

REVIEW

Endogenous migration modulators as parent compounds for the development of novel cardiovascular and anti-inflammatory drugs

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Development of novel cell migration modulators for anti-inflammatory and cardiovascular therapy is a complex task since any modulator will necessarily interfere with a balanced system of physiological regulators directing proper positioning of diverse immune cell types within the body. Whereas this shall serve efficient pathogen elimination, lack of proper control over these processes may result in counterproductive chronic inflammation and progressive tissue injury instead of healing. Prediction of the therapeutic potential or side effects of any migration modulator is not possible based on theoretical considerations alone but needs to be experimentally evaluated in preclinical disease models and by clinical studies. Here, we briefly summarize basic mechanism of cell migration, and groups of synthetic drugs currently in use for migration modulation. We then discuss one fundamental problem encountered with single-target approaches that arises from the complexity of any inflammation, with multiple interacting and often redundant factors being involved. This issue is likely to arise for any class of therapeutic agent (small molecules, peptides, antibodies, regulatory RNAs) addressing a single gene or protein. Against this background of studies on synthetic migration modulators addressing single targets, we then discuss the potential of endogenous proteins as therapeutic migration modulators, or as parent compounds for the development of mimetic drugs. Regulatory proteins of this type commonly address multiple receptors and signalling pathways and act upon the immune response in a phase-specific manner. Based on recent evidence, we suggest investigation of such endogenous migration modulators as novel starting points for anti-inflammatory and cardiovascular drug development.

Abbreviations

APC, antigen-presenting cell; CCN1, cysteine-rich angiogenic inducer 61; cRGD, cyclic RGD peptide; DCM, dilated cardiomyopathy; DCMi, inflammatory cardiomyopathy; EAM, experimental autoimmune myocarditis; ECM, extracellular matrix; FAK, focal adhesion kinase; mAb, monoclonal antibody; MCP-1, monocyte chemoattractant protein-1; MI, myocardial infarction; MIP-1 α , macrophage inflammatory protein-1 α ; MNC, mononuclear cell; PI3K, phosphoinositide 3-kinase; TSP, thrombospondin; TXA, thromboxane; VSMC, vascular smooth muscle cell

Basic considerations in cell migration modulation

Pharmaceutical companies have targeted a broad spectrum of molecules for the treatment of inflammatory diseases. A number of immune cell migration-modulating drugs were highly efficient in animal models of inflammatory disorders, and some of these were also successful in clinical trials. The search for novel mechanistic principles and approaches in cell migration modulation continues, with increasing attention to the fact that single-target drugs have inherent limitations due to the complexity of inflammatory processes with multiple interacting and often functionally redundant factors being involved (Mackay, 2008).

The development of novel immune cell migration modulators for anti-inflammatory and cardiovascular therapy is a complex task, since they will necessarily interfere with a delicately balanced system of multiple migration regulators that direct proper positioning of diverse immune cell types within the body (Luster *et al.*, 2005). In principle, this shall

serve efficient pathogen elimination and resolution either by background immune surveillance or by non-destructive short-term inflammation (Wolf *et al.*, 2003) (Figure 1). On the other hand, lack of control over these primarily beneficial processes may result in 'counterproductive' chronic inflammation associated with insidious chronic tissue injury, instead of proper healing within a short time period. It should be emphasized that it is impossible to predict, based on theoretical considerations alone, the therapeutic potential or side effects of any new immune cell migration modulator for different disease settings. Instead, its overall effects need to be investigated experimentally in detail (e.g. application route, dosage regime) for every envisaged target disease separately. The necessity to conduct detailed *in vivo* studies addressing, for example drug dose issues, is illustrated by the paradoxical stimulation of malignant tumour growth by low concentrations of a RGD-mimetic integrin inhibitor primarily developed as an anti-tumour agent (Reynolds *et al.*, 2009). Nevertheless, the identification of basic *in vitro* effects of a new agent may serve as a guideline for the design of *in vivo* experiments in disease models, that is which cell

