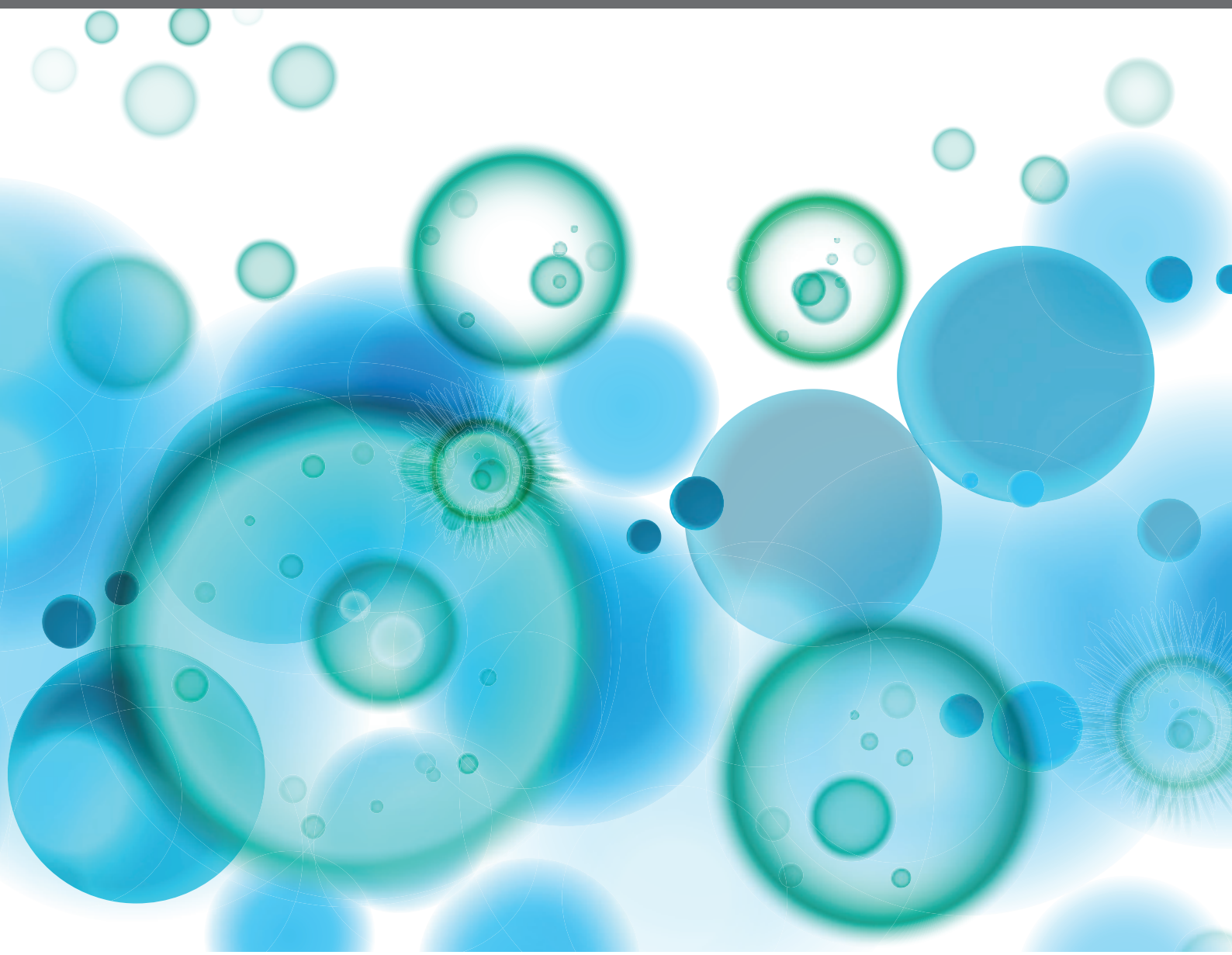
CELL-CELL AND CELL-MATRIX ADHESION IN IMMUNOBIOLOGY AND CANCER

EDITED BY: Toshiyuki Murai, Hiroto Kawashima and David Naor
PUBLISHED IN: Frontiers in Immunology and Frontiers in Oncology



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ISSN 1664-8714
ISBN 978-2-88963-526-9
DOI 10.3389/978-2-88963-526-9

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CELL-CELL AND CELL-MATRIX ADHESION IN IMMUNOBIOLOGY AND CANCER

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Citation: Murai, T., Kawashima, H., Naor, D., eds. (2020). Cell-Cell and Cell-Matrix Adhesion in Immunobiology and Cancer. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-526-9

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frontiers in Immunology

EDITORIAL
published: 23 January 2020
doi: 10.3389/fimmu.2019.03126

Editorial: Cell-Cell and Cell-Matrix Adhesion in Immunobiology and Cancer

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Keywords: cell adhesion molecule, extracellular matrix, immune system, cancer progression, cytoskeleton, cell dynamics

Editorial on the Research Topic

Cell-Cell and Cell-Matrix Adhesion in Immunobiology and Cancer

Cell-cell interactions and cell-extracellular matrix (ECM) interactions guide complex cellular decisions in various physiological processes including immune regulation such as leukocyte trafficking via blood and lymphatic vascular system. The interactions are also critical for the development of diseases and disorders including immune disorders, cancers, cardiovascular diseases, and neurodegenerative diseases. These interactions are mediated at the cell surface by adhesion molecules including integrins, selectins, CD44, and lectin molecules such as sialic acid-binding immunoglobulin-like lectins (Siglecs) as well as cell-cell interaction molecules such as cadherins. In addition, these interactions are mediated by ECM molecules such as hyaluronan and proteoglycans. Recent experimental evidence has indicated that these processes are finely tuned by the supramolecular assembly of adhesion molecules and cytoskeletal proteins that sense and recognize the physical properties of environmental cues. In light of the current need for new approach to treating and preventing diseases such as immune disorders and cancer and due to the complex nature of the molecular sociology of the cell, it has been increasingly evident that a multidisciplinary approach to evaluate them is crucial. We invited leading international researchers in the field to this Research Topic, which comprises seven reviews and two original articles and provides a timely and comprehensive view of recent advances in the understanding of cell-cell and cell-ECM interactions in the regulation of immune cell function and cancer particularly for potential cancer immunotherapy.

Cancer immunotherapy has shown great progress in the past decade, particularly focusing on immune modulation by immune checkpoint blockade (ICB), which has revolutionized cancer treatment and has fundamentally changed the outcome for certain groups of patients with advanced cancer. The 2018 Nobel Prize in Physiology or Medicine was awarded to Tasuku Honjo and James P. Allison, for their work on unleashing the body's immune system to attack cancer by targeting immune checkpoint molecules PD-1 (1) and CTLA-4 (2), respectively. Current attempts include the introduction of combination therapies of such novel therapeutic strategies with the conventional therapies, including chemotherapy. Si et al. performed a meta-analysis involving clinical trials with cancer patients and showed that incidence of peripheral neuropathy related to PD-1/PD-L1 inhibitors was significantly lower compared with chemotherapy group, while the risk was increased when it was combined with chemotherapy. On the other hand, at present, several trials to combine ICB with other emerging immunotherapies including adoptive cell therapy are exploring to further improve the outcomes.

Edited and reviewed by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *09 December 2019*
Accepted: *23 December 2019*
Published: *23 January 2020*

Citation:
Murai T, Kawashima H and Naor D (2020) Editorial: Cell-Cell and Cell-Matrix Adhesion in Immunobiology and Cancer. Front. Immunol. 10:3126. doi: 10.3389/fimmu.2019.03126

Chimeric antigen receptor (CAR)–modified T cell therapy is also the emerging therapy to cancer. However, while CAR-T therapy has had success in the treatment of hematological tumors, it is much harder for CAR-T cells to attack solid tumors (3). One of the most important adhesion molecules for T cell homing is L-selectin, and Watson et al. reported that increasing L-selectin on anti-cancer T-cells improved immune control of solid tumor progression. This study revealed a novel role for L-selectin in cancer immunotherapy for solid tumors and offers great promise to serve as a novel methodology to enhance the efficacy of CAR-T therapy for solid tumors. The review by Ivetic et al. provides a detailed review for the biochemical properties of L-selectin in immune cell function and its diverse functions in leukocyte-endothelial interactions. Other lectins such as Siglecs also regulate immune responses by binding to cancer cell-derived glycans, yet the potential to target these molecules' interactions are currently poorly investigated. Läubli and Borsig describes the role of Siglecs in immune suppression in cancer with an original view that highlights three families of cell-cell interaction molecules: selectins, Siglecs and integrins that support cancer development. Authors further discuss the mechanisms during immune evasion and metastasis that can be therapeutically targeted by blocking these cell-cell interactions.

Dendritic cells (DCs) are key regulators of tumor immunity and thus DC cancer immunotherapy is another emerging method (4). Particularly, integrins play a pivotal role in various aspects of immune cell function by transmitting signals bidirectionally. Harjunpää et al. put forward an updated perspective of current knowledge on integrin function in immune cells including DCs and its relevance in cancer, and provide lists on anti-cancer clinical trials targeting integrins which have published results on clinical efficacy. Integrins are known to serve as mechanoreceptors for outside-in signaling through two transcriptional activators, yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) operating in the Hippo pathway. An original article by Guenther et al. describes a novel mechanosensing link between β2 integrin and myocardin related transcription factor/serum response factor (MRTF/SRF) pathway that regulates DC migration and adhesion by using emerging techniques of three-dimensional cultures and quantitative imaging and physical measurement of traction force during cell adhesion.

Bidirectional communication between cancer cells and their tumor microenvironment (TME) is critical for cancer progression, and thus cancer cell-ECM interaction in TME may be a new target for immunotherapy (5). ECM is composed of two classes of macromolecules: (1) glycosaminoglycans and proteoglycans, and (2) fibrous proteins including collagens and laminins, among which the glycosaminoglycan hyaluronan has an impactful role in TME. Bourguignon focused on the role of hyaluronan and its receptor CD44 on RNA function, particularly on the function of micro-RNAs and long non-coding RNAs (lncRNAs). Liu et al. overview the complexity of hyaluronan and its interactions with receptors in TME in promoting or inhibiting cancers. Tzanakakis et al. presented a review on the role of proteoglycans including versican, syndecan and endocan in cancer development focusing on possible targets for cancer therapy. Classically, the generation of an efficient adaptive immune response against cancer occurs in secondary lymphoid organs (SLOs), while recent studies on TME revealed that they also occur directly at the tumor site called ternary lymphoid structures (TLSs) where lymphoid organs ectopically induced (6). Since evidence suggests that TLSs use a similar set of adhesion molecules to SLOs, the information on cell-cell and cell-matrix adhesion collected in this issue might be useful in understanding TLS-mediated anti-cancer immune responses and for modulating therapeutic response to ICB.

We wish that this collection will provide in-depth insights into current research on cell-cell and cell-matrix adhesion in immunobiology and cancer, and serve to discover and develop novel therapeutic approaches and the methods for prevention of diseases in the near future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 24 April 2019
doi: 10.3389/fimmu.2019.00875

Proteoglycans and Immunobiology of Cancer—Therapeutic Implications

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Disparity during the resolution of inflammation is closely related with the initiation and progression of the tumorigenesis. The transformed cells, through continuously evolving interactions, participate in various exchanges with the surrounding microenvironment consisting of extracellular matrix (ECM) components, cytokines embedded in the ECM, as well as the stromal cells. Proteoglycans (PGs), complex molecules consisting of a protein core into which one or more glycosaminoglycan (GAG) chains are covalently tethered, are important regulators of the cell/matrix interface and, consecutively, biological functions. The discrete expression of PGs and their interacting partners has been distinguished as specific for disease development in diverse cancer types. In this mini-review, we will critically discuss the roles of PGs in the complex processes of cancer-associated modulation of the immune response and analyze their mechanisms of action. A deeper understanding of mechanisms which are capable of regulating the immune response could be harnessed to treat malignant disease.

Keywords: proteoglycans, immunobiology, cancer, extracellular matrix, remodeling

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *31 January 2019*
Accepted: *05 April 2019*
Published: *24 April 2019*

Citation:
Tzanakakis G, Neagu M, Tsatsakis A and Nikitovic D (2019) Proteoglycans and Immunobiology of Cancer—Therapeutic Implications. Front. Immunol. 10:875. doi: 10.3389/fimmu.2019.00875

INTRODUCTION

Cancer initiation is a multi-faceted process with a contribution of genetic, metabolic and environmental factors. Tumorigenesis is closely associated with chronic inflammation, with approximately 20% of cancer incidences being directly correlated to chronic infections (1). Indeed, all tumors independently of etiology are distinguished by an early inflammatory milieu and characterized by discrete interactions with the immune system at all stages of disease progression (2, 3). During malignant transformation, cells obtain complex biological characteristics correlated with more efficient survival, invasion, metastasis and the ability to evade the immune response. The transformed cells, through continuously evolving interactions, communicate with and alter the surrounding microenvironment consisting of extracellular matrix (ECM) components, cytokines embedded in the ECM, and the stromal cells (e.g., fibroblasts, endothelial cells, adipocytes, and immune cells) (4, 5). The resulting ECM remodeling crucially contributes to the abnormal tumor inflammatory pattern (5, 6).

Proteoglycans (PGs) are complex molecules consisting of a protein core into which one or more glycosaminoglycan (GAG) chains are covalently tethered. The bound GAGs can be the heparan sulfate (HS), the chondroitin sulfate/dermatan sulfate (CS/DS), or the keratan sulfate (KS) type. In mammalian cells PGs are associated to the plasma membranes, released into the ECM or intracellularly localized. Presently, 45 PGs have been identified with each member characterized by immense alterability attributed to the modifications of the protein core and by the type and different stoichiometry of the GAG chain substitutions (7, 8). Thus, PGs have highly specific

and multifaceted biological roles, including: (i) contributing to ECM superstructure (9); (ii) defining ECM biochemical and physicochemical properties (9); (iii) acting as receptors of diverse responsiveness as well as a pool of various biologic effectors such as growth factors (10–12).

It is well-established that malignant tumors have discrete PG expression profiles, which are immediately correlated with their behavior and differentiation status. Thus, epithelial tumors exhibit a different PG characterization as compared to mesenchymal tumors (13, 14). Proteolytic cleavage of PGs, due to the action of matrix metalloproteinases (MMPs), cathepsins, and bone-morphogenetic protein-1, can release bioactive fragments or matrikines with roles in the propagation of tumorigenesis separate from that of parent molecules (15). Importantly, PGs are highly implicated in the processes of cancer-associated inflammation (16, 17). Indeed, PGs are suggested to modulate key events respective to both innate and adaptive immunity (18, 19).

In this review, we provide a critical overview of PGs' roles in the inflammatory cancer milieu and consecutively extrapolate to potential options for the development of targeted cancer therapies.

IMMUNOBIOLOGY OF CANCER

The input of the immune system, introduced as cancer immunoediting, consists of three phases: elimination (i.e., cancer immunosurveillance), equilibrium, and escape (20). Importantly, the tumor ECM contributes to the development of an immunosuppressive network where stromal cells intertwine with inflammatory immune cells and with cells appending to the vascular system. Within this complex, a newly formed network, secreted cytokines and chemokines can sustain the tumor immune escape (21, 22). The chain of tumorigeneis can be initiated by injury of normal tissue, independently of the causative agent, and concomitant triggering of acute inflammation (23, 24). The maintenance of inflammatory conditions due to various effectors leads to chronic inflammation which may evolve to precancerous lesion (25–29). The evolvement of the pre-cancerous lesion can be attenuated by an active immune defense or driven to primary tumor development (30), as schematically presented in **Figure 1**. Thus, the immune system has the ability to perceive and destroy many tumors early on in their development, whereas during the stage of equilibrium, a restraint of the tumor is attained. Some tumors will succeed in escaping from the growth restriction maintained by the immune system, and become clinically apparent (20, 31).

The resolution of disease and response to therapy is likewise affected by the tumor microenvironment as two major subsets of tumors with distinct mechanisms of resistance to immune-mediated destruction have been recognized. Tumors exhibiting the inflamed immunophenotype present with the recruitment of $CD8^+$ cytotoxic T cells, B cells and macrophages. Immune resistance in this case is due to the action of microenvironment-originating negative immune regulators. In non-inflamed tumors an absence of T-cells and innate immunity regulators is evident and leads to immediate immune failure (32, 33). Indeed, the ability of the tumor cells to escape impairment by the immune system has been suggested as a novel "hallmark of cancer" (6).

REMODELING OF THE CANCER ENVIRONMENT

During tumor progression, an extensive remodeling of the ECM with correlated release of pro-tumorigenic factors and orchestration of surrounding "stroma" cells is initiated. This remodeling of the tumor microenvironment is closely associated to the modulation of the immune response (34, 35). Thus, in cancer pathogenesis the resulting "desmoplastic reaction" among resident fibroblasts, endothelial cells, pericytes, leukocytes and surrounding ECM is directly correlated with invasion and poor patient prognosis (36, 37). The restructuring of the ECM is due to: (i) modulation in the synthesis and release of ECM components; (ii) degradation of the ECM owing to enzyme action or chemical degradation due to radical oxygen species (ROS) (4, 38). Specific ECM remodeling will ultimately result in: (i) the detachment of tumor cells from each other, from adjacent stromal cells or from matrix; (ii) enhanced growth and mobility of tumor cells; (iii) modulation of the immune system and finally in sustaining of the tumorigenic microenvironment (39, 40).

PGs ARE ACTIVE MEDIATORS OF CANCER-ASSOCIATED INFLAMMATORY MILIEU

Small Leucine Rich Proteoglycans Have a Dual Role in the Regulation of Cancer Inflammatory Setting

The family of small leucine rich PGs (SLRPGs) was initially correlated with the regulation of innate immunological responses, noteworthy due to the fact that the triggering of these very responses can lead to the initiation of tumorigenesis (41). The role of a pro-inflammatory molecule has been designated to a class I SLRP member, biglycan (17). Importantly, this SLRP is overexpressed and secreted by various cancers including gastric (42), pancreatic (43), ovarian (44), and colon cancer (45). This SLRP can be either freed from the ECM through proteolytic degradation initiated upon tissue injury or *de novo* produced by activated macrophages and resident cells with various immunological roles (**Figure 2**) (46). Soluble biglycan can undertake the role of signaling mediator by binding to the Toll-like receptors (TLR)-2 and -4 on the surface of macrophages. The formation of the ligand-receptor complex initiates sterile inflammation and can facilitate pathogen-mediated inflammation through the production of pro-inflammatory cytokines and chemokines, including the tumor necrosis factor (TNF)-α, interleukin (IL)-1β or chemokine (C–C motif) ligand (CCL)2 (46, 47). It was shown that soluble biglycan utilizes TLR2/4 signaling pathways to activate adaptor molecule myeloid differentiation primary response 88 (MyD88), for the recruitment of neutrophils and macrophages or to initiate Toll/interleukin (IL)-1R domain-containing adaptor inducing interferon (IFN)-β (TRIF) activities for T-lymphocyte

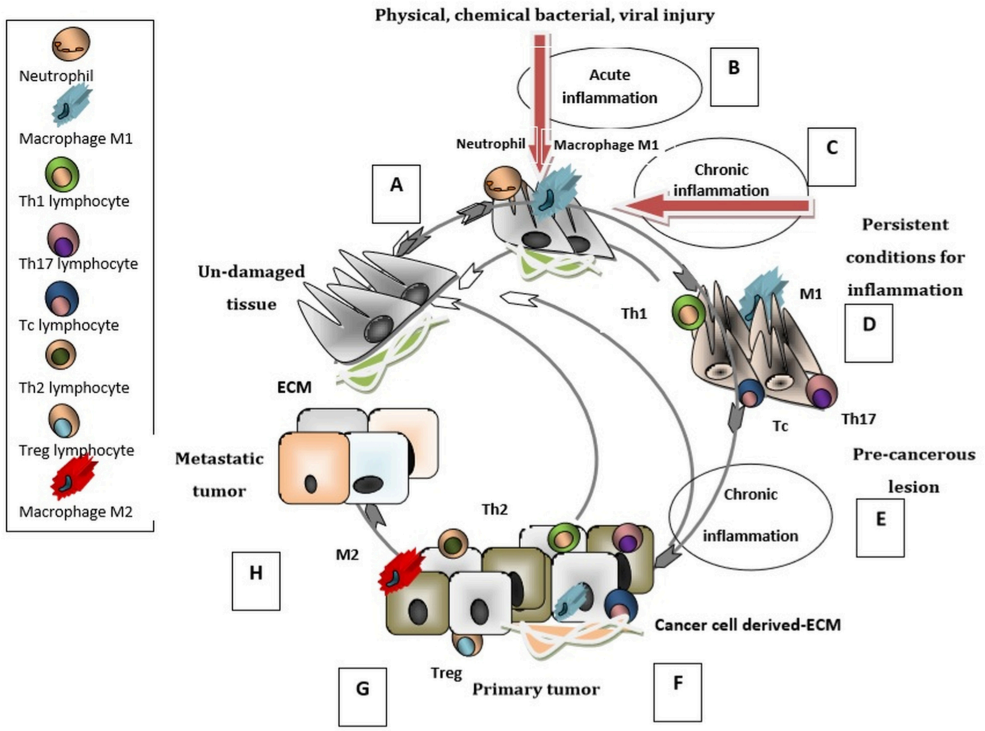

FIGURE 1 | Immunobiology of cancer cycle. When un-damaged tissue **(A)** is subjected to injury, innate immunity cells (neutrophils and macrophages type M1) infiltrate the tissue and generate an acute inflammation **(B)** that triggers the repair cascade guiding the tissue to its un-damaged status. ECM is altered and contributes to inflammatory processes. If the inflammation is sustained and has chronic characteristics **(C)**, in addition to M1 macrophages the tissue is infiltrated with adaptive immunity cells (Th1, Tc, Th17); the pre-cancerous lesion **(D)** can return to its normal un-damaged status. At these stages the elimination of tumor development is possible. If the chronic inflammation persists **(E)** then the pre-cancerous lesion evolves into a primary tumor. The primary tumor can be infiltrated by M1, Th1, Tc, Th17 cells **(F)** with intense anti-tumoral activity which can restrain the tumorigenesis process and establish the immune equilibrium stage. In contrast, when the infiltrating cells are M2 type macrophages Th2, Treg lymphocytes **(G)** a pro-tumorigenesis milieu is enhanced and the primary tumor evolves into an aggressive/metastatic tumor. The ECM is modulated by tumor/stromal -cells and gains new immunosuppressive characteristics that favor metastasis **(H)**. This last stage of the tumor-immune cycle is characterized by the escape of tumor cells from the immune control.

recruitment (48). Moreover, the role of a danger signal (DAMP) that triggers the NLRP3 inflammasome through upstream TLR2/4 and P2X receptors signaling has been attributed to biglycan (49), by specifically regulating the crosstalk between TLR2/4- and $P2X_7$-NLRP3-caspase-1 (50, 51). Increasing data proposes that the initiation of TLR2/TLR4 signaling enhances tumor cell growth, downregulates apoptosis, and upregulates the synthesis of growth factors and inflammation-associated cytokines by tumor and stromal cells (50, 52, 53). Recently, biglycan was shown to bind with high affinity to macrophage CD14, an established GPI-anchored TLRs co-receptor. CD14 is mandatory for biglycan-dependent TLRs activation, where biglycan seems to have the role of a re-router as complexing with specific TLR members induces a discrete response. Thus, in macrophages, the biglycan/CD14/TLR2,4 complex induces the TNF-α expression, thebiglycan/CD14/TLR2 co-localization results in HSP70 release, whereas the biglycan/CD14/TLR4 complex initiates CCL5 secretion (54). Moreover, in a mouse model of renal injury, a deficiency of CD14 prevented biglycan-mediated cytokine expression, recruitment of macrophages, and M1 macrophage polarization (54).

Moreover, biglycan has been implicated in the regulation of important to tumorigenesis (4), ROS generation (55). Indeed, biglycan through activating discrete TLR receptors discriminately stabilizes NADPH oxidase (NOX) 1, 2, and 4 enzymes and regulates their downstream ROS production, resulting in either a positive or negative modulation of IL-1β synthesis (18). Furthermore, TLR2/4 induced ROS generation by biglycan (56) stimulates macrophages and dendritic cells to express chemokine (C-X-C) ligand 13 (CXCL13), the major chemoattractant for B and B1 lymphocytes (56). In addition to chemoattracting B cells, biglycan through TLR2/4 stimulates RANTES, MCP-1, and MIP-1α secretion, inducing macrophages and T cells recruitment into the kidney. Thus, these developments indicate that biglycan signaling can bridge innate and adaptive immunity (56), with the ability to act at different check points of the tumor immune cycle.

On the other hand, by enhancing NOX-generated ROS, biglycan induces genomic volatility coupled with chromosomal DNA modifications, which results in increased tumor cell growth, viability, and a metastatic ability of inflammation-associated tumors (57). Even though the majority of reports

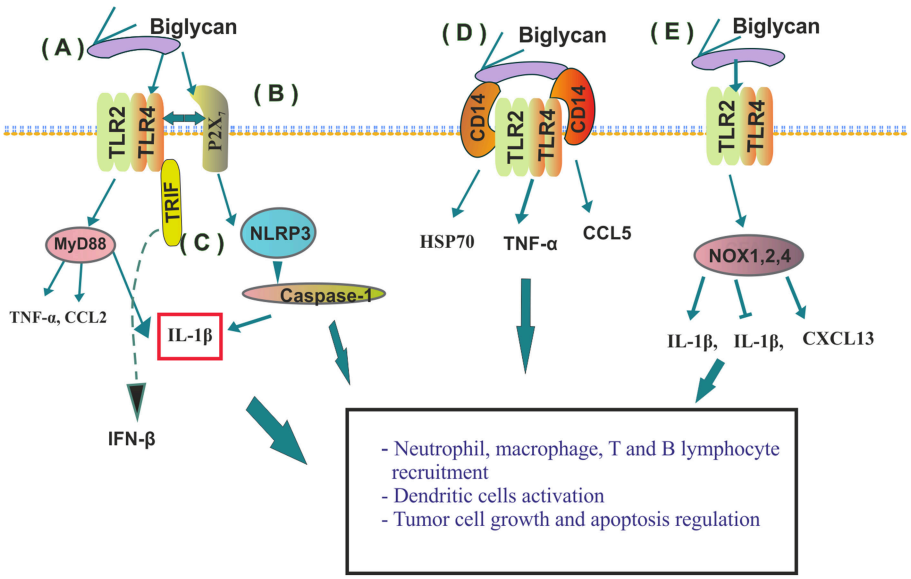

FIGURE 2 | Immunomodulatory roles of biglycan. **(A)** By binding to TLR2 or 4 on macrophages' biglycan induces cytokine (TNF-α, CCL2 or IL-1β) release; **(B)** Crosstalk between TLR2/4- and P2X induces NLRP3 inflamozone and IL-1β activation and consequent neutrophil recruitment; **(C)** Soluble biglycan initiates Toll/interleukin (IL)-1R domain-containing adaptor activation inducing interferon (IFN)-β (TRIF) activities for T-lymphocyte recruitment; **(D)** Biglycans' binding determines CD14/TLR2/TLR4 complex formation and discrete downstream signaling; **(E)** Biglycan binding to specific TLR receptors discriminately activates NADPH oxidase (NOX) 1, 2, and 4 enzymes and regulates their downstream ROS production resulting in both positive or negative modulation of IL-1β synthesis; or in the stimulation of macrophages and dendritic cells to express chemokine (C-X-C) ligand 13 (CXCL13), the major chemoattractant for B and B1 lymphocytes.

suggest that the activities of biglycan are pro-oncogenic, recent studies provide evidence that biglycan can promote an anti-inflammatory response that is key for the resolution of acute inflammation and hence the switch to acquired immunity response (18). Indeed, it is now proposed that biglycan, even though exhibiting affinity to both TLR2 and 4, will discriminately bind to only one TLR, which will, in combination with specific TLR adaptor molecules, lead to a discrete biological response (17, 18). The summarized regulation of immunity responses by biglycan can conceivably affect the development and resolution of early pre-cancerous lesions as well as the progression of inflamed tumor subtypes. Decorin, likewise an SLRP class I member, has established anti-tumorigenic properties (58). This SLRP member has the ability to initiate the TLR2/4 downstream signaling and cytokine release with an outcome different to that of biglycan. Specifically, utilizing a transforming growth factor –β (TGF-β)/oncogenic microRNA (miR)-21/tumor suppressor programmed cell death protein 4 (PDCD4) axis decorin downregulates the release of IL-10, which is an anti-inflammatory mediator and thus inhibits tumor growth (59). Moreover, decorin mobilizes mononuclear cells to the region of damaged tissue by enhancing CCL2 release and thereby, effectively upholding the inflammatory state (60). In a breast cancer xenographic model it was demonstrated that the decorin protein core inhibits genes obligatory for the regulation of the immunological response. Therefore, Buraschi et al. conclude that the "systemic administration of decorin protein core reveals a fundamental basis of action for decorin to modulate the tumor stroma as a biological mechanism for the ascribed anti-tumorigenic properties." Indeed, the authors' gene ontology data indicated an inhibitory role in the regulation of proteins implicated in immunomodulatory responses when assigned to decorin protein core (61). The SLRP lumican has been postulated to modulate tumor-associated inflammation by affecting peripheral monocyte extravasation, and Fas–FasL signaling (14). Interestingly, lumican enhances LPS-dependent activation of TLR4 (62).

The Hyalectan Versican Is Crucial to Both Innate and Adaptive Immunity

Versican, a member of the hyalectan family of large chondroitin sulfate PGs (CSPGs) has also been implicated in inflammatory processes (19, 63). These pericellular PGs are localized, among other, to the sub-endothelial compartment where they encounter the infiltrating leukocytes and modulate their biological activities by binding to specific receptors including TLR2, and P- or L-selectins (19). Once bound to the versican-containing ECM, leukocytes degrade the ECM to generate pro-inflammatory fragments that further drive the inflammatory response (64). Versican has been established to be overexpressed in a number of cancers, including prostate, breast, malignant myeloma, glioblastoma, laryngeal, pancreatic ovarian, gastric, testicular germ-cell, and cervical cancer, as recently discussed (65).

Importantly, in cancer-associated inflammation, versican can affect the production of cytokines by both lymphoid and myeloid cells. Thus, Lewis lung carcinoma (LLC) cells overexpress versican in that, by binding to TLR2 and its co-receptors on macrophage cell membranes, it activates the latter and facilitates their TNF-α secretion (66). The initiation of this cascade by

versican and the induction of TNF-α by myeloid cells enhances LLC cell metastatic growth (66). Furthermore, an increased expression of versican V1 and V3 isoforms was positively correlated to lung metastasis in a bladder cancer murine model due to the increased release of CCL chemokine by macrophages (67). Specifically, versican was shown to facilitate bladder tumor cell migration, resulting in lung metastasis due to a mechanism involving CCL2/CCR2 secretion by macrophages. Said & Theodorescu propose that the aforementioned cytokine release generates a permissive lung inflammatory environment (68). Likewise, in mesotheliomas, versican downregulates macrophage M1 phenotype and decreases their ability to phagocytose tumor cells (69) contributing to the process of immune "escape."

Interestingly, monocytes cultured in versican-containing supernatants of colon cancer cells secrete pro-inflammatory cytokines, including IL-12, TNFα and ROS, whereas monocytes cultured in versican-free supernatants gathered from breast cancer tumors' culture exhibited a different profile of secreted cytokines. Thus, versican seems to have the ability to specifically direct the inflammatory monocyte response (19), conceivably correlated to the differential recruitment of lymphocytes, and differentiation into tumor-associated macrophages and dendritic cells (DC) (70).

DCs are proposed to engage tumor antigens and relocate to draining lymph nodes, where they trigger tumor-specific T cells (71). Importantly, versican derived from tumor ECM activates DCs' TLR2, resulting in the production of immunosuppressive IL-10 and DC dysfunction. This was associated with a TLR2-induced increase of IL-6 and IL-10 cell-surface receptors and a strongly decreased cytokine concentration threshold is needed to trigger STAT3 (72, 73). Intact versican, thus, dampens DC activation (72, 73) and conceivably downstream Th and cytotoxic lymphocyte (CTL) differentiation. Likewise, intact versican secreted by macrophages was shown to have anti-inflammatory properties in a mouse model of acute pulmonary inflammation (74). On the other hand, in myelomas, versican is proteolyticaly processed to the DAMP versikine, which induces the secretion of IL-1β and IL-6 by human myeloma marrow-derived macrophages (MAMs). Importantly, MAMs chiefly synthesized V1, the precursor to versikine, whereas stromal cells secreted the versican-degrading protease. This interplay is suggested to enhance the "T-cell inflammation," response and downregulate the "tolerogenic consequences of intact versican accumulation" contributing to tumor restraint (75).

Syndecans, Modulators of the Inflammatory IL-6/STAT3 Pathway

Syndecans (SDCs), cell membrane HS containing PGs (76), have also been implicated in tumor immunomodulation. SDC1-defficient mouse model of colitis-related colon carcinoma exhibited higher susceptibleness to malignant transformation due to the enhanced topical release of IL-6, downstream activation of STAT3 and target genes with important roles in colonic tumorigenesis (77). Likewise, in inflammatory breast cancer, SDC1 was shown to upregulate the inflammatory IL-6/STAT3 pathway (78), a crucial part of tumor-stimulating signaling in epithelial-origin tumors (79). The well-described role of the enzyme heparanase, responsible for the cleavage of the HS chains may partly elucidate the role of the HS-bearing SDCs (80). Thus, due to the fact that HS regulates inflammatory processes at various checkpoints, e.g., including the segregation of inflammatory cytokines to the ECM, the configuration of leukocyte binding to the endothelial cells and ECM components, as well as the generation of responses respective to innate immunity by interacting with the TLRs, the reconfiguration of HS due to enzymatic heparanase affects the propagation of inflammatory events (80, 81).

Endocan, A Unique Plasma PG Blocks Leukocyte Trafficking

Endocan is an endothelial-derived soluble PG, initially identified in human umbilical vein endothelial cells (82). Interestingly, endothelium activated by inflammatory processes and/or tumor pathogenesis exhibits a strong augmentation in endocan expression correlated to poor patient prognosis (81, 83–85). Glycanated human endocan has been shown to strongly promote tumor growth (86) through its ability to bind to the integrin CD11a/CD18 (LFA-1) and thus to block leukocyte binding to endothelium and subsequent infiltration to tumor tissues (87); or through its promotion of growth factor actions (83, 88). On the other hand, the non-glycanated mouse or human endocan polypeptide was shown, in a murine model, to delay tumor expansion through the induction of pan-leukocytic infiltration of CD122+ expressing cells within tumor and stroma tissues (89).

The Intracellular PG, Serglycin, Is Implicated in Heamotological Malignancies

Serglycin, the only known intracellular PG is shown to be overexpressed in various tumor tissues and cell lines (90, 91). Importantly, serglycin is a key compound of secretory particles produced by CTL/NK cells, suggested to facilitate the safe storage of particle toxins, granzymes and perforin and affect CTL/NK cells' cytotoxic ability (92). Even though no evidence is available as such, these data implicate a plausible contribution of serglycin to processes of cancer-associated inflammation.

THERAPEUTIC IMPLICATIONS

The modulation of PGs' activities, as endogenous mediators of cancer-associated inflammation, is a novel approach in the field of cancer immunotherapy. Research efforts up to date have demonstrated the plausibility of this strategy as discussed below. Thus, the administration of the versican fragment characterized as DAMP, versikine, is suggested to "facilitate immune sensing of myeloma tumors and modulate the tolerogenic consequences of intact versican accumulation" which may facilitate tumor restraint and enhance T-cell-activating immunotherapies (75). Blocking the versican/CCL2/CCR2 signaling axis is indicated to purvey novel adjuvant strategies for detaining the emergence of clinical metastasis in bladder cancer patients (68). The non-glycanated mouse or human endocan polypeptide

restrains tumor growth by increasing leukocyte infiltration *in vivo*, enhancing *de facto* innate immunity response (89). Suppressing the shedding of SDC1 from intestinal epithelial cells decreases intestinal inflammation and putative malignant transformation by augmenting NF-κB, respective downstream signaling, and neutrophil transmigration in ulcerative colitis (93). Indeed, the modulation of SDC1 expression could facilitate immunesurveillance and the positive resolution of precancerous lesions. Biglycan is suggested to bridge innate and adaptive immunity through TLR2/4 downstream signaling due to its' ability to regulate neutrophil, macrophage, T and B lymphocyte, and dendritic cells activities (17, 18, 46–56). Thus, selective inhibition of biglycan-TLR2/TLR4 axis could be a novel therapeutic approach targeting at various checkpoints of the cancer immunobiology cycle. Modulations of PGs post-transcriptional modifications, including their respective sulfation pattern, seems to be a therapy option as inhibition of sulfatase-2 activity had cytotoxic and partial hepatoprotective activity in both *in vivo* and *in vitro* hepatocellular carcinoma models (94, 95). Indeed, a clinically relevant pattern of PG and GAG expression and structural modifications was recently determined for PGs and GAG synthesizing enzymes in glioma and breast cancer, which might help in the development of personalized therapy (96).

