concentration of 250 µl/ml and protein at a concentration of 100 mg/ml. Separation of proteins was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Besides bromphenole as coloring Laemmli buffer contained SDS which is an anionic detergent that applies a negative charge to each protein in proportion to the protein's mass. Further, it denatures secondary structures as well as tertiary structures apart from disulfide bounds. This process was facilitated by heating the samples to 95℃ for 3 minutes followed by vortexing and quickly cooling them down on wet ice. Proteins with a continuous negative charge and a uniform secondary structure could then be separated by size using gel electrophoresis. A voltage of 130 V for 90 minutes was applied causing that proteins migrate through the gel towards the anode. Gels had 10 lanes, each containing 35 µl of the prepared samples. As the migration speed is proportional to the protein's size and a standard with proteins of known sizes was applied, it is possible to range the band that later can be detected. Gels had two phases, a stacking gel, that oriented and concentrated proteins, and a resolving gel, where the separation took place. Gels that were used contained polyacrylamide (5% in the stacking, 12% in the resolving gel) and were produced as follows. 16.0 ml acrylamide (30%), 10.8 ml lower buffer, 13.2 ml distilled water (a. dest.) were added to a vessel, stirred, and after adding 200 µl ammoniumpersulfate (APS, 10%) and 80 µl tetramethyl-ethylenediamine (TEMED) the liquid was immediately pipetted in gel cassettes and leveled out. APS and TEMED initiate polymerization and the concentration of acrylamide determines the pore size. During 60 minutes at room temperature autopolymerization took place. On top of the resolving the stacking gel was put, produced in a similar way using 2.5 ml acrylamide, 3.8 ml upper buffer, 8.7 ml a. dest, 60 µl APS and 20 µl TEMED. The electrophoresis was performed in a chamber containing Tris buffer. Afterwards gels were carefully taken out of their cassettes and put onto polyvinylidene fluoride

(PVDF) membranes in a stack of tissue papers for transferring (or blotting) proteins. The blotting process took place in a blotting chamber in Tris transfer buffer containing 20% methanol during 80 minutes at a voltage of 110 V. Proteins in the original separated organization were so transferred onto PVDF membranes. The membranes were put into a blocking solution for 1 h at room temperature. Blocking solution contained PBS supplemented with 0.1% Tween 20 (PBS-T) and 5% nonfat dried milk and has its purpose in preventing non specific binding of antibodies to the PVDF membrane. Afterwards they were incubated in blocking solution containing 1 µg/ml polyclonal rabbit anti RGM-A antibody (Abcam) that was raised against a synthetic peptide derived from mouse RGM-A. The incubation took place overnight at 4℃. Membranes were then washed in PBS-T three times for 20 minutes. The washing process is meant to dissolve antibodies that did not specifically bind to the target protein's epitope. After washing the membranes were incubated using either goat anti rabbit IgG or donkey anti goat IgG depending on the origin of the primary antibody, in a dilution of 1:2000, which was conjugated to horseradish peroxidase, for 45 minutes at room temperature. The washing process was repeated and proteins were detected by enhanced chemiluminescence. Horseradish peroxidase catalyzes a reaction between luminol and hydrogen peroxide precisely at those regions where secondary antibody is bound to primary antibody that detects a specific protein, in this case RGM-A. The detection reagent was produced from two solutions, one containing 4.4 ml a. dest, 500 µl Tris (1M)-HCl (pH 8.5), 50 µl luminol solution (440 mg dissolved in 10 ml DMSO) and 22 µl coumeric acid solution (150 mg dissolved in 10 ml DMSO), the other containing 4.5 ml a. dest, 500 µl Tris (1M)-HCl (pH 8.5) and 3 µl hydrogen peroxide. Once mixed, the solutions were kept in the dark, the membrane was incubated for 1.5 minutes, and emitted radiation was detected using Diana CCD Camera.

To double-check for protein loading every blot was done in duplicates and simultaneously examined for ß-actin using rabbit monoclonal anti ß-actin antibody in a dilution of 1:1000 and the same secondary antibody at the same concentration.

2.2.3 Immunohistochemistry

The principle of immunohistochemistry is, similar to immunoblotting, indirectly visualizing a specific protein using antibodies which are now applied to sections of

tissue samples. Again, the visualization is based on interactions between a protein, a primary and a secondary antibody. While the primary antibody binds to an epitope of the protein to detect, the secondary antibody connects to the primary and is itself coupled to a detectable marker. Therefore, detecting the secondary antibody reflects the location of the protein the primary antibody is directed against. In contrast to immunoblotting experiments immunohistochemistry provides a method to localize a specific protein within tissue. Immunohistochemical staining of murine tissue sections was performed additionally to immunoblotting experiments in order to verify the expression of RGM-A in extraneuronal tissue.

Samples of murine tissues (spleen and lymph node as secondary lymphatic organs) were immediately after harvesting fixated using formaldehyde (4%) and embedded into paraffin to be able to produce sections of 2 µm thickness. The embedding was performed using a tissue processor: samples were put into ethanol (96% twice for 15 min, 100% 4 times for 60 min), xylol (for 80 min and 45 min) and finally paraffin (twice for 45 min, once for 90 min). After cooling down to 8℃ the paraffin had hardened. While paraffin embedding is a precondition to be able to cut tissue into thin sections it yet alters the protein's tertiary structure. Therefore, sections were rehydrated (xylol: 5 min, 3 min, 3 min, 100% ethanol: 3 min, 3 min, 96% ethanol: 3 min, 70% ethanol: 3 min, 50% ethanol: 3 min, PBS: 30 min), boiled and gradually cooled down to allow the protein to assume its original structure. Samples were boiled 7 times for 5 minutes in citrate buffer containing 2.1 g/l sodium citrate (pH 6.0) using a 600-W microwave oven.

Before applying the primary antibody (polyclonal anti RGM-A diluted 1:10 in 0.1% TBS BSA) sections were incubated for 15 min in methanol containing hydrogen peroxide (2.5%) and in 10% porcine serum diluted in 0.1% TBS BSA. Hydrogen peroxide was used in order to inhibit endogenous peroxidase which would otherwise interfere with the detection method described below. Porcine serum was used as a blocking agent to avoid unspecific binding of the primary antibody. The incubation with the primary antibody took place overnight at 4℃. As a secondary antibody biotinylated swine anti rabbit IgG was applied for 30 min at a dilution of 1:400. A linkage to biotin makes it possible to detect the secondary antibody using peroxidase conjugated streptavidin that binds to biotin. The activity of the enzyme is then visualized by a chromogen, diaminobenzidine, which is a substrate for peroxidase. Oxidized diaminobenzidine forms a brown visible precipitate. A counterstaining was

performed using Mayer's hemalum diluted 1:2 in a. dest. Sections incubated in the absence of primary antibody were used as negative controls.

Beside sections, a similar staining of granulocytes was performed in order to examine the expression of the RGM-A receptor neogenin on their cell surface. Therefore, neutrophil leukocytes were isolated out of human venous whole blood as described below. Polymorphonuclear leukocytes (PMN, 10^7 each) were fixated in 4% formaldehyde solution and exposed to a blocking solution (3% BSA in PBS) for 30 min at room temperature. PMN were then incubated for 30 min at room temperature using anti neogenin antibody as a primary antibody dissolved in 300 µl BSA 1.5% (1:100). CD 45 antibody (1:50) was added to the solution which was incubated for another 45 min. Staining CD 45 was performed to confirm the cell type and the integrity of the cell as leukocytes are CD 45 positive. Next, samples were washed three times using PBS. The secondary antibody used in this immunohistochemical staining was instead of peroxidase linked to a fluorescence marker and used in a dilution of 1:500 in 1.5% BSA in PBS. A green fluorescing antibody (wavelength of emitted light 519: nm) was to detect the neogenin antibody, while a red fluorescence (wavelength of emitted light: 617 nm) reflected the localization of CD 45. To distinguish both primary antibodies was possible since they were of different origin having different Fc fragments. Incubation took place for 30 min at room temperature keeping all samples in the dark to avoid fading of the dye. The washing process was repeated and samples were counterstained using 4',6-diamidino-2-phenylindole (DAPI), a blue fluorescent stain to visualize nuclei. Further, the identical experiment was performed using nonspecific IgG at the same concentration as negative control. Cell suspensions were smeared onto an object holder and fluorescence was microscopically assessed.

2.2.4 Histopathology

Preparing sections for histopathological evaluation followed the steps described above until the rehydration process. Now sections were stained for 10 min using Mayer's hemalum, a solution containing hematoxylin as well as potassium aluminum sulfate staining basophilic structures like the nucleus. After rinsing with a. dest. and briefly exposing to hydrogen chloride solution sections were blued in water for 30 min and counterstained with eosin for 5 minutes Eosin is an acidic stain to visualize

cationic structures like many proteins. Further, sections were fixated using ethanol (70%: 1 min, 3 min, 96%: 3 min, 100%: 3 min, 3 min) and xylol (3 min, 3 min). The sections that were examined derived from murine abdominal wall after induced peritonitis as described below. Hematoxylin-eosin(HE)-stained sections were microscopically assessed to evaluate the extent of the inflammation.

2.2.5 Cytology and immunofluorescence

An alternative way to confirm the expression of neogenin on leukocytes is provided by fluorescence activated cell sorting (FACS). Belonging to a technique called flow cytometry a FACS analysis can be used to count microscopically small particles like white blood cells (diameter approximately 12 µm) in a suspension and detect fluorescence of particles. Flow cytometers are able to bring particles one by one into a stream of fluid that passes through a laser light beam. Scattered light is then detected and analyzed to estimate the size of each particle. Simultaneously light emission at a certain wavelength is measured.

Here, leukocytes that were labeled with antibodies against surface proteins were analyzed. Granulocytes suspended in PBS were labeled using non-specific IgG, anti CD45 antibody or anti neogenin antibody. The process of isolating human granulocytes is described below in the context of transmigration assays. Non-specific IgG was applied as a negative control, CD 45 was marked to simultaneously confirm cells to be intact leukocytes. 10^5 cells each in 100 µl PBS were put into tubes and antibodies were added (IgG, CD 45: 10 µl stock solution, anti neogenin: 2 µl stock solution). Incubation took place at room temperature for 60 minutes As a washing procedure 2 ml PBS were added and cells centrifuged at 950 g for 5 minutes After repeating the washing twice samples containing anti neogenin antibody were incubated with fluorescing secondary antibody (2 µl stock solution) for 30 min at room temperature keeping all samples away from light. This secondary antibody was connected to the fluorescein derivate fluorescein isothyocyanate (FITC) that emits light after excitation at 488 nm wavelength. The antibodies directed against CD 45 as well as the control antibodies were connected to a fluorescent dye so that no marking with a secondary antibody was needed. After repeating the washing process cells were stabilized in a fixation buffer (250 µl diluted 1:10, Cytofix™) containing saline and formaldehyde and analyzed in a flow cytometer (Becton Dickinson).

2.2.6 ELISA analysis of cytokine levels

Cytokines are messenger proteins between immune cells that act in an autocrine or parakrine manner and play a central part in mediating inflammation. To answer the question if RGM-A influences pro-inflammatory cytokine secretion, an enzyme-linked immunosorbent assay (ELISA) was performed. In this method antigens that need to be detected are adsorbed onto the plastic surface of a 96 well ELISA plate. After rinsing all unbound proteins soluble antibodies can mark the bound antigens and can itself be detected by a ligand, streptavidin that is coupled to horseradish peroxidase. Free ligand is washed away and the amount of bound ligand can be measured in an enzymatic reaction.

Human whole venous blood (anticoagulated with 10 U/ml sodium heparin) was collected from healthy non-smoking volunteers. Informed consent was obtained from each volunteer, and the protocol was approved by the Institutional Review Board. Heparinized whole blood (500 µl) was then incubated with RGM-A at a concentration of 500 ng/ml in the presence of zymosan-A (100 µg/ml) for 4 h, and plasma was collected by centrifugation at 209 g for 15 minutes. As control, blood was incubated with NaCl or with ZyA, but not containing RGM-A. The amounts of cytokine levels were determined by ELISA (SearchLight Proteome Array custom designed by Pierce Boston Technology Center). Following a standard sandwich ELISA procedure (DuoSet® ELISA Development System, R&D) the entire plate was imaged to capture chemiluminescent signals generated at each spot within each well of the array. The SearchLight CCD Imaging and Analysis System features image analysis software that calculates cytokine concentrations (pg/ml) using predetermined standard curves. Additionally, cytokine levels were measured using mouse peritoneal lavage following peritonitis. In a model of self-limiting sterile peritonitis as described below the abdominal cave of each animal was rinsed with 5 ml PBS after 4 h to capture the inflammatory exsudate. Before, mice were partly treated intravenously with RGM-A in order to examine its impact on cytokine levels. In a further subset of experiments neogenin knockout (neogenin -/-) mice were treated and examined similarly.

2.2.7 CaCo-2 cell culture

CaCo-2 is a cell line derived from colon epithelial cells, it was isolated from human colon carcinoma cells in 1974 and purchased from the American Type Culture Collection. CaCo-2 is an established cell line to investigate epithelial barrier functions(53). Cells were cultured in medium containing 100 ml fetal calf serum, 5 ml sodium pyruvate, 5 ml amino acid solution and 5 ml antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B. CaCo cell cultures were passaged weekly using accutase. Before use cells were microscopically assessed regarding vitality and confluence.

2.2.8 Isolation of human polymorphonuclear leukocytes and transepithelial migration assays

Transepithelial migration assays provide a possibility to assess the transmigration of polymorphonuclear leukocytes (PMN) in-vitro. Induced by a chemoattractant (formylmethyl-leukyl-phenylalanine, fMLP) PMN migrated from an upper compartment of a transwell insert through a monolayer of epithelial cells into a lower compartment. To detect an influence of chemotactic proteins on the transmigration rate PMN can be incubated before. The number of cells that reaches the lower compartment was measured using a myeloperoxidase (MPO) assay since this enzyme is specific for PMN. The experiment consisted in preparing transwell inserts, isolating PMN out of whole blood (and incubating them if required), the transmigration process and the assessment of MPO.