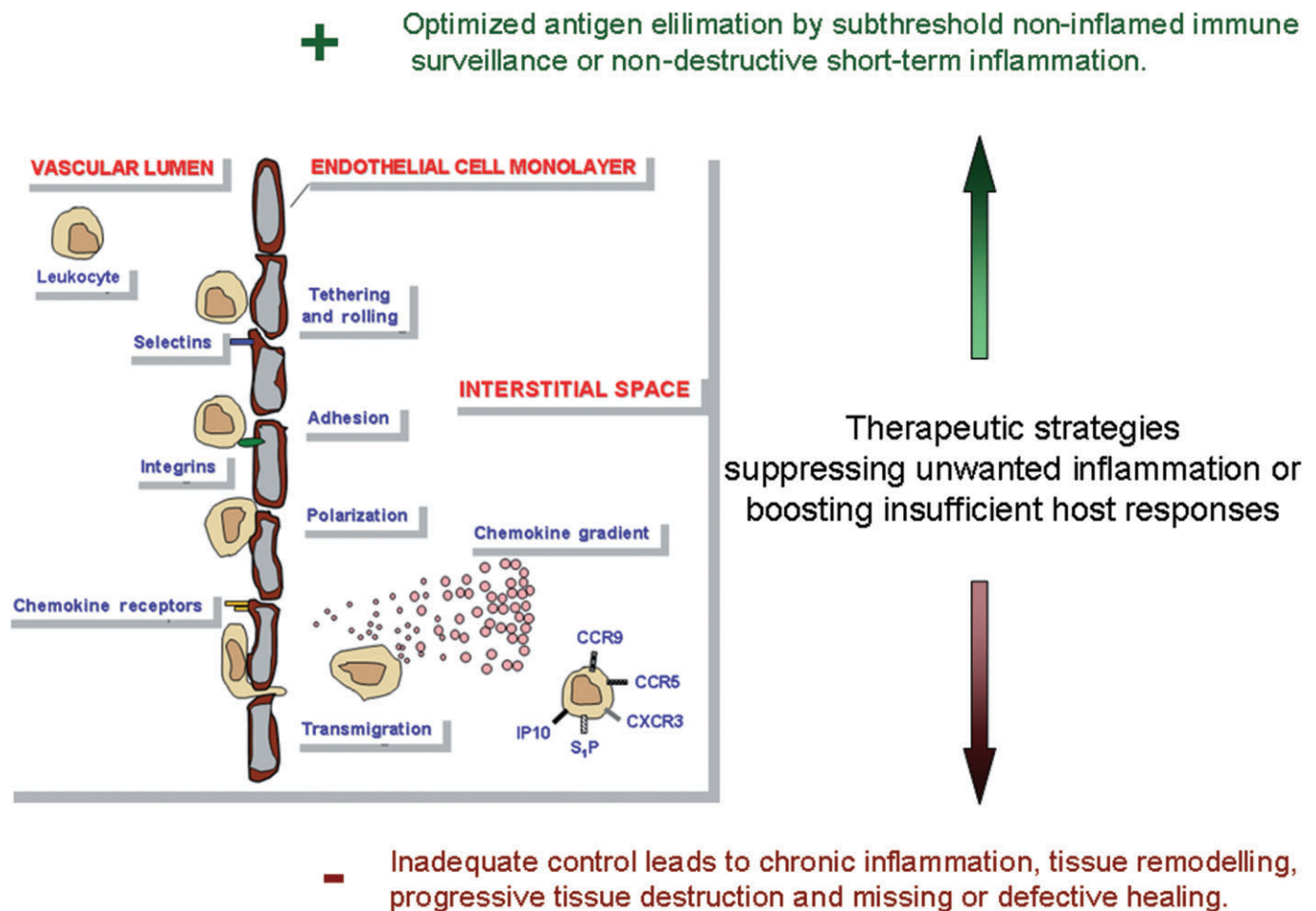


Figure 1

Immune cell migration and its therapeutic modulation: Addressing a delicate balance between efficient pathogen elimination with non-destructive and short-term inflammation versus chronic inflammation associated with progressive and often insidious tissue injury.

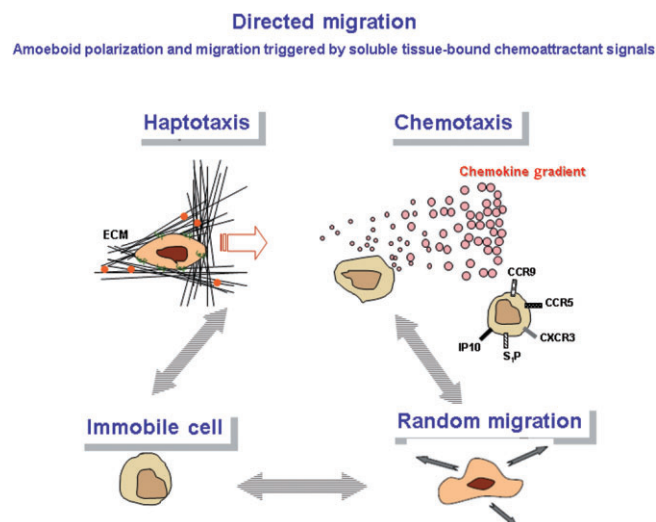


Figure 2

Directional motility: Haptotaxis is the directional movement along a gradient of cellular adhesion sites or substrate-bound chemoattractants as present in the extracellular matrix. Chemotaxis is the cell movement following a gradient of soluble molecules such as chemokines.

functions should to be measured during treatment and which side effects might be anticipated.

Molecular mechanisms of cell migration

Our knowledge about the fundamental molecular mechanism of cell migration (Figure 2) and their regulatory factors has been greatly expanded by numerous groundbreaking studies (Schulz *et al.*, 2009; Mempel *et al.*, 2006; Alvarez *et al.*, 2008; Laird *et al.*, 2008; Mora and von Andrian, 2008) and novel analytical methods (Wolf *et al.*, 2003; Sumen *et al.*, 2004; Halin *et al.*, 2005; Wolf *et al.*, 2009) during the last decade. This includes the molecular mechanisms of amoeboid migration of leucocytes including intracellular polarization processes, influences from the microenvironment of the migrating cells and the balance between immigration and egress (Matloubian *et al.*, 2004) versus local confinement and residency. Live-cell microscopy has refined the conception of immune processes, from abstract models of leucocyte trafficking, into a more immediate understanding of how and in which sequence immune cells interact with each other, and eliminate pathogens or repair tissues. We only briefly summarize basic cell migration mechanisms since excellent reviews on this topic are available (Friedl and Weigelin, 2008; Wong *et al.*, 2010).

Migration modulators may be roughly classified according to a partial process that is strongly affected (Figure 3), but any modulator may have different biological effects in immune cell subtypes, and the overall effect therefore needs to be determined in relevant disease models *in vivo*. Com-

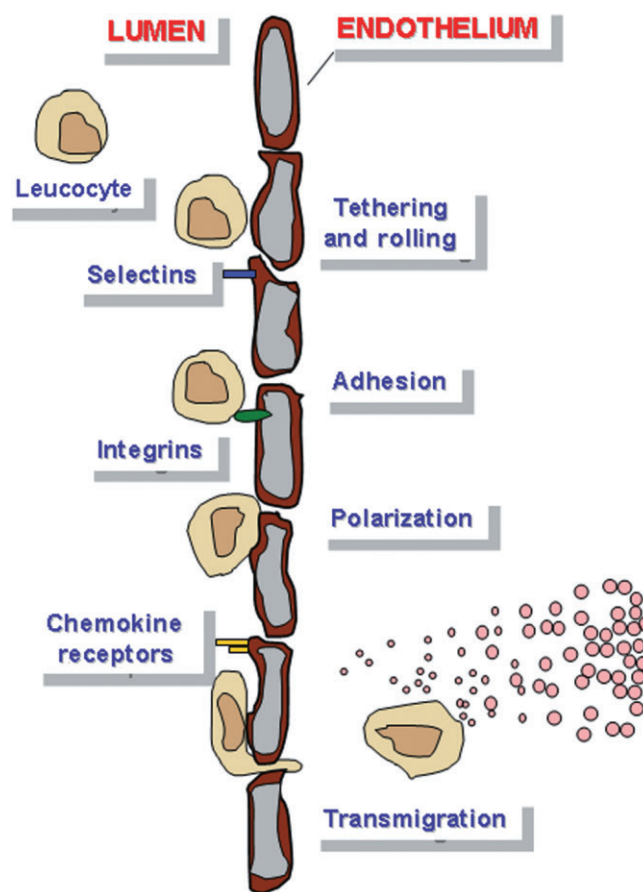


Figure 3

Checkpoints to target migration: Molecular targets of drugs in clinical development include selectins (TBC1269, *Revotar* by Texas Biotechnology), integrins (*Eftaluzimab* by MerckSerono, *Natalizumab* by Elan and Biogen Idec, *Cilengitide* by Merck) and chemokine receptors (INCB3284 by Incyte, MK0812 by Merck, CCX282 by ChemoCentryx, *Maraviroc* by Pfizer, TAK-779 by Takeda, *Fingolimod* by Novartis, MDX-1100 by Medarex).

monly, the effect of a new migration modulator is characterized for individual leucocyte subclasses only. Because of their important role in mediating inflammatory reactions, interstitial cell migration offers interesting therapeutic targets, both for the attenuation of uncontrolled inflammation or the enhancement of inadequate host immune responses. Although a number of fundamental molecular mechanisms of cell migration are active in any type of cells and tissues, beyond this common basis, there are significant differences in the trafficking behaviour of leucocytes to and from distinct organs. The molecular foundations for these differences are incompletely understood. For obvious reasons, it would be preferable, however, to have tissue-specific cell migration inhibitors (Marsolais and Rosen, 2009) targeting the diseased organ only, instead of modulating migration throughout the body. An example for tissue-specific migration inhibition in late-stage clinical evaluation for Crohn's disease is the CCR9 inhibitor CCX282 (Mackay, 2008).