Despite these advances, however, many issues need yet to be addressed for implementation to clinical practice (97). Furthermore, an understanding of tumor microenvironment biology and its PG component is necessary for therapy development.

AUTHOR CONTRIBUTIONS

DN designed the concept and completed the final editing of the manuscript. All authors contributed to writing of the manuscript. DN and MN prepared the figures. All authors read and approved the final version of the manuscript.

FUNDING

DN was supported by ELKE KA10038. MN was supported by PN 19.29.01.01, PN-III-P1-1.2-PCCDI-2017-0341/2018 and 7PFE/16.10.2018.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 10 May 2019
doi: 10.3389/fimmu.2019.00947

Dissecting the Dual Nature of Hyaluronan in the Tumor Microenvironment

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Hyaluronan (HA) is a glycosaminoglycan with a simple structure but diverse and often opposing functions. The biological activities of this polysaccharide depend on its molecular weight and the identity of interacting receptors. HA is initially synthesized as high molecular-weight (HMW) polymers, which maintain homeostasis and restrain cell proliferation and migration in normal tissues. These HMW-HA functions are mediated by constitutively expressed receptors including CD44, LYVE-1, and STABILIN2. During normal processes such as tissue remodeling and wound healing, HMW-HA is fragmented into low molecular weight polymers (LMW-HA) by hyaluronidases and free radicals, which promote inflammation, immune cell recruitment and the epithelial cell migration. These functions are mediated by RHAMM and TLR2,4, which coordinate signaling with CD44 and other HA receptors. Tumor cells hijack the normally tightly regulated HA production/fragmentation associated with wound repair/remodeling, and these HA functions participate in driving and maintaining malignant progression. However, elevated HMW-HA production in the absence of fragmentation is linked to cancer resistance. The controlled production of HA polymer sizes and their functions are predicted to be key to dissecting the role of microenvironment in permitting or restraining the oncogenic potential of tissues. This review focuses on the dual nature of HA in cancer initiation vs. resistance, and the therapeutic potential of HA for chemo-prevention and as a target for cancer management.

Keywords: hyaluronan, hyaluronan receptors, tumor microenvironment, cancer resistance, tumor initiation, CD44, RHAMM

OPEN ACCESS

Edited by:
Hiroto Kawashima, Chiba University, Japan

Reviewed by:
Vincent Charles Hascall, Cleveland Clinic Lerner College of Medicine, United States
Suniti Misra, Medical University of South Carolina, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *04 December 2018*
Accepted: *12 April 2019*
Published: *10 May 2019*

Citation:
Liu M, Tolg C and Turley E (2019) Dissecting the Dual Nature of Hyaluronan in the Tumor Microenvironment. Front. Immunol. 10:947. doi: 10.3389/fimmu.2019.00947

INTRODUCTION

Cancer is the second leading cause of mortality worldwide according to GLOBOCAN estimates, accounting for ∼9.6 million death in 2018 (1). Two decades ago, Hanahan et al. proposed the six classic hallmarks during the multi-step development of cancer (sustained proliferative signaling, evasion of growth suppressors, limitless replicative potential, resistance of apoptosis, sustained angiogenesis, and invasion/metastasis) (2). Conceptual advancements in the past two decades have added evasion of immuno-surveillance, elimination of cell energy limitation, genome instability, and the participation of host cells in facilitating tumor initiation and progression (3, 4). Emphasis has historically been placed on the key role of mutations and genomic instability as drivers of tumor initiation and progression. However, the somatic mutation burden in physiologically normal tissues can approach the level of many cancers (5). These somatic mutations increase with age, and include cancer driver genes, which appear to be under strong positive selective pressure. They

occur in the absence of evidence for tumorigenic conversion (5), suggesting that driver gene mutations by themselves are insufficient for cancer initiation. Experimental data also predict that the microenvironment can block or permit the oncogenic potential of mutations (6–9). For example, teratocarcinomas transplanted into early embryos do not form tumors but participate in the development of normal tissues (10). Moreover, the tumorigenic potential of genomically unstable breast cancer cell lines can be curtailed by blocking specific signaling pathways from the extracellular matrix (11–13) suggesting the paracrine interactions between the host cells, tumor cells, and the extracellular matrix can disable or enable the oncogenic potential of mutations. Therefore, dissecting the nature of such interactions is critical to identify therapeutic targets that manage tumor initiation, tumor progression, and post-treatment tumor recurrence.

The tissue polysaccharide, hyaluronan (HA), is one example of an extracellular matrix component that participates in cancer initiation and progression (5, 14–16). HA, like many factors, is multifunctional and has both tumor-promoting and -suppressing properties. HA is an anionic, linear, non-sulfated glycosaminoglycan (GAGs) composed of repeating disaccharide units of glucuronic acid, and N-acetylglucosamine that are synthesized by hyaluronan synthases (HAS1-3) and that can achieve molecular weights in excess of 1,000 kDa. Fragmentation of these large polymers by free radicals and hyaluronidases (HYAL1-3) generate low molecular-weight HA varying from 1 to 500 kDa. Despite its simple primary chemical structure, the large range of polymer sizes generates diverse physiochemical functions and signaling properties (14, 15). High molecular weight HA (HMW-HA, defined here as *>500 kDa* and in text) predominates in normal tissues, where it provides a scaffold for protein interactions and is essential in maintaining tissue homeostasis. The viscoelastic properties of HMW-HA contribute to porosity and malleability of extracellular matrices (e.g., stem cell niches) (17–20), which are important for resistance to somatic mutation, protection against mechanical damage, and regulating cell trafficking. HMW-HA is also anti-inflammatory and anti-proliferative, which may contribute to tumor resistance in normal tissues (21–24). Fragmentation of HMW-HA into low molecular-weight polymers (LMW-HA, defined here as 7–200 kDa, **Figures 1A–D** and in text) is minimal or absent in homeostatic tissues, but is increased during response-to-injury and remodeling events in the embryo and adult (25, 26) due to expression of HYAL1-3 and generation of ROS/NOS. LMW-HA activates signaling cascades that promote cell migration, proliferation, immune cell influx, and mesenchymal cell trafficking (27, 28) (**Figures 1A–C**). This review highlights the consequence of these opposing functions of HA polymer sizes to tumor resistance, initiation and progression. The potential of HA polymers and the processing/signaling machinery for these polymers as therapeutic targets in cancer prevention and management is emphasized.

THE HYALURONOME

HA is uniquely synthesized by plasma membrane HAS. Polymers are initiated on the cytoplasmic face of these proteins, then extruded through pores created by aggregated HAS into the ECM (29–32). The three HAS isoforms are encoded on different chromosomes and exhibit distinct tissue distribution and enzymatic properties (31, 33, 34). HA polymers are degraded by three major hyaluronidases (HYAL1-3). HYAL1 and 3 are primarily located in the lysosome and, together with glucosaminidases and glucuronidases (35), degrade HA polymers into monomers. HYAL2 is located at the cell surface, which together with extracellular reactive oxygen/nitrative species (ROS/NOS), generate extracellular LMW-HA (36). These HMW- and LMW-HA polymers bind to CD44, RHAMM, LYVE1, TLR2/4, STAB2, and LAYILLIN (37). HA receptors lack kinase activity and signal by associating with growth factor receptors (e.g., EGFR, PDGFR, and TGFBR) (38, 39). One function of HA:HA receptor interactions is to regulate receptor clustering and membrane localization (40). These critical HA functions are mediated by polymer size and the size preference of HA receptors. For example, while CD44, RHAMM and TLR2,4 bind to a range of sizes, RHAMM and TLR2,4 preferentially bind to very low molecular-weight HA (<7 kDa) (4, 37). The functional complexity of HA polymer signaling is additionally generated by the extensive and often tissue/stimulus-specific alternative splicing of CD44 (41, 42), tissue-specific expression of LYVE1 (lymphatics), and STAB2 (liver) (43–46). The multi-compartmentalization, stress- and stem cell-specific expression of RHAMM, as well as cell context-dependent interaction of HA receptors with each other (e.g., RHAMM/CD44, RHAMM/TLR2,4) also influence HA-mediated signaling (37, 38).

How this HA signaling machinery functions in tumorigenesis can be complex and unpredictable. For example, CD44 can perform tumor-promoting or -suppressing functions depending on its association with protein partners (47). Despite its elevated expression on progenitor-like tumor-initiating cells, deletion of CD44 in a mouse model of luminal breast cancer susceptibility increases rather than decreases metastases. Similarly, either increasing or decreasing intracellular RHAMM expression will de-regulate its functions in centrosome and spindles, increasing the potential for generating genomic instability and enhancing tumorigenesis (48). Such dual properties make direct therapeutic targeting of HA receptors challenging. Nevertheless, de-regulated HA metabolism/signaling typifies many cancers and often predicts poor clinical outcome (49–56). Therefore, developing an understanding of HA metabolism/signaling as a microenvironmental factor that contributes to cancer susceptibility, initiation and progression is likely to identify therapeutic avenues for managing this disease.

HYALURONAN: A DOUBLE-EDGED SWORD IN CANCER?

The tumor microenvironment is emerging as a critical factor in cancer progression and recurrence. HA production and accumulation in both the stroma and tumor parenchyma is characteristic of prostate, bladder, lung, breast and other cancers, and is linked to poor clinical outcome (49, 51, 52, 57–59).

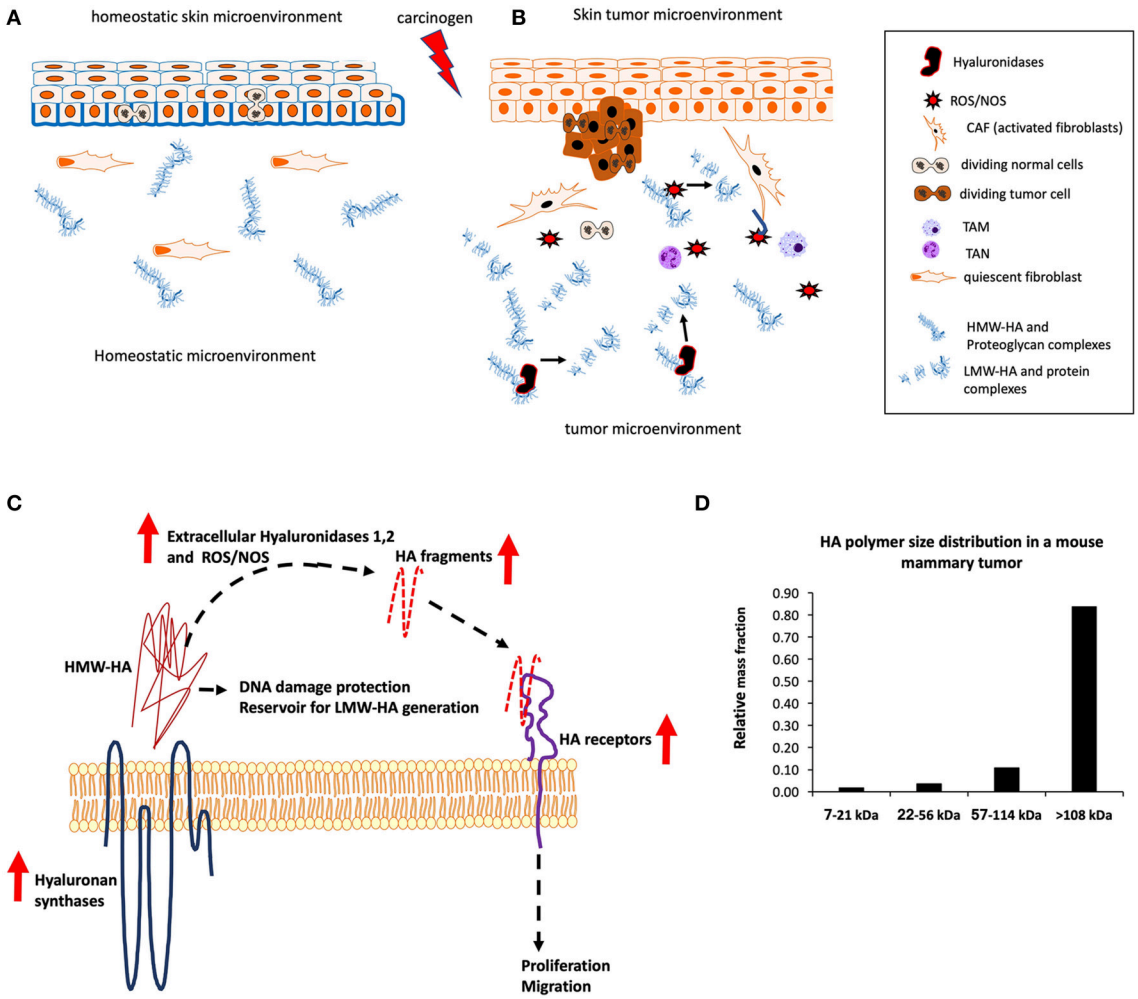

FIGURE 1 | Hyaluronan synthesis and fragmentation in normal and tumor microenvironments. **(A)** Homeostatic skin is characterized by an organized epidermis with the regulated symmetric and asymmetric division of basal keratinocytes. These are covered by layers of HMW-HA coats (blue outline). Dermal fibroblasts are quiescent and HMW-HA is organized into complexes with proteins and proteoglycans. Extracellular LMW-HA accumulation is restricted. **(B)** Tumor-initiating events result in disorganized growth of epidermal cells and changes to HA organization and processing. HA synthesis increases, hyaluronidases are expressed/released and reactive oxygen/nitrogen species (ROS/NOS) production is high, resulting in HA fragmentation and reduced organization of macromolecular complexes. HMW-HA coats around epidermal cells are reduced. LMW-HA activates fibroblasts (CAFs, cancer-associated or activated fibroblasts) and attracts immune cells [tumor-associated neutrophils (TANs) and tumor-associated macrophages (TAMs)] that produce ROS/NOS. The fragmented and disorganized tumor microenvironment supports tumor proliferation and evasion of immune surveillance. **(C)** In disease states such as tumors or chronically inflamed tissues, native or HMW-HA synthesis is increased by constitutively elevated HAS expression. LMW-HA accumulates due to increased expression and activity of extracellular hyaluronidase activity and reactive oxygen/nitrogen species (ROS/NOS) produced by stressed tissues. LMW-HA activates pro-migratory and proliferation pathways through CD44, RHAMM and TLR2,4, whose expression is also increased. In a disease such as cancer, HMW-HA contributes to a stem cell-like microenvironment that is immuno-suppressive and may protect tumor cells from DNA damage. **(D)** The continued accumulation of HMW HA polymers also provides a source for generating LMW-HA that accumulates in tumor microenvironments as shown in **(A)**. These fragments can be targeted by peptide mimetics that bind and sequestering them, preventing their activation of pro-migration and proliferation signaling pathways.

The majority of these studies do not report the molecular-weight ranges of HA present in the tumor samples. However, the increased expression of HA receptors that preferentially bind to LMW-HA (e.g., RHAMM), increased HYAL expression (e.g., HYAL1,2) (49, 50, 55, 57, 60, 61), and elevated ROS levels predict that LMW-HA is elevated. However, It is likely that both HMW and LMW-HA are increased in tumor microenvironments and that both contribute to tumor survival, growth and aggression (**Figures 1B,C**). Data mining using TCGA data sets in the cBioPortal for Cancer Genomics (www.cbioportal.org) provides unbiased evidence the association of increased HA production and fragmentation with cancer patient survival. For example, increases in hyaluronidases, HAS and HA receptors are significantly linked to reduced survival in colorectal (e.g., HAS2, HMMR, CD44, HYAL1, $p = 0.013$) (62), and breast [e.g., HAS2, HMMR, HYAL2, $p = 0.023$, (63) and $p = 5.24\text{e-}3$(64)] cancers.

Experimental studies provide direct evidence for the importance of elevated HA production in tumor initiation and progression. As examples, MMTV-Neu mice over-expressing a HAS2 transgene exhibit increased mammary tumor incidence and growth compared to wildtype animals (65). HAS2-mediated acceleration of tumor progression results in part from resistance to apoptosis caused by constitutive activation of PI3K/AKT signaling (65, 66). Conversely, HAS2 knockdown in metastatic breast cancer cell lines arrests tumor cells in G0/G1 as a result of reduced cyclin A, B, and cdc2 expression (67). Stable knockdown of this HAS in MDA-MB-231 breast cancer cells decreases their invasion, which is rescued by HAS2 re-expression (66). Conversely, cell sorting of breast tumor cell lines that bind to high levels of HA identifies highly invasive, metastatic subpopulations (68). Experimental evidence from these types of studies suggests that elevated HMW-HA accumulation in either the peri-tumor stroma or tumor parenchyma performs several key functions that favor tumor growth in addition to protection from apoptosis. These include reducing uptake of chemotherapeutic drugs by regulating drug transport proteins, reducing neo-angiogenesis, and reducing drug diffusion into the tumor by increasing local tissue hydrostatic pressure (69–71). HMW-HA reduces immune surveillance at least in part by blocking neo-angiogenesis. Importantly, abundant HMW-HA polymers also provide a stable source for generating LMW-HA.

An example of evidence supporting this latter function is provided by the enhanced invasion of tumor cells when they are transfected with both HAS2 and hyaluronidases (72). HYAL1 knockdown reduces tumorigenicity of breast cancer cell lines (73), while forced expression of HYAL1 promotes tumor cell growth and migration in culture and *in vivo* (74, 75). LMW-HA polymers promote neo-angiogenesis, tumor cell migration, invasion, and proliferation (16). LMW-HA also attracts macrophages, which polarize into subpopulations that protect tumor cells from adaptive immune cell killing (76). This fragment-specific signaling is mediated by HA receptors, the best studied of which are RHAMM and CD44. Tumor cell migration stimulated by LMW-HA is often associated with an epithelial-mesenchymal transition mediated by CD44 through activating PI3K/AKT and TGFβ signaling (65), while RHAMM is linked to increasing tumor cell migration via regulating ERK (77–79) and AURKA/beta-catenin pathways (80, 81). This signaling not only impacts the functional properties of tumor cells but also regulates stromal cells properties. Thus, LMW-HA/CD44/RHAMM binding promotes angiogenic capillary invasion into the tumor microenvironment. In MMTV-Neu mice, elevated LMW-HA production induces intra-tumoral microvessel formation through promoting angiogenic factor secretion, and enhanced type I collagen, fibronectin, bFGF, CD31 mRNA expression (65, 74). Further, bFGF-induced angiogenesis is accelerated in the presence of LMW-HA (74).

LMW-HA in the tumor microenvironment also promotes monocyte recruitment and differentiation of cytotoxic M1 subtypes into the M2 subtype by altering the Th1/Th2 cytokine balance, thereby enhancing local ROS level, which increases LMW-HA generation and M2 monocyte accumulation (76). This appears to have clinical relevance since both increased M2-like tumor-associated macrophages (TAMs) and LMW-HA accumulation correlate with tumor metastatic potential and poor prognosis in breast cancer patients (54). Both CD44 antibodies and LMW-HA binding peptides reduce monocyte activation and M2 polarization predict that these effects are modulated by LMW-HA:CD44 interactions(76). Moreover, tumor-derived LMW-HA promotes tolerance of tumor cells to infiltrating macrophages (82). This tumoricidal neutralization is suggested to involve IRAK-M (interleukin-1 receptor-associated kinase M), with LMW-HA as an extracellular modulator through both TLR2,4 and CD44 (82).

Collectively, clinical and experimental studies show the importance of tumor or peri-tumor stromal cell HA production and fragmentation for tumor progression. Targeting HA may therefore provide promising therapeutic approaches in cancer management and treatment.

THERAPEUTIC APPROACHES FOR REDUCING HA PRODUCTION AND FRAGMENTATION TO CONTROL TUMORIGENESIS

Inhibiting HA Synthesis

4-Methylumbelliferone (4-MU) is an inhibitor that depletes one of the building blocks (glucuronic acid, GA) of HA synthesis (83, 84). In 4-MU treated mammalian cells, UDP-transferase catalyzes the transfer of GA onto 4-MU, thus depleting the pool of cytoplasmic UDP-GA and inhibiting HA synthesis (83, 85). 4-MU also decreases HAS2/3 expression (60–80% in cancer cell lines) (86). This suppression is accompanied by reduced CD44 and RHAMM expression, suggesting a feedback loop between HA synthesis and receptor expression (86). HA-mediated downstream signaling is therefore inhibited following 4-MU administration with a consequent reduction in proliferation, migration, and invasion of cancer cells. 4-MU reduces metastasis in skin, lung, osteosarcoma, and breast cancer xenograft models(85, 87–91). Dietary incorporation of 4-MU is also associated with enhanced chemo-prevention: daily ingestion of 4-MU (450 mg/kg) for 28 weeks abrogated prostate tumor initiation and metastasis in experimental models (91). Nevertheless, this approach is not specific for tumor-associated HA and would also block the desirable functions of HMW-HA in normal tissues.

Targeting LMW-HA

Blocking Hyaluronidase Activity

Natural derivatives (i.e., heparin, glycyrrhizic acid, and sodium aurothiomalate) and drugs (i.e., fenoprofen) targeting HYAL isoforms have been investigated as therapeutic agents (26, 85, 88). Originally described as urinary HYAL inhibitors, O-sulfated HA derivatives (sHA) exhibit potent inhibition of HYAL1 activity (IC_{50}: 0.0083–0.019 μM) through binding to allosteric sites (92, 93). Moreover, sHA exhibits more effective non-competitive than competitive inhibition, suggesting its efficacy would not be impeded by elevated HA concentration in tumor tissues. In a prostate cancer animal model, sHA-application inhibited HYAL1

activity and triggered apoptosis through the extrinsic pathway (70). The downregulation of HYAL1 correlated with reduced CD44 and RHAMM expression, and PI3K/AKT signaling, suggesting a feedback mechanism between HA degradation and signaling in tumor cells. The proliferative and invasive capacities of prostate cancer cells are also decreased significantly after sHA-treatment (69).

Conversely, increased degradation of stromal HA by PEGPH20 (a pegylated hyaluronidase) in pancreatic cancer degrades the HA capsule that accumulates around these tumor cells to allow better exposure of tumor cells to chemotherapy and release suppression of neo-angiogenesis (94). PEGPH20 efficacy in facilitating a response of pancreatic tumor cells to chemotherapy is currently being assessed in Phase 3 clinical trials for metastatic pancreatic ductal adenocarcinoma (ClinicalTrials.gov) (94). PEGPH20 administration in animal models of pancreatic cancer induced vascular collapse in tumors and dramatically elevated the interstitial fluid pressure, enhancing perfusion, and delivery of chemotherapeutic drugs into the solid tumor (71). Thus, combined administration of PEGPH20 and other chemotherapy (i,e: gemcitabine) promoted survival and reduced tumor growth in mouse models through inducing apoptosis and suppressing proliferation (71).

Sequestering HA Fragments

The use of peptides or small molecules that bind to the HA binding regions of HA receptors, such as CD44 and RHAMM, is another therapeutic approach that attempts to sequester LMW-HA, thus preventing these from interacting with receptors to abrogate signal activation. To date, extensive use of peptides to block tumorigenesis has not yet been reported. However, RHAMM- or HA-binding peptides can inhibit invasion and survival of prostate and breast cancer cells in culture and *in vivo* (95, 96). LMW-HA binding peptides have been isolated by screening phage display libraries (97, 98) and by designing RHAMM-like peptides that bind to LMW-HAs with nM affinity (95). These have been shown to block inflammation and fibrosis in both cell culture and animal models (95). Since some cancers originate from progenitor-like cancer-initiating cells, which commonly express CD44, small molecule inhibitors of CD44:HA binding are also being developed (99). However, polysaccharide:protein interactions in general and HA-CD44 binding in particular occur over large surfaces, making design of small molecule inhibitors challenging (95). The amounts of LMW-HAs that accumulate in the peri-tumor stroma is surprisingly small (**Figure 1D**). These low amounts predict the use of LMW-HA binding peptides may be one of the most effective approaches for targeting the tumorigenic properties tumor microenvironments.

HMW-HA, HYALURONIDASE, AND CD44 AS TUMOR PREVENTION MECHANISMS

HMW-HA can also suppress tumor initiation in cancer-prone species by inhibiting proliferation, migration/invasion and ROS-mediated DNA damage. HMW-HA blocks proliferation by signaling through CD44 to promote G1/G0 arrest (100) and, as noted above, alter tumor growth kinetics by suppressing neo-angiogenesis and immune responses (101). Consistent with these

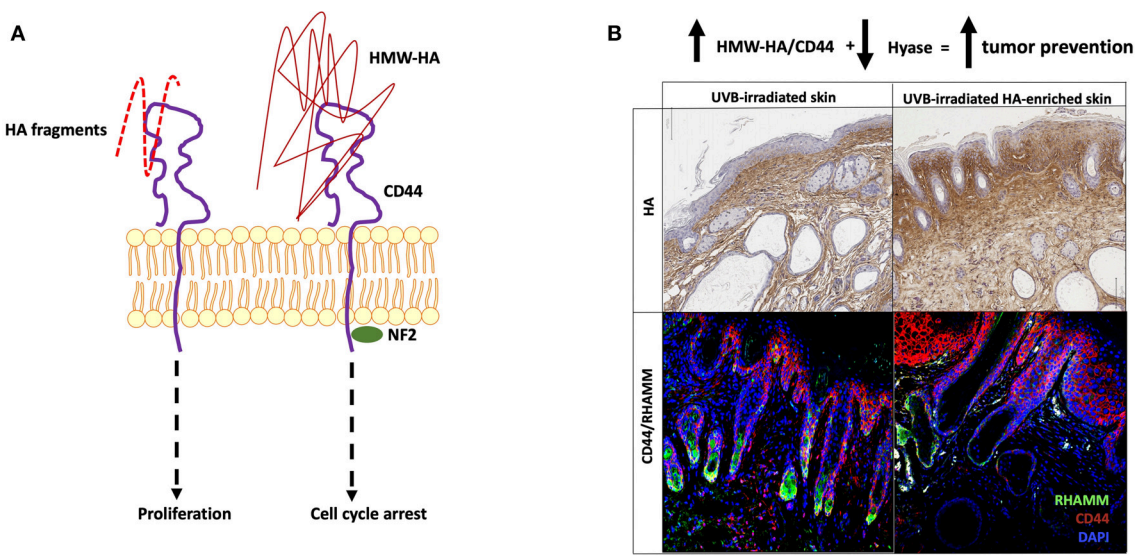

FIGURE 2 | HMW-HA functions in tumor prevention. **(A)** LMW-HA:CD44 interactions promote proliferation while HMW-HA:CD44 interactions suppress proliferation, which can be mediated by an association of CD44 with the tumor suppressor, Merlin (NF2). HMW-HA:CD44 signals result in cell cycle arrest and are one mechanism for reducing susceptibility to cancer. **(B)** Dorsal skin of UVB-exposed keratinocyte tumor-susceptible mice treated topically with HMW-hyaluronan-phosphatidylethanolamine (HA-enriched) do not form tumors, accumulate higher levels of HMW-HA (top panels) and CD44 (bottom panels), and lower levels of hyaluronidases than UVB-irradiated controls. Epidermal HA (brown) is detected via an HA-binding biotinylated protein on paraffin-embedded tissue counterstained with hematoxylin (blue). CD44 (red) and RHAMM (green) are detected using pan-antibodies in dorsal skin of mice counterstained with DAPI (blue).

reported functions, HMW-HA suppresses growth of murine astrocytoma cell lines, glioma and colon carcinoma xenografts. HMW-HA has also been reported to reduce the migratory and invasive capacity of aggressive cancer cells (23, 102), which could occur by several mechanisms. These include: (1) HMW-HA strengthens cell-cell junctions and decreases the permeability of ECM, thus preventing invasion (103); (2) HMW-HA promotes tight junction formation and myosin polymerization in lymphatics by displacing LMW-HA from the LYVE-1 receptor, which restricts invasion into lymph nodes (103); (3) HMW-HA blocks migration by enhancing NF2 and CD44 co-association, which activates the tumor suppressor properties of CD44 (24); and (4) HMW-HA modifies the motogenic effects of growth factor signaling. As an example, bFGF-treatment decreases sarcoma cell migration as a result of increased HAS1, HAS2, and CD44 expression combined with a reduced expression of HYAL2 (74), which favors accumulation of HMW-HA(55). Further, the addition of HMW-HA reduced fibrosarcoma cell migration, which can be overridden by exogenous LMW-HA or HAS1 knockdown (74). In addition to these growth- and migratory-suppressing effects, HMW-HA also reduces ROS-mediated DNA damage, which lessens mutational burden and thereby reduces the risk of neoplastic transformation (36, 104).

While the above studies predict functions of HMW-HA that could contribute to tumor resistance in cancer-prone species, such as mice and humans, the cancer-resistant naked mole-rat (*Heterocephalus glaber*) provided the first direct evidence for HMW-HA in suppressing carcinogen-induced tumorigenesis(24, 105). In *H. glaber*, the combination of HAS2 overexpression, strongly reduced hyaluronidase activity, and signaling through CD44 provides resistance to several carcinogenic insults, including UVB and activated RAS (24). Interestingly, HMW-HA accumulates in some tissues, notably skin, and remains high throughout the lifetime of this animal (24). In contrast, HMW-HA declines in skin and most tissues of human and mouse with age (106–110) and after chronic exposure to UVB, which is the predominant carcinogen causing keratinocyte tumors.

The naked mole-rat utilizes many tumor resistance mechanisms, including the HMW-HA-regulated mechanism termed "early contact inhibition" (ECI), a robust form of contact inhibition that involves expression of a novel isoform of $p16^{INK4A}$ (105). HA induces early expression at the $p16^{INK4A}$ locus through CD44 binding, resulting in blocked phosphorylation of Rb and attenuation of cell cycle. Naked mole-rat fibroblasts cultured in the presence of hyaluronidase display no ECI and downregulate $p16^{INK4A}$ expression (16). This effect of HMW-HA is mediated by CD44:NF2 signaling (24, 105) (**Figure 2A**).The growth-inhibiting non-phosphorylated form of NF2 is predominant in cultured naked mole-rat cells, but the addition of hyaluronidase stimulates NF2 phosphorylation, which promotes cell growth (24).

HMW-HA AS A CHEMOPREVENTION STRATEGY

To date, HMW-HA has not been used as a chemoprevention strategy, although oral consumption has been shown to restrict tissue inflammation, notably in the bowel (111). The barrier to topical application of HMW-HA has historically been its restricted passage through the outer cornified layer of the epidermis. However, the recent development of high molecular-weight hyaluronan-phosphatidylethanolamine polymers [E. Turley (2010), Patent number: *US20130059769A1*] that cross the epidermis and form coats around keratinocytes and dermal cells (112) will permit an assessment of whether or not increasing skin HMW-HA can reduce the predisposition of skin to tumorigenesis in cancer-susceptible species (**Figure 2B**).

CONCLUSIONS

In summary, LMW-HA augments the proliferative and migratory capacities of tumor cells, while HMW-HA reduces tumorigenicity and confers cancer resistance by restricting proliferation, limiting inflammation, neo-angiogenesis, and possibly DNA damage. Further research is required to harvest the full therapeutic potential of targeting LMW-HA polymers and utilizing the tumor resistance properties of HMW-HA. Improved understanding of the mechanisms augmenting the size-dependent biological effects of HA is likely to advance new therapeutic development to limit tumorigenesis.

AUTHOR CONTRIBUTIONS

ML provided the first draft of the manuscript, which was edited by ET and CT. CT and ML provided the data for figures. ET assembled all figures presented in this review.

ACKNOWLEDGMENTS

This work was supported by a Cancer Research Society Grant (#22491) to ET. ET and ML were supported by the Translational Breast Cancer Research Unit through the Breast Cancer Society of Canada. ML was also supported by the Interdisciplinary Development Initiative in Stem Cells and Regenerative Medicine Scholarship (R3170A15) through Western University.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 14 May 2019
doi: 10.3389/fimmu.2019.01068

L-selectin: A Major Regulator of Leukocyte Adhesion, Migration and Signaling

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L-selectin (CD62L) is a type-I transmembrane glycoprotein and cell adhesion molecule that is expressed on most circulating leukocytes. Since its identification in 1983, L-selectin has been extensively characterized as a tethering/rolling receptor. There is now mounting evidence in the literature to suggest that L-selectin plays a role in regulating monocyte protrusion during transendothelial migration (TEM). The N-terminal calcium-dependent (C-type) lectin domain of L-selectin interacts with numerous glycans, including sialyl Lewis X (sLe^X) for tethering/rolling and proteoglycans for TEM. Although the signals downstream of L-selectin-dependent adhesion are poorly understood, they will invariably involve the short 17 amino acid cytoplasmic tail. In this review we will detail the expression of L-selectin in different immune cell subsets, and its influence on cell behavior. We will list some of the diverse glycans known to support L-selectin-dependent adhesion, within luminal and abluminal regions of the vessel wall. We will describe how each domain within L-selectin contributes to adhesion, migration and signal transduction. A significant focus on the L-selectin cytoplasmic tail and its proposed contribution to signaling via the ezrin-radixin-moesin (ERM) family of proteins will be outlined. Finally, we will discuss how ectodomain shedding of L-selectin during monocyte TEM is essential for the establishment of front-back cell polarity, bestowing emigrated cells the capacity to chemotax toward sites of damage.

Keywords: L-selectin (CD62, SELL), migration, inflammation, signaling, adhesion, monocyte, lymphocyte, Neutrophil (PMN)

OPEN ACCESS

Edited by:
Toshiyuki Murai, Osaka University, Japan

Reviewed by:
Francis William Luscinskas, Harvard Medical School, United States
Ronen Alon, Weizmann Institute of Science, Israel

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *15 February 2019*
Accepted: *26 April 2019*
Published: *14 May 2019*

Citation:
Ivetic A, Hoskins Green HL and Hart SJ (2019) L-selectin: A Major Regulator of Leukocyte Adhesion, Migration and Signaling. Front. Immunol. 10:1068. doi: 10.3389/fimmu.2019.01068

L-SELECTIN GENE EXPRESSION

The human L-selectin gene (*sell*) is located on the long arm of chromosome 1 (1q24.2), and is arranged in tandem with its family members (in the order: L-, P-, and E-selectin). L-selectin consists of ten exons spanning a region of ∼21.0 kb. FOXO1 regulates transcription of the human *sell* gene (1, 2), whereas chromosome immunoprecipitation experiments identify other transcription factors (Mzf1, Klf2, Sp1, Ets1, and Irf1) in regulating the mouse *sell* gene (3). Splicing of the exons into mature mRNA is translated to form a protein product with a predicted molecular weight of 30 kDa. However, the actual molecular weight of L-selectin differs between cell types—ranging from 65 kDa in lymphocytes to 100 kDa in neutrophils and is due to cell type-specific glycosylation (4–10). It is highly likely that altered glycosylation patterns in L-selectin could dictate cell-specific functions, but this has not been explored in any detail. L-selectin is organized into distinct structural domains:

a ligand binding calcium-dependent (C-type) lectin domain (CTLD), an EGF-like domain, two complement-like repeat sequences and an extracellular cleavage site (**Figure 1A**).

Splice Variants of L-selectin

Splice variants of L-selectin have been identified and characterized in both mice (11) and humans (12). The mouse *sell* gene is composed of 9 exons. The two splice variants, termed L-selectin-v1 and L-selectin-v2, possess an additional exon, nested between exons 7 and 8. The splice variants share the first 49bp sequence, whereas L-selectin-v2 extends for an extra 51bp that is immediately 3' to this region. Both splice variants possess longer cytoplasmic tails (WT = 17 aa; v1 = 30 aa; v2 = 32aa—see **Figure 1B**). The overall mRNA levels of L-selectin-v1 and -v2 constitute 2–3% of the total L-selectin mRNA, so its impact in endogenous leukocyte trafficking and signaling is not fully understood. However, over-expression of these variants in cells lacking L-selectin reveal altered capacities in adhesion to sLe^x under flow, ectodomain shedding in response to cellular activation, and signaling to p38 MAPK following antibody-mediated clustering (AMC) (11).

The human splice variant lacks exon 7, which codes for the transmembrane domain (12), so transcripts lacking this exon are secreted and soluble. Patients with rheumatic disease presented an increased prevalence of the splice variant transcript, which is thought to contribute to the increase in soluble L-selectin. Based on the relatively low abundance of the variant transcript, it is currently not clear what proportion of circulating soluble L-selectin is represented by either the cleaved form (e.g., through "basal shedding"—see later) or the spliced transmembrane-less forms.