PMN were isolated from whole blood obtained by venipuncture and anticoagulated with acid citrate/dextrose. The approval by the Institutional Review Board and a written informed consent from all donors was obtained. PMN were isolated by density gradient centrifugation using two solutions (72%, 63%) of Percoll™. Percoll™ consists of silica particles coated with polyvinylpyrrolidone, is almost chemically inert and does not adhere to membranes. By diluting Percoll™ to concentrations having a density above and below the density of PMN it is possible to separate a cell population by centrifugation. Test tubes were filled with 4 ml of both solutions in separated phases and covered with an equal amount of anticoagulated

whole blood that had been cooled down to room temperature. Tubes were centrifuged for 29 minutes at 500 g so that the band containing PMN could be separated and transferred to 50 ml Falcon tubes. In each tube PMN of no more than 16 ml whole blood were put. From now on PMN were cooled on wet ice to avoid previous activation of immune cells. To receive a cell population of more than 95% PMN red cells that could be found in the cell suspension were lysed adding 30 ml lysis buffer (containing EDTA, sodium bicarbonate, ammonium chloride) per tube for 10 minutes. After spinning down (10 minutes at 209 g) PMN were washed in 20 ml DPBS (without $MgCl_2$ or $CaCl_2$), centrifuged again (10 minutes at 209 g) and dissolved in Hanks Balanced Salt Buffer (HBSS+) so that a concentration of 4×10^7 per ml was achieved. The concentration of PMN had been estimated before by a cell counter (Coulter® Z2) and confirmed afterwards to rule out an unexpected loss of cells. Microscopic evaluation ruled out a contamination with more than 5% red cells. Isolated PMN were used within 2 h.

CaCo-2 cells were grown on the basolateral aspect of permeable transwell inserts (3µm pore size) until confluency was achieved (7-10 days after seeding) so that the PMN transmigration could be performed in a physiological basal-to-apical direction. Monolayers were washed and placed in HBSS+ for further studies. $1x10^6$ PMN in a volume of 100 µl were then placed in the upper compartment. Chemotaxis was induced through fMLP in a concentration of 10 ng/ml in the lower compartment of the permeable transwell that contained 900 µl HBSS+. Formylmethyl-leukyl-phenylalanine (fMLP) is a potent activator of both PMN and macrophages. All inserts were incubated for 60 minutes at 37°C.

The transmigration rate was assessed using an established MPO assay. Transmigrated PMN in the lower compartment were lysed adding 50 µl of citric acid to reduce adhesion as well as 50 µl Triton-X to the samples (20 minutes). 28 µg of azino-bisethylbenzothiazoline-sulfonic acid (ABTS) and 50 µl hydrogen peroxide were dissolved in 45 ml a. dest. and 5 ml citric acid to receive an ABTS solution that was pipetted on each aliquot of sample (50 µl aliquots and 75 µl ABTS solution). ABTS is an azurophilic marker of myeloperoxidase that could then be quantified on a plate reader at a wavelength of 405 nm. Myeloperoxidase catalyzes a reaction between ABTS and hydrogen peroxide resulting in a green-colored product.

To assess the absolute amount of transmigrated PMN a standard was pipetted using a defined amount of isolated PMN (10^5 cells, lysed as described and 10 times

1:2 diluted in HBSS+). This experiment was also performed with the lower compartment not containing fMLP to demonstrate the transmigration assay as a reasonable model for cell migration.

Further, PMN were preincubated with decreasing concentrations of RGM-A (500 ng/ml, 50 ng/ml, 5 ng/ml; 20 minutes at 37℃). To distinguish a specific effect from an unspecific this experiment was repeated with RGM-A being substituted by bovine serum albumin (500 ng/ml, 50 ng/ml, 5 ng/ml) and with RGM-A that had been heat inactivated (500 ng/ml, inactivation for 60 minutes at 95℃). As another possibility to demonstrate specificity the added RGM-A was inactivated by simultaneously adding an anti RGM-A antibody (2 µg/ml, R&D).

So far PMN were incubated with RGM-A and pipetted in the upper compartment of the permeable transwell. In addition, the described experiment was performed adding RGM-A (500 ng/ml) selectively to the lower compartment, or to both compartments. PMN that were incubated with RGM-A (500 ng/ml) could further be incubated additionally adding an antibody that interacts with possible target receptors of RGM-A. By interfering in that way it was examined whether an effect of RGM-A was reversible or not. The used antibody was directed against neogenin (10 µg/ml). All experiments were at least done in triplicates each containing 3 transwell inserts for every condition. On every transwell plate a control neither containing RGM-A nor antibodies was used to assess transmigration rates.

2.2.9 Cell adhesion assay

A cell adhesion assay investigates the ability of a substance to alter the capability of leukocytes to adhere to an artificial surface. A protein is immobilized in circular spots onto plastic dishes (35 mm diameter) and granulocytes are allowed to adhere to that surface. Adhesion is then evaluated microscopically. Here, RGM-A was applied using different concentrations (20 ng/µl, 10 ng/µl, 1 ng/µl) and, as control, BSA 0.1% in PBS. 1 µl of each protein solution was pipetted onto defined spots and immobilized by air-drying.

Human granulocytes were isolated as described above and allowed to adhere to the RGM-A coated surface for 15 min at 37℃ at a concentration of 10^6/ml. Then, rinsing the dishes with warm PBS removed all nonadherent leukocytes. The evaluation of the amount of adherent leukocytes was done taking photographs under

a microscope. To investigate the role of neogenin as target receptor, cells were further preincubated (20 min at 4℃) using anti neo genin and W6/32 mouse anti HLA-1 antibody as control (10 µg/ml each).

2.2.10 ZyA induced peritonitis model

Peritonitis evoked in mice can be used as model to study cellular migration and inflammation in-vivo. The model of zymosan-A (ZyA) induced peritonitis in mice was chosen as it is established, fail-save and self-limiting. ZyA was applied intraperitoneally to induce peritonitis. Either RGM-A or its vehicle was injected intravenously at the same time. At defined points of time mice were killed, a peritoneal lavage was performed and cells that had migrated into the abdominal cavity were counted and characterized. Further, MPO levels in the peritoneal exsudate were assessed. All animal protocols were in accordance with the German guidelines for use of living animals and were approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen. Mice were male and 6-8 weeks old (C57BL/6N, The Jackson Laboratory).

1 mg ZyA dissolved in 1 ml DPBS (without $MgCl_2$ or $CaCl_2$) was administered intraperitoneally to evoke sterile acute peritonitis. ZyA is a glucan prepared from yeast cell walls and provides a stimulus for non-specific immune cells via toll like receptor 2(54). Simultaneously either RGM-A or vehicle was injected intravenously, therefore 1 µg RGM-A was used dissolved in 150 µl DPBS containing 0.1% BSA. To inject intravenously a vein (V. jugularis) was freed after mice had been anaesthetized using pentobarbital dissolved in NaCl (0.9%) that was applied intraperitoneally at a concentration of 100 mg per kg bodyweight.

A peritoneal lavage was done at either 4 h, 8 h or 24 h after injection to assess the number of recruited leukocytes. Therefore 5 ml of ice-cold DPBS (containing 10 U/ml unfractionated heparin) was injected, carefully spread in the abdominal cavity and extracted again. Lavages were instantly put into tubes on wet ice to reduce the cells' metabolism. Leukocytes were then studied and counted microscopically using a Neubauer counting chamber. Aliquot samples of the lavage were taken to assess the relative amount of PMN using an MPO assay similarly to the description above: Cells in a volume of 900 µl were lysed by Triton-X (50 µl, after adding 50 µl citric acid) for

20 minutes, 500 µl of this lysate were incubated with ABTS solution for 5 minutes at 37℃, and 2 aliquots (100 µl) of each sample were pipetted on a 96 well plate to be measured at 405 nm. To visualize the leukocytes of the peritoneal lavage cells were spinned down according to Cytospin® protocol: 70 µl cell suspension and 30 µl BSA (7.5%) were pipetted into a conical top piece that is attached to an object holder and spinned down (150 g, 5 minutes). The addition of BSA was due to reducing shear forces. Prepared in such a way leukocytes were stained in order to microscopically distinguish different types of leukocytes and to visualize the relative amount of cells. The staining method of Diff-Quick® was chosen containing a xanthene stain and, secondary, a thiazine solution. All microscope slides were dipped into the staining solutions 30 sec each after very brief heat fixation using a Bunsen burner and chemical fixation with methanol. After rinsing with distilled water slides were air-dried.

In addition to the described subset of experiments three control groups (4 animals at all times) that only received 1 ml DPBS intraperitoneally (without ZyA) were evaluated for a better possibility of comparison.

2.2.11 ZyA induced peritonitis model in neogenin -/- mice

A well established method for investigating gene functions in-vivo is provided by using knockout mice with targeted repression of a certain gene. Differences in the physiology of knockout mice compared to wild type mice can point out the function of the missing gene. Here, suspecting neogenin to be the target receptor for RGM-A, the ZyA induced peritonitis was repeated as described above in order to evaluate differences between wild type and neogenin -/- mice. A neogenin -/- mouse was provided. Crossing it with wild type mice it becomes necessary to select the homozygous and heterozygous offspring in the second following generation. Therefore, genotyping was performed. Knocking out a gene is often combined with transferring a marker to be able to determine whether or not the foreign sequence was integrated. In this case all neogenin -/- mice were homozygous for the gene of ß-galactosidase, an enzyme that catalyzes the hydrolysis of galactosides. X-gal (bromo-chloro-indolyl-galactopyranoside) is a galactoside containing indol and therefore a chromogen substrate for ß-galactosidase providing a blue color when hydrolyzed. A tissue sample (tail) of the mice was placed into X-gal solution (1 mg/ml in PBS) further containing 5 mM ferricyanide, 5 mM ferrocyanide, 2 mM magnesium

chloride, 0.01% sodium deoxycholate and 0.02% NONIDET P40 as a detergent. Samples were incubated for 60 min at 37℃. The inte nsity of the color that begins to develop at the cut edge allowed to distinguish wild type, heterozygous and knockout mice.

3 Results

3.1 Summary of results.

Before presenting results in detail an overview of the principal findings is given here:

1. Expression analysis of RGM-A and neogenin.

   Extraneuronal expression is a biological prerequisite for RGM-A as physiological immune modulating protein. RGM-A expression was observed in various tissues outside the CNS, it was highly expressed in lung and spleen. Its receptor in the CNS, neogenin, is also expressed on the surface of human leukocytes.

2. Attenuating effects of RGM-A on leukocytes in-vitro.

   Preincubation of human leukocytes with RGM-A led to a dose dependent decrease in transmigrational activity in transmigration assays (decrease of 60% at a maximum concentration of 500 ng/ml RGM-A). This effect was shown to be dependent on the neogenin receptor.

   Leukocytes were furthermore less able to attach to a surface coated with RGM-A in a cell adhesion assay. This effect was also dependent on the neogenin receptor.

   RGM-A suppresses the release of pro-inflammatory cytokines in stimulated whole blood samples.

3. Anti-inflammatory effects of RGM-A in-vivo.

   RGM-A inhibited leukocyte migration in-vivo. In a mouse model of peritonitis leukocytes were less likely to infiltrate the peritoneal cave due to sterile inflammation if exposed to RGM-A. This was also demonstrated to be neogenin dependent. RGM-A application resulted in decreased release of pro-inflammatory cytokines.

3.2 RGM-A is expressed in extraneuronal tissues.

RGM-A was expressed both on the level of transcription and translation in various murine organs (Figure 4). As brain tissue is known to express RGM-A in a relevant amount all data were evaluated in relation to the detected expression in whole brain samples. mRNA was found in lung and spleen in similar amounts and, to a considerably lower extent (about 20 % in relation to brain tissue), in intestine and kidney (Figure 4a). Liver samples contained 40% RGM-A mRNA compared to brain samples.

The expression on protein level was measured highest in whole brain lysate. The expression in lung, spleen and intestine was lower but still showed a specific band (Figure 4b). In kidney and liver tissue the protein appeared to be rarely expressed. It can be assessed that RGM-A is very likely to be expressed in tissues apart from the central nervous system, respectively in spleen, lung and intestine. This was verified by immunohistochemical staining of lymphoid tissues (lymph node and spleen, Figure 5).

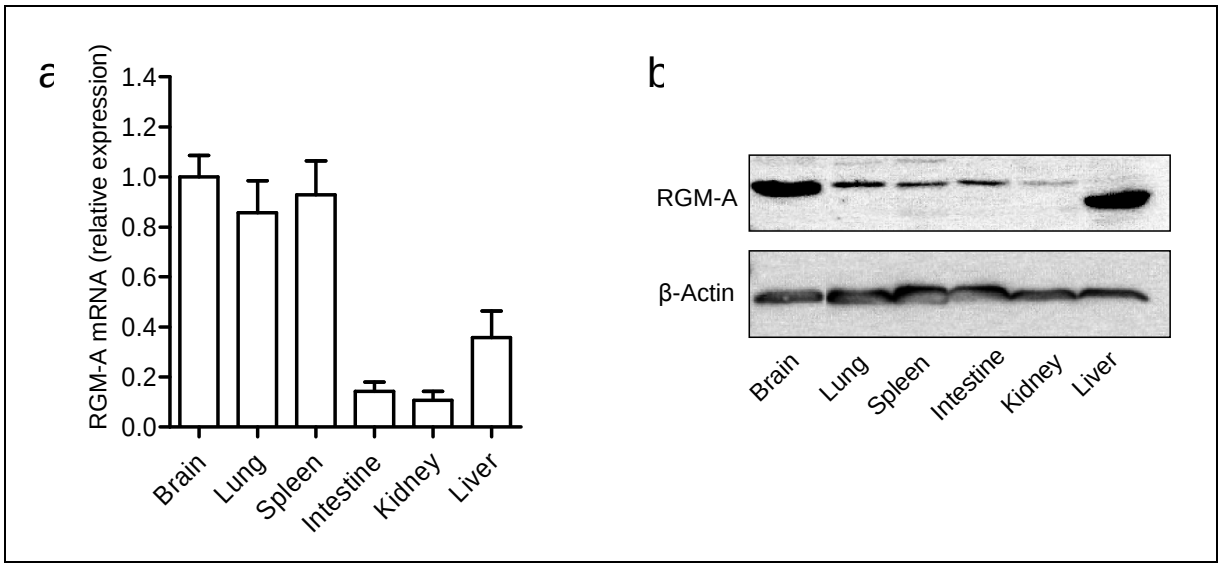

Figure 4. RGM-A is expressed post-developmentally outside the central nervous system. a. Real time PCR quantifies RGM-A mRNA compared to RGM-A mRNA present in murine brain tissue. Pronounced detectable levels could be found in lung and spleen tissue. (mean±SEM, n=6 per organ) b. Western blot analysis of pooled murine tissues shows a significant expression in lung, spleen and intestine. One representative experiment of 3 is shown.