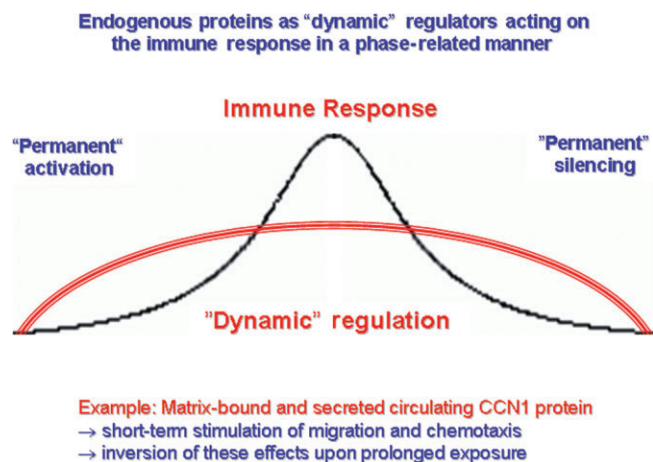


Figure 4

A coordinated immune response encompasses both activating and phase-related counterbalancing mechanisms. The latter are essential for proper responses to injury and include (i) down-regulation of chemokine receptors and desensitization of receptor-dependent signalling, (ii) termination of chemoattractant activity by neutralizing receptors and proteolytic degradation and (iii) chemoattractant receptor internalization and recycling or storage in vesicles.

'Dynamic' regulators acting in an immune response phase-related manner

Multiple new inducers of cell migration have been identified, but counterbalancing mechanisms (Friedl and Weigelin, 2008; Steevens and Meyaard, 2011; Steevens *et al.*, 2011) have also been intensely studied, including down-regulation or desensitization of chemokine receptors, ligand competition, termination of chemoattractant activity through capture by neutralizing chemoattractant receptors and proteolytic degradation. Intracellularly, chemoattractant receptors are internalized, recycled to the leading edge or stored in vesicles, thus controlling the availability of both the chemoattractant and its receptor. Activation-induced down-regulation of the S1P1 receptor in T cells is another well-known example of chemoattractant signal tuning and was successfully targeted by drugs in multiple sclerosis (Kappos *et al.*, 2006; Carroll, 2011; Cohen *et al.*, 2011), organ transplantation (Habicht *et al.*, 2006; Brinkmann, 2007; Lan *et al.*, 2008) and allergic diseases (Marsolais *et al.*, 2011).

Whereas many drugs have permanent silencing effects (e.g. conventional immunosuppressive drugs), this is not a common mode of action of endogenous regulators of the immune response, which instead act in a phase-related manner (Figure 4). This type of physiological regulators is commonly integrated into regulatory networks that have evolved to coordinate a sequence of processes towards optimal repair of injuries. Numerous cell migration and differentiation-regulating proteins are active during fetal development, become almost completely silenced in the healthy adult organism, but are typically re-induced in injured tissues. This type of re-induction of developmental

proteins (Fechner *et al.*, 2003; Perbal, 2004; Kubota and Takigawa, 2007; Hamilton, 2008; Llera *et al.*, 2010), and also of fetal microRNA (miR) patterns (Thum *et al.*, 2007), is often remarkably monomorphic irrespective of the specific type of injury or organ involved. Many typical examples are found in the large family of matricellular proteins (Vilmos *et al.*, 2001), commonly characterized by re-expression during tissue injury and repair (Schellings *et al.*, 2004; 2009; Leask and Abraham, 2006; Okamoto, 2007; Chen and Lau, 2009; Kyriakides and Maclauchlan, 2009; Norris *et al.*, 2009; Chiodoni *et al.*, 2011; Dobaczewski *et al.*, 2011) and by multiple interactions with immune cells (Kuznetsova and Roberts, 2004; Frangogiannis, 2008; Sangaletti and Colombo, 2008). As opposed to dedicated chemokine–chemokine receptor or other specific ligand–receptor pairs, proteins of this type are commonly multimodular in structure and exert complex, context-dependent functions through multiple interacting proteins and signalling pathways. A following chapter on *Endogenous Migration-Inhibiting Molecules as Parent Compounds for Drug Development* discusses how a matricellular protein of this type, and a mimetic peptide, may have therapeutic potential in cardiovascular and other diseases associated with pathogenic inflammation. Whereas that chapter focuses on CCN1 as one paradigm for endogenous immune cell migration modulators to be considered as parent compounds for drug development, the full spectrum of proteins that dynamically and physiologically regulate immune responses is unknown. Remarkably, context dependency of endogenous immunomodulating proteins (such as the CCN protein family) has also been observed for adipocytokines (leptin, adiponectin and others). For a full discussion of their properties, we refer to excellent recent reviews (Lago *et al.*, 2007; 2009; Lang and Ratke, 2009) and papers investigating the complex role of adiponectin in dilated (DCM) and inflammatory (DCMi) cardiomyopathy (Wittchen *et al.*, 2007; Skurk *et al.*, 2008; Bobbert *et al.*, 2011). Briefly, leptin and adiponectin are involved in the regulation of migration of both leucocytes and tumour cells.

Problems arising from biological redundancy and limited knowledge

Beyond specific issues relating to individual drugs, one general consideration relates to the fact that single-target approaches may be problematic due to the multitude of interacting and redundant factors involved in inflammatory processes. Any acute or chronic inflammation in the tissue in response to mechanical microbial injury, autoimmune disease or allograft rejection triggers tissue infiltration by effector cells: neutrophils, monocytes, T cells and in chronic states also B cells. This creates a highly complex network of interacting cells and signalling cascades that involve far more players than can be addressed by targeting of a single molecule. Serious problems of limited or lacking efficacy of single target drugs have indeed been encountered in multiple pre-clinical or clinical trials (Horuk, 2009a,b). It appears that multiple interactions and redundancy are commonplace in the biological systems regulating and balancing proper immune cell migration. It should be emphasized that the

resulting limitation of narrowly targeted agents is likely to apply to any class of therapeutic agent (small molecule drugs, peptides, monoclonal antibodies, regulatory RNAs), unless a single unique process of outstanding importance can be identified.