REGULATION OF L-SELECTIN PROTEIN EXPRESSION IN DIVERGENT LEUKOCYTE SUBSETS

The identification of human neonatal bone marrow $CD10^-CD62L^{hi}$ cells reveals that L-selectin is one of the earliest surface markers to be expressed on lymphoid-primed hematopoietic stem cells (13). Similar observations have been made in mice, suggesting L-selectin plays a pivotal role in stem cell trafficking and differentiation (14, 15). $CD10^-CD62L^{hi}$ cells can differentiate into B cells, dendritic cells, monocytes, NK cells and T cells. It is likely that the L-selectin expressed in these early progenitor cells is required for trafficking from the bone marrow toward peripheral lymphoid organs. Whether L-selectin-dependent signaling also contributes to subsequent differentiation is not known.

The average leukocyte will express ∼50,000–70,000 molecules of L-selectin at the plasma membrane (16, 17). Numerous reports have shown that L-selectin is anchored on finger-like projections called microvilli, which increases tethering efficiency during recruitment (18–20). Protein expression of L-selectin is constitutive on most circulating leukocytes, and is slowly turned over at the plasma membrane through a process of ectodomain shedding (commonly referred to as "shedding"). A variety of artificial or physiological agonists of cell activation can, within minutes, promote robust L-selectin shedding in numerous leukocyte sub-types (21–24). The zinc-dependent metalloproteinase, a disintegrin and metalloproteinase (ADAM)17, is the major enzyme responsible for L-selectin shedding in leukocytes (25–28). However, ADAMs 8 and 10 have also been reported to cleave L-selectin in specific settings (29, 30). From a clinical perspective, the soluble circulating form of L-selectin (released as a consequence of ectodomain shedding) is sometimes used as a surrogate plasma/serum biomarker for leukocyte activity triggered during acute or chronic inflammation (31–35). Similarly, leukocytes expressing low levels of surface L-selectin (judged by flow cytometric analysis) are a classic indicator of cellular activation (24, 34). Paradoxically, a drop in soluble L-selectin can also be detected in certain diseases, such as sepsis (36). The drop in detectable soluble L-selectin could be due to its adsorption to luminal vascular ligands that are upregulated during sepsis. Alternatively, L-selectin could be cleaved from transmigrated neutrophils within abluminal/non-luminal regions of vessels (see later). Although soluble L-selectin can compete for cell-associated L-selectin, little is understood about how the two forms buffer leukocyte recruitment during inflammation. Soluble L-selectin is detected in the plasma of healthy humans (0.7-−1.5 μg per mL), suggesting cell-associated L-selectin is cleaved from resting circulating leukocytes at low levels. Indeed, mouse neutrophils lacking ADAM17 express higher than average surface levels of L-selectin (26, 37). This phenomenon is also observed when broad-spectrum synthetic inhibitors of ADAM17 are used, or when the cleavage site of L-selectin is mutated and rendered "sheddase-resistant" (38–40). The manner in which L-selectin expression is regulated, either at the translational or post-translational level, will be unique in different leukocyte subsets. For example, the lifespan of a central memory T-cell (T^{CM}) far exceeds the lifespan of a neutrophil (i.e., 6 weeks compared to 8 days, respectively) (41, 42). Aging neutrophils possess waning levels of L-selectin, which contrasts with T^{CM} (43–45). The sections below provide a few examples of how L-selectin is expressed in different leukocyte subsets and the impact that this might have on immune cell behavior.

The Impact of L-selectin Expression in Lymphocytes

Lymphocytes constitute ∼78–88% of the total white blood cell count in mice. These values are quite different in humans (20–40%). L-selectin-deficient mice expose the absolute requirement for L-selectin in the trafficking of naïve T-cells to lymph nodes (46). Immunofluorescence imaging of frozen mouse lymph node sections reveals that L-selectin is cyclically expressed in recirculating naïve T-cells as they adhere to and extravasate across high endothelial cell (HEC) monolayers and enter the lymph node parenchyma (47). These observations would suggest ectodomain shedding is triggered during adhesion and TEM, and that re-expression of L-selectin occurs upon their entry into the lymphatics, via the thoracic duct. Increasing L-selectin expression in naïve T-cells does not enhance homing of T-cells

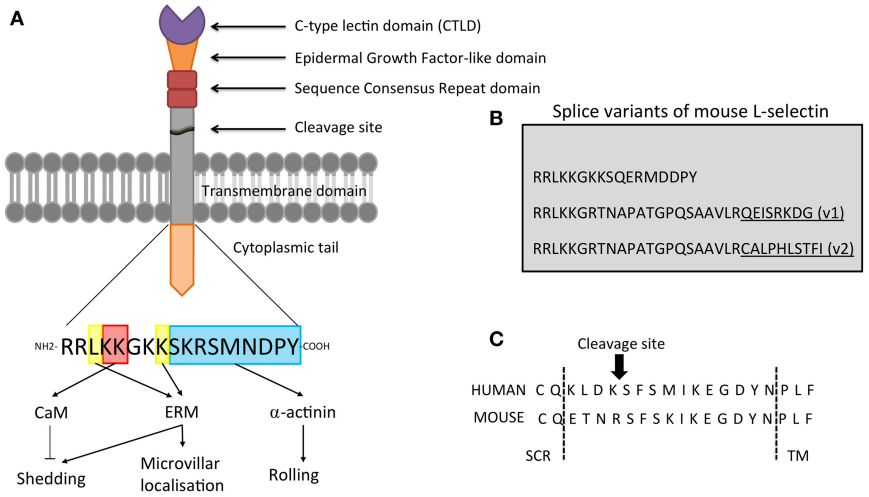

FIGURE 1 | Domain organization of L-selectin. **(A)** L-selectin is a type I transmembrane glycoprotein. Going from N-terminal to C-terminal, it is broken down into: C-type lectin domain (CTLD), Epidermal Growth Factor (EGF)-like domain, two sequence consensus repeat (SCR) domains, a cleavage site, transmembrane domain and a 17 amino acid cytoplasmic tail. Amino acid sequence of the cytoplasmic tail of human L-selectin is depicted, highlighting the amino acids that support binding to calmodulin (CaM), ERM proteins, and alpha-actinin. **(B)** The three sequences correspond to the mouse L-selectin tail (note the mouse L-selectin tail has a single serine at position 364, whereas the tail of human L-selectin possesses an extra serine residue at position 367). Sequence conservation at the membrane-proximal region that support binding to ERM and CaM (RRLKKG) is 100% conserved. The amino acid sequences of two splice variants of mouse L-selectin (v1 and v2) are provided in the sequences below. Underlined residues represent sequences that are unique to v1 and v2. **(C)** Amino acid sequences surrounding the cleavage site of human and mouse L-selectin. Arrow indicates the position of cleavage. TM, transmembrane domain; SCR, sequence consensus repeat region.

to lymph nodes, suggesting that L-selectin densities can reach saturation within specific plasma membrane domains (e.g., on microvillar tips) (48).

Signaling downstream of the T-cell receptor triggers the activation of PI3Kδ, which leads to ectodomain shedding and concomitant inhibition of Klf2-dependent expression of L-selectin in mouse T-cells (49). The terminal differentiation of naïve T-cells into effector memory T-cells (T^{EM}) involves both L-selectin shedding and the transcriptional shutdown of the L-selectin gene. Collectively, these events drive the trafficking of T^{EM} away from secondary lymphoid organs toward peripheral tissues (39, 40). T^{CM} are smaller in size and have longer telomere lengths than T^{EM}, but it is not clear if the respective presence or absence of L-selectin expression contributes to the phenotype (45). Expression of L-selectin in T^{CM} is essential for trafficking toward, and the immune surveillance of, peripheral lymph nodes (39, 40).

Regulatory T-cells (Tregs) play important immunosuppressive roles in diverse immune responses in health and disease [e.g., graft-vs.-host disease (50)]. Tregs are characterized by the presence of the transcription factor FoxP3, which can be further subdivided according to the expression of surface epitopes, including L-selectin. Tregs expressing L-selectin have been shown to have superior immunosuppressive roles in different disease models (51–54). Again, it is not clear if L-selectin-dependent trafficking and/or signaling contributes to improved immunosuppressive function.

The surface level of L-selectin is significantly reduced in naïve CD4 T-cells following HIV entry (55–59). The HIV accessory protein, Vpr, can increase L-selectin mRNA expression in primary human naïve T-cells and Jurkat T-cells, but its impact on the pathobiology of HIV is unclear (59). In contrast, the other accessory proteins, Nef and Vpu, can sequester L-selectin into subcellular compartments and decrease cell surface expression (57, 60). Such post-translational mechanisms of reducing L-selectin surface expression by HIV is thought to prevent trafficking back to secondary lymphoid organs, redirecting infected T-cells to the systemic circulation to increase viremia. L-selectin expression on naive CD4 T-cells has recently been defined as a novel receptor for HIV entry (58), and work from this study reveals that L-selectin shedding ensues 4–6 days post-infection. Moreover, HIV preferentially infects T^{CM} over T^{EM} cells – positively correlating with L-selectin expression. Blocking ectodomain shedding of L-selectin with (broad-spectrum) synthetic inhibitors to ADAM17 (BB-94, TAPI-1, TAPI-2) does not increase HIV attachment or entry, but reduces the release of viral particles (58). These findings suggest that blocking ectodomain shedding of L-selectin may alter membrane/cytoskeleton dynamics that are dependent on the release of HIV particles from infected T-cells.

One study has recently shown that increasing L-selectin expression in cytotoxic CD8 T-cells is causal to viral clearance (61). L-selectin expression is rapidly lost when antigen-presenting cells prime naïve recirculating CD8 T-cells in lymph nodes. Re-expression of L-selectin is detected when antigen-primed CD8 T-cells egress the lymph node, which is now known to be essential for trafficking toward visceral or mucosal virus-infected organs. Bestowing antigen-primed CD8 T-cells with higher levels of L-selectin, by genetically rendering the molecule non-cleavable, increases clearance of viral-infected sites without obvious signs of altering cytokine secretion profiles or clonal expansion. Interestingly, L-selectin shedding in tumor

antigen-primed human CD8 T^{CM} cells inversely correlates with the upregulation of the degranulation marker, CD107a, and enhanced tumor lytic activity (62). It is not exactly clear how L-selectin contributes to tumor or virus killing, and whether conserved mechanisms exist between mice and humans. Previous studies have shown that L-selectin clustering can augment T-cell receptor signaling, suggesting roles beyond just trafficking (63, 64).

Although L-selectin can act as a co-stimulator of specific leukocytic responses, it is much harder to model this experimentally without the prior stimulation of L-selectin—e.g., via AMC. In this regard, AMC of L-selectin on mouse splenic T-cells (CD4 and CD8) and B-cells can increase their responsiveness to the chemokine CCL21, via CCR7 (65). Interestingly, the density of surface-expressed L-selectin within different T-cell subsets (i.e. $CD62L^{lo}CD44^{hi}$ [T^{EM}] or $CD62L^{hi}CD44^{lo}$ [T^{CM}] subsets within CD4 cells, or CD8 cells) positively correlated with enhanced chemotaxis toward CCL21. This model suggests that clustering of L-selectin by endothelial expressed ligands (e.g., within HEV) can facilitate the transmigration of T-cells into lymph nodes. Spleen tyrosine kinase (Syk) was shown in this study to have *in vivo* importance in this mechanism.

Manipulating cell surface levels of L-selectin on leukocytes can impact the pathogenesis of atherosclerosis. Mice lacking L-selectin will develop accelerated atherosclerosis (66), suggesting that L-selectin-dependent trafficking to atherosclerotic lesions is somehow protective. Flow cytometric phenotyping of immune cell infiltrates within aortae of $CD62L^{+/+}ApoE^{-/-}$ and $CD62L^{-/-}ApoE^{-/-}$ mice, revealed that two B-cell subsets, B1a and Bregs, provided an anti-inflammatory role in the pathogenesis of atherosclerosis (67). As atherosclerotic lesions mature, the microvascular network of the vasa vasorum can innervate into late developing lesions – acting as a portal for increased leukocyte trafficking (via "the back door") into lesions (68). Moreover, the increased microvascular density within late developing regions is deemed a predicator for unstable plaque rupture (69, 70). Endothelial cells lining the microvessels within these late lesions were shown to upregulate P-selectin proteoglycan ligand-1 (PSGL-1), a known ligand for L-selectin (68). The data, collectively, suggests that L-selectin-dependent recruitment of leukocytes into early atherosclerotic lesions is protective, but, as the disease progresses, L-selectin-dependent recruitment of leukocytes can be pathogenic.

Regulation of L-selectin Expression in Neutrophils

Human neutrophils represent 50–70% of the total white blood cell count, which contrasts with values of 10–25% in mice (71). L-selectin regulates neutrophil trafficking to sites of inflammation, but our understanding of the intracellular mechanisms underpinning this process is still incomplete. The first *in vitro* transmigration experiments involved the addition of primary human neutrophil suspensions onto cytokine-activated monolayers of human umbilical vein endothelial cells (HUVEC). In such assays, L-selectin is rapidly cleared from the neutrophil plasma membrane through ectodomain shedding (72). L-selectin shedding is a classic readout for neutrophil activation and/or "priming" (i.e., partial activation), which is concordant with an upregulation in CD11b expression (αMβ2 or Mac-1) (24). Blocking L-selectin shedding in human neutrophils with broad-spectrum inhibitors for ADAM17 (e.g. Ro-31- 9790, KD-IX-73-3, TAPI-0, TAPI-1, TMI005, and GM6001) does not impact rolling velocity or the kinetics of TEM (72), which is contrary to what has been observed in mice (73).

AMC of L-selectin on human neutrophils can result in its own ectodomain shedding (74), acting as a self-limiting mechanism by which L-selectin can behave as a cell adhesion molecule and signaling receptor. Prolonged perfusion of neutrophils over immobilized sLe^x can increase rolling velocity over time, which is directly due to L-selectin shedding. The activation of p38 MAPK drives L-selectin shedding during rolling, and the term "mechanical shedding" was coined to explain this phenomenon (75). Mechanical shedding has only been characterized in human neutrophils rolling on sLe^x, but blocking L-selectin shedding in mice with KD-IX-73-3 also reduces neutrophil rolling velocities (73, 76). These studies collectively indicate that mechanical shedding could have *in vivo* significance. However, it should be noted that L-selectin on human neutrophils is decorated itself with sLe^x, acting as a ligand for E-selectin (8, 9), so this would invariably lead to altered mechanisms of L-selectin-dependent recruitment of human neutrophils (see later).

A recent study has challenged the view that L-selectin shedding is a classic outcome of neutrophil transmigration (77). Neutrophils harvested from synovial fluid of arthritic patients registered L-selectin positive and showed little signs of priming. L-selectin shedding is therefore not a strict prerequisite for neutrophil emigration into inflamed tissue. Interestingly, neutrophils aspirated from the fluid of acutely formed skin blisters were shown to be primed (e.g., CD11b-positive) and L-selectin-negative. These observations suggest that the mechanisms of neutrophil migration toward acute or chronic inflammation could differ in the expression and turnover of adhesion molecules. Indeed, chemokine receptor expression on the neutrophils and the array of cytokines and chemokines in synovial vs. blister fluid microenvironments appear to be radically different (77).

L-selectin-null mice display significant reductions in neutrophil recruitment to a 4 h model of thioglycollate-induced peritonitis (46). Monitoring the rolling flux of neutrophils in post capillary venules of exteriorised cremasteric muscle (induced by surgical trauma) showed no differences between L-selectin-null mice and WT controls at early time points. However, a significant reduction in neutrophil rolling flux of L-selectin-deficient neutrophils was detected after 50 min of cremaster exteriorisation. Superfusing exteriorised cremasteric tissue with platelet activating factor (PAF), a potent neutrophil chemoattractant, showed only modest reductions in the rolling flux of L-selectin-null neutrophils (78). This observation supports the idea that L-selectin ligands might be absent in endothelial cells lining cremasteric post-capillary venules (79). Although no difference in neutrophil adhesion to post-capillary venules of either WT or L-selectin-null mice was

observed, neutrophil emigration from post-capillary venules was significantly reduced in neutrophils lacking L-selectin. Moreover, interstitial chemotaxis toward a gradient of the chemokine CXCL1 (or Keratinocyte-derived Chemoattractant (KC)—another potent neutrophil chemoattractant) was severely impaired in the small proportion of emigrated neutrophils (78). These findings led to the hypothesis that L-selectin may regulate neutrophil chemotaxis, suggesting extended roles beyond tethering and rolling. Neither of these studies explored whether L-selectin was shed in response to PAF superfusion or chemotaxis toward KC. Interestingly, subjecting neutrophils that express non-cleavable L-selectin to a model of KC-induced interstitial chemotaxis phenocopied the behavior witnessed in neutrophils lacking L-selectin. These results seem counterintuitive, but suggest that the membrane-retained fragment (MRF - i.e., the "stump" of L-selectin retained after shedding) may be responsible for guiding neutrophil interstitial chemotaxis, as the MRF can only be processed from WT L-selectin.

The migration of circulating neutrophils into the inflamed peritoneum results in a significant reduction in L-selectin expression, matching what has been observed *in vitro* with human neutrophils crossing activated HUVEC monolayers (72). Knock-out studies reveal that this mechanism is through ectodomain shedding via ADAM17 (26). Mouse neutrophils lacking ADAM17 express 10 times more surface L-selectin, but will still lose a significant amount of this when entering the peritoneum (26). Whether the observed loss is through the action of a related ADAMs protease member (e.g., ADAM10 or ADAM8) (29, 30) or through the loss of membrane fragments (e.g., exosomes) has not been fully determined. The higher L-selectin expression in ADAM17-null neutrophils drives slower rolling velocities in inflamed cremasteric post-capillary venules.

Neutrophil aging in the circulation leads to the progressive increase in CXCR4 expression, with a corresponding decrease in L-selectin expression (80). An increase in CXCR4 expression is required for homing of aged neutrophils to the bone marrow for macrophage-mediated clearance (81). However, it is not clear if the reduced L-selectin expression is causal to, or an epiphenomenon of, neutrophil aging. Furthermore, it is not clear if the reduction in L-selectin expression is at the translational or post-translational level (e.g., increased basal shedding or exosome-like mediated release).

The impact of L-selectin expression on neutrophil behavior has been extensively reviewed elsewhere (82).

L-selectin in Monocytes

Monocytes constitute ∼2–8% of all circulating white blood cells in the adult human, which contrasts with 0.9–1.5% in mice. Within the human population, a further three monocyte subsets are characterized according to the relative abundance of two surface markers: $CD14^{++}$ $CD16^{-}$ or "classical" (80–95%), $CD14^{++}$ $CD16^{+}$ or "intermediate" (2–11%), and $CD14^{+}$ $CD16^{++}$ or "non-classical" (2–8%). Classical monocytes express high levels of L-selectin, which is in stark contrast to the lesser abundant intermediate and non-classical monocytes (83).

L-selectin mediates the recruitment of human monocytes to activated endothelial monolayers under flow conditions (84, 85). Subsequent experiments showed that L-selectin/PSGL-1 interactions in trans are responsible for the adhesion of monocytes through the process of secondary tethering and rolling (i.e., the interaction of bystander leukocytes with already adherent leukocytes) (86). Imaging of L-selectin on classical monocytes crossing TNF-α-activated endothelial monolayers shows that L-selectin shedding is triggered specifically during TEM and not during earlier phases of the multi-step adhesion cascade (87). These observations challenge current perceptions of L-selectin shedding occurring during the phase of "firm adhesion" in the multi-step adhesion cascade. Two recent articles suggest L-selectin plays a role in regulating monocyte pseudopod protrusion during TEM (87, 88). Interestingly, expression of L-selectin in monocyte-like THP-1 cells (which do not express endogenous L-selectin) bestows them with a more invasive phenotype (i.e., possessing a higher propensity to protrude through inflamed endothelial monolayers) (87, 88).

The secreted mucin AgC10, released from the parasite *Trypanosoma cruzi*, can bind to L-selectin on human monocytes, which over a 4–6 h period can induce a reduction in L-selectin expression via ectodomain shedding (89). Consequently, monocytes treated with AgC10 have a reduced capacity to interact with activated endothelial monolayers under shear stress conditions.

The equivalent of classical monocytes in mice is characterized by: $CX3CR1^{low}$, $CCR2^{+}$, $Ly6C^{+}$, and $CD26L^{+}$. On the other hand, "patrolling monocytes" are considered to be the mouse equivalent of non-classical monocytes ($CX3CR1^{hi}$, $CCR2^{-}$, $Ly6C^{-}$, $CD62L^{-}$), which will typically remain in the circulation and patrol the endothelium for infection and/or damage (90). In both human and mice, the classic monocytes share L-selectin and CCR2 expression. It would be interesting to test if the expression of L-selectin in patrolling monocytes would bestow a more pro-invasive phenotype.

Invading human cytotrophoblasts, which are tasked to bridge a gap between the placenta and the uterus, are thought to employ L-selectin to mediate interstitial migration (91, 92). These findings further support extended roles for L-selectin beyond classic leukocyte tethering and rolling—moreover within microenvironments that are devoid of hydrodynamic shear stress.

Using a mouse model of thioglycollate-induced peritonitis, researchers showed that the turnover of L-selectin is radically different between monocytes and neutrophils: L-selectin expression drops in emigrated neutrophils, but remains unchanged in monocytes (26). The differences underpinning these L-selectin dependent and independent modes of leukocyte emigration are not understood, nor are their relevance to human monocyte TEM (87, 88).

The exposure of isolated human monocytes to selenium promotes metalloproteinase-dependent shedding of L-selectin (93), leading to reduced adhesion on immobilized ligand under flow conditions. Furthermore, the observed shedding could be reversed using synthetic inhibitors of ADAM17 (i.e., GM6001). Administering selenium to mice led to a significant increase in soluble circulating L-selectin. Reducing agents such as dithiothreitol have been shown to increase ADAM17 activity

(94), but whether the anti-oxidant properties of selenium could be directly/indirectly linked to reducing cysteines on ADAM17 has not been explored.

DOMAIN ORGANIZATION OF L-SELECTIN

L-selectin shares a number of common extracellular domains with its family members, E-, and P-selectin (**Figure 1A**). In contrast, the cytoplasmic tails of the selectin family members bear no resemblance to one another and likely transduce unique intracellular signals. In this section we will discuss the roles that each domain plays with respect to adhesion, signaling, and ectodomain shedding.

CTLD and the EGF-Like Domain

L-selectin possesses an N-terminal CTLD (95), which belongs to a large superfamily of metazoan proteins with diverse functions (96, 97). The CTLD of L-selectin directly regulates leukocyte tethering and rolling by interacting with the minimal determinant tetrasaccharide, sLe^x (98). A unique feature of the selectins is to stabilize bond lifetimes through conformational changes in the CTLD that occurs in response to an external force, such as hydrodynamic shear stress. Mutagenesis of E-selectin showed the existence of coordinate bonds between Ca^{2+} and amino acid residues within the upper face of the CTLD stabilizes the interaction (99, 100). Note that these residues are also common to L-selectin. The 3- and 4-hydroxyl groups of fucose in sLex also form a coordinate bond to Ca^{2+}, which collectively stabilize selectin/ligand binding (101). Binding of L-selectin to sLe^x occurs within a critical threshold of shear stress (typically between 0.3 and 1.0 dynes per cm^2). L-selectin binding obeys a 'catch' and 'slip' bond mechanism, where optimal shear stress conditions can expose more of the ligand-binding domain and increase adhesiveness. The bond 'slips' as the tethered cell maneuvers over the ligand-bound site, where an increase in tensile force eventually exceeds the threshold of the catch-bond (102). The catch-slip bond phenomenon is described in more detail elsewhere, elaborating on the existence of a triphasic (slip-catch-slip) behavior of binding (103–105).

X-ray crystallographic evidence of the selectins reveals that the CTLD folds onto a region of the EGF-like domain, linked by a hydrogen bond between Y37 and N138 (101, 106). This inter-domain interaction constrains L-selectin into a less adhesive conformation whilst in the circulation. The functional significance of this interaction was characterized in neutrophils, where uncoupling the Y37/N138 hydrogen bond increases the bond lifetime, which manifests in increased neutrophil priming within the circulation (107). Neutrophils bearing an N138G knock-in mutation within L-selectin displayed enhanced bacterial killing and worsened outcomes in models of sterile injury. Both of these phenotypes were directly linked to the increased priming of neutrophils, confirming causality to the knock-in mutation. It has been suggested that this mode of "mechanochemistry" exists in other immune cells that express L-selectin (107).

SCR Domains

All three selectin family members possess a varying number of SCRs (in humans L-selectin has 2, E-selectin has 6, and P-selectin has 9), which bear homology to complement regulatory proteins. SCRs are also termed "sushi domains" and are present in a number of different cell adhesion molecules (108). In respect of the selectins, the SCR serves to distance the CTLD from the plasma membrane, placing it in a strategically advantageous position, reaching out beyond other cell adhesion molecules, to support tethering and rolling behavior. L-selectin is thought to have fewer than its family members as it is anchored to microvilli, which bestows advantageous positioning for tethering under flow.

Cleavage Domain

L-selectin undergoes ectodomain shedding at a specific membrane-proximal location, eleven amino acids above the transmembrane domain—between positions K321 and S322 (109) (see **Figure 1C**). Alanine scanning mutations surrounding the cleavage site would suggest that there is redundancy in ADAM17 recognition of the cleavage site. Deletion of multiple amino acids suggests that the actual distance of the cleavage site from the plasma membrane is more important (110). Studies in ADAM17 knock-out mice reveal the accumulation of L-selectin on the surface of neutrophils and monocytes, strongly suggesting that both the turnover and induced ectodomain shedding is mediated by a similar enzyme. Due to the relaxed specificity of the L-selectin cleavage site, it is difficult to definitively state that both basal and activated shedding target K321/S322. Binding of calmodulin to the L-selectin cytoplasmic domain is thought to regulate the conformation of the cleavage site (see later), where binding confers protection from shedding and dissociation renders susceptibility to proteolytic attack (111).

Transmembrane Domain

Swapping the transmembrane domain of L-selectin with that of CD44 has been shown to alter the subcellular location away from microvilli and toward the cell body, suggesting that amino acids within this region are responsible for the anchoring L-selectin to microvilli (112, 113). It's currently not understood what the amino acids within the transmembrane domain might interact with to retain L-selectin in microvilli, but intramembrane proteases [such as presenilins (114)] could be involved. For example, CD44 is a known target of γ-secretase (115–117), the active site of which is resident within the plasma membrane. It's currently not known if γ-secretases can bind to CD44 without cleaving it and whether its interaction could influence its subcellular localization on the plasma membrane. More subtle mutations within the L-selectin tail, which have been shown to abrogate ERM binding, can phenocopy the microvillar displacement (20).

Cytoplasmic Tail

The tail of L-selectin is only 17 amino acids long, yet it has been documented to bind up to 6 intracellular proteins, which include: alpha-actinin (118), calmodulin (111), ezrin, moesin

(119), protein kinase C (PKC) isozymes (120) and μ1alpha/AP-1 (121). Given the size of the L-selectin tail, not all of these proteins can bind simultaneously, but are likely to interact under tight spatio-temporal constraints—e.g., during tethering, rolling, firm adhesion, and TEM. More of this will be discussed in the section below.

Deleting the C-terminal 16 amino acids of the L-selectin tail can dramatically impact its lateral mobility along the plane of the plasma membrane, brought about by a lack of anchoring to the underlying cortical actin-based cytoskeleton (122). Such large truncations can disrupt tethering dynamics under flow conditions (123) and reduces the efficiency of L-selectin shedding (38, 110, 124).

The L-selectin tail is highly basic, with a pI of approximately 11.0, increasing its attraction toward negatively charged phospholipids within the inner leaflet of the plasma membrane. Recent evidence, both *in vitro* and *in silico*, support the view that the tail of L-selectin folds onto the inner leaflet of the plasma membrane to form an "L-shaped configuration" (125–127). Binding of ERM proteins to the tail of L-selectin is thought to desorb the tail from the inner leaflet of the plasma membrane and render it competent for calmodulin binding (126).

BINDING PARTNERS OF THE L-SELECTIN TAIL

Numerous proteins have been shown to co-precipitate with L-selectin in biochemical experiments (mainly through standard immunoprecipitation or "pull-down" techniques). These observations do not imply direct binding, but may represent proteins belonging to higher-ordered complexes, and would require further validation by interdisciplinary means. As mentioned above, the cytosolic tail of L-selectin is highly basic and can attract false-positive binders from whole cell lysates. Experiments using the L-selectin tail peptide should undertake a "pre-clearing" step, where the exposure of the cell lysate to a scrambled form will attract false binders based on charge without sequence specificity. The sections immediately below list known and validated binding partners of the L-selectin tail.

Alpha-Actinin

Alpha-actinin is a classic actin filament cross-linking protein (128). There are four isoforms of alpha-actinin (1 to 4), each expressed from a different gene. Isoforms 1 and 4 are expressed in non-muscle cells and have molecular weights of approximately 100 kDa on polyacrylamide gels. In contrast, isoforms 2 and 3 are expressed in skeletal and cardiac muscle cells. Solid phase binding experiments between smooth muscle-purified alpha-actinin (specifically from chicken gizzard) and L-selectin peptide revealed that the interaction was specific, and further confirmed by immunoprecipitation (118). Deletion of the C-terminal 11 amino acids of L-selectin (called 'LΔCyto') abrogates interaction with alpha actinin binding (118). Injection of cell lines expressing LΔCyto L-selectin into the circulation of rats revealed a significant reduction in rolling efficiency within inflamed mesenteric venules (129). These studies strongly imply the requirement for the cytoplasmic tail of L-selectin in regulating rolling interactions. However, little is known about how alpha-actinin interacts with L-selectin and whether the interaction is regulated by (serine/tyrosine) phosphorylation of the L-selectin tail or the production of secondary messengers (e.g., Ca^{2+}). Isoforms 1 and 4 of alpha-actinin possess CaM-like EF hands, which can bind Ca^{2+} and inhibit actin cross-linking activity (128). Interestingly, isoforms 1 and 4 expressed in smooth muscle cells (as in chicken gizzard) exist as EF domain splice variants that are Ca^{2+}-insensitive (128). As Ca^{2+}-sensitive and insensitive forms of alpha-actinin were likely used by Pavalko et al. (118), it is currently unclear if Ca^{2+} binding to alpha-actinin might play an active role in regulating L-selectin binding. Despite its conserved identity with CaM, the EF-hand of alpha-actinin is not believed to interact with L-selectin as the amino acids that support binding of CaM and alpha-actinin are located at opposite ends of the L-selectin tail (130) (see **Figure 1A**). Non-muscle alpha-actinin is known to bind to other tails of cell adhesion molecules and is reviewed extensively elsewhere (131).

Calmodulin (CaM)

CaM is an 18 kDa calcium binding protein, which was first identified to bind L-selectin in immunoprecipitation experiments, and subsequently confirmed using *in vitro* solid-phase binding assays (109). CaM binds constitutively to L-selectin in resting cells. Its interaction is thought to render the membrane-proximal cleavage site resistant to proteolytic attack. Upon cell stimulation, CaM dissociates to promote an allosteric change in the cleavage site to drive ectodomain shedding by ADAM17. Serine 364, but not S367, on human L-selectin has been shown to be responsible for the dissociation of CaM from L-selectin (87). Indeed, mutation of S364 to alanine significantly reduces phorbol myristate acetate (PMA)-induced shedding (132). Biophysical experiments show contradictory models for how CaM interacts with L-selectin. One report suggests that CaM binds to both a membrane-proximal region of the L-selectin tail, and a region within the transmembrane domain (133). This arrangement of binding was shown to be calcium-dependent and is thought to act in a ratchet-like mechanism, bringing the cleavage site close to the plasma membrane and displacing some of the transmembrane domain into the cytosol. However, this hypothetical view is deemed thermodynamically unfavorable. Another report used lipid bilayers to address a similar question and found that CaM binds to L-selectin in an "extended conformation," leaving one domain available for interacting with other binding partners. Biochemical and molecular modeling analyses confirmed that it is possible for the tail of L-selectin accommodate CaM and another protein (Ezrin-Radixin-Moesin—see section below). These experiments corroborated with the aforementioned biophysical data that hypothesized an extended form of CaM could accommodate ERM (134). Some suggestions of how different binding partners could be configured during different stages of the multi-step adhesion cascade is described elsewhere (135). It is still not clear whether CaM binding to the L-selectin tail occurs in a calcium-dependent or independent manner, and experiments have been able to support both views (130, 134).

Ezrin-Radixin-Moesin (ERM) Proteins

The ERM proteins are a 3-member family of cytoskeletal proteins ranging from 75 to 80 kDa. They all contain very similar domains—a globular N-terminal domain, belonging to the band 4.1 ezrin-radixin-moesin (FERM) superfamily, a central alpha-helical domain and an acidic actin-binding domain (see **Figure 2A**). The primary role of ERM is to serve as membrane cytoskeleton linkers, however it is becoming increasingly apparent that they play essential roles in mediating signal transduction. In respect of cell adhesion molecules, such as L-selectin, ERM binding restricts their lateral mobility across the plane of the plasma membrane. Anchoring cell adhesion molecules to an underlying cortical actin framework would facilitate receptor clustering and support tethering under flow conditions. Over-expression of L-selectin in fibroblasts can drive the formation of filopodia-like extensions (134). Indeed, ERM proteins are actively involved in forming microvilli (143, 144). ERM adopt an autoinhibited folded conformation when inactive and become "open" when in contact with phosphatidylinositol 4,5-bisphosphate (88, 145–148). Phosphorylation of a conserved threonine residue stabilizes the open conformation (149), which can be targeted by a number of serine/threonine kinases (150–154). Dephosphorylation is triggered by chemokine stimulation and Rac activity in T-cells (140–142) (see **Figure 2B**), however the mechanism remains poorly understood. Moreover, chemokine induced stimulation of T-cells leads to microvillar collapse that is thought to increase membrane contact between T-cells and endothelial cells.

ERM proteins can also act as adaptors of signal transduction via a number of different pathways (145, 155). In immune cells, ERM possess a cryptic immunoreceptor tyrosine-based activation motif (ITAM) nested within the FERM domain (156–158) (**Figure 2A**). Studies have shown that the cryptic ITAM can recruit Syk, which in turn can impact numerous downstream pathways—including the activation of integrins toward an "intermediate" state (159, 160). Ezrin is unique from moesin in that it possesses a tyrosine at position 353 (138), which, when phosphorylated by Src, can recruit the p85 regulatory subunit of PI3K. The conversion of phosphatidylinositol (4, 5)-bisphosphate (PIP2) to phosphatidylinositol (3–5)-trisphosphate (PIP3) is essential for the recruitment for guanine nucleotide exchange factors (GEFs—e.g., Vav), which serve to locally activate the Rho family of RhoGTPases, such as Rac (see later in **Figure 4A**).

The ERM proteins were first shown to interact with the L-selectin tail using affinity chromatography, where the 17 amino acid tail was synthesized and immobilized onto sepharose beads (119). Clarified extracts, derived from T-cells stimulated with or without PMA, were passed through these affinity columns to identify novel binders in activated and resting cells. Interestingly, moesin only bound to the column if T-cells were stimulated with PMA. In contrast, ezrin bound to the affinity column in a stimulus-independent manner. These results suggest that ezrin and moesin switch in their binding to L-selectin in a stimulus-dependent manner. Fluorescence Lifetime Imaging Microscopy (FLIM) was used to measure Fluorescence Resonance Energy Transfer (FRET) between L-selectin-GFP and ezrin/moesin-RFP, confirming that L-selectin/ezrin interaction was more readily detectable in resting cells than L-selectin/moesin interaction (88). The biological significance underlying these specific interactions is discussed later.

Protein Kinase C (PKC) and Serine Phosphorylation of the L-selectin Tail

By tagging the tail of human L-selectin with Glutathione-S-Transferase (GST), Kilian et al. discovered that a 60 kDa PKC-like kinase activity could be precipitated in classic "pull-down" experiments from clarified extracts of Jurkat T-cells (120). Further investigations led them to show that at least 3 isozymes of PKC (α, ι, and θ) are able to phosphorylate the tail of L-selectin. PKC-α and PKC-θ bind more readily to serine phosphorylated L-selectin tail. Given that purified PKC-α can directly phosphorylate serine residues on L-selectin strongly supports the view that PKC is a direct binder of the L-selectin tail. As phosphorylation of S364 drives CaM dissociation (87), it is highly likely that serine phosphorylation of the L-selectin tail occurs during or after shedding. The turnover of the MRF (i.e., the cleavage product of L-selectin that remains in the plasma membrane) has been shown to take ∼30 min to be fully degraded (161). There is a potential window of opportunity that the MRF/PKC complex may transduce unique intracellular signals. In support of the MRF/PKC complex, clustering of the T-cell receptor (TCR) has been shown to drive L-selectin shedding in a PKC-dependent manner (162). Importantly, increased retention of PKC onto the tail of L-selectin was observed only after TCR stimulation (120).