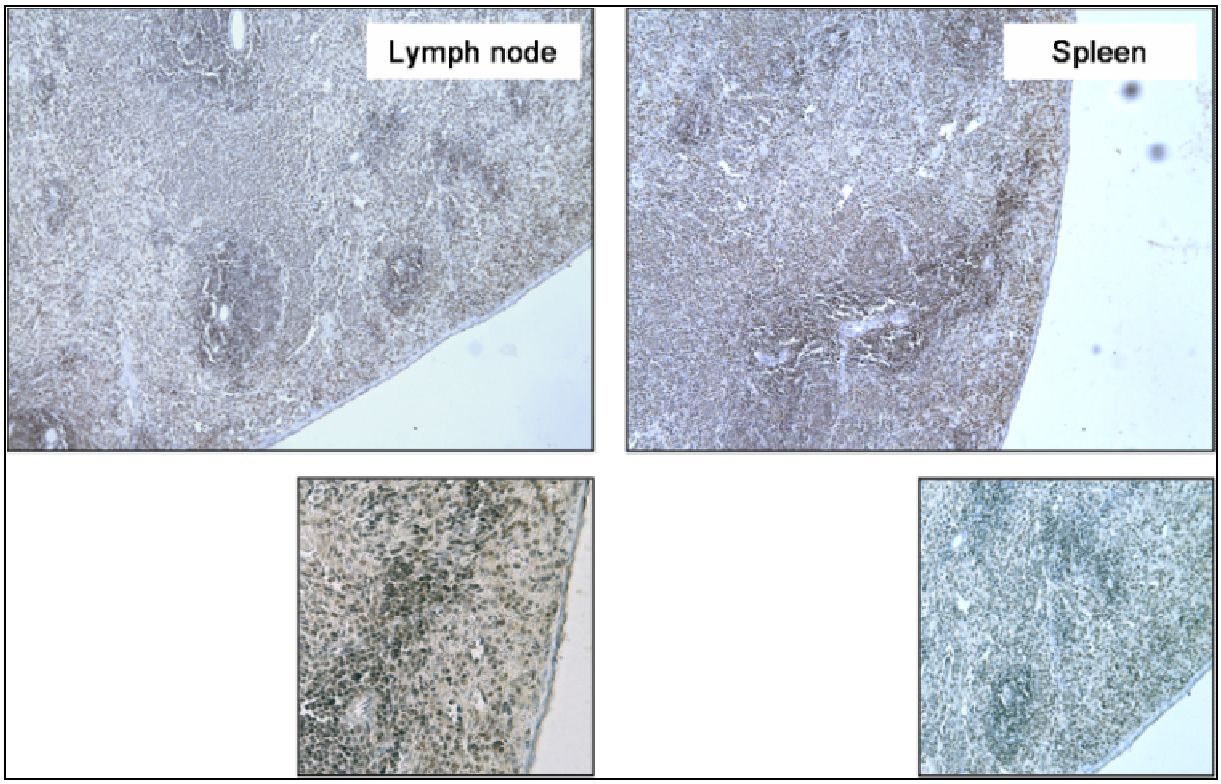

Figure 5. RGM-A is expressed in murine lymphatic tissue. Immunohistochemical staining shows RGM-A positive leukocytes in representative sections of lymph node and spleen. (magnification 200x, 400x)

3.3 RGM-A attenuates polymorphonuclear leukocyte migration in-vitro in a dose dependent fashion.

It was first tested whether a model of fMLP induced chemotaxis would be valid to evaluate migration of polymorphonuclear leukocytes (PMN) in response to a chemoattractant. As expected, it was found that fMLP very significantly induced migration of PMN through a monolayer of CaCo-2 epithelial cells placed on transwell inserts (Figure 6a). Migration of PMN induced in this way through a layer of epithelial cells was reduced in a dose dependent fashion through RGM-A (Figure 6b). 500 ng/ml RGM-A reduced the migration of PMN in response to fMLP to 38% compared to a control (±8%, $P < 0.05$), RGM-A at a concentration of 50 ng/ml still led to a significant decrease (66%±8%, $P < 0.05$), while at a further reduced concentration of 5 ng/ml no significant effect could be observed anymore (97%±17%). At the same concentrations bovine serum albumin (BSA) did not alter the rate of cell migration. PMN incubated with heat inactivated RGM-A or with RGM-A and a neutralizing

antibody (R&D Systems) resulted in loss of the specific action of RGM-A on neutrophil migration (Figure 7).

Further, PMN were exposed to RGM-A on the apical or basalolateral aspect of the semipermeable membrane. Figure 8 shows the different degrees in reduction of migration if RGM-A was added selectively to either side of the transmigration chamber. Preincubation and exposure to RGM-A in the upper chamber significantly reduced the migration of PMN compared to when RGM-A was selectively placed in the lower compartment. Simultaneous application in the lower and upper compartment did not show a significant additional effect compared to when RGM-A was only added in the upper chamber.

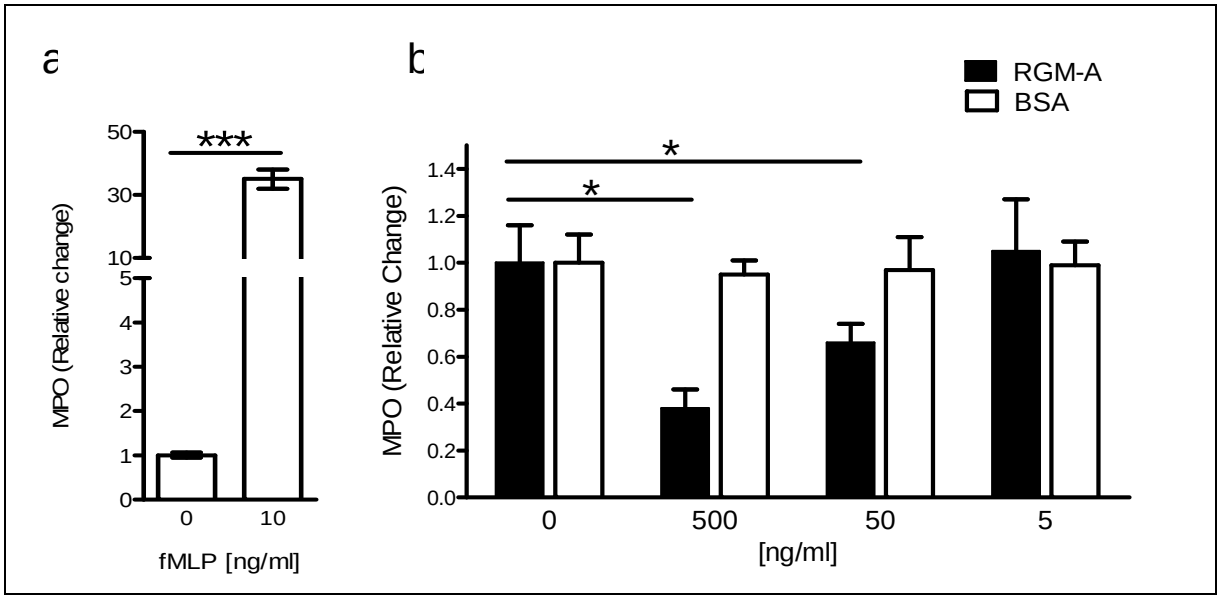

Figure 6. RGM-A inhibits PMN migration to chemotactic stimuli in-vitro. a. fMLP (10ng/ml) induces chemotaxis of PMN through a monolayer of CaCo cells. $1x10^6$ granulocytes were placed in the upper compartment and transmigration into the lower compartment was measured after 60 minutes at 37℃. b. RGM-A reduces PMN transmigration in a dose dependent fashion. PMN were preincubated with decreasing doses of RGM-A, then chemotaxis was induced through fMLP (10ng/ml, 60 minutes, 37℃). Under the same circumstances bovine serum albumin (BSA) did not show an influence on PMN migration. (n=6 per condition, *P < 0.05, ***P < 0.001)

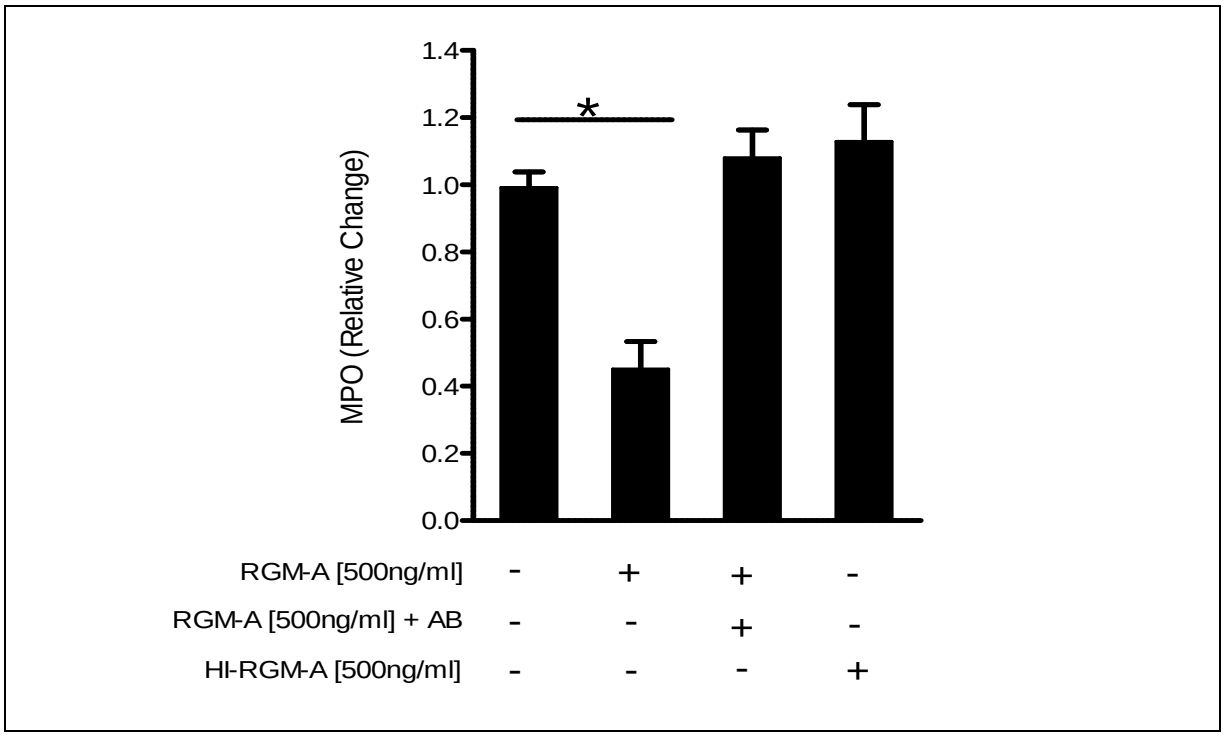

Figure 7. The effect of RGM-A on PMN migration can be abolished by antibody neutralization and heat inactivation. RGM-A inhibited migration of PMN at a concentration of 500 ng/ml. By adding anti RGM-A antibody (AB, R&D Systems, 10 µg/ml) this effect was not significant anymore. A similar result is reached after heat inactivation (HI) of RGM-A at 95℃ for 60 minutes. (n=6 per condition, *P < 0.05)

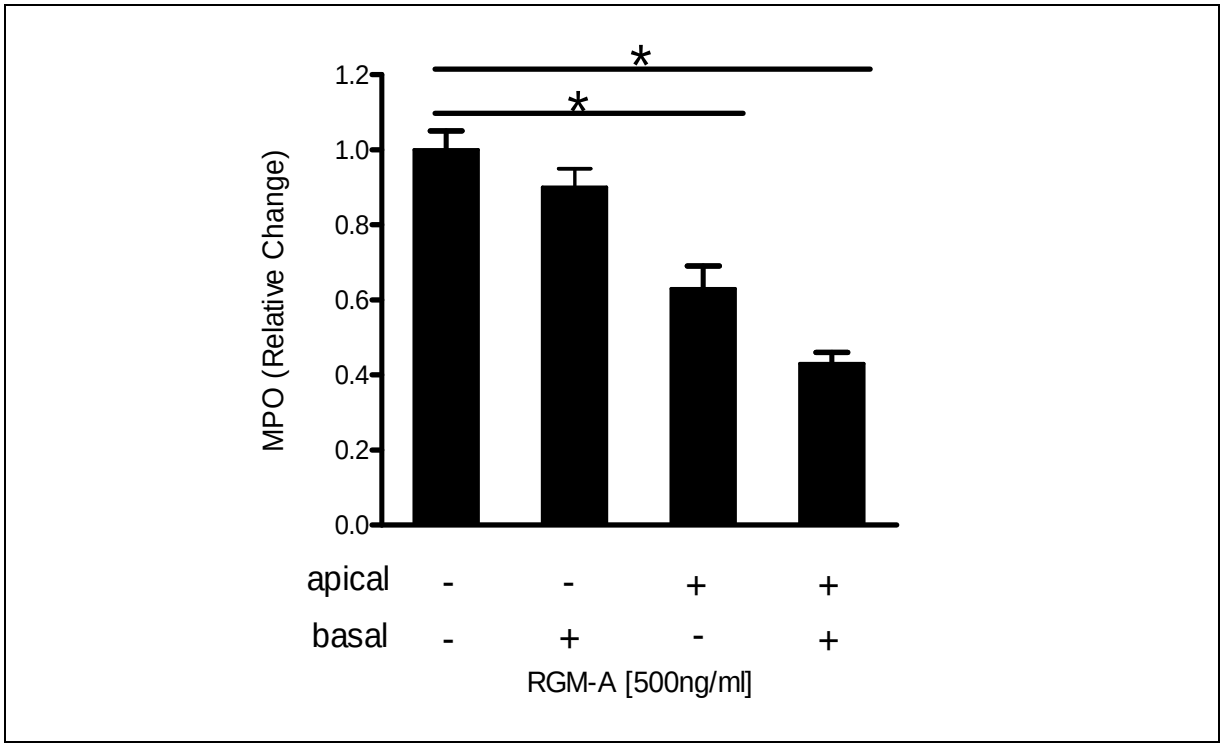

Figure 8. RGM-A affects leukocyte migration only when present in the apical compartment of the barrier. RGM-A inhibits fMLP induced PMN migration when present in

either the upper chamber or both chambers. PMN were placed in the upper chamber and migration to 10 nM fMLP in the lower chamber was measured in the presence and absence of RGM-A (500 ng/ml) in the upper chamber, the lower chamber or both. (n=6 per condition, *P < 0.05)

3.4 The RGM-A receptor neogenin is expressed on human leukocytes.

FACS analysis and immunostaining were performed using anti neogenin and anti CD 45 antibodies to label leukocytes. CD 45 was used as marker for intact human leukocytes and to show coexpression with neogenin. Granulocytes were demonstrated to express both proteins on their surface (Figure 9).