The existence of unidentified subsets of immunological target cells may also confound the conclusions drawn from narrowly defined *in vitro* studies. One example are the monocytic cells that consist of 'inflammatory' and 'resident' subsets with differential functions and trafficking properties (Kamei and Carman, 2010). Notably, the spleen has recently been identified as a peculiar reservoir of 'inflammatory' monocytes that are readily recruited to injured myocardium and other tissues. In general, the complex architecture of the interstitial space and the full spectrum of phenotypic and functional changes of leucocytes resulting from their interactions with the endothelium during adhesion and transmigration cannot be modelled by any current *in vitro* system (Wong *et al.*, 2010). Moreover, a single chemoattractant is frequently used *in vitro*, whereas multiple chemoattractants are regularly involved *in vivo*, which act upon intracellular signalling cascades in a hierarchical manner.

Focusing on chemokine receptors, Horuk has recently discussed possible reasons for the failures of multiple trials using single-chemokine receptor antagonists. He suggests that they may be attributable to trying to target a complex disease with an antagonist to a single receptor, whereas the more than 40 different chemokines and 19 chemokine receptors so far identified create an immensely complex immunoregulatory network. As an alternative approach, he suggests promiscuous non-peptide antagonists inhibiting multiple targets (Horuk, 2009a,b).

Current drug classes and clinical relevance in cardiac disease

Current single-target pharmacological approaches (Figure 3) include selectin inhibition (Ley *et al.*, 2007; Barreiro *et al.*, 2011; Fernandez-Borja *et al.*, 2011), integrin targeting (Hehlhans *et al.*, 2007) and chemokine or chemokine receptor blockade (Horuk, 2009a,b; Zernecke and Weber, 2011), which aim at cell surface targets involved in leucocyte rolling, adhesion and transendothelial migration. One important example for successful therapeutic integrin targeting is a monoclonal antibody (mAb) to $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrin for the treatment of multiple sclerosis and inflammatory bowel disease. The results of two phase 3 clinical trials showed that natalizumab markedly reduces the number of relapses in individuals with multiple sclerosis (Polman *et al.*, 2006; Havrdova *et al.*, 2009; Hutchinson *et al.*, 2009).

In preclinical models in the context of cardiac diseases, the usage of monoclonal antibodies against integrins $\beta 2$ and $\alpha 4$, either alone or in combination, showed a blockade of inflammatory cell migration into the ischaemic myocardium after myocardial infarction (MI) (Legare *et al.*, 2007). Cai *et al.* investigated the role of T-cell selectin ligands on cardiac recruitment of CD8⁺ T cells during myocarditis and allograft rejection. Here, selectin ligand-deficient CD8⁺ T cells showed a reduction in their ability to interact with P- and E-selectins

and a blockade of heart-directed migration (Cai *et al.*, 2006). One of the earliest steps of an acute inflammatory response is the selectin-dependent rolling of leucocytes. Hicks *et al.* (2003) suggested recombinant P-selectin glycoprotein ligand-1-immunoglobulin (rPSGL-Ig) as characteristic ligand to influence leucocyte rolling in living blood vessels for an inhibition of neutrophil migration. Baron *et al.* further compared the effect of the chimeric antibody c7E3 Fab (abciximab) with the antibody LM609, which is directed specifically against integrin $\alpha v\beta 3$, and observed comparable results in the prevention of smooth muscle cell adhesion to the extracellular matrix (ECM) proteins osteopontin and vitronectin, and of cell migration during the development of restenosis. Overall, combined administration of both antibodies represented the most effective treatment (Baron *et al.*, 2000). Furthermore, the fibrin-derived peptide B β 15-42 (FX06) was shown by Wiedemann *et al.* (2011) to reduce infarct size in a coronary artery occlusion/reperfusion model by inhibition of leucocyte migration and preservation of endothelial barrier function.

Intracellular migration-related signalling pathways have been directly addressed by inhibition of specific phosphoinositide-3-kinase (PI3K) isoforms (Barber *et al.*, 2005; Camps *et al.*, 2005), and significant progress has recently been made in understanding their differential functions with respect to cell migration. Pharmacological inhibition of PI3K- γ by synthetic small molecules has promoted infarct resorption and prevented adverse cardiac remodelling after MI in mice (Seropian *et al.*, 2010). Loss of PI3K- γ has enhanced cAMP-dependent MMP remodelling of the myocardial N-cadherin adhesion complex and the ECM in response to biomechanical stress (Guo *et al.*, 2011). At the molecular level, the interplay between class I PI3Ks and Rac signalling in phagocytic functions has been dissected (Costa *et al.*, 2011), and negative feedback regulation of Rac in leucocytes from mice expressing a constitutively active PI3K- γ has been demonstrated (Costa *et al.*, 2007). Leucocyte transmigration is also modulated by chemokine-mediated PI3K- γ -dependent phosphorylation of vimentin (Barberis *et al.*, 2009). Signalling through PI3K- γ has functional relevance beyond immune cell migration, since it appears to be one common platform for leucocyte, platelet and cardiovascular stress sensing (Hirsch *et al.*, 2006). For an overview on the role of PI3Ks in cardiovascular diseases and current PI3K-targeting drugs, see Eisenreich and Rauch (2011).

A therapeutic approach against autoimmune myocarditis was further suggested by Goser *et al.* (2005) who demonstrated that blockade of the chemokines MCP-1 or macrophage inflammatory protein-1 α (MIP-1 α) with monoclonal antibodies attenuates the pathogenesis of experimental autoimmune myocarditis (EAM), by inhibiting mononuclear cell (MNC) migration via the receptors CCR2 and CCR5.

Other preclinical studies have investigated drugs with migration-inhibiting properties. PPAR- γ ligands inhibit monocyte chemotactic protein-1 (MCP-1)-directed migration of monocytes (Kintscher *et al.*, 2000), endothelial cells (Goetze *et al.*, 2002) and vascular smooth muscle cells (VSMCs) (Goetze *et al.*, 2001). In a chronic cardiac transplant rejection model, Ogawa *et al.* showed that clarithromycin, a macrolide antibiotic involved in MMP regulation, suppressed the development of graft arterial disease and myocardial remodelling. This treatment led to inhibition of MMP-9 and

suppression of smooth muscle cell migration and proliferation (Ogawa *et al.*, 2008). Positive effects on myocardial infarct size following left coronary ligation were observed by Nichols *et al.* after treatment of animals with the thromboxane A2 (TXA2) synthetase inhibitor U-63557A, and the TXA2 receptor antagonist SQ-29.548. Reduced neutrophil accumulation in the infarcted zone, together with a decrease in myocardial myeloperoxidase activity as a specific marker of neutrophil infiltration, and blockade on f-MLP-directed chemotaxis *in vitro* marked the protective effects in this study (Nichols *et al.*, 1989).