Metabolic labeling of cell lines expressing L-selectin with radiolabelled pyrophosphate has shown that S364/S367 are indeed phosphorylated following stimulation with chemoattractants (such as C5a and formyl peptides), IgE or PMA (163). Serine-to-alanine mutagenesis of both serine residues does not show any further transfer of radioactive label to L-selectin, suggesting that Y372 is not phosphorylated—at least in response to the agonists tested in this study.

AP-1 Adaptin

The binding of L-selectin to μ1A—a 45 kDa protein of the clathrin-coated vesicle AP-1 complex—was identified in classic pull-down experiments and later verified by protein-protein interaction (121). Interestingly, binding was only witnessed in response to PMA-induced cell activation. Although the cellular mechanisms have yet to be defined, it is likely that L-selectin/μ1A interaction drives transport of de novo synthesized L-selectin from the trans golgi network toward the plasma membrane. The PMA-induced mode of interaction might suggest that the MRF is internalized by virtue of μ1A binding, once L-selectin shedding is complete. μ1A may therefore be responsible for sorting the MRF toward endosome-like vesicles for degradation. The cyclical expression of L-selectin in recirculating naïve T-cells may also suggest that, instead of L-selectin shedding, full-length L-selectin may be internalized by μ1A and taken to an endosomal recycling compartment for re-expression back to the plasma membrane at a subsequent stage during lymph node trafficking.

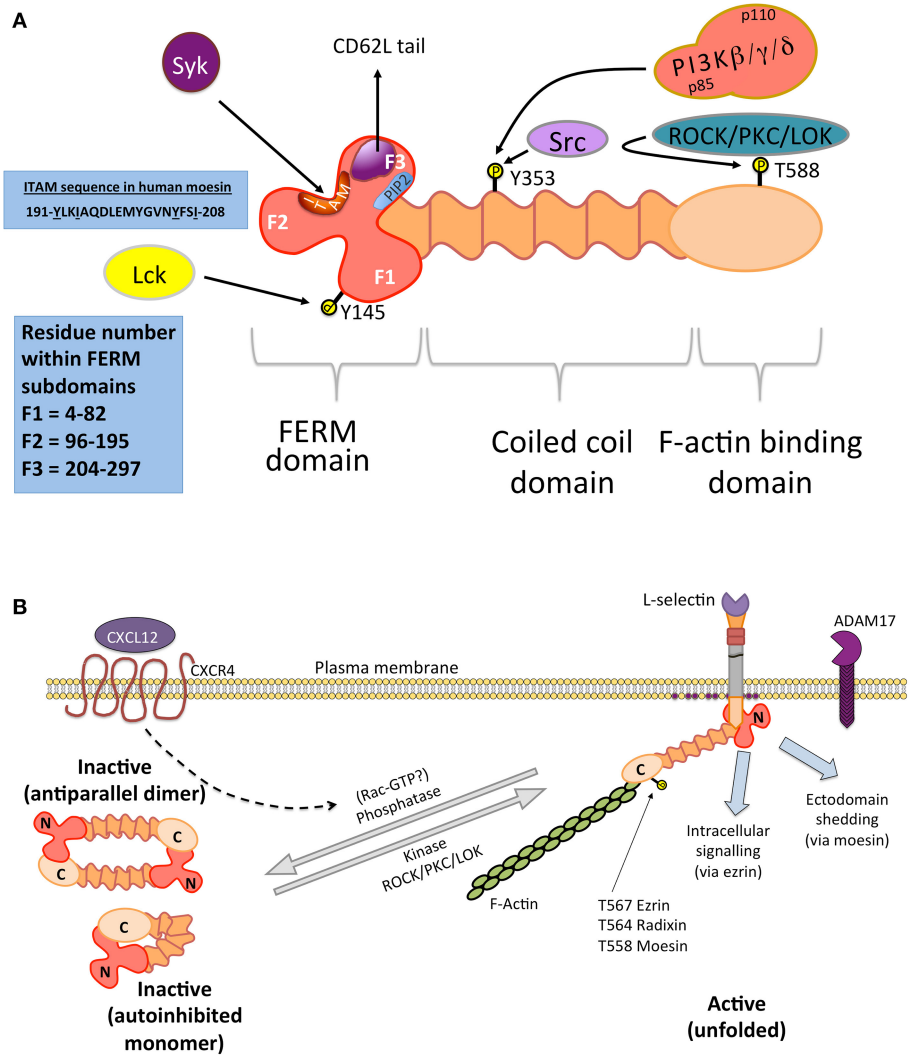

FIGURE 2 | ERM structure and function in immune cells. **(A)** Domain organization of ERM: X-ray crystallographic studies reveal that the N-terminal FERM domain contains a globular clover leaf-shape (136), classified into 3 distinct subdomains (blue box indicates residue numbers that make up each F subdomain). The FERM domain contains multiple interaction sites: a cryptic ITAM (sequence shown), which can recruit Syk, a PIP2 binding site and a region responsible for binding to L-selectin. The tyrosine kinase Lck is responsible for phosphorylating Y145 of the FERM domain in T-cells (137). Src phosphorylation of Y353 in the central coiled-coil domain of ezrin can lead to the recruitment of class I PI3K, via the p85 regulatory subunit (138). Although originally identified in columnar epithelial cells, confocal microscopic colocalisation of PI3K with phospho-Y353-ezrin is witnessed in T-cell receptor-stimulated T-cells (139). These observations suggest that a PI3K/ezrin interaction is possible in leukocytes. **(B)** ERM proteins reside in the cytosol as inactivate parallel homodimers or in an autoinhibited conformation. Binding of ERM to PIP2 within the inner leaflet of the plasma membrane (purple circles) alleviates the inactive conformation, allowing the C-terminal domain to interact with filamentous actin (F-actin). In this manner, ERM are typically classified as membrane/cytoskeleton cross-linkers. The kinase activity of serine/threonine kinases (e.g., ROCK, PKC, or LOK) target a conserved threonine residue in all unfolded ERM (T567 in ezrin, T564 in radixin, and T558 in moesin), stabilizing the open conformation. Recent evidence suggests that ezrin might be involved in regulating intracellular signaling, whereas moesin regulates the clustering of L-selectin prior to its ectodomain shedding by ADAM17 (88). One pathway in which ERM are inactivated has been shown in T-cells, where binding of CXCR4 to CXCL12 leads to the rapid dephosphorylation and microvillar collapse of ERM (140). Although not fully understood, Rac is known to act upstream of the ERM phosphatase (141, 142).

Indirect Binding Partners of L-selectin

Son of sevenless (SOS)/Grb, p56 Lck and the Src family kinases (Fgr, Hck, Lyn, and Syk) can co-precipitate in anti-L-selectin immunoprecipitates and/or pull-down assays (164–166), but this observation does not confer direct interaction. Given that some of these proteins have already been shown to bind to ERM proteins or CaM (134, 137, 156–158, 167), it is plausible that they form indirect, higher-ordered, complexes with the tail of L-selectin. The cytoplasmic tail of L-selectin is highly basic in nature (e.g., the tail of human L-selectin possesses a predicted pI of 11.1). Based on this property, affinity chromatography experiments (such as pull-down assays) can attract false-positive binders if extracts are not pre-cleared with a scrambled peptide control. Such pre-clearing steps eliminate

binders based on charge alone and selectively enrich for sequence-specific binders.

L-SELECTIN CLUSTERING BY LUMINAL AND ABLUMINAL LIGANDS

Glycans form a fundamental biological interface between adhering/migrating cells and their immediate microenvironment. Moreover, cell adhesion molecules, such as L-selectin, have the capacity to transduce intracellular signals downstream of glycan binding. There is a consensus of thought that unbound L-selectin is monomeric, which is clustered when bound to glycan (87, 134). This mode of glycan binding drives "outside-in" clustering (and signaling). Inside-out clustering of L-selectin can be driven by other input signals (168, 169). Outside-in L-selectin clustering, facilitated by monoclonal antibodies or the elegant engineering of cytoplasmic tail clustering modules, enhances bond lifetime through increased avidity, which can stabilize and/or decrease rolling speed (170–172). Both outside-in and inside-out modes of L-selectin clustering require association with the underlying actin-based cytoskeleton, which is likely to be through the ERM proteins but this has not been formally tested. How inside-out and outside-in modes of L-selectin clustering inter-relate to one another during a specific cellular process (e.g., TEM) has never been explored.

Given the diversity of L-selectin ligands, and their compartmentalisation within anatomically defined locations within vessel walls, the signals running downstream of L-selectin provides information to the leukocyte's position within the multi-step adhesion cascade (tethering, rolling, adhesion, and transmigration). Whilst L-selectin ligands on the apical aspect of the endothelium have long been characterized as tethering and rolling receptors, the glycans that are enriched in the basolateral aspect, and within the basement membrane, are likely to drive completely different signals. The bond lifetime of L-selectin with apical ligands will be within the order of subseconds [due to rapid catch-slip bond dynamics (104)]. In contrast, L-selectin-dependent adhesion within microenvironments devoid of hydrodynamic shear stress (e.g., within transmigrating pseudopods) will extend from seconds to minutes. The glycan scaffold within regions devoid of shear stress can amplify signaling downstream of L-selectin. Moreover, ectodomain shedding can also fine-tune the magnitude of input signals downstream of L-selectin—limiting signals beyond a critical threshold. In support of this view, clustering L-selectin on Jurkat T-cells can promote ectodomain shedding (173), a phenomenon that can be recapitulated in neutrophils when monoclonal antibodies are used to cluster L-selectin (74). Therefore, understanding the nature of L-selectin ligands is important for appreciating how a changing glycan scaffold can impact leukocyte behavior, for example during transmigration. This section will discuss the nature of luminal and abluminal ligands, providing some insight into how they were identified and characterized.

An excellent in-depth review on the biosynthetic pathways of glycosylation can be found in the following reference (174). Suffice to say, the majority of glycoproteins are subjected to either N- or O-linked glycosylation. N-glycosylation refers to the attachment of glycans to the amine nitrogen of asparagine (N) side chains within the N-X-S/T consensus sequence, where "X" is any amino acid except proline. In O-glycosylation, glycans are linked to the hydroxyl oxygen of serine (Ser) or Threonine (Thr) side chains. Sulfotransferases mediate the covalent attachment of sulfate groups to selected saccharide residues within specific oligosaccharide chains and therefore modulate glycan structure and function.

Luminal Ligands of L-selectin

The vital role that L-selectin plays in the recirculation of naïve T-cells (46) led researchers to first explore the glycans presented in secondary lymphoid organs. Classic Stamper Woodruff assays (175) revealed that naïve T-cells would accumulate around vessels of thin frozen lymph node sections, which could be functionally blocked by the anti-L-selectin MEL-14 antibody (51). High endothelial venules (HEVs) are specialized microvessels that are unique to secondary lymphoid organs, allowing the entry of blood-borne naïve T-cells into the lymph node parenchyma (in search for antigen priming by resident dendritic cells). The endothelial cells lining HEVs (called high endothelial cells—HECs) can be isolated and grown *in vitro*. Ligands for L-selectin have also been purified and characterized from HEC culture supernatants (176).

Sialyl Lewis X (sLe^x) is the minimal structural determinant for luminal ligands of L-selectin, which is composed of sialic acid, galactose, fucose, and N-acetylglucosamine (Siaα2,3Galβ1,4 (Fucα1,3)GlcNAc) (see **Figure 3**). sLe^x is typically O-linked to protein backbones, such as peripheral lymph node addressin (PNAd), CD34, glycosylation-dependent cell adhesion molecule (GlyCAM-1), mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1), podocalyxin-like protein and spg200 (95, 179–185). Monoclonal antibodies to specific glycan moieties, such as MECA-79 (see below), revealed an apical distribution of these L-selectin ligands in HECs. L-selectin ligands decorating HEV is not solely employed by recirculating naïve T-cells. NK cells are guided to lymph nodes via the same addressin "postcode" to intercept the metastatic spread of B16 melanoma cells to other sites, via local lymph nodes (186, 187). Most recently, neutrophils have been shown to access lymph nodes via the same route as recirculating naïve T-cells, during *Staphylococcus aureus* infection of the lymphatics. The requirement of PNAd (for L-selectin-dependent adhesion) and platelet-derived P-selectin (interacting with neutrophil P-selectin glycoprotein ligand-1—PSGL-1) were equally critical for trafficking to the infected lymph node (188). Since their identification and characterization in secondary lymphoid organs, L-selectin ligands have also been identified in tertiary lymphoid organs surrounding solid tumors (189, 190) and in microvessels within rejecting kidney (191) and heart (192) allografts.

The pan-selectin ligand, PSGL-1, is typically expressed on the surface of leukocytes to mediate a range of complex interactions with other leukocytes, platelets or endothelial cells (193, 194). However, endothelial-specific PSGL-1 expression has been reported in microvessels sprouting into advanced

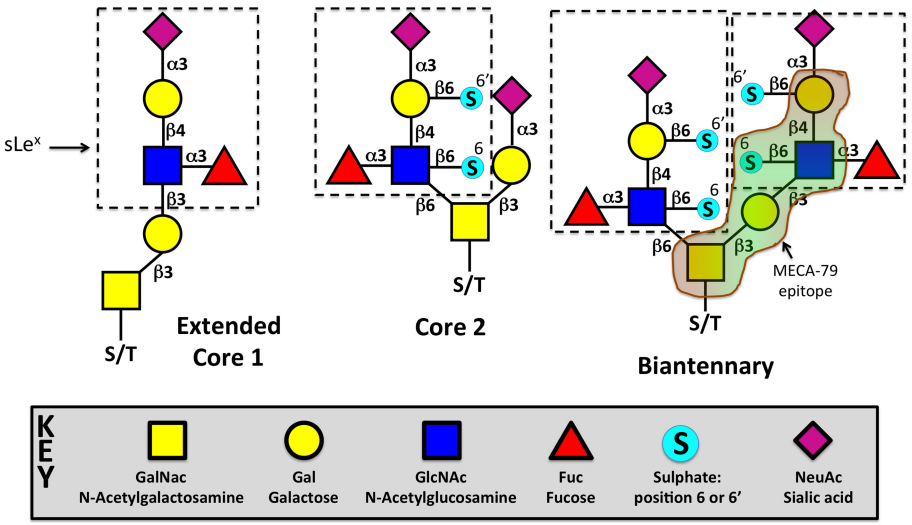

FIGURE 3 | Different glycoforms of O-linked sialyl Lewis X (sLex). sLe^x can exist in multiple configurations: extended core 1, core 2, or both (biantennary). Sulfotransferases can attach sulfate esters to Gal or GlcNAc, respectively at positions 6 or 6′. Sulfates can be added to sLe^x one or both positions in either extended core 1, core 2, or the biantennary configuration. Recognition of the MECA-79 epitope is provided in the biantennary structure, but can exist in any of the 3 glycoforms. N-linked sLex has also been shown to act as bonafide L-selectin ligands, but are not depicted in this figure. More information can be found in a short concise commentary (177). The glycoforms are drawn in accordance with GlycanBuilder (178). S/T = serine/threonine.

atherosclerotic lesions (68), chronic murine ileitis (195) and benign prostatic hyperplasia (196). These examples of atypical PSGL-1 expression represent unique sites for L-selectin-dependent leukocyte trafficking. PSGL-1/L-selectin interactions were first characterized to support secondary tethering and rolling between bystander leukocytes and adherent leukocytes (197). There is some debate over whether L-selectin ligands are expressed at all in the inflamed post-capillary venules of the cremasteric muscle, and whether L-selectin-dependent recruitment is purely through secondary tethering and rolling on adherent leukocytes or deposited exosome-like structures (79, 198, 199).

sLe^x can be linked to lipids, N- or O-linked glycans. The most comprehensively characterized biosynthetic pathway of L-selectin ligands is O-linked sLe^x, which will be discussed below in further detail. A concise and excellent review on N-linked sLe^x is provided in the following review (177).

O-linked sLe^x is decorated on protein backbones as single or biantennary branches that are respectively called "core-1 extended" and "core-2" glycans (**Figure 3**). Both branches extend from N-acetylgalactosamine (GalNac), which itself is covalently attached to a serine/threonine residue by polypeptide N-acetylgalactosamine transferase. sLe^x can be modified by the addition of a sulfate ester on C-6 of N-Acetylglucosamine (GlcNAc), which is specific to extended Core 1 branches, or C-6 on Galactose (Gal) of Core 2 branches—respectively termed 6-sulfo or 6'-sulfo (see **Figure 3**). GlcNAc-6-sulfo is recognized by the MECA-79 monoclonal antibody and is the dominant L-selectin ligand expressed in HEVs and chronically inflamed microvessels (200, 201). Unsulfated sLe^x is capable of supporting some lymphocyte homing, as demonstrated in mice lacking the enzymes GlcNAc 6-O-sulfotransferase (GlcNAc6ST)-1 and GlcNAc6ST-2 (201). The core 1 extension enzyme and the core 2 branching enzyme are respectively called: Core1-β3GlcNAcT and Core2GlcNAcT, which have been systematically knocked down in mice to show little impact on lymphocyte homing to lymph nodes *in vivo* (199, 202). Core1-β3GlcNAcT/ Core2GlcNAcT double knockout mice, which lack any detectable MECA-79 expression within HEV, reduced lymphocyte trafficking by only 50%, suggesting other ligands distinct from 6-sulfo sLe^x could support lymphocyte recruitment to HEV (203). Mass spectrometry discovered the presence of N-linked 6-sulfo sLex in HEV, which was responsible for the residual lymphocyte recruitment in mice lacking functional O-linked 6-sulfo sLe^x.

Interestingly, no recruitment of leukocytes to inflamed cremasteric venules was observed in Core2GlcNAcT knockout mice (199). As mentioned before, no detectable ligands for L-selectin are present on endothelial cells of inflamed cremasteric venules (79). It is possible that L-selectin/PSGL-1 secondary tethering and rolling at these sites is highly dependent on Core2GlcNAcT-mediated glycosylation of sLe^x onto PSGL-1, which has indeed been reported (204).

sLex on L-selectin: A Ligand for E-selectin

It has been known for some time that L-selectin is itself decorated by N-linked sLe^x (8, 9). This post-translational modification is exclusive to L-selectin in human neutrophils, as mouse neutrophils lack the expression of fucosyl transferase 9 (205). Clustering of L-selectin with E-selectin under flow conditions can drive the co-localization of L-selectin with ADAM17 to the uropod (the rear of the cell), where it is thought that enzyme/substrate coalesce to mediate ectodomain

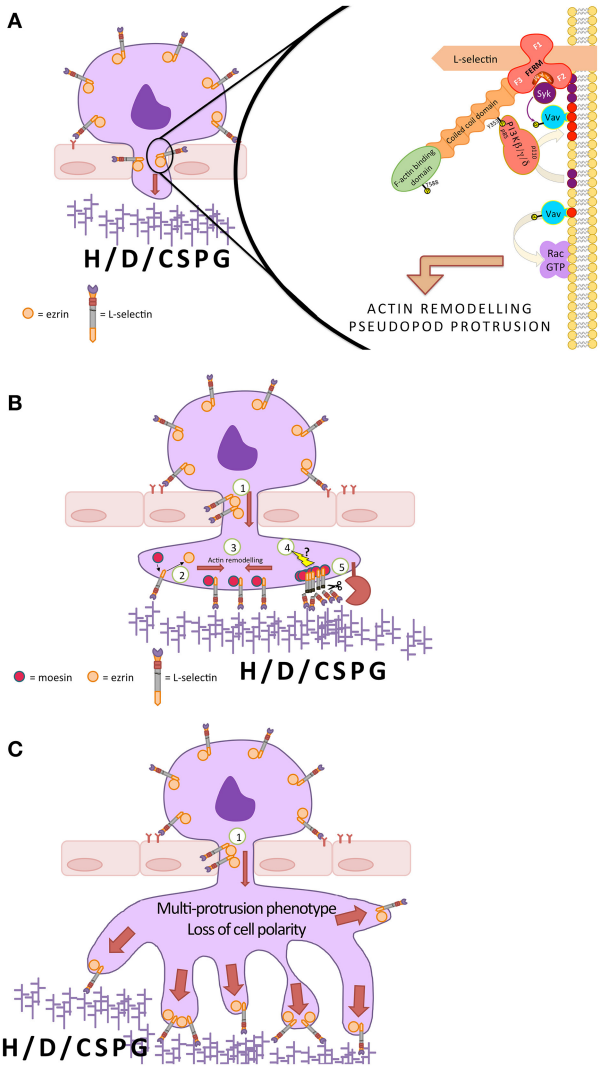

FIGURE 4 | A possible mechanism underlying L-selectin-dependent pseudopod protrusion during monocyte TEM. **(A)** In early monocyte TEM, L-selectin interacts preferentially with ezrin. Moesin and ezrin are the most abundant ERM proteins in monocytes, with little to no expression of radixin. Ezrin can interact with the p85 subunit of class I PI3Ks via phospho-Y353 (a known target of Src kinase). The ezrin-anchored PI3K catalytic subunit (p110) converts PIP2 (purple spheres) into PIP3 (red spheres) within its local vicinity. The most common p110 isoforms in leukocytes are β, δ, and γ. The plekstrin homology domain of guanine nucleotide exchange factors (GEFs), such as Vav, can bind to PIP3 and mediate their effects on the small Rho family of GTPases, such as Rac. The FERM domains of ERM contain a cryptic ITAM, which, upon phosphorylation, can support the binding of spleen tyrosine kinase (Syk). Syk can phosphorylate Vav, increasing its GEF activity significantly toward GTPases like Rac. PI3K and Rac play pivotal roles in pseudopod protrusion, but the molecular mechanism in the context of TEM is currently not known. **(B)** As described in **(A)**, during early TEM, the interaction between L-selectin and ezrin is thought to contribute to pseudopod protrusion (1). As TEM proceeds, the L-selectin within transmigrating pseudopods exchanges affiliation from ezrin to moesin (2). This exchange could happen when L-selectin makes contact with high-density heparan, dermatan or chondroitin sulfate proteoglycan families (H/D/CSPG) within the subendothelial space, such as the basement membrane. Phospho-cycling of serine residues within the L-selectin tail could also be contributing to the exchange mechanism, *(Continued)*

FIGURE 4 | where phosphorylation drives ezrin dissociation and dephosphorylation allows binding of moesin. The moesin/L-selectin interaction leads to the remodeling of L-selectin into clusters (3), possibly by the remodeling of the cortical actin cytoskeleton. The clustered L-selectin may also trigger as yet unexplored signals (4) that lead to ectodomain shedding by ADAM17 (5). Currently, it is thought that the exchange of ezrin for moesin inhibits further protrusion and rapidly shuts down L-selectin-dependent adhesion in this region by activating shedding. **(C)** Blocking L-selectin shedding in monocytes during TEM sustains the L-selectin/ezrin interaction, which exacerbates the signaling mechanism proposed in **(A)**, leading to the formation of multiple pseudopodial extensions and a loss in cell polarity of fully transmigrated cells.

shedding (206). This form of ectodomain shedding is distinct from "mechanical shedding" (75), where neutrophils are perfused exclusively over immobilized sLe^x rather than E-selectin (discussed above). E-selectin-mediated clustering of L-selectin on human neutrophils promotes cell priming (i.e., degranulation and superoxide production), via the activation of p38MAPK (207, 208). Two more recent reports have extended our understanding of the molecular mechanisms underlying L-selectin-mediated neutrophil arrest under flow conditions, which have recently been reviewed (209). Perfusion of human neutrophils over immobilized E-selectin leads to the secretion of MRP8 in rolling cells. The secreted MRP8 then binds to TLR4 and promote integrin LFA-1 activation toward an intermediate activation state, driving the transition from rolling to slow rolling on ICAM-1 (210). A separate study showed that L-selectin-dependent adhesion to E-selectin under flow conditions could drive the clustering of L-selectin and transition LFA-1 toward an "active" state (211). Collectively, these studies suggest that signals downstream of L-selectin/E-selectin interaction and PSGL-1/E-selectin interaction converge to arrest neutrophils. Although mouse neutrophils express L-selectin that lack sLe^x modification, one study has shown that L-selectin and PSGL-1 co-cluster *in cis* in resting cells, which increases when perfused over P- or E-selectin to activate LFA-1 independently of chemokines (164). It is clear that L-selectin-dependent signaling is important in activating LFA-1 in mouse and human neutrophils, but whether any of the signaling pathways are conserved between species is yet to be determined.

Abluminal Ligands of L-selectin

It is increasingly appreciated that L-selectin ligands are present in perivascular and extravascular locations (78, 185, 212–215). This itself challenges the previously held view that the role of L-selectin is restricted to early events in the leukocyte adhesion cascade such as tethering and rolling (216), and supports more recent findings that L-selectin may have additional roles beyond luminal interactions (78, 87, 88). The nature of L-selectin/ligand interactions differs from intravascular ligands, as they occur in regions devoid of flow. Early evidence for extravascular L-selectin ligands was demonstrated by presence of the MECA-79 epitope within the abluminal aspect of HEVs in peripheral lymph nodes (185). Furthermore, in HEVs of Peyer's patches, abluminal expression appeared more abundant than luminal expression,

alluding to a role for L-selectin within spatially distinct regions of the vessel wall (185).

The majority of identified extravascular ligands are basement membrane proteoglycans, including members of the heparan, dermatan, and chondroitin sulfate proteoglycan families (HSPGs, DSPGs, CSPGs) (212, 215, 217, 218). Using immunohistochemistry in kidney sections, HSPGs were identified as L-selectin ligands, using a chimeric protein consisting of the L-selectin extracellular domain fused to the Fc portion of IgM (L-selectin-IgM). L-selectin-IgM binding was abrogated by heparitinase I but not sialidase treatment (which removes sialic acid from sLe^x) (212, 213). However, although L-selectin co-localizes with the HSPGs perlecan, agrin, syndecan-4 and collagen type XVIII in the kidney, only collagen type XVIII was responsible for L-selectin binding, which was blocked in collagen XVIII-, but not perlecan-deficient mice (213). Interestingly, despite the presence of collagen type XVIII in some renal structures, L-selectin-IgM was unable to bind in some regions, suggesting that not all collagen XVIII molecules are permissive for L-selectin binding. This indicated that specific GAG chains may be necessary, which was further characterized as requiring O-sulphation (213). Supporting evidence was shown in heparin-mediated inhibition of L-selectin, requiring 6-O-sulphation of the glycolsaminoglycan (GAG) chains in heparin (219). GAG chain length is also an important determinant of L-selectin binding, as *in vitro*, L-selectin binding efficiency was reduced for lower molecular weight heparin relative to full length heparin (213).

Binding of other GAGs to L-selectin has also been demonstrated. In cartilage, L-selectin binding was removed by chondroitinase ABC but not heparitinase I, suggesting the presence of CSPG or DSPGs (213). Similarly, chondoritinase ABC reduced L-selectin binding in particular regions of the kidney, suggesting the contribution of CSPGs or DSPGs. The CSPG, versican, has been identified as an L-selectin ligand in the kidney (212, 220, 221). Like for HSPGs, these interactions depend on particular GAG chains and, similarly, require sulphation (220). Furthermore, the DSPG biglycan on microvascular endothelial cells of the endometrium binds L-selectin (218), and *in vitro* assays have demonstrated the ability of biglycan to induce L-selectin clustering (87). An increasing gradient from the apical to basal aspect of the endothelium has been observed for HSPGs and subsequently for biglycan (87, 222), which may influence L-selectin clustering, and its subsequent shedding, at high ligand densities (173, 223).

Interestingly, whilst binding of L-selectin to sLe^x modified vascular ligands occurs optimally at physiological blood pH 7.4, binding to extravascular ligands is optimal at pH 5.6 (224). This could reflect an altered conformation of L-selectin in acidic inflammatory environments that allows binding to different types of ligand, for example by protonation of histidine residues at low pH. Indeed, the CTLD of selectins contain clusters of basic amino acid residues that bind sulfate groups on GAGs via hydrogen bonds (225). At pH 5.6, the CTLD is additionally protonated, increasing the number of H-bonds between the groups (225). Perhaps, under inflammatory or hypoxic conditions in which an acidic environment is generated, the lowering of pH could act as a "switch" for L-selectin binding with greater affinity to subendothelial ligands to facilitate leukocyte tissue infiltration. *In silico* modeling suggests that the sulfate density of the GAG chain is also an important determinant of ligand binding. However, in contrast to Celie et al. who suggest the importance of 6-O-sulphation, here, 3-O-sulfation of HSPGs and 4,6-O-sulfation of CSPGs are reported to increase affinity (225). Whilst the binding site for sulphated proteoglycans is unknown, it seems plausible that binding may involve a cationic region of the CTLD distinct from the sLe^x site, as has been shown for sulphated glycolipids (226).

In a rodent model of renal ischaemia-reperfusion (I/R) injury, L-selectin binding to HSPGs was evident in the subendothelial region of interstitial capillaries, which was not detectable in healthy kidneys (214). Mice lacking functional collagen XVIII and perlecan displayed reduced monocyte influx to the kidney 24 h post-I/R injury. Consistent with this, human kidney post-transplant biopsies and acute allograft rejection biopsies showed greater HSPG dependent L-selectin binding to the basement membrane of peritubular capillaries than control kidneys (214). The mechanisms of regulation of HSPG binding of L-selectin are not fully understood, but HSulf 1 and 2 enzymes have been identified in humans to cleave 6-O-sulfate residues (227). In acute allograft rejection, HSulf 1 was downregulated (214), leading to the speculation that HSulf1 may cleave 6-O-sulphated HSPG chains constitutively under basal conditions, but, under inflammatory conditions, its downregulation causes HSPGs to retain 6-O-sulphation and bind L-selectin. The relevance of subendothelial L-selectin ligands may be of importance in inflammatory situations involving endothelial injury where basement membrane proteoglycans are exposed.

Extravascular Ligands of L-selectin

In addition to L-selectin ligands in luminal and subendothelial environments, putative ligands have been identified in extravascular locations. Using recombinant L-selectin fused to IgG as a histological probe, binding of L-selectin to ligands was identified in myelinated regions of the cerebellum and spinal cord, but not peripheral nervous system, in human, mouse or rat tissues (228, 229). L-selectin ligands were subsequently shown to localize specifically to myelin (230). Ligands were also located in the renal distal tubule, and upon obstruction of the ureter, were found to relocate to peritubular capillaries where infiltration of inflammatory cells ensued (229, 231). Binding of L-selectin-IgG to ligands in the cerebellum and renal distal tubule was abolished by organic solvents and could not be detected by SDS-PAGE, suggesting that they may be glycolipid, rather than protein ligands (229).

Whilst it seems that such ligands would seldom encounter L-selectin due to its shedding from leukocytes during extravasation, incomplete L-selectin shedding, or restoration of surface expression could allow this interaction to initiate an inflammatory response. Indeed, it has been speculated that the interaction between L-selectin on leukocytes and ligands on myelin may provoke an inflammatory response to drive demyelinating diseases (230). This is supported by

the finding that L-selectin deficiency prevents macrophage-mediated destruction of myelin in the experimental allergic encephalomyelitis animal model which has clinical similarities to human multiple sclerosis (232). Thus, L-selectin appears to be involved in non-migratory functions. Shedding of L-selectin may serve, in part, to limit the interaction of leukocytes with such extravascular ligands to avoid inappropriate leukocyte activation under normal circumstances. Interestingly, L-selectin is also expressed on trophoblasts and interacts with ligands upregulated on the uterine luminal endothelium during the receptive phase for embryo implantation (233), perhaps performing a role in epithelial "capture" of the blastocyst, analogous to its role in leukocyte capture from blood.

NON- CANONICAL ROLES FOR L-SELECTIN IN REGULATING PSEUDOPOD INVASION AND PROTRUSION DURING TEM

Most textbooks will depict ectodomain shedding of L-selectin occurring on leukocytes that have adhered to the apical aspect of the endothelium, following chemokine-dependent integrin activation. Recent evidence in monocytes would suggest that this is clearly not the case. Live imaging of $CD14^{++}$ $CD16^{-}$ classical human monocytes crossing TNF-activated HUVEC under flow conditions led to the observation that L-selectin undergoes ectodomain shedding exclusively during TEM, and not before (87). Suggestions have been proposed that L-selectin is involved in transducing signals (likely co-stimulatory signals alongside integrin- and chemokine-dependent signaling) to drive pseudopod protrusion in TEM. The window within which L-selectin can transduce signals in TEM is limited by its ectodomain shedding, which is maximal when monocytes have fully entered the subendothelial space. In support of L-selectin transducing signals prior to completion of TEM, blocking ectodomain shedding in $CD14^{++}$ $CD16^{-}$ monocytes did not impact the kinetics of TEM per se, but monocytes entering the subendothelial space lost front-back polarity – producing unstable and excessive protrusions.

The molecular mechanism underpinning L-selectin signaling during TEM, whilst difficult to define in primary human monocytes, was determined in cell lines. Genetic engineering of THP-1 (monocyte-like) cells co-expressing L-selectin alongside a known binding partner (e.g., CaM, ezrin, or moesin), tagged to either green/red fluorescent proteins (RFP/GFP), enabled close inspection of protein-protein interactions within transmigrating pseudopods and non-transmigrated uropods. Fluorescence lifetime imaging microscopy (FLIM) was used to quantify Förster resonance energy transfer (FRET) between the GFP and RFP tags to understand the spatial organization of L-selectin with its binding partners within sub-10 nm distances. THP-1 cells captured in mid-TEM revealed that L-selectin interacts with CaM in both transmigrated pseudopods and non-transmigrated uropods (87). As TEM proceeds, CaM dissociates from the L-selectin tail, exclusively within transmigrating pseudopods, driven by phosphorylation of S364. Using similar FLIM/FRET approaches, ezrin and moesin were shown to bind sequentially with L-selectin as TEM proceeds (88). Ezrin binds initially to L-selectin, which is swapped-out by moesin, exclusively within transmigrating pseudopods. The change-over in L-selectin binding from ezrin to moesin is likely to reflect when signaling downstream of L-selectin is halted and ectodomain shedding ensues. Blocking L-selectin shedding leads to the sustained interaction of ezrin with L-selectin, where no exchange with moesin occurs. These outcomes would strongly suggest that it is the L-selectin/ezrin interaction that drives signaling for pseudopod protrusion. Interestingly, knocking out moesin, but not ezrin, in mouse T-cells can increase the surface expression of L-selectin (139, 234). These observations imply that in T-cells moesin acts as a "pro-shedding factor," but ezrin does not.

L-selectin-Dependent Signaling During TEM

Unlike the processes of rolling, slow rolling and firm adhesion, little is known about L-selectin-dependent signaling with respect to pseudopod protrusion in TEM. One can speculate that the contribution of luminal, abluminal and interstitial ligands will play a major role in modulating signals downstream of L-selectin/ligand interaction and clustering. Luminal glycoprotein ligands must contain the sLe^x glycan core to participate in singular catch bonding of L-selectin, a biomechanical property that is critical for leukocyte capture under physiological shear forces. These ligands are likely to favor the binding of monomeric L-selectin on microvilli. In contrast, the subluminal ligands that bind L-selectin under shear free conditions neither contain nor depend on the sLe^x core, and likely share unique highly clustered arrays of sulfates [e.g., in CNS sulfolipids (232)]. These multivalent ligands are therefore specialized in clustering L-selectin and triggering different magnitudes of outside-in signals to facilitate migration or non-migratory processes [e.g., where myelin damage by L-selectin expressing effector T cells proximal to inflamed CNS (232)].

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for human transendothelial migration shows just 4 cell adhesion molecules involved in TEM (https://www.genome.jp/kegg-bin/show_pathway?map=hsa04670&show_description=show), with little insight into the intracellular proteins that mediate this process. In contrast, nearly 20 genes within the leukocyte are required to participate in firm adhesion to the apical aspect of the endothelium. The mechanisms that drive pseudopod protrusion are likely to involve numerous integrin and non-integrin cell adhesion molecules and chemokine (or other G-protein-coupled) receptors. Many reports have documented the contribution of L-selectin in the activation of integrins (164, 211, 235–240), mobilization of chemokine receptors (241–243), and cellular responsiveness to chemokines (65)—all of which play essential roles in TEM. Blocking L-selectin shedding through the expression of ΔM-N L-selectin leads to the sustained and exclusive interaction with ezrin in transmigrated pseudopods. Engineering the R357A mutation [which blocks ERM binding (20, 88)] into ΔM-N L-selectin significantly reduces the multi-pseudopodial extension phenotype, strongly implying that L-selectin/ezrin interaction is driving pseudopod protrusion.