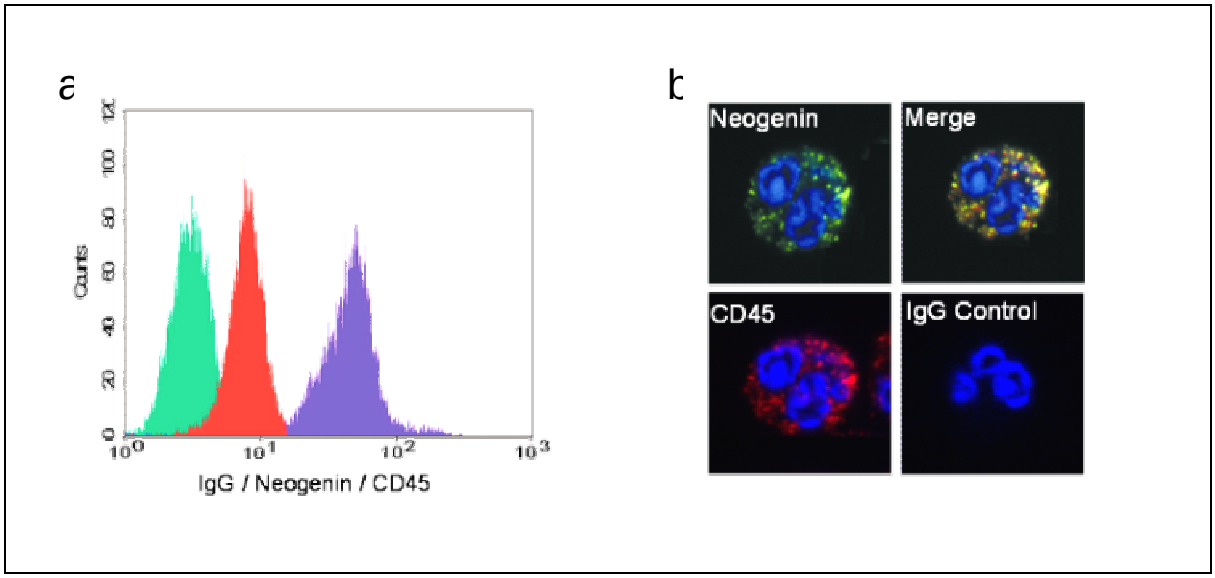

Figure 9. Neogenin is expressed on human polymorphonuclear leukocytes. a. The number of neogenin and CD 45 positive cells was higher than cells binding nonspecific IgG, shown by FACS analysis of labeled leukocytes. b. Leukocytes were stained using anti neogenin antibody (green fluorescence) and CD 45 (red fluorescence). The overlay demonstrates coexpression of both proteins, a control using unspecific immunoglobulines (IgG) was negative.

3.5 Attenuation of PMN migration by RGM-A in-vitro is neogenin dependent.

In the assumption that RGM-A mediates its attenuating effect via a specific receptor on cell surfaces the neogenin receptor was examined. Therefore, transmigrational

assays were modified: anti neogenin receptor antibody was added to RGM-A and PMN during incubation. In a dose dependent mode the inhibitory effect of RGM-A was neutralized as shown in Figure 10: The transmigration rate is shown in relation to the transmigration without RGM-A exposure (left bar). Application of RGM-A led to a decrease in migration even if anti neogenin antibody was added in a concentration of 1 µg/ml. In a fivefold antibody concentration the inhibitory effect was still significant. At the highest concentration of 10 µg/ml yet no difference could be measured (fourth bar from the left) compared to PMN that were not preincubated with RGM-A. Control samples being incubated with immunoglobulin of the same isotype did not influence the previously observed effect of RGM-A.

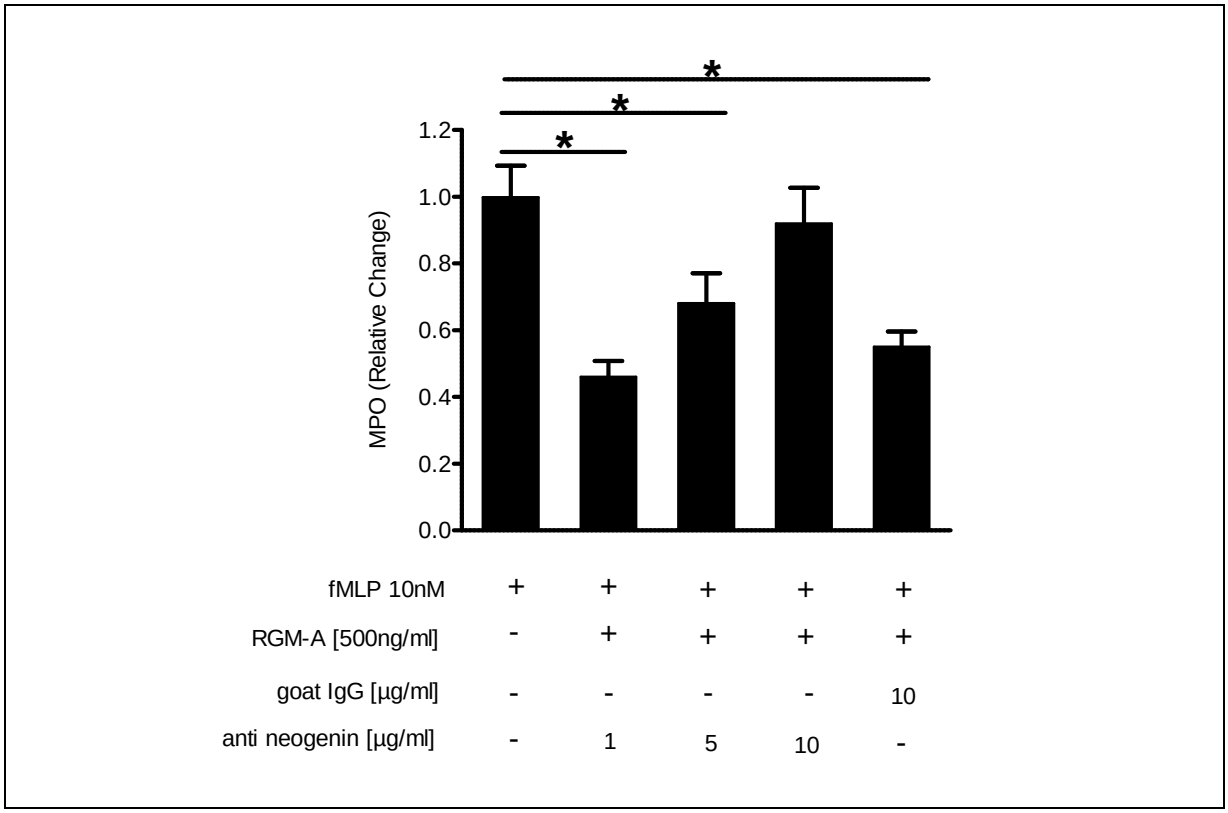

Figure 10. Anti neogenin antibody dose dependently blocks the effect of RGM-A on PMN migration. Transmigration assays were performed as described above. PMN were incubated with RGM-A (500 ng/ml). Additionally, a functionally blocking antibody directed against the neogenin receptor was added at concentrations of 10 µg/ml, 5 µg/ml or 1 µg/ml. At a concentration of 1 µg/ml and 5 µg/ml the RGM-A induced attenuation of migration was still significant. Incubating the sample with 10 µg/ml antibody the effect mediated by RGM-A was not detectable anymore. In contrast to this, using IgG of the same isotype did not influence the effect of RGM. (n=6 per condition, *P < 0.05)

3.6 RGM-A reduces PMN adhesion in-vitro dependent on neogenin.

In a cell adhesion assay the capability of seeded PMN to bind to an RGM-A coated surface was investigated. Different concentrations of RGM-A and BSA were used. It was observed that PMN were less able to bind and attach to the RGM-A coated surface in a dose dependent way (Figure 11, upper row). In spots containing the highest amount of RGM-A only sporadic leukocyte attachment was detected. Similarly to transmigration assays this effect was reversible when PMN were pre-incubated with a neogenin blocking antibody but not using a nonspecific antibody (anti HLA).

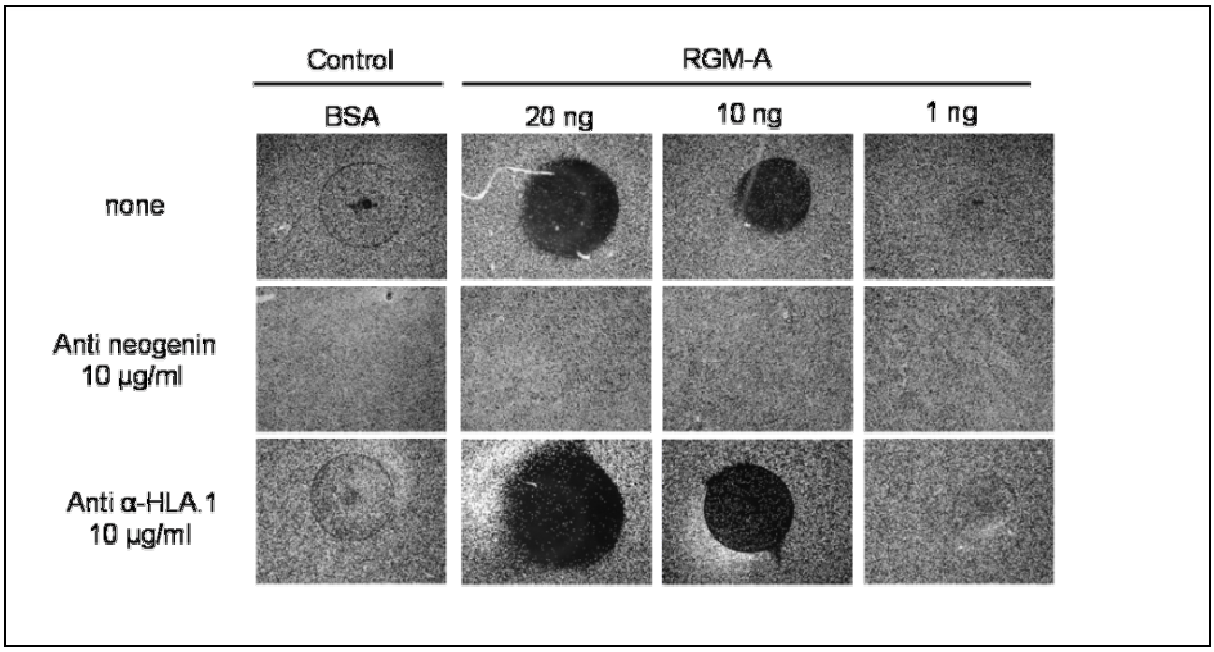

Figure 11. RGM-A shows contactrepulsive effects dependent on neogenin. PMN binding to a plastic surface is dose dependently blocked by RGM-A coating (upper row) and completely reversible upon preincubation of PMN with anti neogenin antibody (middle row) whereas anti HLA did not influence cell adhesion (lower row). One representative experiment of 3 is shown.

3.7 RGM-A suppresses ZyA induced cytokine release in-vitro.

Cytokine levels in human whole blood samples were raised by adding zymosan-A (ZyA), a derivative of yeast cells and inducer of inflammatory responses. In Figure 12 grey bars in relation to black show this distinct increase of TNFα, IL-1ß, IL-6 and

MIP1α due to ZyA. In the presence of RGM-A (500 ng/ml) the increase of cytokine levels could not be observed.

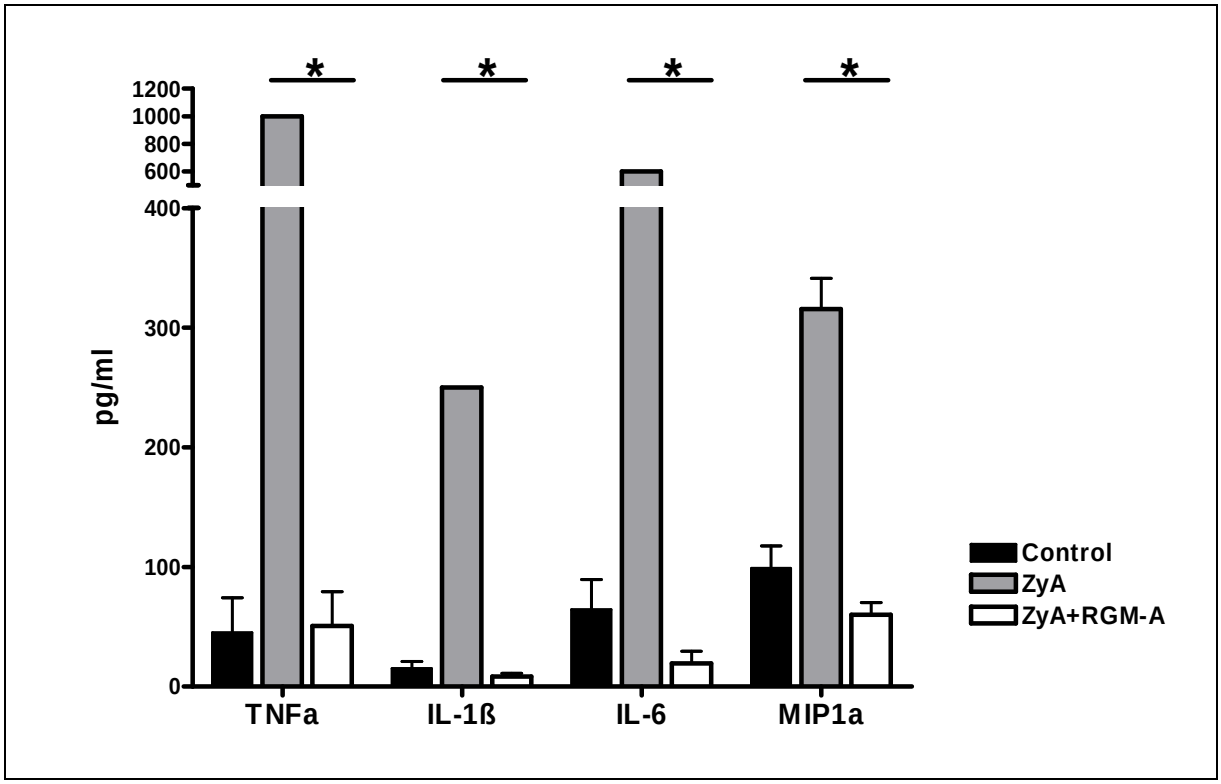

Figure 12. The release of pro-inflammatory cytokines in-vitro is reduced by RGM-A. Human whole blood was stimulated by ZyA (100 µg/ml) in order to provoke a release of TNFα, IL-1ß, IL-6 and MIP1α (ZyA). Addition of 500 ng/ml RGM-A abolished this response (ZyA+RGM-A). Base amount of cytokines was measured without ZyA application (control). (mean±SEM, n=5 per condition, *P < 0.05).