A different therapeutic strategy is exemplified by the drug fingolimod (FTY-720) (Chiba *et al.*, 1999), a receptor modulator that mimics the serum component sphingosine-1-phosphate (S1P) and acts as an agonist for four of the five members of the S1P family of GPCRs. The physiological role of S1P receptors on lymphocytes is control of their exit from lymphoid tissues (Schwab and Cyster, 2007), which is tightly regulated. Stimuli such as antigen challenge can block lymphocyte egress from lymph nodes. FTY-720 thus sequesters lymphocytes in lymphoid organs, prevents their migration to sites of inflammation and limits T-cell access to organ grafts and autoimmune lesions. In contrast to the synthetic selectin, integrin, chemokine or chemokine receptor blockers that *antagonize* endogenous migration activators, S1P-mimetic drugs mimic the action of an endogenous molecule that physiologically *limits* immune cell migration. In rodent models, S1P administration or FTY720 treatment were cardioprotective against ischaemia (Jin *et al.*, 2002; Zhang *et al.*, 2007; Karliner, 2009), improved recovery during reperfusion and reduced infarct size after coronary artery ligation (Hofmann *et al.*, 2009; 2011). In clinical phase 2 and 3 trials of patients with multiple sclerosis, FTY-720 significantly reduced clinical relapse rates and infiltration of autoreactive lymphocytes into the CNS (Kappos *et al.*, 2006). Side effects included an enhanced risk for respiratory tract infections and a reduction of total circulating lymphocytes.

Another example for endogenous *limitation* of migratory processes was found in the suppressors of cytokine signalling (SOCS). The eosinophil chemoattractant CCL11 interacts with CCR3, a chemokine receptor expressed by multiple cell types including macrophages (Menzies-Gow *et al.*, 2002), resulting in SOCS induction and thereby blunted response to proinflammatory cytokines and microbial products (Yoshimura *et al.*, 2007). SOCS controls signalling pathways downstream of integrins, for example, inhibiting focal adhesion kinase (FAK) that is indispensable for cell migration (Liu *et al.*, 2003). Stevenson *et al.* (2011) showed that SOCS1 and 3 enhanced cell adhesion but strongly inhibited migration towards CCL11, and that inhibition of SOCS1 and 3 signalling pathway via FAK and RhoA blocked of immune cell infiltration to the site of allergic inflammation.

Endogenous migration-inhibiting molecules as parent compounds for drug development

Therapeutic tools employed for migration modulation by the various principles outlined above mainly comprise synthetic

compounds: traditional small molecule drugs (Barber *et al.*, 2005) (Camps *et al.*, 2005), synthetic peptides (Rother *et al.*, 2010; Jahns *et al.*, 2011), monoclonal antibodies (Goser *et al.*, 2005; Chan and Carter, 2011; Lee *et al.*, 2011; Weiner *et al.*, 2011) or bispecific protein-monoclonal antibody recombinant molecules (Langer *et al.*, 2011). As possible future tools, we discuss here the possible use of endogenous proteins with inflammation-modulating, cell migration and chemotaxis blocking, or immune signalling properties, as parent compounds for the development of drugs mimicking effects of the endogenous protein or its subdomains. Such therapeutics comprised of, or mimicking, naturally occurring and often evolutionary ancient proteins are one approach towards the exploitation of biological resources. Of note, a number of biological systems such as innate immunity, or miRs with their dependent genes, may be considered as master regulators of fundamental biological processes including tissue repair and regeneration. Not surprisingly, such systems are often conserved during evolution back to ancient species, as illustrated impressively by phylogenetic studies of innate immunity components (Hoffmann *et al.*, 1999; Christophides *et al.*, 2002; Rast *et al.*, 2006; Haine *et al.*, 2008; Lee *et al.*, 2011).

Against the background of more conventional drug discovery strategies, we illustrate this strategy using recent data from our group on a protein from the CCN family (Perbal, 2004; Rachfal and Brigstock, 2005; Kubota and Takigawa, 2007). The starting point for our study (Rother *et al.*, 2010) was the fact that the CCN1 protein is an evolutionary highly conserved matricellular protein that modulates biological processes associated with tissue repair. Recently, we found first evidence of a novel function of CCN1 as a novel modulator of immune cell migration, with therapeutic potential in diseases associated with chronic pathogenic inflammation. In a proof-of-concept study, we used CCN1 gene transfer to evaluate its therapeutic potential in animal models of human inflammatory cardiomyopathy and of MI. CCN1 therapy significantly reduced immune cell infiltration in both models. Figure 5 shows data for the cardiomyopathy model and mechanistic studies demonstrating that the CCN1 treatment resulted in strongly suppressed random migration of immune cells both *in vivo* and *in vitro*, and in abrogation of their chemotactic response to various chemokines.

These data suggest that CCN1 has potential as a new broad-spectrum immune cell migration inhibitor, in contrast to specific chemokine- or chemokine receptor-blocking agents with their known limitations arising from the fact that in most inflammatory diseases, multiple chemokines and chemokine receptors are involved, and no single target of outstanding pathogenic importance exist (see above). From a clinical translational perspective, it was of particular interest that the effects of the endogenous protein CCN1 on immune cell chemotaxis and migration were partially mimicked by cyclic RGD (cRGD) peptides that are currently being evaluated in clinical trials, although as yet for cancer therapy only (Figure 6). Our proof-of-concept study therefore suggests further investigation of CCN1 as a new parent compound for immune cell migration modulation and of cRGD peptides as partial CCN1 mimetics with immediate potential for clinical evaluation in cardiac diseases associated with chronic pathogenic inflammation. At the same time, this study describes a