The events downstream of L-selectin/ERM interaction, in respect of pseudopod protrusion during TEM, remain elusive. **Figure 4** proposes a mechanistic model that could link L-selectin to pseudopod protrusion. Ezrin is unique from moesin in that it can interact with the p85 regulatory subunit of PI3K (138). AMC of L-selectin in T-cells can activate the small GTPases Ras and Rac (166), which in turn can promote two inter-linked signals. Rac can target downstream effectors to remodel the actin-based cytoskeleton for pseudopod formation in leukocytes and other cell types (244–247). The catalytic activity of PI3K is significantly enhanced in the presence of activated Ras, resulting in increased local pools of PIP3 production (248, 249). Guanine nucleotide exchange factors (GEFs), such as Vav [a known Rac GEF (250, 251)], is targeted to the plasma membrane via its plextrin homology (PH) domain. Running in parallel, Syk can bind to a cryptic ITAM sequence within the FERM domain of ERM (156–158). The Syk, which is anchored to ERM, can phosphorylate GEFs within its immediate vicinity, such as Vav (252), to increase its GDP-to-GTP exchange activity. Localized Rac activation will promote actin remodeling and pseudopod protrusion during TEM. The displacement of ezrin from L-selectin by moesin would invariably shut down PI3K-mediated PIP3 production and Rac-mediated protrusion. Again, these models are purely speculative and will require further research to expose the true underlying mechanism.

CONCLUDING REMARKS

Since its identification and characterization, the expression of L-selectin has been carefully interrogated in numerous leukocyte subsets. The motivation underlying this type of classification has been to unearth the ever-expanding functional diversity of individual leukocyte subsets, and their specialized roles in health and disease. A major unanswered question in many such immune cell subsets is to understand if there is a direct role for L-selectin signaling in bestowing their unique functionality.

This review also highlights how the expression of non-cleavable L-selectin in one immune cell-type may confer protection from viruses (61), whereas its expression in another cell type could block polarity necessary for migration toward inflammatory targets (40, 87). Therefore, blocking L-selectin shedding as a therapeutic target will require better understanding of the immune cells it will impact in specific disease settings.

It is highly likely that L-selectin acts as a co-receptor to many of the cellular outcomes featured in this review. Fresh insights into how L-selectin signaling synergises/antagonizes chemokine receptor signaling, integrin signaling, and signaling from non-integrin receptors during adhesion and migration is what is now required to further understand how unique signals are propagated under steady-state and pro-inflammatory conditions.

Finally, technological advances in microscopic imaging have for the first time allowed the interrogation of spatio-temporal interactions (within a sub-10 nm resolution) between L-selectin and its cytosolic binding partners during specific cellular events, such as TEM. Moreover, the recent identification of L-selectin contributing to monocyte protrusion during TEM suggests a revised view on how cell adhesion molecules contribute to the multi-step adhesion cascade. It seems that the one-molecule-to-one-step paradigm may not be as strict as once perceived.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

All three authors are supported by the BHF Center of Research Excellence (RE/13/2/30182). HLHG and SJH are both supported by BHF PhD studentships (FS/18/60/34181 & RE/13/2/30182, respectively).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 22 May 2019
doi: 10.3389/fimmu.2019.01078

Cell Adhesion Molecules and Their Roles and Regulation in the Immune and Tumor Microenvironment

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The immune system and cancer have a complex relationship with the immune system playing a dual role in tumor development. The effector cells of the immune system can recognize and kill malignant cells while immune system-mediated inflammation can also promote tumor growth and regulatory cells suppress the anti-tumor responses. In the center of all anti-tumor responses is the ability of the immune cells to migrate to the tumor site and to interact with each other and with the malignant cells. Cell adhesion molecules including receptors of the immunoglobulin superfamily and integrins are of crucial importance in mediating these processes. Particularly integrins play a vital role in regulating all aspects of immune cell function including immune cell trafficking into tissues, effector cell activation and proliferation and the formation of the immunological synapse between immune cells or between immune cell and the target cell both during homeostasis and during inflammation and cancer. In this review we discuss the molecular mechanisms regulating integrin function and the role of integrins and other cell adhesion molecules in immune responses and in the tumor microenvironment. We also describe how malignant cells can utilize cell adhesion molecules to promote tumor growth and metastases and how these molecules could be targeted in cancer immunotherapy.

Keywords: cell adhesion, integrin, LFA-1, ICAM-1, VCAM-1, immunotherapy, dendritic cell (DC)

OPEN ACCESS

Edited by:
Toshiyuki Murai, Osaka University, Japan

Reviewed by:
David Calderwood, Yale University, United States
Francisco Sanchez-Madrid, Autonomous University of Madrid, Spain

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *18 January 2019*
Accepted: *29 April 2019*
Published: *22 May 2019*

Citation:
Harjunpää H, Llort Asens M, Guenther C and Fagerholm SC (2019) Cell Adhesion Molecules and Their Roles and Regulation in the Immune and Tumor Microenvironment. Front. Immunol. 10:1078. doi: 10.3389/fimmu.2019.01078

INTRODUCTION

Cancer and the Immune System

The immune system and cancer cells share complex interactions during tumor development. Indeed, all types of immune cells can be found in different tumors (1–3). These include lymphocytes such as T and B cells and natural killer (NK) cells, myeloid cells such as dendritic cells (DCs) and macrophages and granulocytes such as neutrophils, eosinophils, and mast cells. The immune contexture, or the frequency, location and functional orientation of different immune cell subsets, varies substantially between tumor types and also between individuals with seemingly identical cancers (2). Interestingly, correlations between immune contexture in the tumor microenvironment and clinical outcomes have been examined in various malignancies. In general, a strong infiltration of memory $CD8^+$ T cells and T helper 1 (Th1) cells correlates with favorable prognosis while strong T helper 2 (Th2) or T helper 17 (Th17) orientation is associated with poor prognosis in terms of overall survival (2, 3). In addition, high infiltration of regulatory cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in tumors often correlates with decreased survival (4–6). Understanding the role of the immune system for

tumor development has been the central focus of tumor immunology since its inception. It has become evident that immune cells are able to recognize and kill malignant cells and thus suppress tumor growth in a process known as cancer immunosurveillance (7). In addition to directly targeting the cancer cells, the immune system prevents viral infections and thus the growth of virus-induced tumors and inhibits tumor-promoting inflammation by eradicating pathogens and by clearing existing inflammation. However, it is now known that the immune system can also promote tumor growth by maintaining chronic inflammation, by shaping tumor immunogenicity and by suppressing anti-tumor immunity, and thus a concept called cancer immunoediting is currently preferred to cancer immunosurveillance (7).

Cancer immunoediting is a process in which both innate and adaptive immune systems work together to suppress/control and promote/shape tumor growth (8). In order for this process to take place, the DCs need to take up tumor antigens, migrate to lymph nodes, present tumor antigens to T cells in the lymph nodes and then activated T cells need to expand and traffic into the tumor site where they interact with the malignant cells. These events can eventually lead to tumor cell killing or immune cell exhaustion. Cell adhesion molecules such as integrins and receptors of the immunoglobulin superfamily play a crucial role in all these events.

Cell Adhesion Molecules (CAMs)

Adhesion molecules are generally divided into five groups: integrins (discussed in greater detail below), selectins, cadherins, members of the immunoglobulin superfamily (IgSF) including nectins and others such as mucins (9). In addition to the conventional adhesion molecules, certain enzymes such as vascular adhesion protein 1 (VAP-1) also play a role in cell adhesion (10).

Apart from structural differences, cell adhesion molecules also bind to different ligands. Integrins typically bind to the extracellular matrix, while selectins, cadherins, and IgSF members are associated with cell-cell adhesion (9). However, immune cell integrins also bind to soluble ligands and ligands on other cells. The cell-cell adhesion mediating molecules can further be divided by their ligands as selectins bind carbohydrates in a calcium dependent manner (11), cadherins mediate preferably homophilic bonds in a calcium-dependent manner (12) and the IgSF subfamily nectins mediate homophilic and heterophilic bonds (9).

Selectins are further divided into P-, E- and L-selectins originally based on which cell types they were found in: **p**latelets, **e**ndothelial cells and **l**eukocytes (however, P-selectin is also expressed on endothelial cells) (13). Selectins differ in kinetics of expression, as P-selectins are expressed within minutes and E-selectins within hours (13). Selectins are especially important for leukocyte trafficking, migration of lymphocytes to peripheral lymph nodes and to the skin. Their most prominent function is associated with the initial stage of the rolling cell adhesion cascade in which selectin binding enables rolling (14). Selectin binding also initiates the subsequent integrin dependent step of slow rolling and cell arrest as selectin binding together with chemokine receptor activation initiates inside out signaling leading to integrin activation (see later sections) (13, 15). L-selectins in particular display a force dependent binding e.g., L-selectin forms catch bonds with its ligands (bonds that strengthen under force). Catch bonds dominate until the applied force reaches a force threshold upon which slip bonds are formed (bonds that weaken under force) (13, 14). In other selectins this occurs to a lesser extent (13, 14).

Proper functionality of selectins is carbohydrate-dependent as is demonstrated in a disorder called leukocyte adhesion deficiency II (LAD II). LAD II is caused by a mutation in a fucose transporter protein leading to fucose not being incorporated into selectin ligands, which ultimately leads to disruption of selectin-mediated leukocyte adhesion (13). Many selectin ligands, including P-selectin glycoprotein ligand 1 (PSGL-1) which is the main ligand for all three selectins (13), mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and peripheral node addressin (PNAd) belong to a glycoprotein family called mucins which function as major components of the mucous protecting epithelial cells lining the digestive, respiratory and urogenital tracts (16). Interestingly, over-expression of mucins, MUC-1 in particular, have been detected in many human malignancies and seem to promote cancer cell growth and survival (17).

Cadherins are associated with cell-cell adhesive bonds in solid tissues (12). Molecules of this family feature cadherin repeat sequences which are stabilized by calcium ions. The essential role of calcium for cadherin adhesive function is also reflected in the protein family name which is an abbreviation of "**ca**lcium-**d**ependent ad**her**ent prote**ins**" (12). Cadherins in turn can be subdivided into classical cadherins (type I and II), protocadherins and atypical cadherins (9, 12).

The IgSF is one of the largest and most diverse protein families (18). All members of the IgSF contain at least one immunoglobulin or immunoglobulin-like domain and most members are type I transmembrane proteins with an extracellular domain (containing the Ig domain[s]), transmembrane domain and a cytoplasmic tail. The most well-known members include major histocompatibility complex (MHC) class I and II molecules and proteins of the T cell receptor (TCR) complex (18). Intercellular adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs), MAdCAM-1 and activated leukocyte cell adhesion molecule (ALCAM), which are important in leukocyte trafficking events, also belong to this family of adhesion receptors (19–21). Of interest, MAdCAM-1 contains both Ig and mucin domains placing it as a member of both IgSF and mucin families (19).

Yet another subfamily of the IgSF is the nectin family which members mediate cell-cell adhesion in various tissues including endothelium, epithelium and neural tissue (9). The members can form homophilic interactions with each other or heterophilic interactions with other nectins or other ligands. They can also co-operate with cadherins to establish adherens junctions (9, 22). From an immunological point of view, interactions between nectins and immune modulatory receptors such as DNAM-1 (CD226) and TIGIT are of particular interest due to their involvement in regulation of effector cell function and their recently appreciated role in anti-tumor responses (23).

Interestingly, the expression of nectins is also often up-regulated in various tumors (9).

Integrins

Integrins are large heterodimers consisting of α- and β-chains that together form the intact receptor in the plasma membrane. Integrins bind to a wide variety of ligands in the extracellular matrix, on the surface of other cells and also soluble proteins. Leukocytes express various integrins while the β1-integrins (α4β1), β2-integrins (αLβ2, αMβ2, αXβ2, αDβ2), and β7-integrins [α4β7 and αE(CD103)β7] playing particularly important roles in immune cells.

β2-integrins are the predominant integrins on leukocytes (24). The different integrins in this family have different expression in different leukocyte subpopulations (25, 26). LFA-1 (αLβ2, CD11a/CD18) is expressed in all leukocytes and is the predominant integrin in lymphocytes. Mac-1 (αMβ2, CD11b/CD18, CR3) dominates on myeloid leukocytes, especially neutrophils, but is also expressed in NK cells, B cells and some T cells, whilst αXβ2 (CD11c/CD18, CR4) is most abundant on myeloid dendritic cells (DCs). αDβ2 (CD11d/CD18) is the most recently discovered β2-integrin and is expressed on neutrophils, monocytes and NK cells. LFA-1-integrin is more distantly related than the other β2-integrin family members and has a more restricted ligand binding profile compared to the other β2-integrins. It mainly binds members of the ICAM-1-5 and JAM-1 families. In contrast, Mac-1-integrin has a very broad ligand repertoire, with more than 40 reported ligands including ICAM-1-4, iC3b, fibrinogen, fibronectin, factor X, heparin, polysaccharides, and even denatured proteins (26). αXβ2 and αDβ2 are more closely related to Mac-1 than to LFA-1 and have similar, but more restricted, ligand binding properties than Mac-1.

The extracellular domains of integrins are large and consist of several domains (27). In those integrins that contain it, the αI (or A) domain is the most important ligand binding domain, and mediates Mg^{2+}-dependent ligand binding. The I-domain consists of a central β-sheet surrounded by seven α-helices. Ligand binding happens at the MIDAS-site which provides three surface loops to co-ordinate the Mg^{2+} ion, whilst a glutamate or aspartate from the ligand provides the fourth coordination position. This is referred to as the I-domain open conformation. In the closed conformation, the fourth coordination position is replaced by a water molecule – this induces structural changes in the I-domain e.g., rearrangement of the metal-co-ordinating residues and a 10Å shift in the α7-helix. Ligand binding shifts the equilibrium from the closed towards the open state (27).

The I-domain forms part of the ligand binding head domain of the integrin extracellular domain, which also contains the βI-domain (that has a similar structure as the αI domain) and the propeller domain in the α-chain (27). The I-domain sits on top of the propeller domain. Structural signals can be transduced through the integrin β-chain to induce conformational changes in the integrin head-domain or from the ligand-bound head into the cell (27).

In addition to I-domain conformational changes in the ligand-bound and unbound states, integrins can undergo much larger scale structural changes (27–29) (**Figure 1**). When the first integrin ectodomain was crystallized, it was a surprise that the integrin was found in a bent state, with both the α-chain and the β-chain being "bent at the knee" (genu), causing the ligand-binding head to turn down towards the legs of the integrin heterodimer. Indeed, integrins can be found in bent conformation, extended/closed conformation (where the integrin is extended but the I-domain is closed) as well as extended/open conformation (extended integrin/I-domain is in the open conformation) (28).

The transmembrane domains of integrins associate with each other at two motifs, maintaining the integrin in an inactive state (30). The intracellular domains are short and devoid of enzymatic activity. However, they are nevertheless important for regulating integrin function (see below). The cytoplasmic domains of the integrin β-chain are structurally related to each other with several important motifs that are essential for integrin regulation. In contrast, the cytoplasmic domains of the integrin α-chain are more diverse, presumably allowing different integrins to have different functional characteristics.

Inside the cell, these receptors link to the actin cytoskeleton through their cytoplasmic tails (31). In cells such as a fibroblast, they form large multiprotein complexes with intracellular molecules called focal adhesions, the composition of which have been determined by proteomic methods in recent years (31, 32). Integrins also participate in so called outside-in signaling, e.g., transmitting signals into cells through a variety of signaling pathways to change cell behavior (actin reorganization, cell migration, gene expression etc.) (33).

Integrin Regulation

Integrins are not constitutively active and able to bind ligands. Instead, their activity is regulated from inside the cell, through a process called inside-out signaling (24) (**Figure 1**). During this process, cell signaling initiated by other cell surface receptors, induce integrin activation. Receptors such as chemokine receptors, TCR, Toll-like receptors (TLRs), selectins as well as many other cell surface receptors, have been reported to induce integrin activation in immune cells. Integrins can also influence the activity status of other integrins, a process called integrin transregulation (34).

Ultimately, integrin activation in response to inside-out signaling is achieved by cytoplasmic factors that interact with the integrin β-chain cytoplasmic portion (**Figure 1**). Talin is the most well-known integrin activator. Talin binds to the membrane-proximal NPXY motif in the β-chain and is of fundamental importance for integrin activation. Talin binding to the integrin β-chain cytoplasmic domain destabilizes the transmembrane linkage between the α- and β-chain of the integrin, allowing integrin activation (30).

Kindlin is a more recently discovered integrin interaction partner which is nevertheless very important for integrin regulation (35–37). Kindlin binds to the membrane-distal NPXY-motif and a threonine-motif between the NPXY-motifs, but exactly how kindlin regulates integrin function remains incompletely understood. It has been suggested that talin is required for the conformational change of the integrin to the

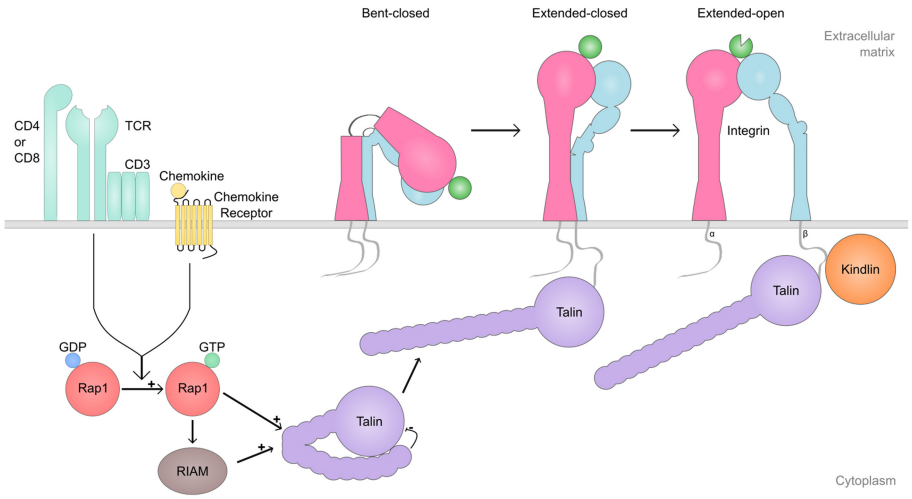

FIGURE 1 | Integrin inside-out signaling. Shown is a simplified representation of the integrin inside-out process, which regulates integrin activation (i.e., integrin conformation switch from bent-closed or extended-closed to extended-open conformation). Cell signaling initiated by receptors such as chemokine receptors, T cell receptor (TCR), Toll-like receptors (TLR), and selectins, among others, trigger the switch from Rap1-GDP to Rap1-GTP which, either dependently of RIAM or not, activate Talin and enable its binding to the β-cytoplasmic tail of the integrin. Finally, Kindlin binds to the β-cytoplasmic tail of the integrin, and together with talin induces the separation of the cytoplasmic tails, and triggers the activation of the ligand-binding domain. The extended-open conformation of the integrin remains stable with Talin and Kindlin bound.

extended, intermediate affinity conformation, but that both talin and kindlin-3 are required for the induction of the high-affinity conformation (38, 39) (**Figure 1**). However, kindlin has also been reported to play a role in integrin clustering, stabilizing the integrin-ligand contact and strengthening cell adhesion (40), by recruiting downstream components such as actin, ILK, and paxillin (41–44).

The small GTPase Rap1 also plays a critical role in integrin activation (45, 46). The effects of Rap1 on integrin activation are at least in part dependent on the Rap1 interacting protein, RIAM (47). RIAM is a Rap1 effector molecule that is important for at least β2-integrin lymphocyte trafficking from blood into peripheral lymph nodes (pLNs) (45), but not for the regulation of all integrins (β3-integrin activation in platelets is independent of RIAM). However, for β2-integrins, the pathway seems to consist of Rap1/RIAM/Talin (45, 48). Very late antigen-4 (VLA-4) (α4β1, CD49d/CD29) -mediated adhesion is dependent on talin but only partly dependent on RIAM (45, 48) suggesting that also RIAM-independent Rap1/talin pathways exist. Recent studies seem to indicate that a RIAM-independent Rap1/talin pathway is of particular importance in cells that rely on quick integrin activation to function efficiently, such as neutrophils and platelets (49).

In addition to talin and kindlin, integrins interact with a multitude of cytoplasmic proteins, for example filamin A, Dok1 and 14-3-3 proteins (50) (**Figure 2**). Filamin A was previously thought to be a negative regulator of integrins, either by inhibiting talin binding (51) and/or by crosslinking integrin cytoplasmic domains (52). However, recent results indicate that it may instead be important for integrin-mediated shear flow adhesion and trafficking of immune cells *in vivo* (53). Integrin cytoplasmic domain phosphorylation has been reported for many integrins and plays a role in regulating interactions with cytoplasmic molecules and therefore further regulates integrin function (24).

The Function of Integrins and Other Cell Adhesion Molecules in Immune Responses

Patients suffering from leukocyte adhesion deficiency type I (LAD-I) have lost or reduced expression of β2-integrins on their leukocytes, and these patients suffer from recurrent bacterial infections (54). Symptoms also include leukocytosis, periodontitis and delayed wound healing. In leukocyte adhesion deficiency type III (LAD-III), integrins are expressed but dysfunctional because kindlin-3 is mutated or absent, and these patients have similar symptoms as LAD-I patients (54). However, they also suffer from a Glanzmann-type bleeding disorder as kindlin-3 is required not only for β2-integrin-mediated leukocyte adhesion but also for β3-integrin-mediated platelet adhesion. These findings show that β2-integrins and their cytoplasmic regulators play fundamentally important roles in immunity (55). Studies with mice deficient for different β2-integrins have further revealed individual contributions to various leukocyte processes (56, 57).

Leukocytes traffic out of the blood stream into the lymph nodes, tissues or tumors by using the leukocyte adhesion cascade, which is regulated by sequential function of adhesion molecules (selectins, integrins, receptors of the IgSF) (58, 59). In brief, selectin-selectin ligand interactions lead to rolling of the leukocyte on endothelial cells, allowing activation of the cell by chemokines present on the endothelium. This leads to activation of integrins on the surface of the immune cell (15). LFA-1 and VLA-4 integrin activation by talin and kindlin allows firm interaction between the immune cell such as a T cell or a

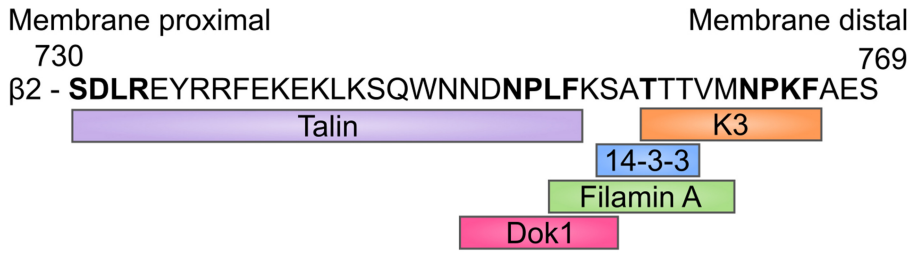

FIGURE 2 | β2-integrin binding sites. Amino acid sequence of the β2-cytoplasmic tail where most of the main integrin binding proteins bind, and the sequences to which they bind. The amino acids highlighted in bold are of particular importance. 14-3-3 proteins only bind to Th758-phosphorylated integrin, whilst phosphorylation of this site inhibits Filamin A binding.

neutrophil and endothelial cells, which express integrin ligands such as ICAMs, VCAM-1, and MAdCAM (37, 38, 58, 60, 61). This is followed by cell spreading, Mac-1-mediated crawling (62), paracellular or transcellular extravasation, and migration into lymph nodes or tissues. In effector T cells, LFA-1 is up-regulated and constitutively activated, which contributes to the trafficking properties of these cells to peripheral tissues (63, 64). In tumors, several steps of the leukocyte trafficking process can be severely disrupted (discussed below).

Adhesion is important also in other immune cell interactions. LFA-1-ICAM-1 interaction, in particular, plays an essential role in the formation of the immunological synapse (IS) between a DC and a T cell (65–67). The structure of an IS is highly organized with key interacting molecules organized in distinct areas called supra-molecular activation complexes (SMACs) (68). The central region of the SMAC (cSMAC) is enriched in TCRs and associated molecules while LFA-1 and ICAM-1 are localized in the peripheral region of the SMAC (pSMAC) and large molecules such as CD45 and CD43 in the distal area of the SMAC (dSMAC). Also VLA-4 is localized at the pSMAC (69). Due to the crucial role for the stabilization of the immunological synapse, LFA-1 is important for T cell activation and proliferation (70, 71). In addition, talin and kindlin-3-mediated activation of LFA-1 has been shown to be important in T cell activation *in vivo* (71, 72). LFA-1 also provides a necessary co-stimulatory signal for T cells lowering the threshold for activation and proliferation following TCR engagement and promotes their IL-2 production (71, 73–75) In addition, LFA-1 has been reported to play a role in Th1/Th2 polarization, development and/or maintenance of Tregs and follicular T cells, and for generation of memory T cells (61, 76–79). Further, LFA-1-aided IS formation is important in the contact between cytotoxic $CD8^+$ T cell/NK cell and the target cell such as a tumor cell and for the release of cytotoxic granules and target cell killing by $CD8^+$ T cells and NK cells (61, 80, 81). Together, these studies show that LFA-1-ICAM-1 pathway plays a crucial role in T cell trafficking, activation and function and thus in the protection of the host from infections and cancer.

Mac-1 and αXβ2 are important receptors for complement iC3b thus mediating phagocytosis of complement-coated particles (82). Also neutrophil degranulation is dependent on Mac-1 integrin (56, 83). Therefore, Mac-1 is vital for the functionality of myeloid cells. However, interestingly, Mac-1 in myeloid cells can also function as a suppressor of immune responses (26) by e.g., inhibiting TLR-signaling in macrophages (84, 85). β2-integrins and the β2-integrin ligand ICAM-1 can also repress DC-mediated T cell activation (86–89) and Th17 differentiation (90, 91) and restrict DC trafficking from peripheral sites to lymph nodes (89, 92).

ICAM-1 is the main ligand for β2-integrins (24). It is expressed at basal levels on several cell types including fibroblasts, keratinocytes, endothelial cells, and leukocytes and its expression increases during inflammation due to inflammatory cytokines such as IFNγ, IL-1β, and TNFα (93, 94). In inflamed tissues, ICAM-1 expressed on endothelial cells binds to β2-integrins LFA-1 and Mac-1 on leukocytes and facilitates their transendothelial migration to the inflammation site. Given the role in mediating leukocyte migration, ICAM-1 up-regulation has been associated to various inflammatory, autoimmune and allergic diseases (94–96). Interestingly, ICAM-1 is also expressed in many types of tumors where it plays a dual role in tumor growth (discussed below).

VCAM-1 is predominantly expressed on endothelial cells and, similar to ICAM-1, its expression increases during inflammation due to inflammatory cytokines such as TNFα (97, 98). VCAM-1 is an important mediator of immune cell mediated rolling, adhesion and extravasation into inflamed tissues by binding to VLA-4 on leukocytes. Thus, VCAM-1 expression has been associated with several autoimmune disorders including rheumatoid arthritis and asthma. In addition, again similar to ICAM-1, VCAM-1 has been shown to play a dual role in cancer development (discussed below).

Integrins and integrin ligands therefore play crucial roles in several immune system functions relevant for tumor rejection, especially in immune cell migration and activation. Indeed, cell adhesion molecules have been shown to play both positive and negative roles in anti-tumor immunity.

Cold Tumors Often Display Dysregulated Expression of Cell Adhesion Molecules on the Tumor-Associated Vessels

Based on the immune landscape, tumors can be divided into inflamed or "hot" and non-inflamed or "cold" tumors. Hot tumors are characterized by transcripts encoding for various T

cell-lineage markers, innate immune cell molecules, chemokines that regulate effector T cell recruitment and also for molecules mediating immune suppression such as PD-L1, Foxp3 and indoleamine-2,3-dioxygenase (IDO) (59, 99, 100). These tumors are highly infiltrated by tumor-infiltrating lymphocytes (TILs) but their function is inhibited due to immune suppressive tumor microenvironment. By contrast, cold tumors have low expression of the before-mentioned transcripts but instead express high levels of factors associated with angiogenesis, molecules involved in Notch and/or β-catenin pathway and serine protease inhibitors. Cold tumors are generally poorly infiltrated by TILs and effective T cell homing into the tumor remains a major obstacle for endogenous anti-tumor immunity and for the success of cancer immunotherapies. Migration of immune cells into tumors can be hampered by many factors, such as impaired chemokine expression at tumor sites, mismatch between chemokines expressed at the tumor site and the set of chemokine receptors being expressed on immune cells, fibrosis around the tumors, and abnormal tumor vasculature (101). Aberrant adhesion molecule expression on leukocytes and cancer cells enables immunoediting and evasion of immune system surveillance while aberrant expression of adhesion molecules on tumor-associated blood vessels can render the whole tumor mass inaccessible for the immune system.

Indeed, down-regulation of adhesion molecules on tumor-associated endothelial cells, a process termed as endothelial anergy, is an effective mechanism utilized by tumors to prevent immune cell trafficking into the tumor site (102, 103). Down-regulation of several adhesion molecules such as ICAM-1/2, VCAM-1, E-selectin, P-selectin, and MAdCAM-1 has been reported in tumor-associated vessels in various human malignancies (104–109) which is at least partly due to high levels of angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in the tumor microenvironment (105, 110). It has been demonstrated both *in vitro* (111) and *in vivo* (112) that low adhesion molecule expression on the endothelial cells due to angiogenic factors leads to diminished leukocyte-vessel wall interactions. Further, diminished leukocyte interactions with tumor endothelium is caused by down-regulation of adhesion molecules on the endothelium and not by decreased expression of LFA-1, VLA-4, or L-selectin on leukocytes (110). From a clinical perspective, up-regulation of ICAM-1 in the tumor microenvironment have been shown to be related to favorable prognosis among patients with various cancers, suggesting an enhancement in cancer immunosurveillance (113–115). Indeed, an increase in TILs was observed in ICAM-1 positive gastric and colorectal cancers compared to ICAM-1 negative tumors (114–116). In addition, Tachimoro et al. showed that more human peripheral blood mononuclear cells (PBMCs) adhered to colon carcinoma (LM-H3) cells *in vitro* that were transfected with ICAM-1 compared to cells with an empty vector (117). PBMC-mediated cytotoxicity was also enhanced towards ICAM-1 expressing LM-H3 cells. Further, when injected into nude mice, a significant reduction was observed in subcutaneous tumor growth and ability to metastases liver with ICAM-1^+ LM-H3 cells compared to ICAM-1 negative cells. In summary, expression levels of integrin ligands on tumor endothelium clearly influences anti-tumor immune responses by affecting immune cell infiltration into tumors.

Expression of Integrins and Other Cell Adhesion Molecules Affect T Cell Infiltration Into Tumors

T cells are important for the recognition of tumor-specific antigens and for the killing of malignant cells (118, 119). Thus T cells, particularly $CD8^+$ T cells, have been demonstrated to be crucial in protecting the host from malignant tumor growth (120, 121). Indeed, $CD8^+$ T cell infiltration and high $CD8^+$ T cell/Treg ratio in the tumor microenvironment has been associated with favorable prognosis in different human malignancies (4, 122, 123). In order for $CD8^+$ T cells to mediate tumor cell killing, they must first become into contact with the malignant cells. However, T cells often fail to infiltrate the tumor tissue, thereby causing a major obstacle for successful treatment of cancer patients with immunotherapy (99, 124). The mechanism of T cell homing to healthy and infected tissues is well-understood but this process may be significantly altered during cancer.

Murine studies have demonstrated that expression levels of both integrin ligands on endothelial cells and integrins on T cells influence T cell tumor infiltration. Fisher et al. showed that ICAM-1 deficiency or blockade decreased $CD8^+$ T cell infiltration into melanoma (B16-OVA) or colon carcinoma tumors (CT26), respectively, demonstrating ICAM-1 expression affecting T cell infiltration into tumors at least in certain tumor models (125). Sartor et al. observed a suppression in tumor growth rate in mice inoculated with fibrosarcoma tumor cells expressing ICAM-1 compared to mice with ICAM-1 negative tumors suggesting an increase in T cell-mediated immunosurveillance in the presence of ICAM-1 (126). Interestingly, also the expression of αE (CD103) on tumor-infiltrating $CD8^+$ T cells has been shown to increase $CD8^+$ T cell trafficking into tumors and thus promoting anti-tumor immunity (127). αE^+ $CD8^+$ T cells had superior capacity to accumulate in tumors in humanized mouse models of breast cancer due to the specific binding of αE on $CD8^+$ T cells to E-cadherin expressed on epithelial cancer cells.

In human patients, high expression of ICAM-1, VCAM-1, and MAdCAM-1 has been shown to correlate with higher density of $CD8^+$ T cells in colorectal cancer (CRC) tumors and also with prolonged disease-free survival (128). Further, human hepatocellular carcinomas (HCC) are more heavily infiltrated with T cells compared to colorectal hepatic metastases (CHM) and that the T cell infiltration was associated with higher expression of ICAM-1 and VAP-1 on tumor-associated endothelial cells in HCC (106). Additionally, a higher proportion of tumor-infiltrating T cells in both tumor types expressed LFA-1 and VLA-4 compared to peripheral blood T cells. In addition, mainly anti-ICAM-1 and anti-VAP-1 and to a lesser extent anti-VCAM-1 mAbs inhibited HCC-derived T cell binding to tumor vascular endothelium *in vitro* suggesting that LFA-1/ICAM-1 and VAP-1 receptor/VAP-1 are crucial pathways mediating T cell recruitment into the tumor site in HCC. Correlation

between levels of cell adhesion molecules (ICAM-1, E-selectin, P-selectin) and T cell infiltration levels has also been shown in melanoma, glioblastoma, Merkel cell carcinoma and squamous cell carcinoma (SCC) (108, 109, 129, 130). Interestingly, when SCC samples were treated with a TLR-7 agonist, imiquimod, tumor vessels up-regulated E-selectin expression causing an increase in CLA^+ $CD8^+$ T cell influx into the tumor, a decrease in Treg frequency and tumor regression. Together, both human and murine studies indicate that integrins and VAP-1 on T cells and integrin ligands on endothelial cells are of crucial importance for T cell infiltration into tumors.

Pre-clinical mouse studies indicate that integrins and integrin ligands may also have other effects on anti-tumor responses besides T cell recruitment into tumors, by influencing T cell priming and effector functions. Schmits et al. showed that LFA-1 deficient mice had defects in $CD8^+$ T cell priming against tumor-specific antigens and thus, failed to reject immunogenic fibrosarcoma tumors (57). Mukai et al. further demonstrated that administration of anti-LFA-1 mAbs abrogated the efficacy of adoptive T cell therapy in mouse models of pulmonary and intracranial fibrosarcomas (131). By contrast, ICAM-1 deficiency or mAbs targeting ICAM-1, VCAM-1, or VLA-4, showed no inhibition on the efficacy of transferred T cells in the same mouse models. Interestingly, LFA-1 blockade and to a lesser extent ICAM-1 blockade caused a decrease in T cell IFNγ production in mixed tumor/T cell cultures but did not affect the level of T cell infiltration into the tumor. These results suggested that the LFA-1 pathway mainly affects T cell effector function but not migration to the tumor site. In addition, given that LFA-1 blockade but not ICAM-1 deficiency/blockade affected the anti-tumor efficacy of adoptive T cell therapy, LFA-1 interactions with other ligands such as ICAM-2 seem to be sufficient to preserve T cell effector function. Accordingly, adoptive T cell studies conducted by Blank et al. further demonstrated that $CD8^+$ T cell infiltration into tumor site is not inhibited in ICAM-1 deficient mice (132). Rather, host ICAM-1 expression affected the priming of adoptively transferred tumor-antigen-specific $CD8^+$ T cells leading to delayed tumor rejection in ICAM-1 deficient mice. In addition to priming, LFA-1 and integrin αE(CD103)β7 expressed on $CD8^+$ T cells play important roles for $CD8^+$ T cell cytotoxicity towards tumor cells expressing ICAM-1 and E-cadherin (133).

In addition to T cell migration into tumors, T cells must also be able to recirculate from the tumor site to draining lymph nodes in order to mount distant responses (134). Interestingly, Yanguas et al. showed that in a mouse model of melanoma, increased number of intra-tumorally injected tumor-specific T cells migrated into the draining lymph nodes in mice treated with anti-ICAM-1 or anti-LFA-1 mAbs compared to mice treated with control IgG (134). Further, activated T cells formed intra-tumoral clusters mediated by LFA-1/ICAM-1 interactions in mouse models of melanoma and breast cancer and similar T cell clusters were also visible in primary human melanoma. These results suggested that LFA-1/ICAM-1 pathway also mediates a mechanism to trap activated $CD8^+$ T cells in the tumor tissue.