3.8 RGM-A has anti-inflammatory potential in-vivo.

A well defined self-limiting model of ZyA induced peritonitis in mice was used in order to examine a possible impact of RGM-A on leukocyte migration. After intraperitoneal injection of ZyA the inflammatory response was quantified by the number of cells that migrated into the abdominal cavity. Peritonitis induction with ZyA was followed by a singular immediate i.v. injection of either vehicle (BSA 0.2% in PBS) or 1 µg RGM-A. Interestingly, mice that received RGM-A did not similarly show the otherwise observed increase in immune cells in the peritoneal cavity: Analysis of peritoneal exudates after 4 h, 8 h and 24 h demonstrated a significant, yet transient reduction of infiltrating leukocytes by RGM-A. Already and most profoundly by 4 h RGM-A treated

animals demonstrated reduced recruitment of inflammatory cells (0.77±0.47 x 10^6 cells) compared to vehicle controls (2.08±0.5 x 10^6 cells, $P < 0.05$, Figure 13a, Figure 15). This reduction was observed until 8 h post injection, but not after 24 h. Using MPO as a marker for leukocyte activity, these results were confirmed by the measurement of reduced MPO activity levels in the exsudate (Figure 13b). RGM-A was found to reduce all populations, besides granulocytes also lymphocytes and monocytes, within the inflammatory exsudate (Figure 14). Histopathological analysis revealed that RGM-A injection attenuated histological signs of acute inflammation such as hyperemia, edema formation, tissue destruction and leukocyte infiltration compared to excessive inflammation in vehicle controls (Figure 16).

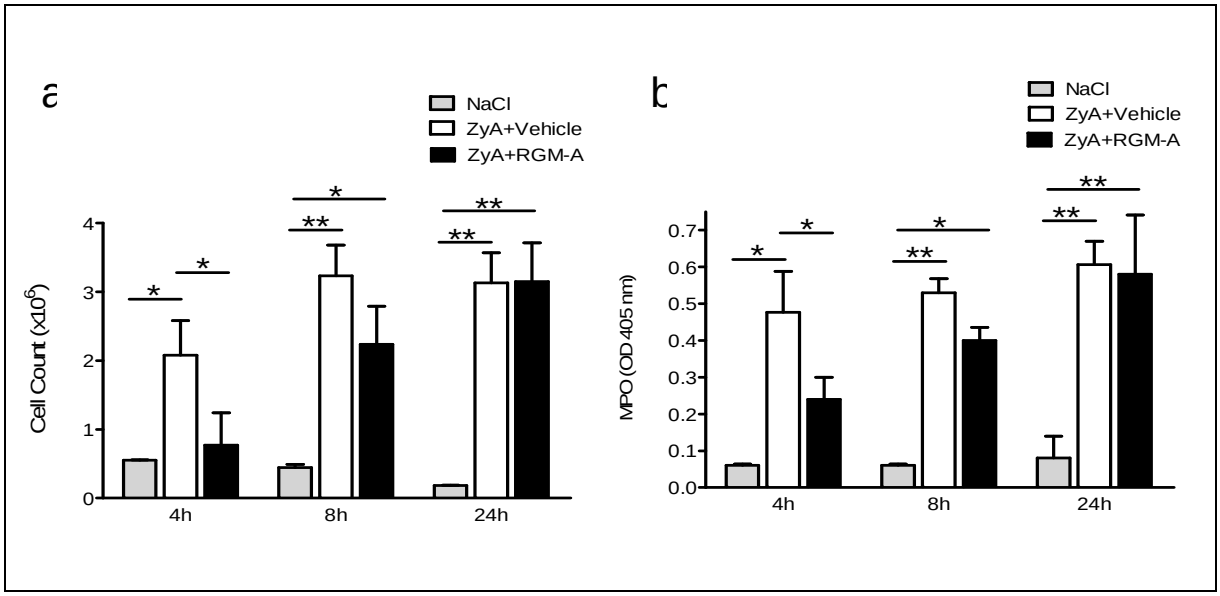

Figure 13. RGM-A induced reduction of leukocyte migration in a mouse peritonitis model. a. RGM-A inhibits leukocyte recruitment in-vivo in a mouse model of ZyA induced peritonitis. ZyA (1 mg in 1 ml) was administered intraperitoneally to induce peritonitis. Animals were treated with either vehicle (150 µl PBS, 0.1% BSA) or recombinant murine RGM-A (1 µg in 150 µl PBS, 0.1% BSA) i.v. A peritoneal lavage was performed after 4 h, 8 h or 24 h and the cell count was evaluated. After 4 h the cell count was significantly lower in the group of animals treated with RGM-A, an effect that was not detectable equally distinct after 8 h or 24 h (mean±SEM, control: n=5 per condition, ZyA: n=16 at 4 h, n=8 at 8 h, 24 h). b. Measurement of MPO activity confirms the reduction in cell count. Cells in the peritoneal lavage were lysed and MPO was assessed by an ABTS assay. (n=5 per condition, *P < 0.05)

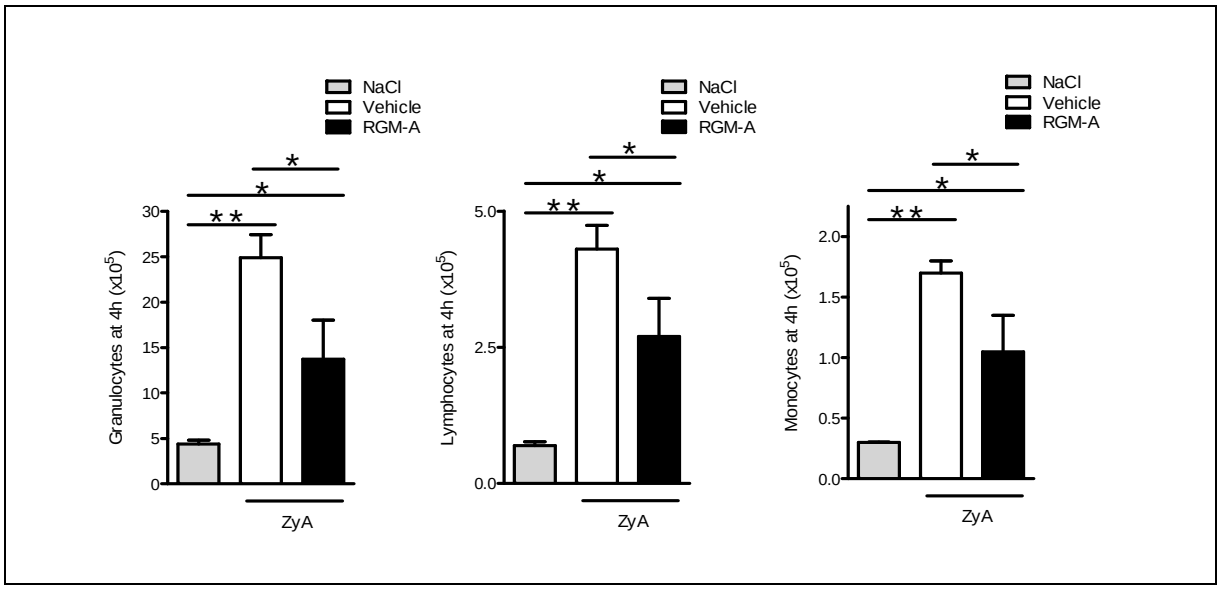

Figure 14. RGM induced attenuation equally affects all major subtypes of leukocytes. Microscopically differentiating leukocytes after 4 h peritonitis did show that all fractions (granulocytes, lymphocytes, monocytes) were affected in a comparable way by RGM-A.

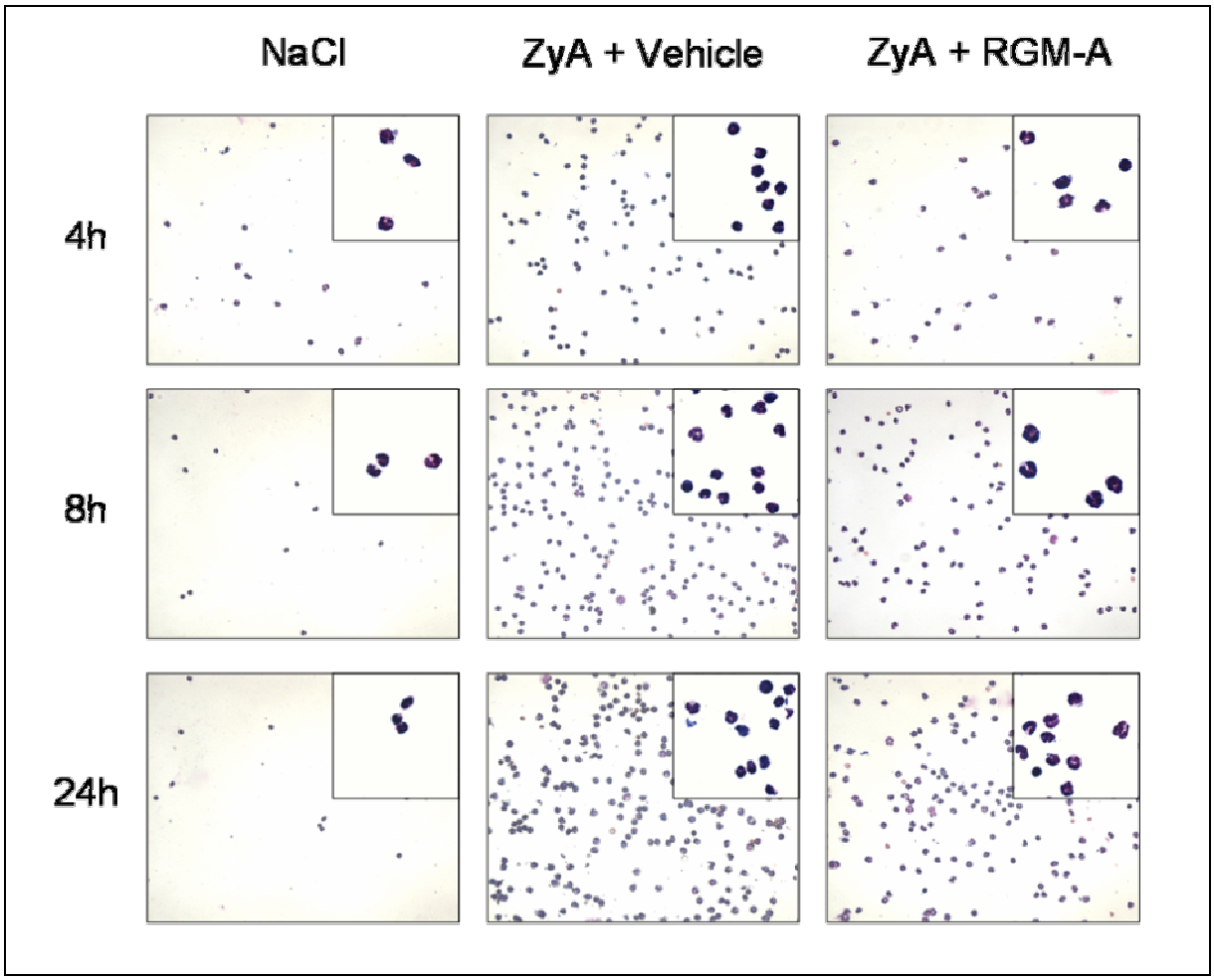

Figure 15. Leukocytes in peritoneal lavage. Cytospin® samples of peritoneal lavage visualized the difference in cell number in the lavage of animals treated with i.v RGM-A or

vehicle injection. Cells were stained using conventional Diff-Quick® staining. (magnification 400x, insert 1000x)