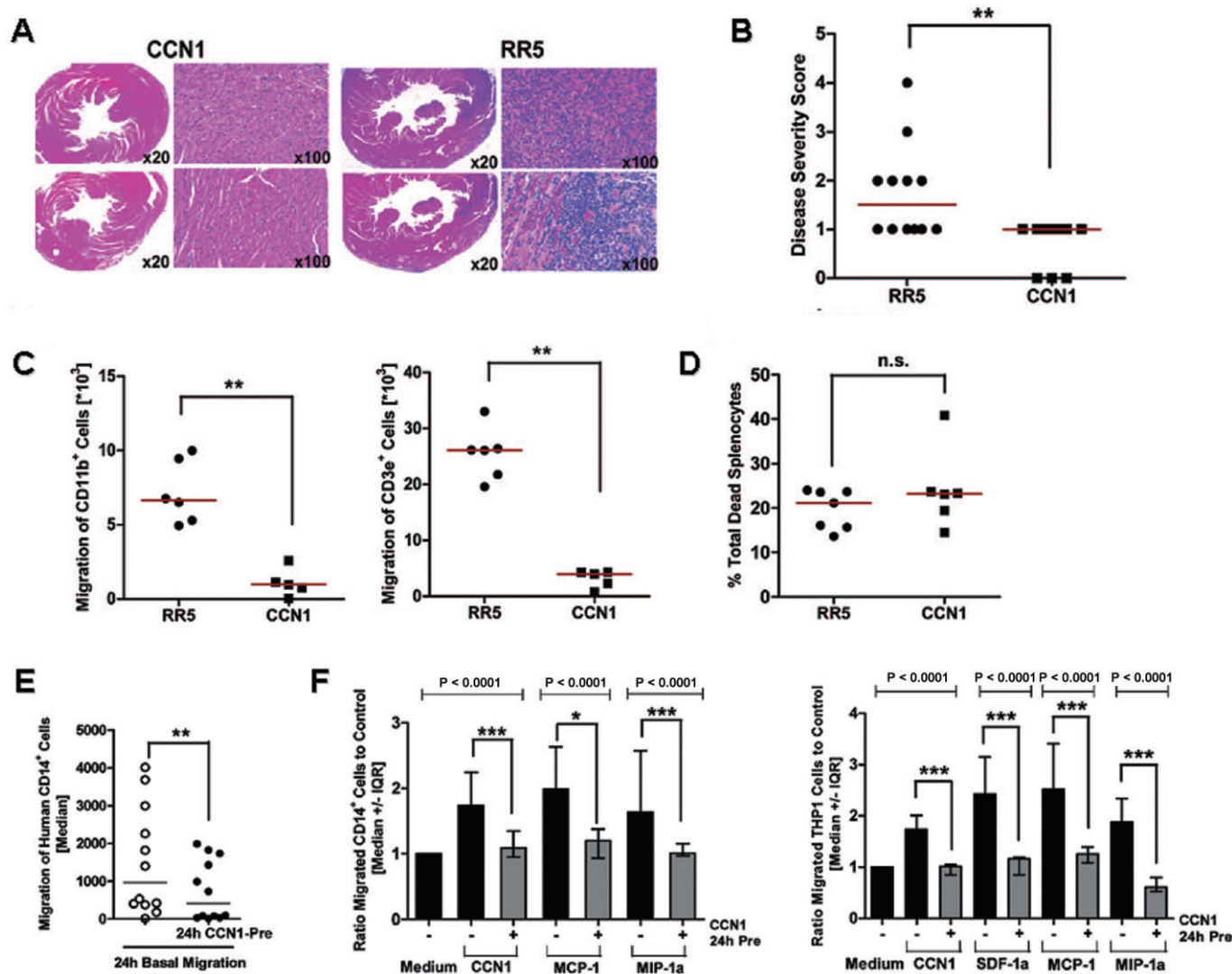


Figure 5

Systemic CCN1 therapy attenuates murine autoimmune cardiomyopathy (Rother *et al.* 2010) (reproduced by permission from Circulation). (A) Treatment with a CCN1 gene transfer vector (AdV-CCN1) significantly reduced cardiac immune cell infiltration as assessed by histological analysis of mouse hearts after 3 weeks, in comparison with RR5 control vector-treated animals. (B) The cardiac disease score was significantly reduced by this treatment. (C) Migration assays of splenocytes isolated from mice at peak inflammation showed significantly reduced *ex vivo* migration of CD11b⁺ macrophages (left) and CD3e⁺ T cells (right) from AdV-CCN1-treated mice compared with controls. (D) No difference in cell viability was detected. (E) Mechanistic studies of CCN1 effects *in vitro* showed a significantly reduced basal migration rate of human CD14⁺ monocytes. (F) Abrogation of the chemotactic response to chemokines (SDF-1 α , MCP-1, MIP-1 α) important in the pathogenesis of diverse cardiovascular and inflammatory diseases. SDF-1 α , stromal cell-derived factor-1 α ; MIP-1 α .

novel migration-inhibiting effect for cRGD peptides, which should be relevant for both anti-cancer and anti-inflammatory treatment.

As for most other migration modulating drugs, a full elucidation of the mechanisms by which CCN1 and cRGD peptide modulates immune cell migration at the molecular level has not yet been achieved. However, current data indicate that both random migration and directed migration along a chemotactic gradient are affected by these agents. We do not yet know if there are differential effects of CCN1 or cRGD peptide on the migration of immune cell subpopula-

tions *in vivo*, if a T-cell response is generated in draining lymph nodes and if antigen-presenting cells (APCs) migrate there normally during these treatments. Differential effects of CCN1 and cRGD dose and timing with respect to disease course need to be evaluated further in the future. Despite these limitations of our current knowledge, however, CCN1 is likely to expand the spectrum of tools for chemotaxis modulation and offer new therapeutic perspectives for cardiovascular and autoimmune disorders. Beyond this proof-of-principle study using recombinant protein and a mimetic peptide, it certainly is necessary to better understand the