In summary, integrins, integrin ligands and other cell adhesion molecules expressed on T cells and endothelial cells mediate $CD8^+$ T cell trafficking into tumors at least in some tumor models, but may also play crucial roles in T cell priming and effector functions, thereby affecting anti-tumor immunity in a multitude of ways.

Integrins and Other Cell Adhesion Molecules Affect DC Function During Anti-Tumor Immunity

DCs orchestrate immune responses and it is now widely appreciated that DCs also play a crucial role in regulating the host immune responses to cancer. Indeed, DCs have been found in various types of tumors both in humans and mice (2, 135–140). In the tumor microenvironment, many tumor cells die naturally or as a result of anti-cancer therapies such as chemotherapy and thus DCs often interact with dying tumor cells enabling them to acquire tumor antigens (141). In addition, immature DCs can also interact with live cells, including other immature or mature DCs and acquire tumor antigens by transferring parts of plasma membrane and intracellular proteins in a process termed "nibbling" (142). Further, immature DCs can also directly interact with live tumor cells and acquire tumor antigens by nibbling (143). Following antigen capture, DCs will ultimately become activated and migrate to the draining lymph node via the lymphatic vessels to present tumor-antigens to T and B cells (141) (**Figure 3**).

Adhesion receptors on DCs are involved in many of the processes involved in DC-mediated anti-tumor responses. Several receptors such as αVβ5-integrin expressed on immature DCs are involved in the interaction with and phagocytosis of dying cells (144, 145). In addition, given that dying cells often become opsonized by complement component iC3b, DCs can also interact with dying tumor cells via the β2-integrins Mac-1 and αXβ2 (146, 147). However, as described above, β2-integrins often have anti-inflammatory effects in myeloid cells such as DCs, and these interactions lead to suppression of DC activation and, thus, tolerance. Further, since inflammation of various levels has often been associated with tumor development (148), and ICAM-1 expression is up-regulated in lymphatic vessels during inflammation (94), interaction between Mac-1 and ICAM-1 expressed on DCs and inflamed lymphatic endothelium, respectively, may lead to decreased ability of DCs to activate T cells (87). Therefore, integrins on DCs may be involved in the uptake of dying tumor cells via adhesion receptors such as αVβ5-integrin, and in the subsequent initiation of DC-mediated anti-tumor responses. However, β2-integrins expressed on DCs may instead be involved in suppressing DC function. How these processes influence the anti-tumor responses mediated by DCs *in vivo* is currently unclear.

Following tumor-antigen capture, DCs need to enter the lymphatic vessels in order to migrate to the draining lymph node to prime tumor-specific T cells. The role of integrins and other adhesion molecules in the migration of DCs to lymphatic vessels is currently under debate. Ma et al. elaborated anti-ICAM-1 or anti-LFA-1 mAbs effectively inhibiting antigen-bearing epidermal DCs from migrating to regional lymph nodes *in vivo* (149). Studies conducted by Xu et al. further demonstrated the importance of ICAM-1 expression on lymphatic endothelium

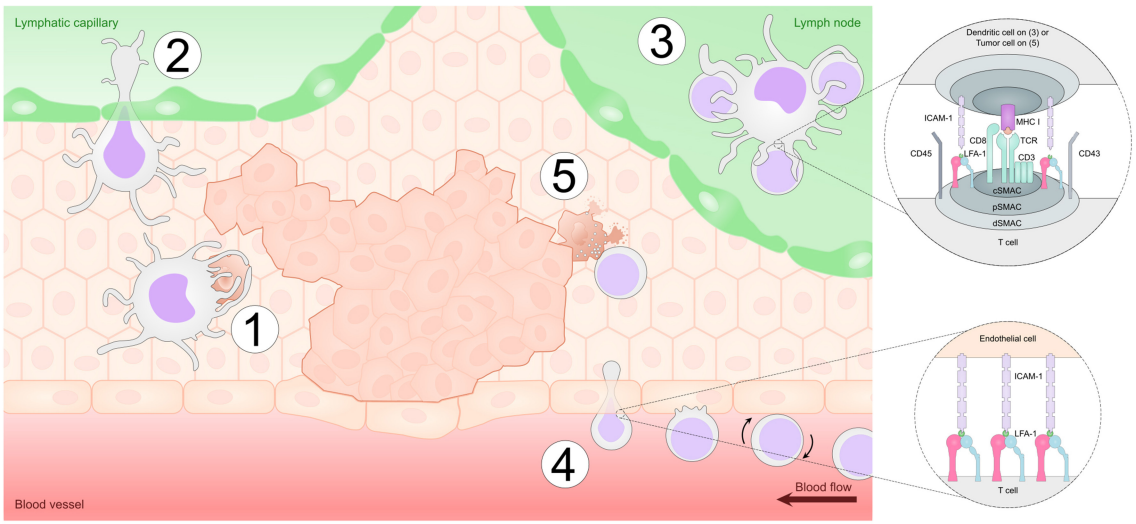

FIGURE 3 | Integrins play a vital role in anti-tumor immunity. Dendritic cells (DCs) take up tumor antigens in the tumor microenvironment by phagocytosing dying tumor cells in a process mediated by adhesion molecules such as $\alpha_V\beta_5$ integrins (Step 1). DCs then enter the lymphatic vessels partly in an LFA-1/ICAM-1-dependent manner and migrate to the draining lymph node (Step 2). In the lymph node, DCs form an immunological synapse with $CD8^+$ T cells in order to present the tumor antigen. LFA-1-ICAM interactions mediate adhesion in the immunological synapse and also provide an additional co-stimulatory signal to the T cells (Step 3). Once activated, T cells travel via the blood stream and enter the tumor site by interacting with adhesion molecules including E-selectin, ICAMs and VCAM-1 on endothelial cells in a process termed leukocyte adhesion cascade. This process is regulated by sequential expression of selectins (L-selectin) and integrins (LFA-1, VLA-4) on the migrating T cell (Step 4). Finally, after reaching the tumor microenvironment, $CD8^+$ T cells form an immunological synapse with tumor cells and kill the malignant cells via the release of cytotoxic granules (Step 5).

for the migration of hapten-bearing Langerhans cells into the draining lymph node (150). In addition, Johnson et al. demonstrated the up-regulation of ICAM-1 and VCAM-1 on dermal lymphatic endothelial cells at the presence of inflammatory cytokines which then mediated DC trafficking into the lymph nodes via afferent lymphatic vessels (151). However, according to Grabbe et al., hapten-bearing DC migration from the blood to inflamed skin or from skin into the regional lymph node is similar in β2 (CD18) deficient mice compared to WT mice suggesting that β2-integrins are not required for DC migration into the regional lymph nodes (152). Furthermore, Morrison et al. reported that a mutation in β2 which disrupts the integrin/kindlin interaction and thereby renders the integrin inactive, resulted in increased rather than decreased DC migration to peripheral lymphoid organs through an effect on gene transcription in these cells (37). Accordingly, Podgrabinska et al. further showed that the rate of antigen-bearing DC migration into the lymph nodes was similar between ICAM-1 deficient mice and WT mice (87). Interestingly, according to Thompson et al. naïve tumor-specific $CD8^+$ T cells can also become activated and gain effector cell phenotype directly at the tumor site, suggesting that cross-presenting DCs are also able to prime $CD8^+$ T cells in the tumor. These results indicate that DC migration into lymph nodes may not even be completely necessary for DC-mediated anti-tumor responses (153). In conclusion, as the role of β2-integrins in DC migration into lymph nodes is currently unclear, whether β2-integrins on DCs influence DC migration and anti-tumor responses in humans is currently not known and requires further study.

In contrast to the unclear role of β2-integrins in DC biology and DC-mediated anti-tumor responses, the αE-integrins may promote DC anti-tumor responses. Several studies have shown that mouse DCs expressing the integrin αE (CD103) are superior in promoting anti-tumor T cell responses (154–157). Indeed, αE^+ DCs are the main cell population carrying tumor-antigens into the draining lymph node and critical for effective anti-tumor $CD8^+$ T cell priming both *in vitro* and *in vivo*. Further, increased tumor growth is observed in the absence of αE^+ DCs in mouse models of cancer (154). In humans, high $\alpha E^+/\alpha E^-$ ratio has been significantly associated with increased overall survival in various malignancies including breast cancer, head-neck squamous cell carcinoma and lung adenocarcinoma (154). However, the expression of CD141 (BDCA-3, thrombomodulin), rather than αE, has been thought to mark human DC population functionally equivalent to mouse αE^+ DCs (158). Indeed, Jongbloed et al. and Bachem et al. demonstrated that $CD141^+$ DCs were superior in their capacity to cross-present various antigens to $CD8^+$ T cells compared to other DC populations (159, 160). Accordingly, a study assessing gene-signatures also associated high intra-tumoral levels of $CD141^+$ DCs with better overall survival in melanoma patients (155). Increased disease-free survival among patients with aggressive triple-negative breast cancer was also associated with gene signature specific for $CD141^+$ DCs (161).

In conclusion, although integrins play a key role in DC biology, the role of cell adhesion molecules in DC-mediated anti-tumor responses is still unclear and clearly requires further study, especially in human patients.

Integrins and Other Adhesion Molecules Affect the Development and Tumor-Infiltration of Regulatory Cells

Tregs, defined as $CD4^+CD25^{hi}CD127^{lo}$ or $CD4^+CD25^+Foxp3^+$ in humans and mice, respectively, are critical in maintaining peripheral tolerance (162). Tregs are able to suppress the effector function and proliferation of various cell populations including T cells, B cells, NK cells, DCs and macrophages by secreting inhibitory molecules such as TGFβ and IL-10, by direct cell-cell contact or by indirect mechanism via antigen presenting cells (APCs) (162–167). For their suppressive function, it is crucial that Tregs are able to migrate to the site of inflammation and thus Tregs express high levels of cell adhesion molecules including ICAM-1, L-selectin, P-selectin, and VLA-4 (168). Interestingly, some adhesion molecules, particularly β2-integrins, also play an important role in Treg development and function (77, 169–171). Wang et al. showed that reduced CD18 (β2) expression in mouse Tregs disrupted the interactions between Tregs and DCs which led to poor Treg proliferation and decreased ability to produce TGFβ1 and thus decreased suppressive function *in vitro* compared to WT Tregs (169). In concordance with *CD18*$^{-/-}$ mice, also *LFA-1*$^{-/-}$ mice showed reduced Treg numbers in secondary lymphoid organs and decreased conversion rate of conventional $CD4^+$ T cells into Tregs in the periphery (170). In addition, LFA-1 deficient Tregs failed to suppress $CD4^+$ T effector cells *in vitro* and were unable to prevent disease development in experimental colitis model *in vivo*. However, on the contrary to *CD18*$^{-/-}$ mice, *LFA-1*$^{-/-}$ mice showed increase in Treg numbers in the thymus suggesting that in addition to LFA-1, other β2-integrin(s) also affects Treg development. Besides β2-integrins, also L-selectin and integrin αE (CD103) have been associated with Treg function. High expression of L-selectin has been shown to mark Tregs with more potent ability to inhibit graft-versus-host disease (GVHD) and bone marrow (BM) graft rejection in mice compared to L-selectin^{lo} Tregs (172) and $αE^+$ Tregs have been shown to be more effective in suppressing acute inflammatory reactions in the induced SCID colitis model and antigen-induced arthritis model compared to αE negative Tregs (173, 174).

Given that Tregs suppress the effector function of various immune cells, they can also inhibit immune responses against cancer cells and thus promote tumor growth (6). Further, the presence of Tregs in the tumor microenvironment can present a major obstacle for successful immunotherapy. Indeed, high number of tumor-infiltrating Tregs has been associated with poor prognosis in several malignancies (4, 175). Conditional depletion of Tregs has been shown to increase anti-tumor immunity in mouse models of cancer suggesting that targeting Tregs could be beneficial approach for cancer patients (176–178). However, the lack of Treg-specific cell surface markers presents a major challenge for this task. Further, targeting Tregs specifically in the tumor microenvironment in order to prevent harmful systemic immune reactions present even a greater challenge. Interestingly, Anz et al. discovered that integrin αE (CD103) is expressed at significantly higher levels in tumor-infiltrating Tregs in several mouse cancer models compared to other peripheral Tregs (90% in CT26-infiltrating Tregs vs. 20% in splenic Tregs) due to increased levels of TGFβ in the tumor microenvironment (179). In addition, $αE^+$ Tregs displayed significantly more suppressive phenotype *in vitro* compared to αE negative Tregs. However, given the high expression also on anti-tumorigenic DCs and $CD8^+$ T cells, αE seems to be an unsuitable target for cancer immunotherapy.

MDSCs and tumor-associated macrophages (TAMs) also represent cell populations capable of efficient suppression of natural anti-tumor immunity. MDSCs are a heterogeneous population of cells consisting of immature myeloid cells and myeloid progenitor cells (180). As other leukocytes, they are generated in the BM but during pathological conditions such infection or cancer they fail to differentiate into mature DCs, macrophages or granulocytes which leads to accumulation of immature myeloid cells with highly immunosuppressive phenotype (180, 181). MDSCs can be divided into different sub-populations in humans ($CD11b^+CD14^-CD15^+$, $CD11b^+CD14^-CD66b^+$ and $CD11b^+CD14^+HLA\text{-}DR^{-/lo}CD15$) and mice ($CD11b^+Ly6G^+Ly6C^{lo}$ and $CD11b^+Ly6G^-Ly6C^{hi}$) based on their phenotypic differences but they all share myeloid origin and the ability to strongly suppress T and NK cell activity (180–182). MDSCs are recruited into the tumor site by the malignant cells by increasing the level of various soluble factors including IL-6, GM-CSF, TGFβ, VEGF and chemokines such as CCL2 and CCL5, in the tumor microenvironment (180, 183). After reaching the tumor site, MDSCs efficiently suppress the anti-tumor immunity by various mechanisms such as by depleting T cell nutrients such as L-arginine, by producing reactive oxygen species (ROS) and nitric oxide (NO) and by promoting the development of Tregs. Indeed, several studies have reported an increase in anti-tumor immunity following depletion or suppression of MDSCs in mouse models of cancer (184–186). Among human patients, an increase in MDSCs has been observed in the tumor tissue and peripheral blood in many cancer types (187–191). Further, an increase in MDSCs in the peripheral blood has been associated with poor prognosis in patients with various solid tumors (5, 192). Interestingly, Jin et al. reported VLA-4 being responsible for recruitment of circulating monocytes and macrophages into the tumor site (193). Later it was found that tumor growth was significantly suppressed in mice lacking activated form of VLA-4 and in WT mice treated with mAbs targeting VLA-4 (194). In addition, tumors derived from these mice had significantly reduced frequencies of total $CD11b^+Gr1^+$ myeloid cells but increased numbers of $CD8^+$ T cells and mature ($CD80^+$) DCs. *In vitro* IL-4 stimulated macrophages derived from mice lacking activated form of VLA-4 also showed decreased levels of *Il10*, *Tgfb1,* and *Arg1* mRNAs but increased levels of mRNAs encoding for IFNγ and IL-12 compared to WT macrophages. These results suggested that VLA-4 regulates MDSC trafficking into the tumor site and also promotes myeloid cell polarization toward immune suppressive phenotype, inhibits anti-tumor immunity and thus promotes tumor growth.

Mac-1 is highly expressed in myeloid cells and plays a key role in various myeloid cell functions including migration, phagocytosis, and chemotaxis (195). In addition, given that

mAbs targeting Mac-1 have been shown to decrease myeloid cell tissue infiltration and inflammation (196), targeting Mac-1 could also reduce the recruitment of suppressive myeloid cells into the tumor site. Indeed, Zhang et al. demonstrated that CD11b (αM-chain of Mac-1) deficiency reduced intestinal tumor growth in mice by reducing myeloid cell trafficking to the tumor microenvironment (197). Further, CD11b blockade also decreased myeloid cell recruitment into tumors in immune deficient mice bearing human squamous cell carcinoma xenografts and thus improved the anti-tumor responses to radiotherapy (198). However, as Mac-1 has been shown to play other roles in immunity than simply affecting cell recruitment, e.g., in immune suppression, it is possible that CD11b blockade also has other, as yet unrecognized effects on anti-tumor immunity.

Integrins and Other Cell Adhesion Molecules Also Have Cell Intrinsic Effects on Tumor Cells

Integrin expression on tumor cells themselves has been associated with tumor progression and metastasis by increasing the proliferation, survival, migration and invasion of the malignant cells (199). Integrin ligation can promote tumor cell survival by several mechanisms such as by inhibiting p53 and caspase-9 via αV (200) and by increasing anti-apoptotic proteins Bcl-2 and Bcl-X_L via αVβ5 and αVβ3 (201). Particularly integrin αVβ3 has been associated with tumor progression in various human malignancies (202–204) and it co-operates with *SRC* oncogene to enhance anchorage-independent tumor growth and promotes lymph node metastases (205, 206). Other integrins have also been shown to co-operate with oncogenes including β4 which amplifies the signaling of ErbB2 to promote mammary tumorigenesis (207). In addition, integrins such as β3 may also function as markers for highly tumorigenic cancer stem cells (208).

Besides on tumor-associated vasculature, aberrant VCAM-1 expression has also been described on many types of tumor cells such as breast, renal and gastric carcinoma cells (97, 209). Up-regulation of VCAM-1 on malignant cells is associated with increased ability to metastasize and recruit tumor-associated monocytes and macrophages. Also ICAM-1 expression has been associated with the malignant potential of tumor cells and has thus been associated with metastases and poor prognosis in several cancers including melanoma, breast, lung and oral cancer (210–213). Given that MUC-1 which is often expressed on tumor cells (214) can also interact with ICAM-1, circulating cancer cells can adhere to endothelial cells which may represent the first step in metastases formation (215). MUC-1-ICAM-1 interaction can then induce cytokine secretion and ICAM-1 up-regulation in the malignant cells thus recruiting macrophages. Indeed, macrophage infiltration has been demonstrated to correlate with ICAM-1 expressing tumor cells in oral squamous cell carcinoma (213). Macrophage-derived cytokines can then further attract neutrophils which secrete proteases promoting extraluminal migration of the malignant cells (215). Further strengthening the role of ICAM-1 in tumor metastasis, tumor cell lines transfected with ICAM-1 showed enhanced invasive capacity and proliferation *in vitro* (211, 213) and ICAM-1 blockade have been shown to decrease tumor cell invasion *in vitro* (211, 216). In addition, an increase in serum levels of soluble ICAM-1 (sICAM-1) has been associated with disease progression, tumor aggressiveness and decreased survival in several malignancies such as melanoma, chronic B-lymphocytic leukemia, lymphoma, and CRC (217–220).

Given the specific expression on hematopoietic cells, LFA-1 plays a role in the development of hematological malignancies such as leukemias and lymphomas. Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western countries (221). It is characterized by clonal mature B cell accumulation in the BM, blood and other lymphoid tissues and advanced disease often manifests itself as lymphadenopathy, hepatomegaly, splenomegaly, BM failure, and recurrent infections. The malignant CLL cells are highly dependent on lymphoid microenvironment where they interact with and receive survival and proliferation signals from the accessory cells (222). In addition to chemokines, malignant cells require the expression of leukocyte adhesion molecules, particularly LFA-1 and VLA-4, in order to migrate into the lymphoid tissues. Interestingly, Montresor et al. reported differences in integrin signaling in human CLL cells compared to healthy B cells (223). Signaling molecules such as PIP5KC, RAC1, and CDC42 regulating integrin conformational change into the active form in normal lymphocytes showed either none or severely decreased regulatory role in CLL cells. By using live cell imaging, Till et al. further showed that in contrast to LFA-1 expressed on normal human B cells, LFA-1 expressed on CLL cells can be in its active conformation without chemokine induced clustering (224). In addition to aberrant integrin signaling, the expression levels of adhesion molecules on CLL cells usually differ from healthy B cells. Generally, the expression of various adhesion molecules including β2- and β1-integrins, CD54, CD62L, and CD44 is very low on peripheral blood and BM CLL cells compared to cells derived from healthy donors (225). Interestingly, Hartmann et al. demonstrated that CLL cells require the same integrins as healthy B cells in trafficking through the BM, spleen, and lymph nodes (226). On the contrary to normal human B cells, CLL cells were unable to arrest in ICAM-1 expressing endothelium *in vitro* and to migrate to lymph nodes of NOD/SCID mice *in vivo* due to low expression of LFA-1. In addition to low LFA-1 expression, VLA-4 expression was variable on CLL cells but yet significantly reduced compared to healthy B cells. Therefore, the ability of human CLL cells to migrate to the BM of NOD/SCID mice *in vivo* was significantly reduced compared to normal B cells. These results suggested that CLL cell migration to the BM and lymph nodes is decreased due to low expression of LFA-1 and VLA-4 thus causing an accumulation in the blood and spleen. Interestingly, the same study also reported that significantly higher expression of LFA-1 and VLA-4 was detected on CLL cells derived from high-risk patients with unfavorable cytogenetic abnormalities such as trisomy 12, deletion 17p or deletion 11q (226). Increased expression of integrins including LFA-1 and VLA-4 on CLL cells among patients with trisomy 12 has also been reported by others and the increase has been associated with up-regulation

of molecules regulating integrin inside-out signaling such as RAP1B and RAPL and with enhanced ligand (ICAM-1, VCAM-1) binding and migration *in vitro* (227, 228). These results suggest that up-regulation of cell adhesion molecules, particularly LFA-1 and VLA-4, could increase CLL cell migration to lymphoid tissues where they would receive more proliferation and survival signals thus leading to more aggressive disease (226, 227). Indeed, higher expression of CD11a (LFA-1 α-chain) on CLL cells has been associated with increase in tumor burden (225) and higher expression of CD49d (VLA-4 α-chain) with disease progression and decreased overall survival (229–231).

Multiple myeloma (MM) is a cancer of the BM caused by malignant, terminally differentiated plasma cells (PCs) (232, 233). It is the second most common hematological malignancy in the world and its symptoms include increased calcium levels, renal insufficiency, anemia, and/or bone lesions. In most cases the malignant plasma cells also secrete monoclonal immunoglobulin proteins (M-proteins) that can be detected in blood and/or urine. Despite the recent improvements in MM therapies, MM is still largely considered to be an incurable disease. As in CLL (222), adhesion molecules play an important role in the interactions between MM cells and accessory cells in the BM thus promoting the proliferation and survival of the malignant cells (234). Indeed, high proportion of human myeloma cells have been shown to express various adhesion molecules including LFA-1, VLA-4, CD44, and ICAM-1 (235–237). LFA-1 expression on myeloma cells in particular has been associated with aggressive disease (236, 237) and to correlate with disease activity (235). Interestingly, by using a mouse MM cell line (5T33MMvt), Asosingh et al. demonstrated LFA-1 playing a role in homotypic cell-cell adhesion and cell proliferation of myeloma cells *in vitro* (238). Further, in contrast to LFA-1 negative cells, only LFA-1 expressing 5T33MMvt cells were able to cause disease *in vivo*. Finally, various adhesion molecules including LFA-1 and VLA-4 have also been associated with drug resistance in MM patients (239).

Anti-Tumor Therapy Targeting Cell Adhesion Molecules

Given that integrin function has been associated with tumor progression, there has been great interest in targeting integrins in the treatment of cancer. However, since integrins also play diverse roles in immunity and anti-tumor responses, blocking or enhancing the function of these molecules *in vivo* may be difficult.

The majority of drugs targeting integrins in the aim to treat cancer inhibit the function of αV- or β1-integrins (240, 241). Both integrin classes have been shown to be highly expressed in many human malignancies and antagonists targeting αV or β1 indeed suppressed tumor growth in preclinical mouse models (199, 202, 242–248). As a result, there have been multiple clinical trials targeting integrins either as a single therapy or in combination with conventional therapies in treating various human cancers (249–289) (**Table 1**). However, in contrast to the pre-clinical studies, the results from clinical trials were rather disappointing with αV or β1 antagonists generally showing either no or only week anti-tumor efficacy (**Table 1**). In one phase II clinical trial with advanced prostate cancer patients, intetumumab (CNTO 95), a mAb targeting multiple αV integrins, in fact resulted in decreased progression-free and overall survival compared to placebo (276). However, it could be possible to improve therapeutic responses of integrin targeting by combining it with other immunotherapies. Indeed, mouse studies conducted by Kwan et al. demonstrated that by combining a fusion protein, consisting of a mouse Fc domain and RGD-binding integrin targeting peptide, with albumin/IL-2 or anti-PD-1 immunotherapy it was possible to enhance anti-tumor immunity and tumor suppression (290).

Due to the specific expression on hematopoietic cells, β2-integrins have mainly been aimed to target to treat inflammatory diseases including liver fibrosis (291) and autoimmune diseases, including arthritis (292), and psoriasis (293). In pre-clinical studies, LFA-1 small-molecule antagonist (BMS-587101) inhibited LFA-1-mediated T cell adhesion to endothelial cells, Th1 cytokine production, and T cell proliferation *in vitro* and also inhibited inflammation *in vivo* (292). In the context of cancer, β2-integrins, mainly LFA-1, would be an attractive target to treat hematological cancers such as leukemias and lymphomas (294, 295). In addition, LFA-1 small molecule antagonists have also demonstrated anti-tumor efficacy against solid tumors in mice (296). However, given that LFA-1 promotes T cell activation and migration, blocking the function of LFA-1 may in fact increase the risk of malignancies and infections (297). Indeed, anti-LFA-1 therapy has been associated with rare but severe systemic adverse events such as immune-mediated thrombocytopenia and hemolytic anemia (298). Further, in 2009, after more than 45,000 psoriasis patients had been treated with efalizumab (humanized anti-CD11a mAb), the drug was withdrawn from the market due to three confirmed cases of fatal viral-based multifocal leukoencephalopathy (PML) (297, 299). One possibility to harness the anti-tumor efficacy of LFA-1 blockade without inducing severe adverse events would be to target LFA-1 specifically on tumor cells. Indeed, Cohen et al. demonstrated that by using bispecific antibody which simultaneously targets LFA-1 and a tumor specific antigen it was possible to specifically block LFA-1-mediated tumor cell adhesion without affecting immune responses in mice (294). In addition, it is also noteworthy that since activation of LFA-1 has been shown to regulate the activity of VLA-4, drugs targeting LFA-1 may also affect the function of other integrins (300).

Given the lack of anti-tumor efficacy and in the case of LFA-1 blockers, the severity of adverse events, it could be more feasible to target the integrin ligands on tumor vessels than the integrins themselves in order to enhance T cell infiltration to the tumor site. As discussed before, previously E-selectin negative tumor-associated vessels in human SCC samples up-regulated E-selectin following treatment with imiquimod (TLR-7 agonist) which resulted in $CD8^+$ T cell influx into the tumor and tumor regression (129). Also systemic thermal therapy (STT) induced the activation of IL-6 trans-signaling causing up-regulation of ICAM-1 on tumor vascular endothelium and thus increased the homing of adoptively transferred $CD8^+$ T cells

TABLE 1 | Clinical trials targeting integrins.

Target	Compound name	Molecule	Study phase	Malignancy	Combination therapies	Clinical outcome	References
αV	Intetumumab (CTNO 95)	mAb (fully human IgG1)	Phase 1	Solid tumors	-	One partial response (4,2% of patients)	(256)
			Phase 2	Melanoma	Dacarbazine ± Intetumumab	No significant differences in the efficacy between treatment groups	(271)
			Phase 2	Prostate cancer	Docetaxel + Prednisone ± Intetumumab	All efficacy end points favoured placebo over intetumumab	(276)
			Phase 1	Solid tumors	Bevacizumab + Intetumumab	No tumor responses	(283)
αV	IMGN-388	Antibody-drug conjugate (intetumumab bound to maytansinoid cytotoxic agent DM4)	Phase 1	Solid tumors	-	No evidence of activity	(267)
αV	Abituzumab (EMD 525797; DI17E6)	mAb (humanized IgG2)	Phase 1	Prostate cancer	-	One partial response (3,8% of patients)	(279)
			Phase 1/2	Colorectal cancer	SoC (Cetuximab + Irinotecan) ± Abituzumab	Primary end point was not met, no significant differences in efficacy was observed between treatment groups	(280)
			Phase 2	Prostate cancer	Luteinizing hormone-releasing hormone agonist/antagonist therapy ± Abituzumab	No significant differences between treatment groups	(286)
αV/α5β1	GLPG-0187	Small molecule inhibitor	Phase 1	Glioma and other solid tumors	-	No tumor responses	(285)
αVβ3/α5β1	ATN-161	Small molecule inhibitor	Phase 1	Solid tumors	-	No objective responses	(251)
αVβ3/αVβ5	Cilengitide (EMD121974)	Small molecule inhibitor	Phase 2	Pancreatic cancer	Gemcitabine ± Cilengitide	No differences in clinical efficacy between the treatment groups	(254)
			Phase 1	Malignant glioma	-	Shows clinical activity	(257)
			Phase 1	Refractory brian tumors	-	Preliminary evidence of clinical activity	(260)
			Phase 2	Glioblastoma	-	Modest antitumor activity	(261)
			Phase 1/2a	Glioblastoma	Temozolomide + radiotherapy + Cilengitide	Promising activity compared to historical controls	(266)
			Phase 2	Prostate cancer	Luteinizing hormone-releasing hormone therapy + Cilengitide	Modest clinical effect	(269)
			Phase 2	Glioblastoma	Surgery + Cilengitide	Modest antitumor activity	(272)
			Phase 2	Melanoma	-	Minimal clinical efficacy	(273)
			Phase 1	Solid tumors	-	No objective responses	(274)
			Phase 3	Glioblastoma	Temozolomide + radiotherapy ± Cilengitide	Addition of cilengitide did not improve outcomes	(277)

(Continued)

TABLE 1 | Continued

Target	Compound name	Molecule	Study phase	Malignancy	Combination therapies	Clinical outcome	References
			Phase 2	Squamous cell carcinoma of the head and neck	Cisplatin + 5-Fluorouracil + Cetuximab ± Cilengitide	No significant differences between the treatment groups	(278)
			Phase 1	Glioblastoma	Cediranib + Cilengitide	Response rates do not warrant further development of this combination	(281)
			Phase 2	Glioblastoma	Temozolomide + Radiotherapy ± Cilengitide	No firm conclusions regarding clinical efficacy were able to be made	(282)
			Phase 2	Non-small-cell lung cancer	Cetuximab + platinum-based chemotherapy (Cisplatin/Vinorelbine or Cisplatin/Gemcitabine) ± Cilengitide	Potential clinical activity	(284)
			Phase 2	Glioblastoma	Cilengitide + radiotherapy + Temozolomide + Procarbazine	Response rates do not warrant further development of this combination	(287)
			Phase 1	Solid tumors	Paclitaxel + Cilengitide	Antitumor activity was observed	(289)
αVβ3	Etaracizumab (abegrin; MEDI-522)	mAb (humanized IgG1)	Phase 1	Solid tumors	-	No objective responses	(250, 259)
			Phase 2	Melanoma	Taracizumab ± Dacarbazine	Etaracizumab had no tumor response when given as a single treatment; Etaracizumab + Dacarbazine similar to historical data for Dacarbazine alone; phase 3 study not reasonable	(264)
αVβ3	Vitaxin (MEDI-523)	mAb (humanized IgG1)	Phase 1	Solid tumors	-	Potential activity	(249)
α5β1	Volociximab (M200)	mAb (humanized chimeric IgG4)	Phase 2	Renal cell cancer	-	Best outcome was stable disease	(253)
			Phase 2	Melanoma	Dacarbazine + Volociximab	Best outcome was stable disease	(252)
			Phase 2	Pancreatic cancer	Gemcitabine + Volociximab	One partial response (5% of patients)	(255)
			Phase 2	Melanoma	-	Insufficient clinical activity to proceed to the second stage of the trial	(258)
			Phase 1	Solid tumors	-	Preliminary activity was observed in two patients (9,5% of patients)	(262)
			Phase 2	Ovarian and peritoneal cancer	Doxorubicin ± Volociximab	No differences between the treatment groups	(263)
			Phase 2	Epithelial ovarian and peritoneal cancer	-	Insufficient clinical activity	(268)
			Phase 1b	Non-small-cell lung cancer	Carboplatin + Paclitaxel + Volociximab	Best outcome was partial response (24% of patients)	(275)
α2	E-7820	Small molecule inhibitor	Phase 2	Colorectal cancer	Cetuximab + E-7820	Objective response rate 3.6%	(265)
			Phase 1	Advanced solid tumors	-	Best outcome was stable disease	(270, 288)

into tumors in mouse models of cancer (125). Further, CpG-ODN (oligodeoxynucleotides (ODN) with cytocine-guanine-rich (CpG) motifs) vaccination caused up-regulation of ICAM-1 and VCAM-1 on tumor-associated blood vessel endothelia leading to strong tumor-infiltration of adoptively transferred tumor-specific T cells and tumor suppression in mouse model of pancreatic islet cell carcinoma (301). These results demonstrated that by increasing inflammatory signals in the tumor microenvironment it could be possible to enhance T cell infiltration into tumors and thus T-cell mediated tumor cell killing. The most straight forward way to increase inflammatory signals would be to administer inflammatory cytokines such as TNFα or IFNγ systemically (302). However, this could lead to severe adverse events as has been demonstrated with the systemic administration of TNFα which was associated with risk of septic shock syndrome leading to multi-organ failure (303). Delivery of inflammatory cytokines or other inflammatory stimuli directly to the tumor site could reduce the risk of side effects (302). Further, systemic administration of TNFα coupled to ACDCRGDCFCG-peptide, a ligand for αVβ3-integrin, has enabled targeting the tumor vasculature and inducing anti-tumor effects in tumor-bearing mice (304). Given that angiogenic factors have been shown to cause decrease in ICAM-1 and VCAM-1 expression on tumor associated vessels (105, 110), targeting angiogenesis could also increase T cell infiltration into tumors. Indeed, treatment with angiogenesis inhibitor anginex significantly up-regulated the expression of VCAM-1 and E-selectin on tumor blood vessel endothelial cells resulting in increase in tumor-infiltrating leukocytes and suppression of tumor growth in mouse models of cancer (305). Further, VEGF blockade significantly increased tumor infiltration of adoptively transferred T cells thus promoting tumor suppression in mice (306).

Among therapies involving adoptively transferred cells, chimeric antigen receptor (CAR) T cell therapy has shown promise in cancer treatment due to measurable responses in several clinical trials across various malignancies (307–310). Given that CAR T cells are usually constructed by joining an antigen-recognition moiety such as single chain antibody (scFv) to TCR/CD3 complex and by adding costimulatory domain such as CD28 or CD137 to improve cell survival and proliferation, CAR T cell therapy has several advantages to conventional T cell therapy such as higher antigen binding affinity and independency of MHC expression on tumor cells (311). However, the major obstacle for CAR T cell therapy is often the inability of the transferred cells to migrate and extravasate into the tumor (312). One way to overcome this obstacle could be to generate a CAR T cell with specificity to tumor-associated vasculature instead of the tumor cells themselves (313). Fu et al. constructed a CAR T cell containing a peptide sequence for echistatin (T-eCAR) which has a high affinity to the integrin highly expressed on tumor vasculature endothelium, αVβ3 (313). Indeed, T-eCAR cells efficiently lysed αVβ3 expressing human umbilical vein endothelial cells and tumor cells *in vitro*. Further, these cells led to extensive bleeding in the tumor tissue and thus tumor growth suppression while sparing normal tissues in mice. In addition to CAR T cells, integrins expressed on the tumor vasculature could also be utilized in order to target drug-delivering nanoparticles to the tumor tissue more efficiently (199). Murphy et al. demonstrated that doxorubicin containing, αVβ3-targeted lipid nanoparticles effectively targeted tumor vessels leading to their apoptosis in mouse models of

TABLE 2 | Adhesion molecule-mediated events promoting tumor growth.

Event	Type of tumor	Adhesion molecule-mediated mechanisms operating in the tumor microenvironment	Consequence for tumor progression	References
1	Solid	Increased secretion of angiogenic factors by the tumor cells reduces the expression of various adhesion molecules including ICAM-1/2, VCAM-1 and E-selectin in tumor-associated endothelial cells	Leukocytes in blood are unable to extravasate to the tumor site (endothelial anergy)	(102, 103, 105, 110–112)
2	Solid	Dying tumor cells become opsonized with iC3b	DCs interact with dying tumor cells via β2-integrins Mac-1 and CD11c/CD18 leading to suppression of DC activation and tolerance	(146, 147)
3	Solid	High expression of adhesion molecules including ICAM-1, VLA-4 and L-selectin on Tregs	Affects Treg trafficking possibly enabling them to reach the tumor site where they suppress effector T cells leading to tumor evasion of the immune system	(168)
4	Solid	High expression of VLA-4 and CD11b on myeloid cells	Myeloid cells are able to reach the tumor site and promote angiogenesis and tumor growth	(193, 195–197)
5	Solid	Expression of various integrins including αVβ3, ICAM-1 and VCAM-1 on tumor cells	Increase in tumor cell proliferation, survival and invasion, recruitment of Tumor Associated Macrophages (TAMs) which allows evasion of the immune system	(97, 201–206, 209–213)
6	Solid	Expression of MUC-1 on tumor cells, which is able to bind to ICAM-1 in endothelial cells	Tumor cells are able to cross the endothelial barrier, which promotes metastasis	(214, 215)
7	Hematological	Upregulation of LFA-1/VLA-4 expression on tumor cells which are able to bind to ICAM-1/VCAM-1 in endothelial cells	Tumor cells are able to cross the endothelial barrier and migrate to lymphoid tissues to receive more proliferation and survival signals promoting tumor progression	(222, 225–227, 229–231, 235–237)

cancer (314). Moreover, αVβ3-targeted nanoparticles showed anti-metastatic effects in spontaneous metastases models without causing adverse events associated with systemic administration of free doxorubicin.