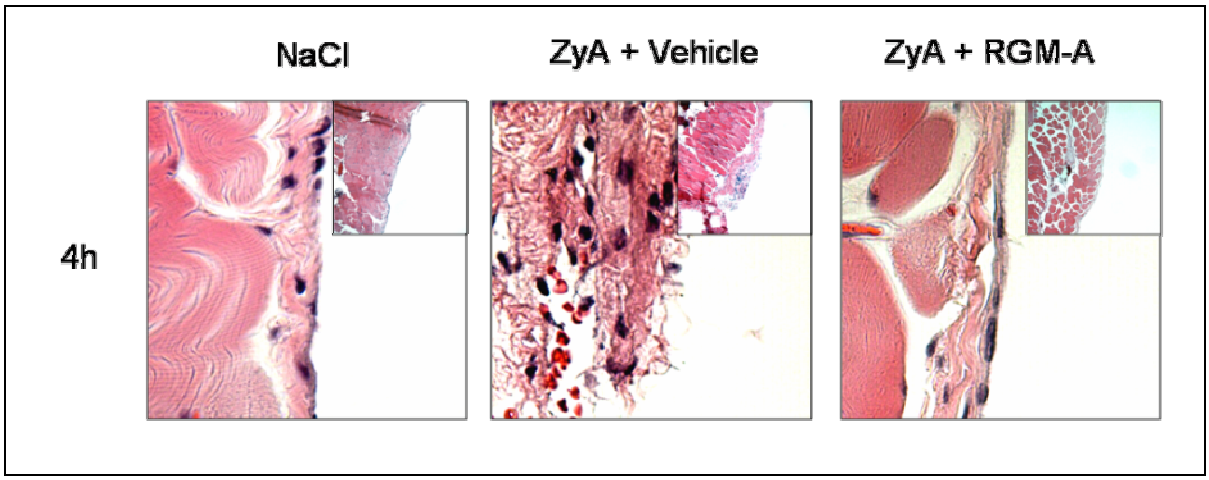

Figure 16. HE stained sections of mouse abdominal wall. To visualize the extent of peritonitis after 4 h paraffin sections of mouse abdominal wall were made. Infiltration with immune cells or hemorrhagic inflammation as seen after injection of ZyA were alleviated if RGM-A was additionally injected i.v. (magnification 1000x, insert 20x)

3.9 Dampening of inflammation by RGM-A in-vivo relies on neogenin.

Furthermore, it was supposed to clarify whether the demonstrated immunmodulatory effect of RGM-A relies on binding to its receptor neogenin in-vivo. Therefore, it was questioned if the observed effect of RGM-A would also be present in mice with gene targeted repression of the neogenin receptor. As demonstrating the maximum effect, the extent of inflammation due to ZyA was assessed after 4 h. Here, compared to wild type (WT) controls, the RGM-A mediated reduction in leukocytes infiltrating the peritoneal cavity was not detectable in the neogenin knockout (neogenin -/-) animals (Figure 17a). Correspondingly, neogenin -/- animals did not demonstrate reduced MPO activity levels upon RGM-A injection compared to WT controls (Figure 17b). Again, findings were visualized by cytospin fixation and microscopy of cells and by histological evaluation of peritoneal tissue. In the neogenin -/- animals, the attenuated acute inflammatory response was not detectable after RGM-A injection (Figure 18).

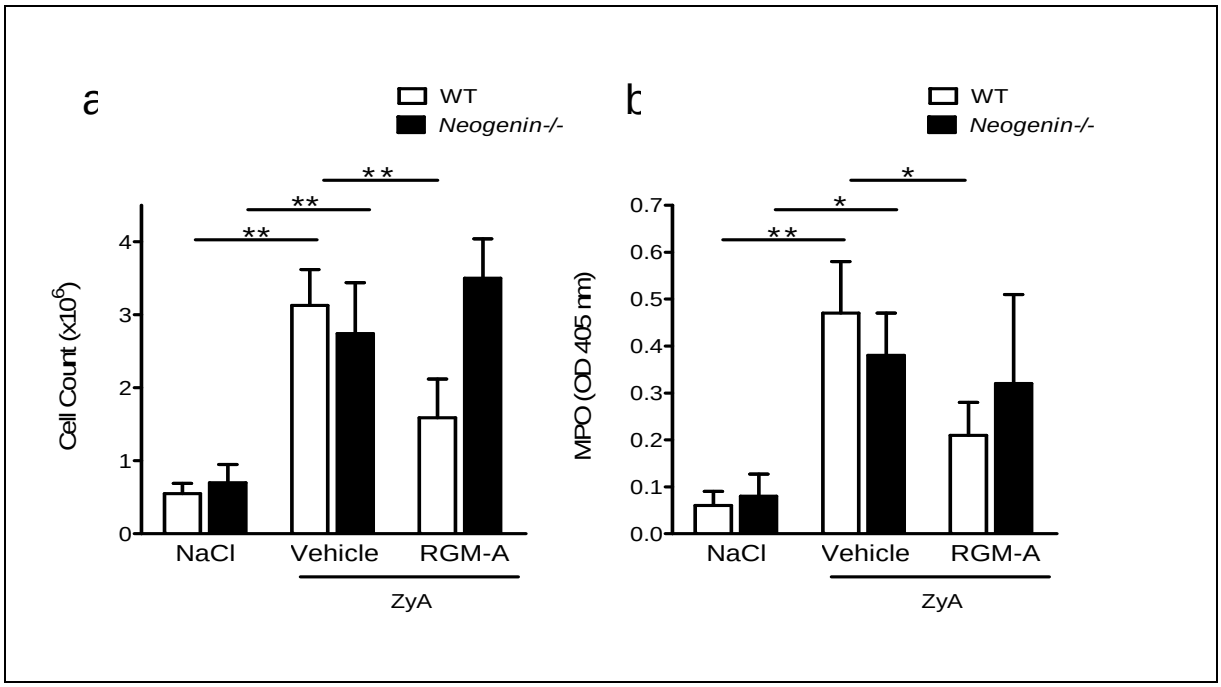

Figure 17. RGM-A failed to induce reduction of leukocyte migration in neogenin -/- mice. a. In contrast to WT mice, neogenin -/- mice did not respond to treatment with RGM-A. After i.p. injection of ZyA (1 mg in 1 ml) mice received either vehicle (150 µl PBS, 0.1% BSA) or recombinant murine RGM-A (1 µg in 150 µl PBS, 0.1% BSA) i.v. After 4 h cells in the inflammatory exsudate were counted, showing a significant reduction after RGM-A application in WT mice, but not in mice lacking neogenin. b. These results were confirmed by measuring MPO activity in the exsudate. (mean±SEM, n=6 per condition, *P < 0.05, **P < 0.01)

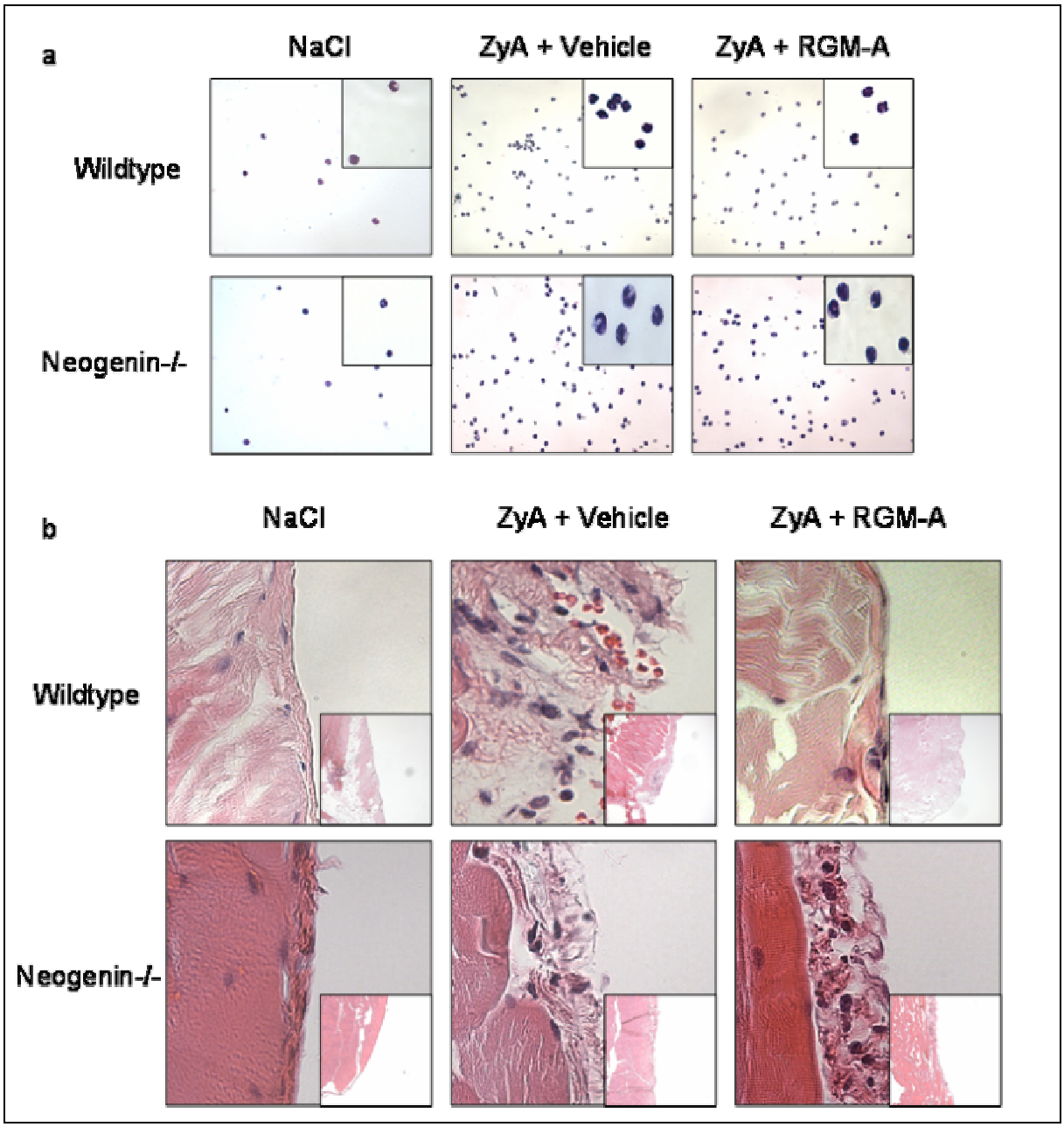

Figure 18. Leukocytes in peritoneal lavage and HE stained sections of mouse abdominal wall, a comparison of WT and neogenin -/- mice. a. Cells that migrated into the abdominal cavity were visualized using Cytospin® and Diff-Quick®-staining. The previously described difference between RGM-A and vehicle treatment in wild type mice is not present in neogenin -/- animals. (magnification 400x, insert 1000x) b. Signs of inflammation in the abdominal wall could be reduced by RGM-A in wild type mice, but not in neogenin -/- mice. (magnification 1000x, insert 40x)

3.10 RGM-A suppresses cytokine production in-vivo dependent on neogenin.

To gain further evidence about the control of local inflammation through RGM-A, the levels of pro-inflammatory cytokine production were valuated. Studying the ZyA induced peritonitis with WT and neogenin -/- mice as described above cytokine levels were measured within the peritoneal exsudate. Similar to the results in-vitro RGM-A application suppressed the levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and MIP-1α in the peritoneal cavity of the WT-animals (Figure 19, white bars). In contrast, the observed reduction of cytokines within the inflammatory exudates was not present in the neogenin -/- animals: RGM-A did not alter the release of cytokines in those animals (black bars).

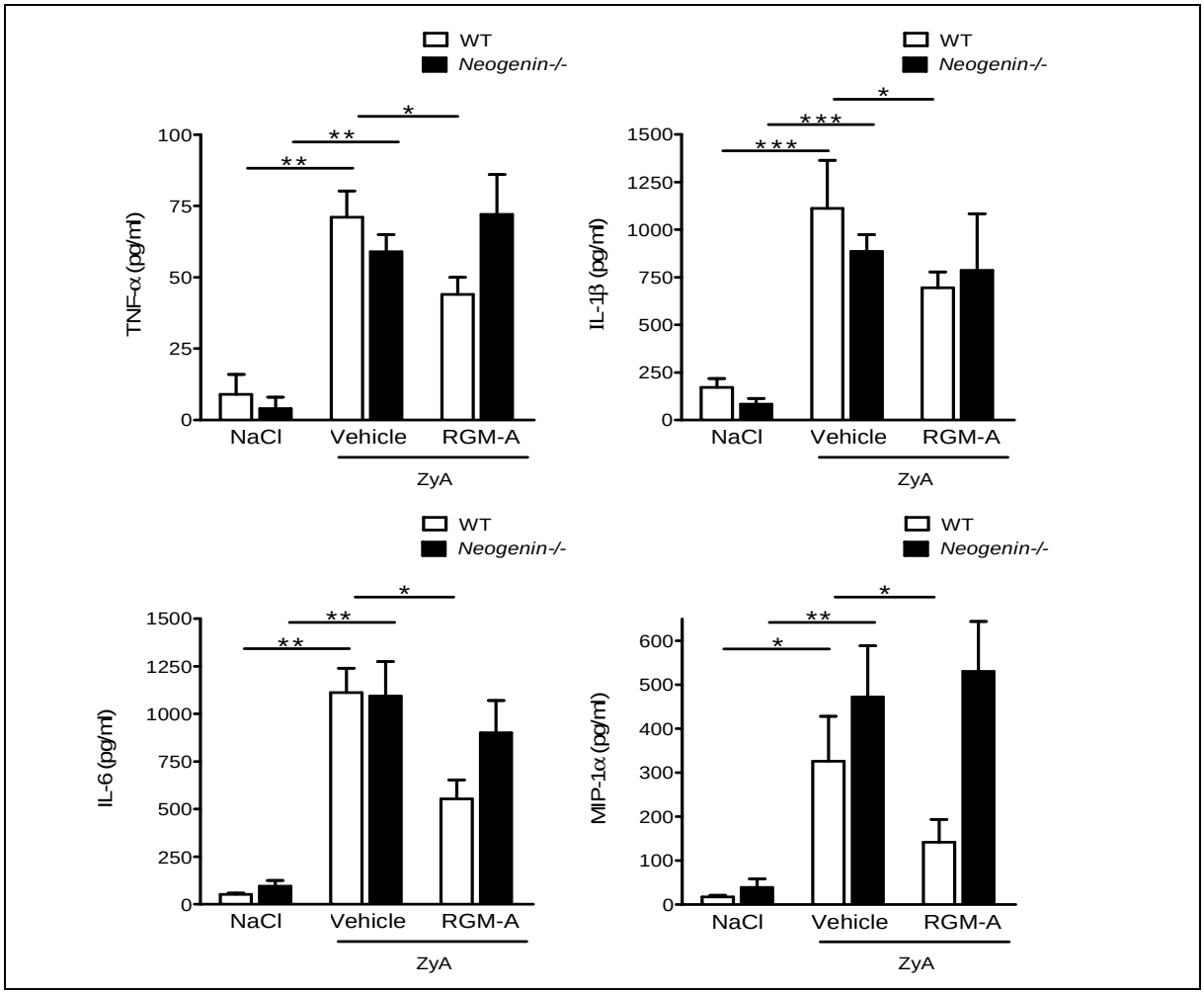

Figure 19. Cytokine release in the context of induced peritonitis is reduced by RGM-A in wildtype mice, but not in neogenin -/- mice. After inducing sterile peritonitis by ZyA a peritoneal lavage was taken after 4 h and cytokine levels measured by ELISA. Concordantly with leukocyte counts, the level of tumor necrosis factor (TNF)α, interleukin (IL)-1ß, IL-6 and

macrophage inflammatory protein (MIP)1α were significantly reduced by RGM-A in wildtype (WT) mice. However, no influence of RGM-A on cytokine release could be observed in neogenin -/- mice. (mean±SEM, n=6 per condition, *P < 0.05, **P < 0.01)

4 Discussion.

This study investigated the expression as well as the immunological impact of the repulsive guidance molecule A. It identifies a previously uncharacterized function for this repulsive neuronal guidance cue outside the central nervous system as inhibitor of leukocyte migration during acute inflammation.