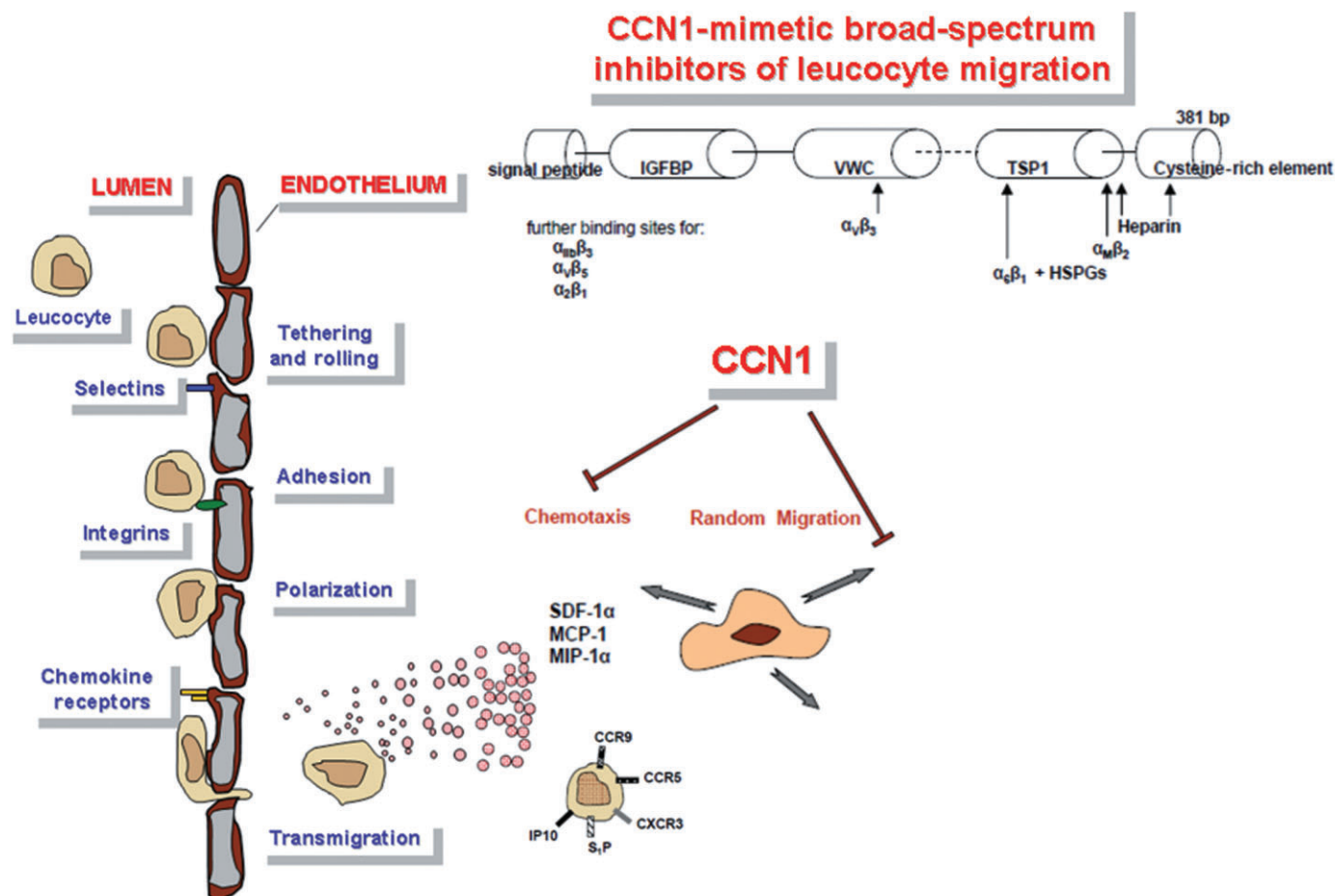


Figure 6

Migration modulation by CCN1: Within the context of other migration-modulating substances, the endogenous protein CCN1 showed significant inhibition of the random migration and chemotaxis of immune cells *in vitro* and *in vivo*. The complex multidomain protein CCN1 does not only interact with a broad spectrum of integrins including $\alpha_6\beta_1$ (Chen *et al.*, 2000), $\alpha_v\beta_3$ (Kireeva *et al.*, 1998), $\alpha_v\beta_5$ (Monnier *et al.*, 2008), $\alpha_M\beta_2$ (Schober *et al.*, 2002), $\alpha_2\beta_1$ (Lin *et al.*, 2007) and $\alpha_{IIb}\beta_3$ (Jedsadayanmata *et al.*, 1999), but also with heparansulphate proteoglycans (HSPGs) (Chen *et al.*, 2000). Nevertheless, immune cell preincubation with a structurally simple cRGD peptide binding selectively to α_v type integrins only inhibited their chemotactic response in a similar way as CCN1 preincubation. Obviously, CCN1 effects on immune cells are in part mediated via integrins, and cRGD peptides may be evaluated as a first class of CCN1-mimetic drugs with immediate potential for clinical evaluation. CCN1 treatment certainly exerts more complex effects on inflammatory processes *in vivo* than cRGD peptides by way of the huge spectrum of integrin heterodimers that can be addressed by CCN1 on multiple cell types, but partial functions of therapeutic use may be extracted by further dissection of the proteins domain and subdomain functions. Regarding most matricellular proteins, our knowledge in this respect is only fragmentary.

metabolism of CCN1 (proteolytic cleavage, products with different biological functions) before a systematic search for the most useful mimetic drugs can be finalized. Interestingly, another group has arrived at a similar general strategy for drug development starting from thrombospondin-1 (TSP-1), another endogenous protein inhibiting angiogenesis (Colombo *et al.*, 2011; Taraboletti *et al.*, 2011). It is quite remarkable that this 'parent compound' TSP-1 is also a matricellular protein that is re-induced in the adult organism by tissue injury, similar to CCN1 with which it interacts.

Therapeutic interest in CCN1 was initially triggered by genomics studies in humans, which resembles the path of other investigations that have used genomic approaches to drive novel compound pipelines. Before any anti-

inflammatory approach can be undertaken, it is crucial to delineate the distinction between acute inflammation supporting tissue repair and regeneration, as opposed to chronic inflammation without reparative advantage but instead inducing progressive tissue injury. With respect to the latter type of inflammation, further investigation of CCN1 as a new parent compound for immune cell migration modulation appears warranted, as well as of cRGD peptides as a first class of partially CCN1-mimetic drugs with immediate potential for clinical evaluation in cardiac disorders associated with chronic pathogenic inflammation. Cardiac transplant rejection and transplant injury in other organ transplants appear as particularly interesting disease targets because in these cases preconditioning of the host by CCN1 or cRGD treat-

ment would be feasible, allowing modulation of the host before its immune response is initiated. Experimental investigations in transplant models (Nykänen *et al.*, 2010) are currently in progress.

microRNAs as novel therapeutic tools and targets

In addition to innate immunity proteins and other ancient proteins involved in tissue repair and regeneration, with the regulatory (non-coding) RNA molecules, a completely new class of biological regulators has been discovered. These RNAs are distinct from the protein-coding messenger RNAs (mRNAs) and other RNAs essential for protein biosynthesis (ribosomal rRNAs, transfer tRNAs) and have long been neglected as dysfunctional molecules. The currently most intensely studied class of regulatory RNAs are miRs, which are capable of regulating complex gene networks, as well as short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs), which mediate gene silencing by the process of RNA interference (Poller and Fechner, 2010; Poller *et al.*, 2010). Both classes, in particular shRNAs, have already been successfully employed as novel therapeutic tools in diverse diseases including cardiovascular (Suckau *et al.*, 2009) and malignant disorders.

It has become evident that naturally occurring miRs are involved in the physiological regulation of the innate (Davidson-Moncada *et al.*, 2011) and adaptive immune system (Baltimore *et al.*, 2008; Carissimi *et al.*, 2009; Sonkoly and Pivarski, 2009; Xiao and Rajewsky, 2009; O'Connell *et al.*, 2011), and are deregulated in various diseases including autoimmune disorders (Pauley *et al.*, 2009; Tang *et al.*, 2009; Fulci *et al.*, 2011; Furer *et al.*, 2011; Liston *et al.*, 2010), antiviral immunity (Cullen, 2006), organ transplant rejection (Harris *et al.*, 2010) and atherosclerosis (Weber *et al.*, 2010). Specifically, miRs are directly involved in immune cell migration (Pucci *et al.*, 2009), function (Kohlhaas *et al.*, 2009; Wei and Pei, 2010) and differentiation, (Schmeier *et al.*, 2009), or mediate the effects of cell signalling molecules such as AKT1 (Androulidaki *et al.*, 2009).

The importance of miRs in the immune response has also been demonstrated in studies that employed miR modulations to induce antigen-specific regulatory T cells (Tregs) and promote immunologic tolerance (Annoni *et al.*, 2009), or to suppress the development of allergic airways disease (Mattes *et al.*, 2009). Selective miR ablation in Treg cells has resulted in uncontrolled autoimmunity (Zhou *et al.*, 2008), and inactivation of the miR-processing enzyme Dicer has disrupted invariant NKT cell development.