It may also be possible to enhance the anti-tumor response of adoptively transferred cells by increasing LFA-1 function in the cells. Simply "locking" LFA-1 in an active state (through mutation) is unlikely to be successful, as T cells expressing such mutated integrins are actually deficient in T cell migration, which requires dynamic modulation of integrin activity (315). Instead, indirect approaches may be required. Indeed, mutation of a phosphorylation site in VLA-4 leads to increased LFA-1 activity (through integrin transregulation) and enhanced tumor immunity (316). Also, modulating LFA-1 activation status through engineering of ALCAM increases tumor rejection of brain tumors (317).

Conclusions and Future Perspectives

Taken together, adhesion molecules play vital roles in the function of the immune system both in health and disease. During cancer development, adhesion molecules, particularly integrins, mediate crucial functions in nearly every step of the anti-tumor response including in tumor antigen uptake, activation of tumor-specific T cells, leukocyte trafficking into the tumor site and tumor cell killing. However, malignant cells can also utilize cell adhesion molecule pathways to promote tumor growth. Expression of various integrins on tumor cells promotes tumor cell proliferation, survival and metastases while increased secretion of angiogenic molecules causes down-regulation of adhesion molecules on tumor-associated blood vessels and thus prevents immune effector cell infiltration into the tumor. Tumor cells also recruit regulatory cells such as Tregs and MDSCs which express high levels of integrins enabling them to reach the tumor site. The main cell adhesion molecule-mediated events promoting tumor growth are listed in **Table 2**.

Immunotherapy including immune checkpoint blockade and CAR T cells has revolutionized the field of cancer therapy during the last decades. Given the various roles in tumor development, integrins also seem like promising targets for cancer therapy. However, clinical trials targeting integrins directly on malignant cells have shown disappointing results with low therapeutic efficacy. Rather, increasing the expression or function of β2-integrins on immune cells or their ligands on tumor-associated blood vessels and enhancing anti-tumor responses may represent a more efficient approach. In addition, considering that still a considerable number of patients do not benefit from current forms of immunotherapy due to the inability of T cells to access the tumor microenvironment, enhancing β2-integrin function could open a possibility to overcome this impediment. For this and also to prevent harmful treatment-related adverse events, it is vital to fully understand the various functions of β2-integrins and how their expression and function are regulated.

AUTHOR CONTRIBUTIONS

All authors contributed to writing the manuscript, with HH and SF playing leading roles in manuscript planning and writing. ML and CG wrote part of the text. ML made the figures.

FUNDING

This study was funded by the Academy of Finland, Sigrid Juselius Foundation, University of Helsinki, Magnus Ehrnrooth Foundation, Liv och Hälsa Foundation, HiLIFE (all to SF).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 May 2019
doi: 10.3389/fimmu.2019.01138

A β2-Integrin/MRTF-A/SRF Pathway Regulates Dendritic Cell Gene Expression, Adhesion, and Traction Force Generation

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β2-integrins are essential for immune system function because they mediate immune cell adhesion and signaling. Consequently, a loss of β_2-integrin expression or function causes the immunodeficiency disorders, Leukocyte Adhesion Deficiency (LAD) type I and III. LAD-III is caused by mutations in an important integrin regulator, kindlin-3, but exactly how kindlin-3 regulates leukocyte adhesion has remained incompletely understood. Here we demonstrate that mutation of the kindlin-3 binding site in the β2-integrin (TTT/AAA-β2-integrin knock-in mouse/KI) abolishes activation of the actin-regulated myocardin related transcription factor A/serum response factor (MRTF-A/SRF) signaling pathway in dendritic cells and MRTF-A/SRF-dependent gene expression. We show that Ras homolog gene family, member A (RhoA) activation and filamentous-actin (F-actin) polymerization is abolished in murine TTT/AAA-β2-integrin KI dendritic cells, which leads to a failure of MRTF-A to localize to the cell nucleus to coactivate genes together with SRF. In addition, we show that dendritic cell gene expression, adhesion and integrin-mediated traction forces on ligand coated surfaces is dependent on the MRTF-A/SRF signaling pathway. The participation of β2-integrin and kindlin-3-mediated cell adhesion in the regulation of the ubiquitous MRTF-A/SRF signaling pathway in immune cells may help explain the role of β2-integrin and kindlin-3 in integrin-mediated gene regulation and immune system function.

Keywords: dendritic cells, adhesion, MRTF-A, SRF, MKL-1, LAD-III, traction force

OPEN ACCESS

Edited by:
Toshiyuki Murai,
Osaka University, Japan

Reviewed by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *07 January 2019*
Accepted: *07 May 2019*
Published: *28 May 2019*

Citation:
Guenther C, Faisal I, Uotila LM, Asens ML, Harjunpää H, Savinko T, Öhman T, Yao S, Moser M, Morris SW, Tojkander S and Fagerholm SC (2019) A β2-Integrin/MRTF-A/SRF Pathway Regulates Dendritic Cell Gene Expression, Adhesion, and Traction Force Generation. Front. Immunol. 10:1138. doi: 10.3389/fimmu.2019.01138

INTRODUCTION

Leukocyte adhesion is an essential process in immunity. Adhesion is necessary for leukocyte surveillance in tissues, phagocytosis, lymphocyte homing, leukocyte extravasation, T-cell priming, and cytotoxic killing. One of the most important group of adhesion molecules on leukocytes are the β2-integrins. β2-integrins are transmembrane proteins consisting of the β-chain CD18 which forms heterodimers with the four α-chains: CD11a, CD11b, CD11c, and CD11d, forming 4 different

receptors e.g., CD11a/CD18 (LFA-1, $\alpha_L\beta_2$, ITAL Ag), CD11b/CD18 (Mac-1, $\alpha_M\beta_2$, CR3, ITAM Ag), CD11c/CD18 (p150p95, $\alpha_X\beta_2$, CR4, ITAX Ag), CD11d/CD18 ($\alpha_D\beta_2$, ITAD Ag) (1).

β2-integrins participate in the leukocyte adhesion cascade where they mediate slow rolling and firm adhesion to the endothelium (2). During this process β2-integrins bind intercellular adhesion molecules (ICAMs) expressed on endothelial cells. In addition to ICAMs, β2-integrins also bind to a vast variety of other ligands such as complement protein iC3b during phagocytosis (3).

Integrins have been found to transmit signals bidirectionally, which means that they mediate inside-out and outside-in signaling during cell adhesion. On resting leukocytes integrins are typically in an inactive, bent conformation which prevents ligand binding (2). Intracellular signaling initiated for example by chemokine receptors eventually leads to integrin conformational changes into an active conformation that has an increased affinity for ligands (inside-out signaling) (4). Subsequent integrin-ligand binding results in further recruitment of integrins and cytoskeletal proteins to the adhesion site, followed by integrin-mediated outside-in signaling leading to further cellular changes.

Integrin conformational changes are regulated by binding of cytoplasmic cofactors to the cytoplasmic tail of the β2-integrin (5). The best characterized integrin cofactor is talin, which is associated with the active (high affinity) integrin conformation and mediates further binding of cytoplasmic proteins such as actin and focal adhesion kinase (6). Talin has been shown to mediate integrin downstream signaling under higher forces. Low and intermediate force transmission is mediated by another cytoplasmic integrin cofactor, kindlin (6).

The importance of adhesion molecules, their ligands and their cytoplasmic interaction partners is demonstrated in a group of immunodeficiencies termed leukocyte adhesion deficiencies (LAD). While LAD-I is caused by reduced or abolished expression of β2-integrins (7), LAD-III is caused by the mutation or absence of kindlin-3. LAD patients experience recurrent bacterial and fungal infections as well as severe bleeding (in LAD-III only) as a result of impaired leukocyte and platelet adhesion.

Kindlin-3-null mice have a similar phenotype as LAD-III patients, demonstrating the importance of kindlin-3 in integrin regulation (8). While it has been demonstrated that only a low level of kindlin-3 is required to enable proper β2-integrin function (9), the exact mechanism by which kindlin-3 regulates adhesion remains incompletely characterized (10). We have previously established a mouse model with a TTT/AAA β2-integrin knock-in mutation, which results in kindlin-3 being unable to bind to β2-integrins and consequently a loss of leukocyte adhesion (11). Furthermore, the reduced β2-integrin/kindlin-3 interaction leads to a range of phenotypic changes in leukocytes (11–13). Interestingly, TTT/AAA-β2-integrin KI dendritic cells (DCs) display an increased maturation phenotype. They express higher level of costimulatory markers such as CD40, CD80, CD86, chemokine receptors (CCR7) and produce more cytokines (IL-12, IL-10) than wild type cells. KI dendritic cells also display increased migration to lymph nodes and induce increased Th1 responses *in vivo* compared to WT dendritic cells (12). While these experiments indicate that active β2-integrins suppress the mature, migratory dendritic cell phenotype, the signaling pathways downstream of β2-integrins that mediate this phenotypic switch have not been identified.

SRF has been termed the master regulator of the cytoskeleton as this transcription factor regulates the expression of many cytoskeletal genes. The majority of SRF-mediated transcription of cytoskeletal genes has been shown to be dependent on its cofactor MRTF-A. In leukocytes, MRTF-A/SRF have been shown to regulate the expression of cytoskeletal proteins as well as β2-integrins (14–16). The MRTF-A/SRF pathway is activated in response to external cell stimuli which initiates F-actin polymerization downstream of RhoA activation. MRTF-A constantly shuttles between the cytoplasm and the nucleus but has been shown to be mainly cytoplasmic in resting cells. In the cytoplasm MRTF-A is bound to G-actin, thus upon F-actin polymerization MRTF-A is released and free to shuttle into the nucleus. Nuclear MRTF-A then initiates gene transcription together with SRF (17).

Here we show that kindlin-3-regulated β2-integrin adhesion is required for signaling via RhoA and actin to initiate MRTF-A nuclear localization in dendritic cells. Furthermore, dendritic cell adhesion, traction force generation and gene expression is regulated by MRTF-A/SRF signaling. These results may help explain the role of β2-integrins and kindlin-3 in gene regulation in leukocytes, leukocyte adhesion processes and immune responses.

METHODS

Mice

Bone marrow was collected from euthanized male and female C57Bi/6NCrl (Charles River), previously described TTT/AAA β2-integrin knock-in mice (11) (8–39 weeks) and full MRTF-A knockout and control mice previously described in Cheng et al. (18). Fetal liver cells were collected from Kindlin-3$^{-/-}$ and control mice. Experiments were performed according to Finnish Act on Animal Experimentation (62/2006) and approved by the Finnish National Animal Experiment Board. Kindlin-3$^{-/-}$ and control mice were handled in strict accordance with regulations in Germany regarding the use of laboratory animals.

Dendritic Cell Culture

Dendritic cells were generated by culturing bone marrow for 9–10 days (media change on day 3; 6 and 8) in 10 ng/ml GM-CSF (Peprotech) RPMI +10% FCS, 100 U/ml Pen/Strep and 2 mM L-glutamine. In some experiments, 10 μM CCG1423 (Cayman) was used to inhibit MRTF-A for 2 days before experiments.

Immunohistochemistry

1x10^6 dendritic cells on uncoated, iC3b (6 μg/ml; Calbiochem) or fibronectin (10 μg/ml; Calbiochem) coated coverslips were serum starved for 1 h with 0.3% FCS/RPMI, followed by serum stimulation (15% FCS 30 min). In adhesion stimulation experiment WT and KI dendritic cells were detached, serum starved in suspension for 1h and stimulated with replating the cells on glass coverslips or on iC3b coated coverslips for 1h. Cells

were fixed with 4% PFA. F-actin content of 25–100 cells/animal was assessed via measurement of corrected total cell fluorescence (CTCF) of TRITC-phalloidin (Sigma) as described in Abashidze et al. (19). All slides were imaged using a Leica SP5 II (Leica Microsystems) LAS AF Lite Software, with 561 Laser (10% laser power). Z-stacks were taken with the following parameters: Spectral Range: 570–779 nm, QD405/488/561/635 mirror, Smart Gain 800 V, Smart Offset 0,0%, Pinhole 111.49 μm, Zoom: 1,00; Objective 63X, z-Distance 8.003μm, 55 steps, Format 512 × 512.

MRTF-A staining was performed on non-starved, serum starved, and serum stimulated cells. After fixation cells were permeabilized with 0,2% Triton-x/PBS for 5 min and stained for 1 h with anti MRTF-A (C-19, sc-21558, Santa Cruz Biotechnology) 1:100 in 1%FBS/PBS.

RhoA Bound GTP Level

The RhoA activity was assessed via RhoA G-LISA Activation Assay Kit (Cytoskeleton Inc, Denver) according to the manufacturer's instructions. Samples were collected from serum starved cells to acquire a non-stimulated baseline level of GTP-bound RhoA (determined for each biological repeat and set to 1). Based on this value the fold change of RhoA activation was calculated for serum-stimulated cells (serum starvation followed by 15% FCS for 10 min). All samples were collected on ice and snap frozen in liquid nitrogen within 10 min after lysing the cells and stored at −80°C until G-LISA was run.

Static Adhesion Assay

Static adhesion assay with resting and activated (200 nM PdBu, Sigma-Aldrich) cells at 0,2 x 10^6 cells/ml on ICAM-1 (6 μg/ml; R&D Systems) and iC3b (6 μg/ml; Calbiochem) was performed as previously described (20). Rescue experiments with MRTF-A were performed after cell transfection as follows: CD11b, CD18 WT, CD18 TTT/AAA, MRTF-A*** (21) in pCDNA3.1 vector or a combination of them were transfected into COS-1 cells using Xfect (Takara) following the manufacturer's guidelines. After transfection, the cells were incubated for 48 h at 37°C before static adhesion and western blot experiments.

Western Blot

Transfected cells were lysed in M-PER lysis buffer (Thermo Scientific) in the presence of phosphatase and protease inhibitors (Pierce), and lysates were analyzed by Western blotting. Primary antibodies against MRTF-A, and against myc-tag were from Santa Cruz.

Flow Cytometry

Following Fc block (eBiosciences, 93) cells were stained with the following antibodies: CD11a-PE (BioLegend, 2D7), CD18-FITC (BD Biosciences, C71/16) CD11c-PE-Cy7 (eBioscience, N418), CD11b-APC (BioLegend, M1/70), CD80-APC (eBioscience, 16-10A1), CCR7-PE (BioLegend, 4B12), CD40-PE (BioLegend, 3/23), CD86-FITC (BD Biosciences, GL1). FITC-conjugated antibodies were used at 1:100 dilution, all other antibodies were used at 1:200 dilution. Data were acquired using a LSRForetessa (BD) and analyzed using FlowJo software (TreeStar, USA).

Traction Force Microscopy

200.000 dendritic cells in 2 ml whole media were incubated for 3h on iC3b (6μg/ml in PBS Calbiochem) -coated silicone-based gel substrates (Young's Modulus = 1 kPa)(Matrigen, USA).

For MRTF-$A^{-/-}$ and control experiments, cells were plated on elastic polyacrylamide (PAA) gel substrates (Young's Modulus = 1 kPa) (22). Substrates were covered with sulfate fluorescent microspheres (Invitrogen, diameter 200 nm; excitation wavelength 488) before coating with iC3b. Sulfo-SANPAH (Sigma-Aldrich) was used as a linker in between the substrate and the coating. Cells were incubated on substrates for 1h prior to imaging. Single cells together with the underlying beads were imaged with 3I Marianas imaging system (3I intelligent Imaging Innovations, Germany) at +37°C in 5% CO_2. A 63x/1.2 W C-Apochromat Corr WD=0.28 M27 objective was used. Following live cell imaging, cells were detached from the gel substrates with 10 × Trypsin (Lonza) and a second set of nanobead images, serving as reference images, were obtained. Spatial maps of cell-exerted nanobead displacements were achieved by comparing the reference bead images together with the experimental images. With the knowledge of the bead displacement fields, substrate stiffness (1 kPa), and a manual trace of the cell boundary, the cell-exerted traction fields were computed by using Fourier Transform Traction Cytometry (23, 24). The root mean squared magnitude was computed from the traction field.

Cell Migration Assays

3D migration assays were performed with μ-Slide Chemotaxis 3D (Ibidi) imaging slides according the manufacturer's protocol. Briefly, dendritic cells were mixed into a 1.5 mg/ml bovine collagen I (cellsystemsSlides) mix and injected into the slide's thin imaging strip. After 45min collagen polymerization, one of two chambers flanking the imaging strip were filled with media, and the other with media containing 1.25 μg/ml mCCL19 (R&D systems). One slide was used for each condition, assays were repeated 3 times. Dendritic cells were imaged using 3I Marianas imaging system (3I intelligent Imaging Innovations, Germany) by utilizing multipoint imaging. Controls were always imaged before treated or knock-in/-out dendritic cells. A 10x/0.30 EC Plan-Neofluar Ph1 WD = 5.2 M27 objective was used, the dish was placed in a heated sample chamber (+37°C), controlled for CO_2. Cells were imaged using bright field, for 4 h every minute.

qPCR

Total RNA was isolated from WT and WT MRTF-A inhibited dendritic cells with Nucleospin RNA kit (Macherey-Nagel) and converted into cDNA with High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) according to the manufacturers' protocols. The quantitative Real-Time PCR (qRT-PCR) was performed by using Taqman chemistry. Briefly, the cDNA was amplified in a 11 μl volume containing 1 x TaqMan Fast Advanced Master Mix and predeveloped TaqMan primers and probes (CCR7 Mm01301785_m1, CD11a Mm00801807_m1). 18S rRNA (Eukaryotic 18S rRNA Endogenous Control, Thermo Fisher Scientific) was used as a reference gene and no template control (NTC) was included in

each assay. Reactions were run with CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with a program consisting of initial steps of 50°C for 2 min and 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Each sample was run in triplicate. qRT-PCR data was analyzed by using CFX Maestro (Bio-Rad) and finalized with Excel(Microsoft). Relative units were calculated by using the comparative C_T method which has been described elsewhere (25).

mRNA Sequencing

The sequencing was performed at the Biomedicum Functional Genomics Unit (FuGU) of the University of Helsinki with the Illumina NextSeq500 using a NextSeq Mid Output 150 cycle flow cell. From 2μg of total RNA from each biological replicate (4 MRTF-A$^{-/-}$ and 4 MRTF-A$^{+/+}$, age matched), Library preparation was done using a NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (E7760) and mRNA enrichment was done by NEBNext® Poly(A) mRNA Magnetic Isolation Module (E7490), according to manufacturer's protocols. 10 PCR cycles were used. Libraries' concentrations were determined by Qubit, while quality and size distribution were analyzed using Bioanalyzer 2100. Fastq files are deposited at NCBI's Gene Expression Omnibus (GEO) database with BioProject ID PRJNA535475 and BioSample accession number SAMN11506732.

RNA-Seq Data Analysis

The sequencing quality of raw RNA-Seq reads from fastq files was evaluated by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing reads were then aligned against the mouse reference genome (*Mus musculus* GRCm38.95) by HISAT2 (26). Transcripts were counted using HTSeq (27). Differential expression analysis was performed by the R Bioconductor package DESeq2 (28) using <0.05 as cutoff for the adjusted *p*-value. Heatmaps were generated from normalized expression (regularized log transformation) values for individual samples that were obtained from DESeq2.

Pathway Enrichment Analysis

All the differentially expressed genes having adjusted *p*-value <0.05 (calculated by DESeq2) were ranked by their log2 fold-changes. Pathway enrichment analysis for GO biological process of the ranked gene list was done with the GSEA software version 3.0 (29) against C5 gene ontology biological process dataset (version 6.2) downloaded from molecular signature database (MSigDB). The GSEA output was further processed and visualized using EnrichmentMap (version 3.2.0) on Cytoscape (version 3.7.1). In addition, pathway enrichment analysis for reactome pathways was performed by g:Profiler (30).

Statistics

The Student's 2-tailed *t*-test and Mann-Whitney test, were used to calculate statistical significance in prism. For traction force microscopy and migration assays Mann-Whitney tests were used. All *p*-values are shown as <0.05*, <0.01**, <0.005***.

RESULTS

RhoA Activation and F-Actin Polymerization Following Extracellular Stimuli Are Reduced in TTT/AAA-β2-Integrin KI Dendritic Cells

β2-integrin-mediated leukocyte adhesion to integrin ligands requires the interaction between β2-integrin and kindlin-3 (11, 12). Reduced β2-integrin/kindlin-3 interaction in TTT/AAA-β2-integrin KI dendritic cells leads to marked changes in gene expression and cellular reprogramming to a migratory phenotype (12). To identify pathways downstream of kindlin-3 that are involved in integrin/kindlin-3-regulated gene expression in dendritic cells, we performed Ingenuity Pathway Analysis (IPA) of gene expression data from WT and KI dendritic cells (12). This software combines algorithms with published datasets of primary literature, public and third-party databases to identify molecular interactions, causations and model pathways. As indicated in **Table 1**, the MRTF-A/SRF pathway was predicted to be inhibited as a consequence of the reduced β2-integrin/kindlin-3 interaction in TTT/AAA-β2-integrin KI dendritic cells.

The MRTF-A/SRF pathway is regulated by actin dynamics in cells. G-actin-bound MRTF-A remains cytoplasmic, whilst RhoA-induced F-actin polymerization releases this transcription factor. MRTF-A is subsequently free to translocate into the nucleus where it initiates transcription of cytoskeletal genes together with SRF (17, 31, 32). Based on the IPA prediction that the MRTF-A/SRF pathway is inhibited in KI dendritic cells, we first set out to investigate RhoA activity and actin polymerization in WT and KI cells. Indeed, RhoA activity in serum-stimulated cells was lower in KI cells compared to WT cells (**Figure 1A**) and in addition, serum-stimulated F-actin polymerization was significantly decreased in KI dendritic cells compared to WT dendritic cells (**Figures 1B,C**). These results show that β2-integrins are upstream of RhoA-mediated F-actin polymerization in dendritic cells.

MRTF-A Nuclear Shuttling Is Impaired as a Result of Mutated CD18 or Deleted Kindlin-3

As F-actin polymerization initiates MRTF-A nuclear shuttling, allowing regulation of gene transcription together with SRF (33, 34) we investigated serum-induced MRTF-A shuttling in dendritic cells. In resting as well as serum starved dendritic cells MRTF-A was mainly cytoplasmic independent of functional β2-integrin/kindlin-3 interaction, as expected. Serum

TABLE 1 | IPA was performed with gene expression profiles from KI, $N = 3$.

Upstream regulator	Molecule type	Predicted activation state	*p*-value of overlap
SRF	Transcription regulator		3,01E-10
MRTF-B/MKL2	Transcription regulator	Inhibited	1,88E-09
MRTF-A/MKL1	Transcription regulator	Inhibited	4,24E-09

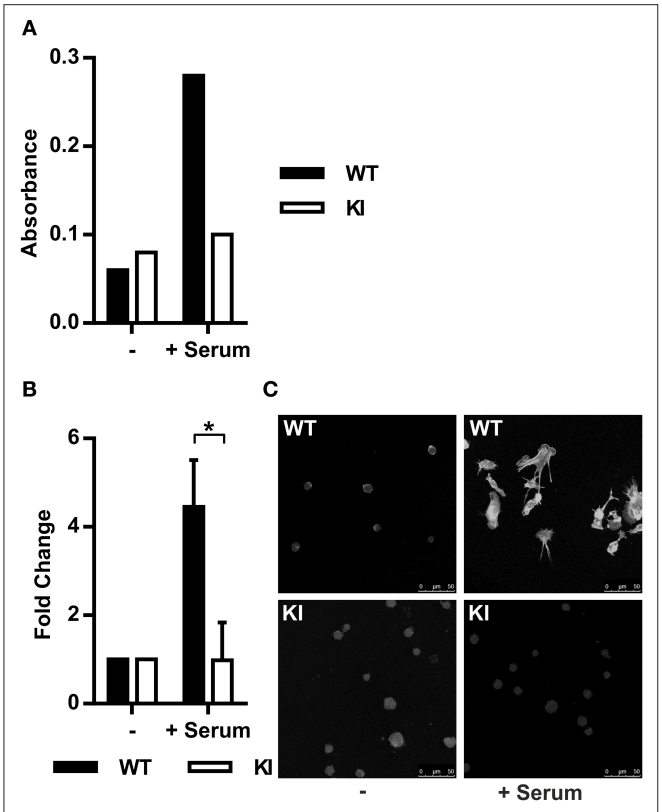

FIGURE 1 | Abolished β2-integrin/kindlin-3 link leads to impaired actin dynamics and impaired nuclear MRTF-A shuttling. **(A)** RhoA activity in WT and KI dendritic cells was measured in serum starved cells (0.3% fetal calf serum (FCS)/RPMI for 1 h) and serum-stimulated cells (15% FCS for 10 min). $N = 3$, one representative result is shown. **(B)** F-actin content was assessed via measurement of corrected total cell fluorescence (CTCF) of TRITC-phalloidin stained cells. Baseline level of F-actin was acquired from serum starved cells and F-actin fold change was calculated for 15% FCS-stimulated cells (30 min stimulation). 25–100 cells per condition were measured, $N = 3$.
(C) Immunofluorescence images of serum starved and serum-stimulated WT and KI cells stained with TRITC-phalloidin. $^*p < 0.05$.

stimulation led to MRTF-A translocation into the nuclei of WT dendritic cells, however in KI dendritic cells MRTF-A remained predominantly cytoplasmic (**Figures 2A,B**).

These results confirm the IPA results and suggest that RhoA activity, actin dynamics and MRTF-A shuttling are dependent on functional β2-integrins in dendritic cells. However, these results do not exclude the possibility that except for kindlin-3, also other cytoplasmic factors that bind to the TTT site in the β2-integrin cytoplasmic tail may regulate this pathway. Therefore, in order to further pinpoint the β2-integrin cofactor involved in activating the MRTF-A/SRF pathway we analyzed F-actin polymerization and MRTF-A shuttling in Kindlin-3 $^{-/-}$ dendritic cells. Indeed, Kindlin-3$^{-/-}$ dendritic cells showed impaired F-actin polymerization and MRTF-A shuttling similar to KI cells (**Figures 2C,D**).

To examine whether integrin/kindlin complexes *per se*, or integrin- and kindlin-3-regulated adhesion, is responsible for regulating MRTF-A nuclear shuttling, we investigated whether the β2-integrin ligand iC3b could induce MRTF-A nuclear shuttling in DCs. Indeed, placing DCs on iC3b allowed for MRTF-A nuclear shuttling in WT but not β2-integrin KI DCs (**Figure 2E**). However, placing the β2-integrin KI cells on fibronectin-coated surfaces, which allows for β1-integrin-mediated adhesion of these cells, allowed for MRTF-A nuclear shuttling both in the absence and presence of serum (**Figure 2F**). These results show that integrin-mediated adhesion, rather than integrin/kindlin signaling, is responsible for the shuttling of this transcription factor in DCs.

These results confirm that MRTF-A nuclear shuttling is regulated by kindlin-3 regulated, β2-integrin-mediated cell adhesion, which in turn mediates RhoA activation, F-actin polymerization and MRTF-A nuclear shuttling in dendritic cells. These results further confirm the bioinformatics data showing that β2-integrin/kindlin-3 regulates MRTF-A/SRF-mediated gene expression in dendritic cells.

Inhibition of MRTF-A in WT Dendritic Cells Does Not Regulate Dendritic Cell Costimulatory Molecule Expression or Cell Migration in 3D

We have previously shown that the β2-integrins negatively regulate dendritic cell maturation, which is associated with increased surface expression of CD40, CD80 and CD86 costimulatory molecules and the chemokine receptor CCR7 in TTT/AAA-β2-integrin KI cells (12). CCR7 is the receptor for chemokines CCL19 and CCL21 which guides dendritic cells to the lymph nodes where they engage in T-cell priming. In order to investigate whether the MRTF-A/SRF pathway is involved in regulating dendritic cell maturation downstream of β2-integrins, we inhibited MRTF-A with a specific inhibitor, CCG1423 in WT dendritic cells and analyzed phenotypic changes by flow cytometry (**Figure 3**). We did not find significant changes in CD40, CD80, or CD86 expression in CCG1423-treated WT dendritic cells as compared to WT non-treated dendritic cells. MRTF-A inhibition, led to a significant increase in CCR7 expression on the cell surface in DCs, although mRNA levels were not affected (**Figures 3D–F**). CCR7 levels were not increased in MRTF-A $^{-/-}$ DCs (**Figure 3E**).

MRTFA/SRF has been previously linked to cell migration in neutrophils, lymphoblasts and hematopoetic stem cells (14, 35, 36). We therefore asked the question whether the MRTF-A/SRF pathway regulates dendritic cell migration. We compared dendritic cell migration speeds in a 3D collagen matrix after MRTF-A inhibition or deletion in the presence of chemokine CCL19 (**Figures 3G,H**). Surprisingly, we did not find a significant difference between migration speed either after MRTF-A inhibition with CCG1423 nor as a consequence of MRTF-A deletion (utilizing MRTF-A$^{-/-}$) in dendritic cells (**Figures 3G,H**). In summary we have found that the MRTF-A/SRF pathway in dendritic cells is downstream of β2-integrins but does not regulate dendritic cell expression of costimulatory molecules, nor cell migration in 3D.

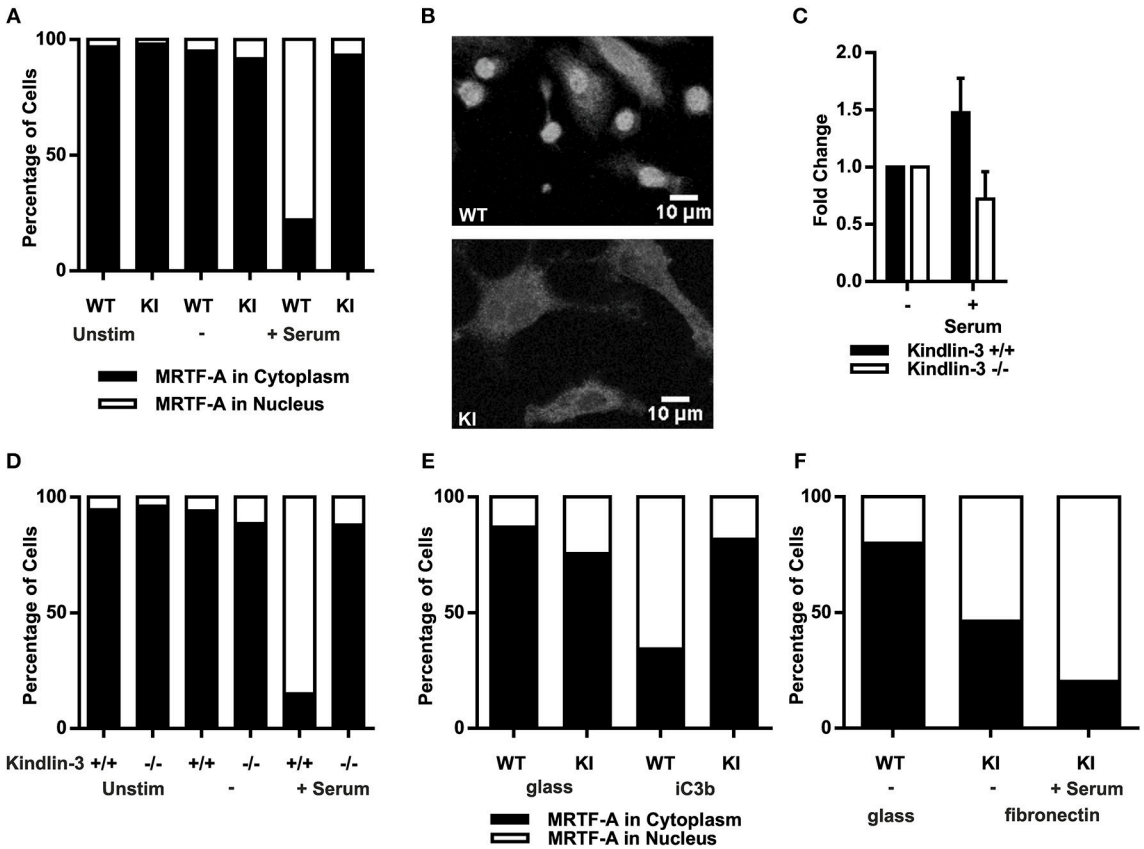

FIGURE 2 | Abolished β2-integrin mediated adhesion leads to impaired nuclear MRTF-A shuttling. **(A)** Total percentages of dendritic cells with nuclear MRTF-A and cytoplasmic MRTF-A are shown. $N = 4$ and 200 cells per condition were analyzed. MRTF-A staining was performed on non-starved, starved and serum stimulated cells and **(B)** immunofluorescence images of WT and KI cells are shown after serum stimulation. **(C)** F-actin content as CTCF of WT and kindlin-3$^{-/-}$ 25–100 dendritic cells per condition were measured, $N = 2$. **(D)** Total percentages of kindlin-3$^{-/-}$ and WT dendritic cells with nuclear MRTF-A and cytoplasmic MRTF-A are shown. **(E)** Total percentages of adhesion on iC3b induced nuclear MRTF-A and cytoplasmic MRTF-A are shown. WT and KI dendritic cells were detached, serum starved in suspension, and stimulated with replating on glass coverslips or on iC3b coated coverslips. **(F)** Total percentages of WT dendritic cells starved on glass compared to KI dendritic cells seeded overnight on fibronectin with nuclear MRTF-A and cytoplasmic MRTF-A. KI dendritic cells were serum starved and stimulated. **(A–F)** If not otherwise indicated: $N = 3$ and 200 cells per condition were analyzed. MRTF-A staining was performed on non-starved, starved, and serum stimulated cells.

MRTF-A Regulates Gene Expression of Cell Cycle, Lipid Metabolism, and Cytoskeletal Genes in Dendritic Cells

Although the MRTF-A/SRF pathway is often described as the "master regulator of the cytoskeleton," the role of the MRTF-A/SRF pathway in regulation of gene expression in dendritic cells has not previously been investigated. To generate more in-depth understanding of the role of the MRTF-A/SRF pathway in dendritic cells, we therefore performed RNA-Seq analysis of MRTF-A$^{-/-}$ cells (**Supplementary Table 1**, **Supplementary Figure 1**). 1401 genes were found to be differentially expressed between WT and MRTF-A$^{-/-}$ cells. 429 of the MRTF-A-regulated, differentially expressed genes are also regulated by β2-integrins in DCs (12) (**Figure 4A**). The main pathways affected were cell cycle-related, but also for example lipid metabolism genes appear to be regulated by MRTF-A. However, also cytoskeletal genes and Rho GTPase signaling were significantly affected by MRTF-A deletion in dendritic cells (**Figures 4B,C**). Several cytoskeletal genes and cytoskeletal modulators, such as Fgr, Hck, Stmn1, Ckap2l, Anln, Tpm2, Tubb5, are downregulated both in MRTF-A$^{-/-}$ and TTT/AAA-β2-integrin KI dendritic cells (**Figure 4D**). Together, these data further indicate that β2-integrin and MRTF-A are on a common pathway regulating gene expression in dendritic cells.