4.1 Expression of RGM-A outside the central nervous system.

In previous studies RGM-A was mainly described as a protein expressed in the central nervous system(34-37). Its initial discovery was made in chick membranes of the posterior tectum in 1990 without differentiating different subtypes at that stage(29,55). In 2004 the first analysis of the expression pattern of RGM-A, -B and -C was made in mammals(36). Messenger RNA of RGM-A could be detected in hippocampus, midbrain, brainstem and spinal cord of mouse embryos. RGM-B did show a similar distribution, while RGM-C was in contrast expressed in skeletal muscle tissue. Recently, examining rat tissues RGM-A RNA was found in heart, lung, liver, skin, kidney and testis(38) and RGM-A was shown to be involved in the development of the mouse intestine(39).

In order to exert an immunological function, RGM-A has to be expressed outside the central nervous system. To demonstrate this biological relevance and confirm previous studies a number of adult murine tissues were screened. RGM-A was shown to be detectable beyond the central nervous system: Significant expression of RGM-A and its mRNA was found in organs like lung, spleen or the intestine. The partly diverging results of mRNA and protein expression remain open questions possibly indicating varying posttranscriptional processing. The expression found in the spleen was inconsistent to a former study not detecting RGM-A in this organ(38). Yet, this has to be seen against the background that different techniques were used (northern versus western blot analysis). Noteworthy, the spleen, but also the lung and intestine, each contain an intrinsic localized immune compartments, white pulp, alveolar macrophages, peyer’s patches(56), that require a well defined regulation of leukocyte migration.

4.2 Attenuation of PMN migration and adhesion in-vitro through RGM-A.

Knowing that RGM-A is expressed peripherally its functional role was examined. Transmigration assays showed an inhibitory effect of RGM-A on leukocyte migration. It had been assumed that RGM-A mediates its effect as a signal molecule that requires a specific tertiary structure in order to interact with target receptors. Therefore, RGM-A was tested in comparison to a protein of similar size (bovine serum albumin, 66 kD) in order to minimize error sources. Albumin showed no influence on migration rates. Other possibilities to demonstrate specificity are neutralizing RGM-A by destroying or altering its tertiary structure. After heat inactivation, a process that changes the tertiary structure of most proteins, RGM-A did not show any attenuating effect anymore. The same was observed after RGM-A was bound to an anti RGM-A antibody that probably blocked parts of the protein necessary to interact with a target receptor. Furthermore, RGM-A did not act as an inhibitor if placed in the lower compartment of the transmigrational chamber, but only if put in the upper compartment and used for preincubation of PMN. This observation supports the hypothesis that it is first of all leukocytes itself that respond to RGM-A and neither the epithelium nor an interaction with the chemoattractant. The central role of adhesion in cell migration was described above. Therefore the role of RGM-A in adhesion assays was investigated that showed contactrepulsive properties. The repelling effect of RGM-A in this subset of experiments strengthens hypotheses: Again, it seems to be leukocytes itself that respond to RGM-A as no other cells were involved. According to transmigrational assays a control protein was not able to repel leukocytes indicating that the effect is due to the specific structure of RGM-A. In both subset of experiments the effect was shown to be dose dependent. A reason to investigate this is to increase the probability that the effect is causative.

These results confirm the inhibitory effects of neuronal guidance molecules on leukocyte migration in-vitro. Slit-2 was a guidance molecule known for its repelling effect in neuron development. Its effect on migrating leukocytes was examined in transmigration assays in a similar set of experiments showing a stronger suppression compared to RGM-A but using a different subtype of leukocytes of a different species and partly a different chemotactic factor. A significant difference depending on in which chamber the repellent was applied was examined but could in contrast to this study not be reported. Netrin-1 was yet another neuronal guidance cue that had been

looked at in this regard(20,21). It showed an inhibition of fMLP induced leukocyte migration in a dose dependent manner very similar to the result of this study. It has to be reminded of the fact that neither netrin nor slit share significant homologies to RGM-A. Netrin-1 shares its target receptor in the CNS with RGM-A, but most probably having a different binding site(45). A question raised is whether or not different subtypes of RGM should also be looked at. This study focuses on the subtype A as it shows the greatest homology to chick RGM that was first described having repelling properties(36). Recently, RGM-B was reported to show very similar effects as RGM-A in the CNS(57,58) indicating that future studies should be worthwhile having a closer look on RGM-B during inflammatory conditions.

4.3 Immunemodulation of RGM-A in-vivo.

A number of studies have assessed the influence of neuronal guidance proteins on leukocytes in-vivo(19,20). Focusing on the previously mentioned slit-2 and netrin-1 it was shown that both of them do have anti-inflammatory properties in-vivo. Slit-2 was recently examined in a model of mouse peritonitis(59) following the study of 2001 that prompted a broad inhibitory effect on cell migration by slit-2(19). Sterile peritonitis was dampened by slit-2 in a highly significant way. Netrin-1 was also reported to influence cell migration in a mouse model of peritonitis, yet being administered in the peritoneal cavity and not, as in this study and the study looking at slit-2, intravenously(20).

The model of ZyA induced peritonitis reflects the previously described transmigrational assay with the peritoneal cave representing the lower compartment of a transmigration chamber and the intravasal space being the apical compartment containing leukocytes. As RGM-A was effective in-vitro if applied in the apical chamber it was now given intravenously. After exposure to RGM-A leukocytes were less likely to migrate into the peritoneal cavity following ZyA stimulation. To define the time course of this effect more precisely, experiments were evaluated 4, 8 and 24 h after injection of RGM-A. As it is a protein it can be assumed that RGM-A will be metabolized and broken down meaning that its effect should not last. Indeed, the observed effect was not significantly present anymore after 24 h: the number of migrated leukocytes was the same in treated and untreated mice.

4.4 Neogenin as receptor expressed on leukocytes and mediating the effect of RGM-A in-vitro and in-vivo.

After the anti-inflammatory role of RGM-A was highlighted the potential mechanisms involved should be questioned. Here, further supporting the original hypothesis, the RGM-A high affinity receptor neogenin was shown not only to be a target receptor in the nervous system but also to be essential for the immunological function of RGM-A. Neogenin was initially shown to be expressed on human leukocytes and 2 techniques were used to increase validity, FACS analysis and immunohistochemical staining. Experiments using knockout mice are regarded one of the best ways to demonstrate the function of the lacking gene. Neogenin-/- mice demonstrated no response to RGM-A injection, indicating that neogenin is necessary to mediate the effect of RGM-A not only in the CNS but also in its new immunological context. These findings had already been suggested in experiments in-vitro as described above. Both in transmigration assays and in adhesion assays a functionally blocking antibody could reverse the effect of RGM-A on leukocytes. Taken together, the examination of neogenin can be seen as a first step to elucidate the mechanisms of RGM-A induced inhibition of leukocyte migration, but future studies should aim at identifying involved downstream pathways.

The result that the receptor neogenin is detectable on the leukocyte surface coincides with a previous study(20). Another study suggested that neogenin is widely expressed in tissues outside the CNS examining the mouse embryo(46). This is supposed to include tissues like lung, heart, pancreas, kidneys. As far as RGM-A and its function as neuronal guidance protein is concerned neogenin is a target receptor in various studies including the CNS and the peripheral nervous system(39,45). An interaction of RGM-A and neogenin in a context other than guiding neuronal development has probably not been shown, yet neogenin is also seen as a receptor involved in processes apart form CNS development, as, interestingly, in the formation of epithelial sheets(46).

4.5 Influence of RGM-A on cytokine expression in-vitro and in-vivo.

As mentioned above there is a number of cytokines, including chemokines, that are already known to influence leukocyte migration. It was focused on four pro-inflammatory cytokines that should be briefly characterized: tumor necrosis factor (TNF)α, interleukin (IL)-1ß, IL-6 and macrophage inflammatory protein (MIP)-1α. TNF is a cytokine playing a central role in mediating both chronic and acute inflammation as a major pro-inflammatory cytokine(60). It further has the ability to cause necrosis of certain cancers which had led to the discovery of TNF in 1975(61). Later TNF was identified to be a mediator of endotoxic shock as well as causing cancer related inflammation. Interleukine-1 α and ß are further pro-inflammatory cytokines. IL-1ß has homeostatic functions regarding sleep or body temperature(62). It is produced by a great number of cells including endothelial cells. IL-1ß binds to interleukin 1 type 1 receptor (IL-1RI) that in combination with interleukin 1 receptor accessory protein (IL-1RAcP) transduces its effect. Interestingly, the action of IL-1 is balanced by an endogenous interleukin 1 receptor antagonist (IL-1ra). IL-1ß is activated depending on an intracellular scaffold called inflammasome that is sensitive to inflammatory stimuli. The cytokine IL-6 was first described as a T cell derived B cell stimulating factor but is known to be involved in inflammation in different ways, too(63). As a key player it induces C-reactive protein by stimulating hepatocytes and increases leukocyte migration by inducing adhesion molecules. Macrophage inflammatory protein-1 having 4 subtypes is another chemotactic cytokine. It is released by most immune cells like macrophages, T and B lymphocytes, neutrophils, dendritic cells, mast cells and natural killer (NK) cells, to a lesser extent even by platelets and epithelial cells(64). Production requires pro-inflammatory agents, for example lipopolysaccharides or zymosan, or cytokines as TNF or IL-1ß. As chemokine MIP-1 binds to chemokine receptors: MIP-1α to CCR1 and CCR5, MIP-1ß to CCR5. Their target cells are T lymphocytes, NK cells, macrophages and dendritic cells, while neutrophil leukocytes are much less attracted by MIP-1α. The ability of RGM-A to suppress cytokine release in-vitro supports the hypothesis of an immunological function. The increase in cytokine levels after stimulation with ZyA was significantly reduced by simultaneous application of RGM-A.

Looking at the complexity of immunological messenger proteins those results raise a number of questions that have to be targeted in future studies. It remains

unclear whether RGM-A itself reduces the release of pro-inflammatory cytokines or this effect should be considered a consequence of the inhibition of cell migration. On the other hand it might be an RGM caused reduction of TNFα, IL-1ß, IL-6 and MIP-1α that leads to attenuation of leukocyte migration. Very little seems to be known about a context of RGM-A and cytokine release. Looking at other guidance molecules, netrin-1 and TNFα showed an inverse relation in a mouse model of lung infection(20). In contrast to this, semaphoring 7A, that was mentioned as a pro-inflammatory neuronal guidance cue, was demonstrated to have a strong positive impact on cytokine release including TNFα, IL-6 and IL-8(65).

4.6 Mechanism of RGM-A function.

The mode of action of RGM-A, which cells synthesize it, which are the primary target cells, how it is released and what biological function it fulfils, can only be hypothesized (Figure 20 and 21). According to this and previous studies, it can be assumed that endothelial expression of RGM-A inhibits leukocyte diapedesis and recruitment towards inflammatory sites. RGM-A might represent a barrier for aberrant leukocyte migration. Since RGM-A is selectively bioactive if applied from the apical compartment and reduced all leukocyte populations in a rather broad, symmetrical way the proposed target of RGM-A bioactivity appears to be leukocytes before and while leaving the luminal vessel wall (diapedesis). Here, RGM-A might interfere with multiple steps such as tethering, rolling (chemorepulsive function, Figure 20a, 21), contact triggered activation and adhesion (contactrepulsive function, Figure 20b, 21).

RGM-A was reported to be GPI-anchored and possibly proteolytically cleaved in order to fulfill its function(34). It remains unclear whether RGM-A primarily acts as membrane bound or soluble protein. Cleavage due to a change in the pH value could at least be imagined as a mode to activate RGM-A, as inflammation tends to lead to a local drop in the pH value. In addition, RGM-A may directly modulate vessel integrity and barrier function reducing infiltrating cell traffic. Endothelial cells possibly release RGM-A to act in an auto- or paracrine manner. Mode of action and confirming target cells for RGM-A are issues that further need to be pursued for a better understanding. In a similar way, the repression of cytokine release observed after RGM-A exposure confirms its immunological relevance, yet needs to be better understood and put into context.

This study pursues an immunological function of RGM-A outside the CNS that has probably not been described yet. Nevertheless, known modes of action of RGM-A in the CNS can be helpful for a better understanding and should be considered in following studies. Here, apart from binding to neogenin an interaction with BMP has been described(38) and a downstream effect via Rho GTPases(52).