With respect to the regulation of cell migration in general, it has recently been discovered that miRs are not only synthesized and processed within individual cells, but that for certain miRs, there is active secretion *via* packaging into microvesicles (MVs) (Hunter *et al.*, 2008; Yuan *et al.*, 2009; Ogawa *et al.*, 2011; Wang *et al.*, 2011; Zhang *et al.*, 2011), and that such vesicles can enter other cells and deliver miR-150 into, for example, endothelial cells where they then promote cell migration (Zhang *et al.*, 2011). Based on these quite unexpected recent discoveries, microvesicle-encapsulated miR mimetics (Poller and Fechner, 2010; Poller *et al.*, 2010) may also be considered as possible migration modulators.

Delivery systems for novel migration modulators

The above discussed novel migration modulators could be delivered as recombinant proteins as long as no mimetic small molecule drugs or peptides are available, or by gene transfer using vectors that continuously produce and deliver a therapeutic protein into the circulation. Use of a gene vector as an inexpensive 'protein factory' with the additional advantage that all posttranslational modifications are exerted by the host itself and thus in the most appropriate way is a rather old idea (Kay *et al.*, 1993; 1994) first promoted in the field of haemophilia with its need for repetitive infusion of expensive blood-derived (and thus infection-prone) coagulation factors or recombinant proteins. Gene therapy has been significantly advanced by haemophilia researchers and with the employment of adeno-associated virus (AAV)-based vectors (Nathwani *et al.*, 2007; Nathwani *et al.*, 2011), an efficient system for long-term production of therapeutic proteins in liver (Mount *et al.*, 2002; Niemeyer *et al.*, 2009; Sabatino *et al.*, 2011) or skeletal muscle (Arruda *et al.*, 2011; Haurigot *et al.*, 2011), has become available. Whereas most studies to date have been performed in rodents and non-human primates, clinical investigations have also been performed in humans (Arruda *et al.*, 2001; Jiang *et al.*, 2006) where vector dose reduction by use of advanced vector systems has been identified as a key determinant of long-term stability (Herzog *et al.*, 2002; Grimm *et al.*, 2008; Zhong *et al.*, 2008). Given the fact that mimetic drugs are not yet available for numerous proteins of high clinical interest, the gene transfer option deserves continued attention.

When it comes to long-term therapies based on regulatory RNA molecules including RNAi-mediating sequences, efficient production and delivery is highly dependent upon gene transfer technology, due to the instability of these novel therapeutic agents in blood and tissues. For long-term treatments, they are either repetitively delivered in short time intervals, or continuously produced from tissue-targeted and long-term stable vectors. As a major breakthrough in the vector field has been achieved with the introduction of AAV vector systems (Muller *et al.*, 2003; Waterkamp *et al.*, 2006; Li *et al.*, 2008; Schnepf *et al.*, 2009; McPhee and Samulski, 2009), in particular the discovery of highly cardiotropic AAV serotypes (Inagaki *et al.*, 2006; Pačák *et al.*, 2006; Gray and Samulski, 2008). This has not only led to successful gene therapy (Sakata *et al.*, 2007a,b; Muller *et al.*, 2008; Raake *et al.*, 2008; Goehring *et al.*, 2009; Rengo *et al.*, 2009) and recently also to the first successful regulatory RNA therapy of a cardiac disease (Suckau *et al.*, 2009) *in vivo*, by direct i.v. vector injection in animal models but is already in the status of clinical translational phase I and II trials (Jaski *et al.*, 2009; Jessup *et al.*, 2011). If perfect physical vector targeting to the diseased tissue cannot be achieved (transductional targeting), additional transcriptional confinement of the transgene may be achieved by using cardiac-specific promoters (Muller *et al.*, 2006). A number of experimental papers on therapeutic cell migration modulation has successfully employed this delivery mode, for example gene transfer for IL-10 or chemokine MCP-1-7ND in autoimmune myocarditis (Goser *et al.*, 2005; Kaya *et al.*, 2011).

An issue of paramount importance before clinical translation of these experimental strategies is the safety of the delivery tools, which is high for AAV vectors and AAV-derived biological nanoparticles (BNPs), as well as an option to shut down the transgene in the case of serious adverse effects. In summary, current vector systems offer a realistic perspective to serve as relatively simple 'drug factories' for the delivery of biologicals such as proteins or RNA drugs in humans. Regulated delivery based on vector systems is still under development and has not yet reached the stage for clinical translation.

Side effects – general and specific

Any novel immune cell migration modulator for anti-inflammatory and cardiovascular therapy will inevitably interfere with a carefully balanced system of multiple migration regulators directing proper positioning of immune cells within the body. This intervention shall not, however, significantly impair pathogen elimination by background immune surveillance, thus provoking infectious complications. Due to the extraordinary complexity of the immune defence and our still limited knowledge of its regulation, it is impossible to predict the side effects of any new immune cell migration modulator by theoretical reasoning, but this needs to be investigated experimentally for each target disease. The need to conduct detailed *in vivo* studies on optimal drug doses and other issues is well exemplified by the stimulation of malignant tumour growth by low concentrations of a RGD-mimetic integrin inhibitor actually developed and clinically employed as an anti-tumour agent (Reynolds *et al.*, 2009).

Conclusions

Because cardiovascular and immunological diseases that are not immediately fatal will be targeted by cell migration modulators as discussed above, issues of safety will be of paramount importance. Since leucocyte migration is an integral to immune surveillance and molecular recognition, including APC and target cell search and scanning of receptors expressed at cell surfaces, avoidance of unspecific chronic immunosuppression requires careful drug dose selection. Furthermore, cell migration targeting should ideally address specific leucocyte subsets and achieve functional effects related to immune response phase, possibly complementing interference with cell cycle progression (cytostatic drugs). In this regard, we have discussed a fundamental problem encountered with single-target approaches that arises from the complexity of any inflammation, with multiple interacting and often redundant factors (chemokines, receptors for chemokines and other ligands, intracellular signal proteins) being involved. Such discordance between simple synthetic tools versus complex natural networks is likely to cause problems for any class of therapeutic agent (small molecules, peptides, antibodies, regulatory RNAs) addressing single targets.

Against this background of single-target agents, we have discussed the potential of more complex, endogenous proteins addressing multiple receptors and signal pathways and

acting on the immune response in a phase-specific manner, either as recombinant proteins or as parent compounds for the development of novel drugs. Based on recent evidence, we specifically suggest further investigation of matricellular proteins with their rich and context-dependent migration-migration modulating properties, and of their interaction partners, as novel starting points for anti-inflammatory and cardiovascular drug development.

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Conflicts of interest

None.

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