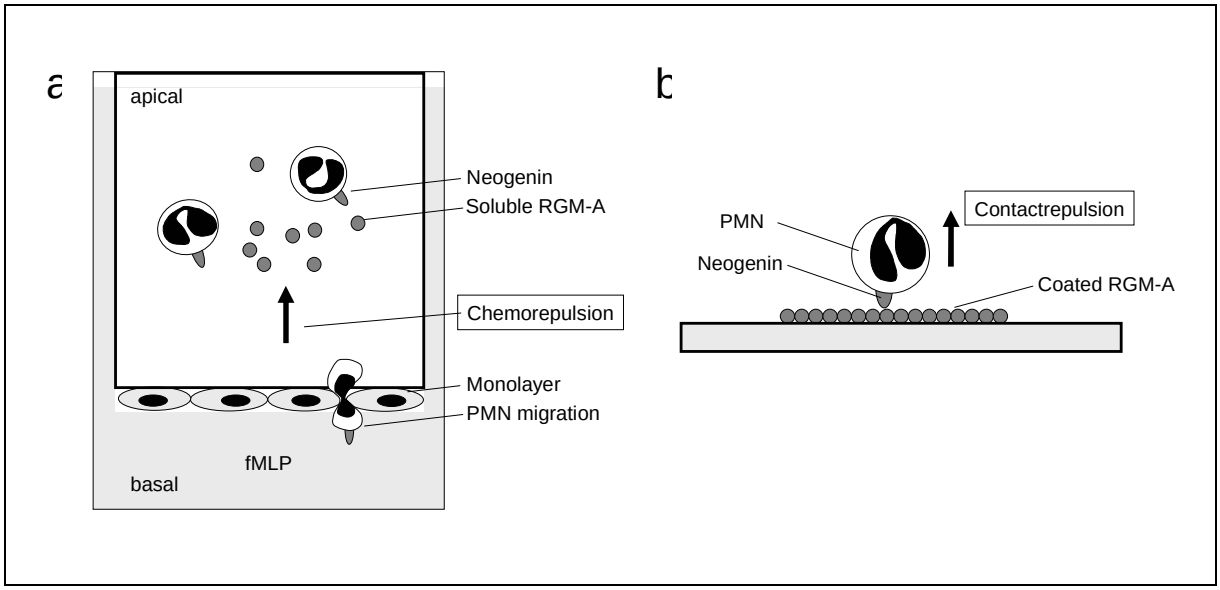

Figure 20. Model of transmigration assay and cell adhesion assay. According to the results of this study RGM-A as a ligand for its receptor neogenin seems to possess both a chemorepulsive (a) and contactrepulsive (b) impact on PMN.

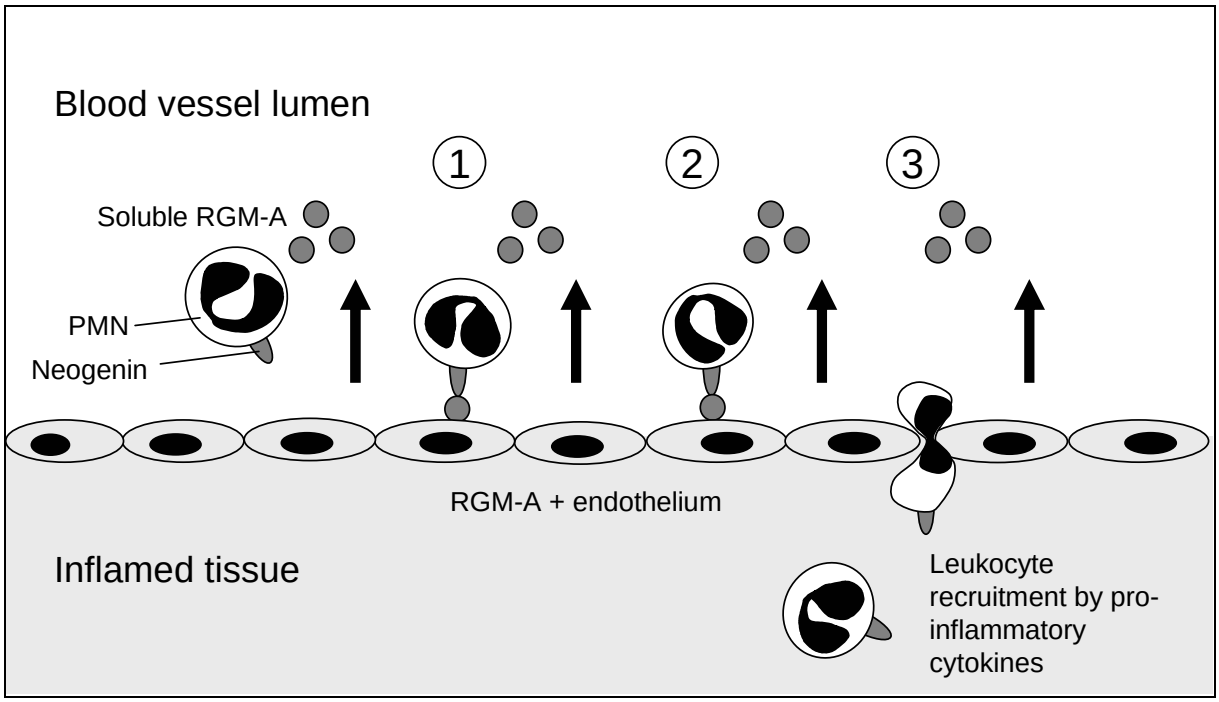

Figure 21. Possible interactions of RGM-A with PMN and endothelial cells. Chemo- (step1-3) and contactrepulsive (step 1-2) properties of RGM-A interfering with leukocyte

recruitment. (1) Tethering, (2) Triggering, (3) Latching and diapedesis. Migration as a multistep process offers different targets for RGM-A to exert its inhibitory effect.

4.7 Concept of shared guidance mechanisms of the nervous and the immune system.

The data of this study support that neuronal guidance molecules at least partly possess immunological relevance. The concept of shared, conserved molecular guidance cues ranging from neurons to leukocytes seems to be corroborated. Since the end of the 19th century neurons were known to migrate during CNS-development(26). Beginning in the 1960s migration was understood to be active rather than passive, radial and tangential mode of migration were distinguished and later, first substances acting as guidance cues could be isolated, as collapsin which was later assigned to semaphorins(66). Parallel to neurobiological research mechanisms of active movement of leukocytes were investigated, and in 2001 the first shared signal molecule (slit-2) was discovered(19). It became apparent that a fundamental conservation of control of cell migration exists, despite the differences between neurons and white blood cells in morphology (neuron migration concerns a single growing axon, leukocyte migration is characterized by multiple pseudopodia), migration speed (neurons migrate by far slower than leukocytes) and their cellular milieu.

Vice versa, interesting effects of known chemokines on neuronal migration could also be reported(67). The first chemokine to be characterized in that way was stromal derived factor (SDF)-1(68). It was originally described as chemoattractant for leukocytes(69) acting via its receptor CXCR4, but a targeted gene knock out of SDF-1 or CXCR4 surprisingly led to disturbed embryological neuronal development. SDF-1 was then reported to influence axon growth cones by modulating their responsiveness to neuronal guidance cues(16).

The concept of shared guidance cues for neurons and leukocytes might be extended looking at current studies about other types of cell movement. Tissue formation is influenced by neuronal guidance proteins including angiogenesis that requires a reorganization of the cytoskeleton and tube formation of endothelial cells(70). Among neuronal guidance molecules primarily netrin-1 and semaphorins have been examined in the context of angiogenesis. While netrin is supposed to

promote vessel formation by an increase in intracellular nitric oxide(71), semaphorin is described as potent inhibitor of angiogenesis(72).

4.8 Potential similarities to the pathology of cancer cells.

Another, yet very different, biological context, in which mechanisms of cell movement seem be conserved, is cancer. A feature of most cancer cells is an inappropriate pathological ability to migrate, infiltrate surrounding tissue and cross vessel barriers using mechanisms that otherwise occur in morphogenesis or inflammation(73). At a molecular level research revealed common mechanisms of migration that were activated in cancer cells, including neuronal guiding cues and their receptors(74-76). In this context, RGM-A was recently described as protein suppressing tumor cell migration(77). RGM-A as well as neogenin were frequently downregulated in colon carcinomas and adenomas, but not in normal colon tissue, where its expression was reported to be localized in basal crypts. Downregulation was due to either gene methylation, loss of heterozygosity or missense mutations. Interestingly, transfecting cancer cells with RGM-A decreased their ability to migrate and built colonies in-vitro as demonstrated by wound assays. Although happening in a different context, those results support the hypothesis of this study. A close relation between neuronal guidance molecules and cancer cell migration was known before, for example looking at netrin and its receptor DCC (hence the name) or at slit-2 or semaphorins(78-80). Conceivably, neuronal migration, leukocyte migration and tumor cell migration are examples for a universal mechanism of evolutionary old proteins like RGM-A attenuating cell movement.

4.9 Conclusion.

Beyond gaining knowledge of the mechanisms of cell movement, the identification of endogenous pathways that downregulate infiltrating cell traffic and dampen inflammation might provide important targets for novel interventional approaches. Interfering with leukocyte traffic and the development of local inflammation appears to be particularly interesting in pathologies characterized by an exaggerated immune response as seen certain conditions of acute inflammation, or also in autoimmune

diseases. In this context, the wide clinical use of TNFα antagonists is a preliminary model of interfering with leukocyte chemotaxis(81). IL-8 provides another example for a pro-inflammatory chemokine whose neutralization can be of clinical value(14). In contrast to identified chemokines, neuronal guidance molecules influence cell traffic by actively abolishing migration. If, and to which extent, the knowledge of RGM-A inhibiting leukocyte traffic will be of clinical benefit remains the major open question of this study.

5 Abstract.

Guiding migrating cells towards their destination is of integral importance in cell biology. Directed migration is found in the development of the central nervous system but also in topically restricted, graded immune responses. In this context attractive environmental cues such as chemokines guiding leukocyte cell traffic have been well characterized in recent years and are likely to be complemented by repulsive cues. One such paradigm exists in the developing nervous system where neuronal migration is balanced by chemoattractive and chemorepulsive cues including the Repulsive Guidance Molecule-A (RGM-A). RGM-A is a GPI-anchored glycoprotein of 33kDa size that guides axon growth cones during neuronal development by repulsion through its receptor neogenin. Its role within the immune response remained unclear. Yet, other neuronal guidance molecules that were shown to influence leukocyte trafficking prompted a possible immunological function of RGM-A. This study reports that RGM-A is expressed in extraneuronal organs as shown by RT-PCR, Western blot and immunohistochemical staining. RGM-A inhibits leukocyte migration by contact- and chemorepulsion dose dependently through its receptor neogenin as demonstrated in transmigration assays (RGM-A 500 ng/ml 0.38±0.08, $P < 0.01$, 50 ng/ml 0.66±0.08, $P < 0.05$, 5 ng/ml 0.97±0.17, RGM-A 500 ng/ml + anti neogenin antibody 10 µg/ml 0.94±0.12). In-vivo RGM-A weakens the inflammatory response in a mouse model of zymosan-A induced peritonitis after 4 hours (migrated cells, 10^6: vehicle 0.55±0.18, vehicle + ZyA 2.08±0.4, RGM-A + ZyA 0.77±0.47, $P < 0.05$). This was confirmed by evaluating MPO levels and histological samples. The RGM-A induced reduction equally concerned all major leukocyte subtypes. RGM-A application further attenuates the pro-inflammatory response reducing cytokine levels (TNFα, IL-1ß, IL-6, MIP-1α). In contrast, the demonstrated anti-inflammatory effects of RGM-A were absent in neogenin -/- mice. Taken together, these results identify RGM-A as a protein with anti-inflammatory functions upon binding to its receptor neogenin. It effectively suppresses leukocyte recruitment supporting the idea of a molecular conserved role as guidance cue for both neurons and leukocytes. The identification of a novel endogenous inhibitor of leukocyte chemotaxis limiting infiltrating leukocyte migration creates opportunities to treat conditions caused by exacerbated or misdirected inflammatory responses.

Zusammenfassung.

Aktive Migration von Zellen spielt eine entscheidende Rolle sowohl während der Entwicklung des Zentralnervensystems (ZNS) als auch bei der Extravasation von Leukozyten, wie sie in Entzündungsreaktionen beobachtet wird. In diesem Zusammenhang wurden in den letzten Jahren Faktoren beschrieben, die die Migration von Leukozyten während entzündlicher Prozesse begünstigen, allen voran Chemokine. Es ist jedoch nahe liegend, dass Chemokine mit migrationshemmenden Faktoren in einem Gleichgewicht stehen, ähnlich wie die Migration von Neuronen während der Entwicklung des ZNS durch chemoattraktive und –repulsive Faktoren balanciert wird. Ein solcher Faktor ist das Repulsive Guidance Molecule-A (RGM-A), ein GPI-verankertes 33kDa schweres Glykoprotein mit abstoßender Wirkung auf axonale Wachstumskegel, die den Rezeptor Neogenin tragen. Die Beobachtung, dass neuronale Leitproteine auch einen Einfluss auf das Wanderungsverhalten von Leukozyten haben können, machte nun auf eine mögliche immunologische Bedeutung von RGM-A aufmerksam. Diese Studie zeigt zunächst mittels RT-PCR, Western Blot und immunhistochemischen Färbungen, dass RGM-A auch in extraneuronalem Gewebe exprimiert wird. RGM-A zeigte eine dosisabhängige hemmende Wirkung auf Leukozytenmigration durch den Rezeptor Neogenin wie in Transmigrationsassays demonstriert werden konnte (RGM-A 500 ng/ml 0.38±0.08, $P < 0.01$, 50 ng/ml 0.66±0.08, $P < 0.05$, 5 ng/ml 0.97±0.17, RGM-A 500 ng/ml + Anti Neogenin Antikörper 10 µg/ml 0.94±0.12). In-vivo, in dem Mausmodell einer Zymosan-A-induzierten Peritonitis, schwächte RGM-A die Entzündungsantwort nach 4 Std. ab (Zellzahl, 10^6: vehicle 0.55±0.18, vehicle + ZyA 2.08±0.4, RGM-A + ZyA 0.77±0.47, $P < 0.05$). Dieses Ergebnis spiegelt sich auch in MPO-Messungen und histologisch wider und betraf alle Leukozytenfraktionen in vergleichbarem Maße. Weiterhin schien RGM-A die Entzündungsantwort durch Zytokine (TNFα, IL-1ß, IL-6, MIP-1α) zu dämpfen. Die Effekte blieben in Neogenin -/- Mäusen aus. Zusammengefasst identifiziert diese Arbeit RGM-A als neuartigen körpereigenen Hemmstoff der Leukozytenmigration mit dem Zielrezeptor Neogenin. Dies unterstützt die Hypothese von evolutionär konservierten, molekularen Mechanismen der Regulation von Zellbewegung, die Leukozyten wie Neuronen betreffen. Mit der Entdeckung werden nicht zuletzt Behandlungsmöglichkeiten für Krankheiten aufgezeigt, die durch eine überschießende Immunantwort charakterisiert sind.

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