MRTF-A Inhibition Results in Decreased Dendritic Cell Adhesion

One of the most prominent phenotypical changes of TTT/AAA-β2-integrin KI dendritic cells is their reduced adhesion. Many cytoskeletal proteins are necessary for cell adhesion. We hypothesized that the reduced adhesion in KI cells may be partly due to the suppression of the MRTF-A/SRF pathway and subsequent changes in expression of cytoskeletal genes. We thus analyzed static adhesion of MRTF-A inhibited dendritic cells to β2-integrins ligands ICAM-1 and iC3b. As shown

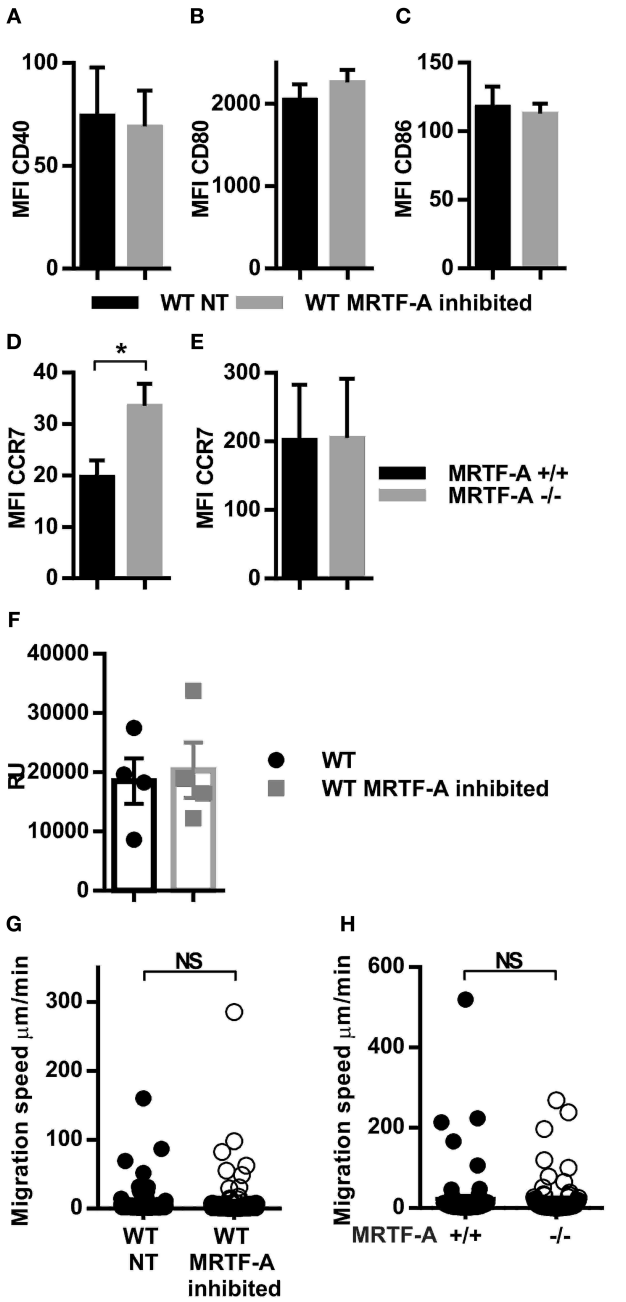

FIGURE 3 | Inhibition but not deletion of MRTF-A leads to altered CCR7 expression and no change in migration speed. **(A)** CD40 **(B)** CD80 **(C)** CD86 and **(D)** CRR7 expression in WT NT and MRTF-A inhibited dendritic cells ($N = 6$); **(E)** CCR7 expression of MRTF-A $^{-/-}$ and MRTF-A$^{+/+}$ ($N = 5$); surface marker expression was determined by flow cytometry. **(F)** CCR7 mRNA level of MRTF-A inhibited and NT WT dendritic cells determined by qPCR ($N = 4$). **(G)** Scatter plots of tracked WT NT and WT MRTF-A inhibited and **(H)** MRTF-A$^{-/-}$ and MRTF-A$^{+/+}$ dendritic cell speeds in a 3D collagen matrix toward CCL19 are shown. Only cells faster than 1 μm/min have been evaluated as migratory and used for the analysis. * $p < 0.05$.

in **Figures 5A,B**, β2-integrin-mediated adhesion of MRTF-A inhibited WT dendritic cells was significantly reduced, although not as drastically as in TTT/AAA-β2-integrin KI cells. A similar reduction in adhesion was seen in MRTF-A$^{-/-}$ dendritic cells (**Figure 5C**). There are two MRTF isoforms present in most cells: MRTF-A and MRTF-B (33). RNA-Seq analysis confirmed that murine dendritic cells express both MRTF-A and MRTF-B, with MRTF-A being expressed at higher levels than MRTF-B (**Figure 5D**). The difference in adhesion of the MRTF-A $^{-/-}$ dendritic cells as compared to inhibitor-treated cells could thus be explained by MRTF-B partly compensating for MRTF-A functions in these cells.

To investigate whether MRTF-A-regulated gene expression by itself could rescue adhesion in TTT/AAA-β2-integrin KI cells, we cotransfected COS-cells with TTT/AAA-β2-integrins and a constitutively active form of MRTF-A that can enter the nucleus without cell stimulation (21) and investigated cell adhesion in these cells. MRTF-A-*** did not rescue TTT/AAA-β2-integrin-mediated adhesion in transfected COS cells, indicating that this pathway alone is not sufficient to drive cell adhesion when kindlin cannot bind to the integrin to induce integrin activation (**Figures 5E,F**). Nevertheless, these results show that the MRTF-A/SRF pathways contributes to β2-integrin-mediated dendritic cell adhesion to ligands.

β2-integrins themselves have been shown to be negatively regulated by SRF and MRTF-A in that lack of either transcription factor results in CD11b overexpression in neutrophils (14, 36). In hematopoetic stem cells SRF deletion results in integrin overexpression (35). We thus set out to clarify if the reduced adhesive phenotype following MRTF-A inhibition was due to downregulation of β2-integrins. We measured surface expression levels of the β2-integrins β2-chain (CD18) and three of the α-chains, CD11a, CD11b, and CD11c (**Figures 5G–K**). Surprisingly we found that none of these β2-integrin subunits was significantly decreased and CD11a was even significantly increased following MRTF-A inhibition (**Figure 5G**), although qPCR showed reduced levels of expression of CD11a (**Figure 5L**). MRTF-A deletion did not affect CD11a surface expression levels (**Figure 5K**). Therefore, reduction of β2-integrin mediated adhesion following MRTF-A inhibition is not due to reduced β2-integrin expression.

The β2-Integrin/MRTFA/SRF Pathway Contributes to Integrin-Mediated Traction Force Generation

Integrins function as bidirectional transmitters of mechanical forces across cell membranes. To investigate whether the β2-integrin/kindlin-3 interaction regulates force transmission through the integrin, we performed traction force microscopy experiments. By comparing the beads' positions on top of which cells were bound to iC3b to the same bead structure without the cell we could measure how much the cell deforms the bead-containing hydrogel. This deformation is due to traction forces generated during adhesion and the force of the traction force can be calculated from bead deformation and hydrogel substrate stiffness. Traction force microscopy experiments comparing TTT/AAA-β2-integrin

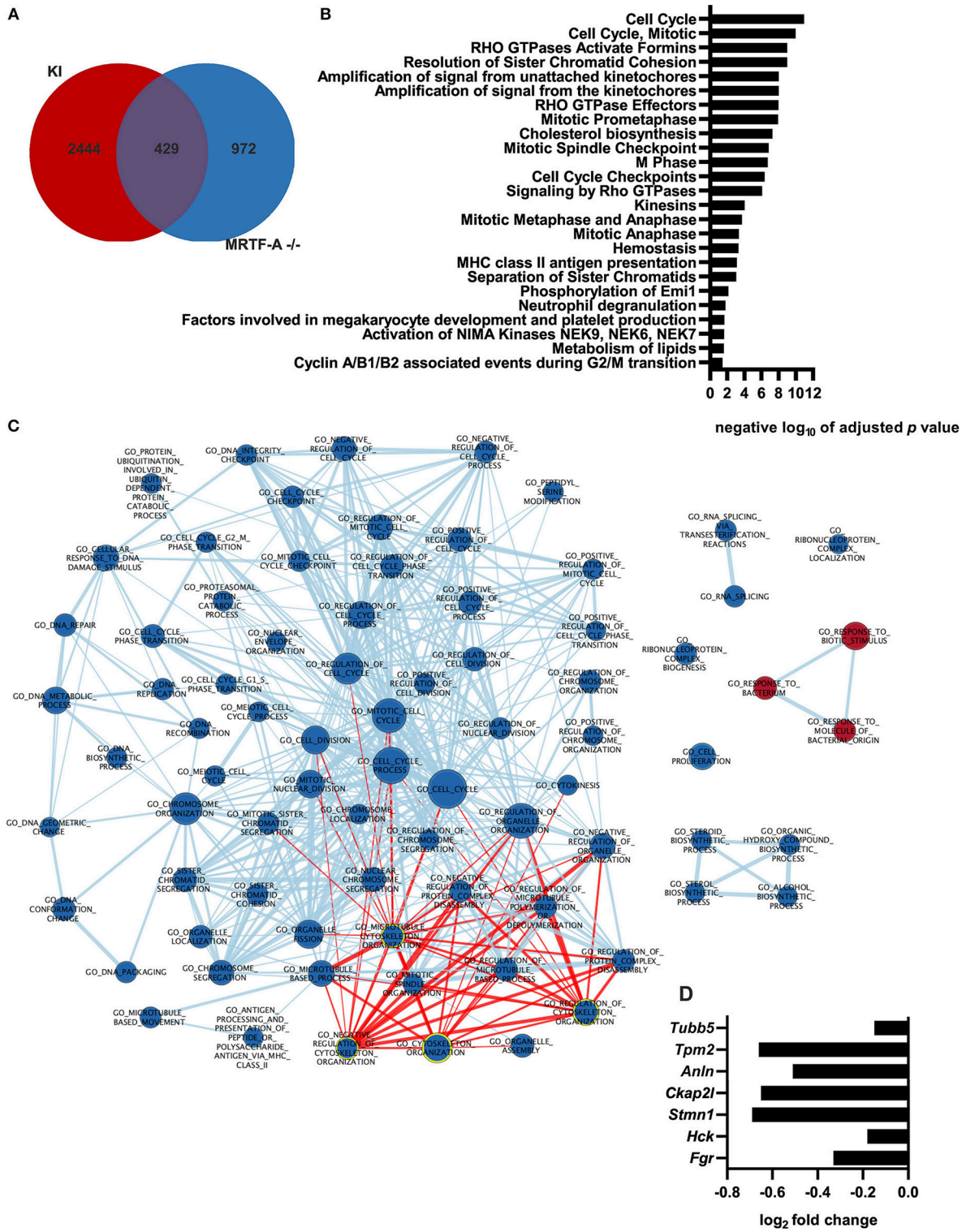

FIGURE 4 | Gene expression profile of MRTF-A$^{-/-}$ cells and comparison with integrin KI. **(A)** Depicted is the overlap of differently regulated genes shared by the MRTF-A$^{-/-}$ vs. $^{+/+}$ and the KI vs. WT RNA-Seq data. **(B)** Ranked list of negative logarithm of adjusted *p*-values from the reactome pathway enrichment analysis. Pathway was performed using g:Profiler. **(C)** Node map of pathway enrichment analysis for GO biological process of the GSEA ranked gene list. Analysis is based on RNA-Seq data derived from MRTF-A$^{-/-}$ and $^{+/+}$ ($N = 4$). Nodes highlighted in red are associated with the cytoskeleton. Nodes highlighted with yellow outline contain word "cytoskeleton." **(D)** Depiction of cytoskeletal genes that are differently regulated in KI and MRTF-A$^{-/-}$.

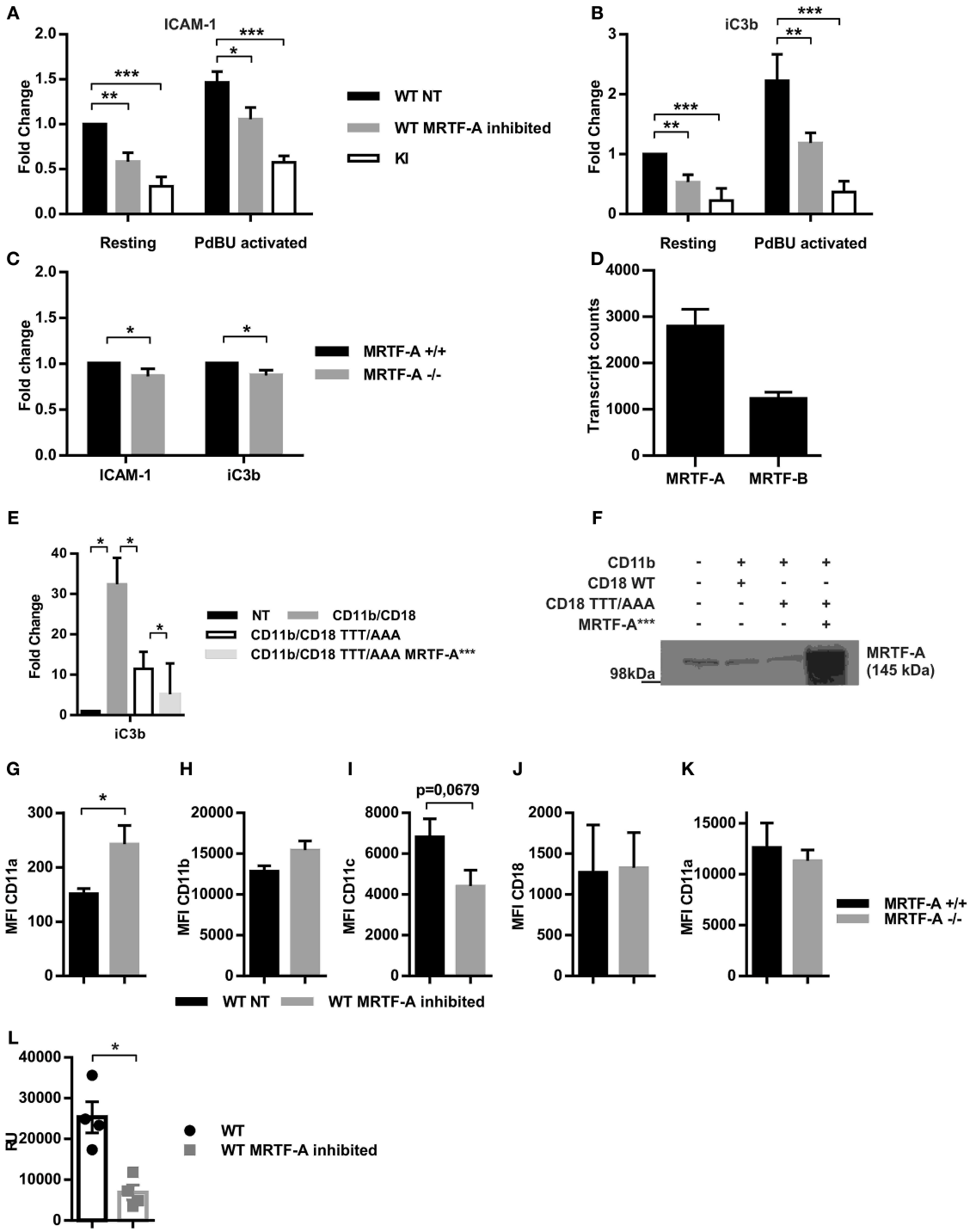

FIGURE 5 | Inhibition of the MRTF-A/SRF pathway leads to reduced adhesion independent of β2-integrin expression. The effect of MRTF-A inhibition on dendritic cell adhesion to **(A)** ICAM-1 and **(B)** to iC3b was compared to adhesion of TTT/AAA-β2-integrin KI cells. Adhesion of resting, non-treated (NT) WT dendritic cells was set to 1 and fold changes for all other conditions was calculated, $N = 5$. **(C)** MRTF-A $^{-/-}$ dendritic cell adhesion to ICAM-1 and iC3b was compared to adhesion of MRTF-A$^{+/+}$ littermates, $N = 4$. **(D)** mRNA level of MRTF-A and MRTF-B in MRTF-A$^{+/+}$ mice ($N = 4$) based on RNA-Seq data. **(E)** TTT/AAA rescue experiment with constitutively active MRTF-A (MRTF-A***). Adhesion of non-treated COS-1 cells was set to 1 and fold change for adhesion of COS-1 cells transfected with CD11b/CD18, CD11b/CD18-TTT/AAA (CD18 featuring the TTT/AAA mutation), and CD11b/CD18-TTT/AAA plus MRTF-A***. **(F)** Depiction of conditions used in rescue experiment. **(G)** CD11a, **(H)** CD11b, **(I)** CD11c and **(J)** CD18 expression in WT NT and MRTF-A inhibited dendritic cells ($N = 6$). **(K)** CD11a expression in MRTF-A$^{-/-}$ and$^{+/+}$ dendritic cells ($N = 5$). **(L)** CD11a mRNA level of MRTF-A inhibited and NT WT dendritic cells determined by qPCR ($N = 4$). *$p < 0.05$, **$p < 0.01$.

KI to WT dendritic cells revealed that KI dendritic cells generate significantly reduced traction forces on β2-integrin ligand coated substrates compared to WT dendritic cells (**Figures 6A,B**).

In order to investigate whether traction force generation was dependent on the MRTF-A/SRF pathway downstream of β2-integrin/kindlin-3, we performed traction force microscopy experiments on MRTF-A inhibited WT dendritic cells. Interestingly, MRTF-A inhibition as well as deletion resulted in a similar decrease of integrin-mediated traction forces on ligand coated surfaces as disruption of the β2-integrin/kindlin-3 interaction (**Figures 6C–F**). Together, these results show that the β2-integrin/kindlin-3/MRTF-A/SRF pathway regulates integrin-mediated cell adhesion as well as integrin-generated cellular traction forces in dendritic cells.

In summary we have found that the MRTF-A/SRF pathway in dendritic cells is downstream of β2-integrin/kindlin-3 interactions and that this pathway regulates dendritic cell gene expression, cell adhesion and traction force generation, but not dendritic cell migration in 3D.

DISCUSSION

Although β2-integrins are abundantly expressed in dendritic cells, the role of β2-integrins in dendritic cell functions is relatively poorly understood. Dendritic cell migration from tissues to lymph nodes, which occurs in a 3D environment, can occur without integrins (37). In addition, β2-integrins have been shown to suppress the mature, migratory dendritic cell phenotype (12). However, the signaling pathways downstream of β2-integrins that mediate this phenotypic switch have not been identified.

In this study we have identified the β2-integrin as an upstream regulator of the MRTF-A/SRF pathway. Our results show that β2-integrin-mediated, kindlin-3-regulated cell adhesion leads to RhoA activation to regulate F-actin polymerization and MRTF-A nuclear shuttling. This allows MRTF-A to coactivate genes together with SRF. Although MRTF-A has previously been implicated as a main player in regulating cytoskeletal genes in other cell types, we show here by RNA-Seq analysis that MRTF-A also regulates gene expression related to cell cycle, lipid metabolism, and many other pathways in dendritic cells. In macrophages, MRTF-A/SRF mediate cytoskeletal gene expression (14, 36, 38), whilst in B cells SRF deletion led to decreased level of IgM, CD19, and CXCR4 expression (39). Thus our results confirm the involvement of MRTF-A/SRF in the regulation of gene expression in immune cells, however the effect on gene expression appears highly cell-type specific.

We show that MRTF-A inhibition or deletion significantly decreases adhesion to β2-integrin ligands ICAM-1 and iC3b but does not impact on dendritic cell 3D migration speed in response to chemokine stimulation. MRTF-A and SRF have been shown to activate gene expression of proteins associated with focal adhesions in fibroblasts and are associated with migration and invasion in a cancer cell line (16). In hematopoietic stem cells SRF regulates adhesion and integrin expression (35). In neutrophils, SRF has been shown to be essential for adhesion and extravasation (36), whilst in macrophages SRF independent gene targets of MRTF-A were predicted to be focal adhesion associated (40). Deletion of MRTF-A in neutrophils leads to impaired 2D migration associated with altered adhesion (14). Therefore, our results together with published studies confirm that MRTFA/SRF transcription factors play an important role in regulating the adhesive phenotype of myeloid immune cells.

We have linked the reduced adhesion to reduced traction forces upon MRTF-A inhibition. This result could be interpreted as that the integrin/kindlin-3 interaction is necessary for overall generation of traction forces, because the integrin/kindlin interaction is required for optimal integrin function. Traction forces are generated by interaction of the (actin-) cytoskeleton with myosin. Both actin and myosin are MRTF-A/SRF target genes. We thus hypothesize that reduced adhesion following MRTF-A inhibition in dendritic cells may be due to altered cytoskeletal organization which ultimately renders the dendritic cells less able to generate integrin-mediated traction forces. An alternative explanation would be that kindlin-3 and talin interaction with the β2-integrin is regulated by SRF, as was shown in $SRF^{-/-}$ neutrophils (36). This alternative would extend the regulatory feedback loop between β2-integrin/kindlin-3, F-actin and MRTF-A back to the integrin regulators kindlin-3 and talin.

Interestingly, like β2-integrin deficiency and kindlin-3 deficiency, which lead to LAD-syndromes in man, MRTF-A deficiency in man has recently been shown to lead to immunodeficiency with prominent susceptibility to bacterial infections (14). Patient neutrophil and lymphocyte functions, such as migration, was impaired, and patient dendritic cells displayed impaired spreading and podosome formation (14), as we have previously reported in TTT/AAA-β2-integrin KI dendritic cells (41). The similarities of symptoms in human MRTF-A deficiency and β2-integrin deficiency, together with the results presented here, indicate that some of the functions of integrins and kindlin-3 in immune cells, which are impaired in LAD patients may involve regulation of transcription through the MRTF-A/SRF pathway. We hypothesize that abnormal expression of actin cytoskeleton genes due to specific defects in the MRTF-A/SRF pathway in LAD cells may contribute to immune defects (e.g., defects in myeloid cell adhesion, migration, phagocytosis, etc) in LAD diseases.

MRTF-A has previously been shown to regulate the development of specific adipocyte subpopulations (42) and is essential for thymocyte development (39). SRF deletion in hematopoetic stem cells led to accumulation of CD11b positive cells and thus an increase in macrophage like cells (35). Interestingly, cell adhesion, which maintains active MRTF-A/SRF signaling, has recently been shown to be essential to prevent progenitor cells from switching to the adipocyte lineage during cardiomyocyte differentiation (43). Mechanistical data illustrating the role of SRF and MRTF-A in the regulation of cellular identity has been gathered from neural and liver cells. In these cells, induced MRTF-A nuclear accumulation resulted in

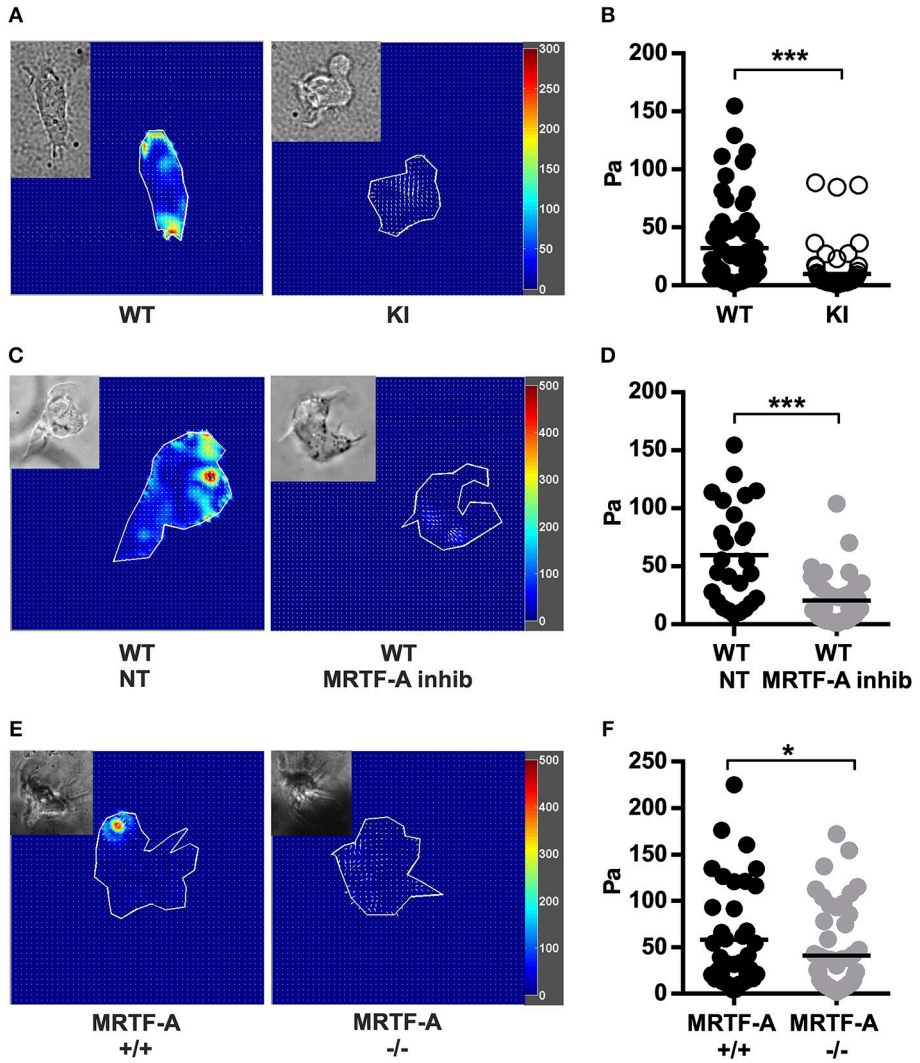

FIGURE 6 | Traction force generation in dendritic cells is regulated by the β2-integrin/MRTF-A/SRF pathway. **(A)** Heatmaps depicting traction force generated by WT and TTT/AAA KI dendritic cells and **(C)** non treated WT and MRTF-A inhibited WT dendritic cells. **(B)** Scatter plots of analyzed WT and KI dendritic cells and **(D)** non treated WT and MRTF-A inhibited WT dendritic cell traction forces (Pa) are shown. Three experiments were performed, 25 cells were measured each time and a total of 75 cells analyzed. **(E)** Heatmaps depicting traction force generated by an example MRTF-A$^{+/+}$ and $^{-/-}$ dendritic cells. **(F)** Scatter plots of analyzed MRTF-A$^{+/+}$ and $^{-/-}$ dendritic cells $N = 4$. $^{*}p < 0.05$, $^{***}p < 0.005$.

downregulation of cell-type specific genes, changes in epigenetic status of regulator elements as well as chromatin organization (44). It is interesting to note that in dendritic cells, our gene expression profiling data now show that MRTF-A regulates genes that are associated with the cell cycle and with lipid metabolism, indicating new roles of this pathway in metabolism and cell proliferation/differentiation in immune cells, topics for further studies in the future.

In summary, our results indicate that the β2-integrin-regulated MRTF-A/SRF pathway is a key regulatory element in dendritic cells that regulates gene expression associated with cell cycle, lipid metabolism and the cytoskeleton. β2-integrins regulate MRTF-A/SRF signaling and expression of cytoskeletal genes, and enables the adhesive dendritic cell phenotype and integrin-mediated traction force generation. We thus propose that the β2-integrin-regulated MRTF-A/SRF pathway may function as a roadblock that needs to be overcome in dendritic cells for them to initiate the phenotypic switch from an adhesive to a mature, migratory phenotype.

ETHICS STATEMENT

Experiments were performed according to Finnish Act on Animal Experimentation (62/2006) and approved by the Finnish National Animal Experiment Board. Kindlin-3$^{-/-}$ and control mice were handled in strict accordance with regulations in Germany regarding the use of laboratory animals.

AUTHOR CONTRIBUTIONS

SF planned the study. CG performed the experiments with assistance of LU and TS. MA performed MRTF-A rescue experiments. HH performed qPCR. ST supervised the traction force microscopy experiments. MM provided kindlin-3$^{-/-}$ and +/+ fetal liver cells. SM provided MRTF-A −/− and +/+ mice. CG, ST, and SY analyzed experimental data. TÖ performed ingenuity pathway analysis of WT and KI and IF analyzed MRTF-A$^{-/-}$ and +/+ RNA-Seq data. CG wrote the paper together with SF.

FUNDING

This study was funded by the Academy of Finland [to SF (project: 315077) and ST (294174)], E-RARE LADOMICS project (Academy of Finland: 326358), Sigrid Juselius Foundation, University of Helsinki, Magnus Ehrnrooth Foundation (to CG and SF), Liv och Hälsa Foundation (all to SF) and the Deutsche Forschungsgemeinschaft (SFB914, project A1; to MM). The University of Helsinki Library covered the open access publication fees for this article.

ACKNOWLEDGMENTS

We thank Maria Vartiainen for useful discussions and for the MRTF-A-*** construct.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01138/full#supplementary-material

Supplementary Table 1 | List of differentially expressed genes from the RNA-Seq data.

Supplementary Figure 1 | Heatmap of gene expression changes.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 12 June 2019
doi: 10.3389/fimmu.2019.01321

L-Selectin Enhanced T Cells Improve the Efficacy of Cancer Immunotherapy

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *26 February 2019*
Accepted: *24 May 2019*
Published: *12 June 2019*

Citation:
Watson HA, Durairaj RRP, Ohme J, Alatsatianos M, Almutairi H, Mohammed RN, Vigar M, Reed SG, Paisey SJ, Marshall C, Gallimore A and Ager A (2019) L-Selectin Enhanced T Cells Improve the Efficacy of Cancer Immunotherapy. Front. Immunol. 10:1321. doi: 10.3389/fimmu.2019.01321

The homing molecule, L-selectin (CD62L), is commonly used as a T cell activation marker, since expression is downregulated following engagement of the T cell receptor. Studies in mice have shown that $CD62L^+$ central memory T cells are better at controlling tumor growth than $CD62L^-$ effector memory T cells, while L-selectin knockout T cells are poor at controlling tumor growth. Here, we test the hypothesis that T cells expressing genetically modified forms of L-selectin that are maintained following T cell activation (L-selectin enhanced T cells) are better at controlling tumor growth than wild type T cells. Using mouse models of adoptive cell therapy, we show that L-selectin enhancement improves the efficacy of $CD8^+$ T cells in controlling solid and disseminated tumor growth. L-selectin knockout T cells had no effect. Checkpoint blockade inhibitors synergized with wild type and L-selectin enhanced T cells but had no effect in the absence of T cell transfers. Reduced tumor growth by L-selectin enhanced T cells correlated with increased frequency of $CD8^+$ tumor infiltrating T cells 21 days after commencing therapy. Longitudinal tracking of Zirconium-89 (^{89}Zr) labeled T cells using PET-CT showed that transferred T cells localize to tumors within 1 h and accumulate over the following 7 days. L-selectin did not promote T cell homing to tumors within 18 h of transfer, however the early activation marker CD69 was upregulated on L-selectin positive but not L-selectin knockout T cells. L-selectin positive and L-selectin knockout T cells homed equally well to tumor-draining lymph nodes and spleens. CD69 expression was upregulated on both L-selectin positive and L-selectin knockout T cells but was significantly higher on L-selectin expressing T cells, particularly in the spleen. Clonal expansion of isolated L-selectin enhanced T cells was slower, and L-selectin was linked to expression of proliferation marker Ki67. Together these findings demonstrate that maintaining L-selectin expression on tumor-specific T cells offers an advantage in mouse models of cancer immunotherapy. The beneficial role of L-selectin is unrelated to its' well-known role in T cell homing and, instead, linked to activation of therapeutic T cells inside tumors. These findings suggest that L-selectin may benefit clinical applications in T cell selection for cancer therapy and for modifying CAR-T cells to broaden their clinical scope.

Keywords: L-selectin/CD62L, T cells, melanoma, adoptive T cell therapy, cancer immunotherapy

INTRODUCTION

Immunotherapy represents a paradigm shift in cancer treatment; instead of directly attempting to kill cancerous tissue through radio- or chemotherapy, the patient's immune system is targeted, with the aim of augmenting the anti-tumor immune response. This may be done via systemic delivery of monoclonal antibodies that inhibit immune checkpoints exploited by tumors to induce immune tolerance, such as α-PD-1 (pembrolizumab, nivolumab) (1) or α-CTLA-4 (ipilimumab) (2). These pioneering immunotherapies are effective alone but behave synergistically to produce the most pronounced clinical effects when used together (3). The clinical outcomes of this approach have been dramatic, but the range of cancers amenable to checkpoint inhibition is limited and is thought to rely heavily on the cancer having a high neoantigen load (4). Many cancers, however, have only low to medium somatic mutation rates, leading to a limited range of neoantigens (4). Furthermore, T cells that are specific for cancer antigens often have only low affinity T cell receptors (TCRs) (5), limiting the efficacy of the endogenous T cell response, even in the presence of checkpoint inhibition.

To circumvent this, another class of immunotherapy involves adoptive transfer of T cells (adoptive cell therapy, ACT) into the patient. Initially, this approach was based on recovering tumor-infiltrating T cells (TILs) from resected cancers, expansion *ex-vivo*, and then re-infusion back to the patient. However, as understanding of the interaction between immune cells and tumor cells has increased, this approach has expanded to include a number of different strategies. Chimeric antigen receptor T (CAR-T) cells circumvent the need for a high-affinity tumor antigen-specific TCR by replacing the extracellular portion of the TCR with a monoclonal antibody receptor. This entirely bypasses the standard antigen presentation pathway, allowing CAR-T cells to be selectively targeted to the tumor tissue. However, to date, most CAR-T cells have been used to treat circulating hematological malignancies (6). Applying this technology to solid tumors has proved challenging, with one key obstacle being trafficking of T cells into tumors that are poorly vascularized (7, 8). The complexity of the tumor microenvironment means that some therapeutic approaches that directly target the tumor can also indirectly improve immune responses. Anti-angiogenic therapies were designed to induce tumor death via hypoxia. However, inhibiting rapid and abnormal angiogenesis had the unexpected effect of normalizing the tumor vasculature (9), and thus allowing improved infiltration of both chemotherapeutic agents and immune cells into the tumor. Indeed, the complexity of cross-talk between the immune system and cancer vasculature is only now being fully appreciated (10).

The leucocyte adhesion molecule L-selectin (CD62L) is a type-I transmembrane lectin receptor expressed on the surface of leucocytes. It is part of the selectin family, which also includes E-selectin (CD62E), expressed on endothelial cells, and P-selectin (CD62P), which is expressed on platelets and activated endothelium. L-selectin regulates entry of naïve and central memory T cells into lymph nodes and activated $CD8^+$ T cells to sites of virus infection (11); L-selectin expression is regulated via proteolytic shedding of the ectodomain and transcriptional silencing of the L-selectin gene (12, 13). Downregulation of L-selectin on T cells is known to take place following engagement of the TCR, and this has led to L-selectin being used as a marker of T cell activation. It has been demonstrated that T cells with a naïve/central memory $CD62L^+$ phenotype are better at controlling solid tumor growth than $CD62L^-$ cytotoxic T cells (CTLs) in mice (14, 15). However, even when matched for differentiation status, T cells from L-selectin knockout mice are less effective than wild type T cells suggesting a direct role for L-selectin in controlling tumor growth (14). We hypothesize that cells which are genetically engineered to express high levels of mutant L-selectin which cannot be shed or gene-silenced would offer improved control of solid tumor growth, through increased T cell recruitment and infiltration of the tumor stroma. In this mutant L-selectin, the membrane-proximal region of CD62L has been replaced with the proteolysis-resistant membrane-proximal region of CD62P. We have therefore designated this modification as LΔP-selectin. In addition, the modified LΔP-selectin is expressed under a human CD2 promoter and locus control region which abrogates transcriptional silencing (16, 17). This study addresses whether these novel constructs serve to improve T cell-based cancer immunotherapy for solid and disseminated tumors.

MATERIALS AND METHODS

Mice

Genotypes of mouse strains used in the study are shown in **Table 1**. C57BL/6J (B6) mice were purchased from Charles River, congenic B6.PL-Thy1a/CyJ mice, designated Thy1.1 hereafter, and $FoxP3^{DTR}$ mice, supplied by Alexander Rudensky and maintained as previously described (18), were bred in house. L-selectin transgenic mice expressing wild type murine L-selectin (CD62Lwt) or a non-cleavable mutant L-selectin (CD62LΔP) on a B6 L-$selectin^{-/-}$ background have been described (16, 19). F5 TCR transgenic mice generated as described previously (20), were backcrossed to B6 mice and maintained as a homozygous colony (F5B6). F5B6 mice were backcrossed to B6 L-$selectin^{-/-}$ mice and maintained as a homozygous colony (F5LselKO). CD62LΔP mice were backcrossed to F5LselKO and maintained as a homozygous colony (F5LΔP). Hemizygous F5 mice were generated by crossing F5B6 with B6 mice and the F_1 generation (F5/B6) used. T-cell donors and tumor-bearing hosts were matched for gender. Mice were housed in a specific pathogen free facility and allowed free access to water and food. Experimental mice were housed in individually ventilated cages. All mouse experiments conformed to the British Home Office Regulations [Animal (Scientific Procedures) Act, 1986 (Project Licenses PPL30/3188 and 30/2635 to AA)] and the protocol was approved by the Animal Welfare and Ethical Review Body at Cardiff University.

Adoptive T Cell Therapy

$CD8^+$ T cells were isolated from pooled splenocytes and lymph nodes of naïve mice using a $CD8a^+$ T cell isolation kit for negative selection, and LS columns, according to the manufacturer's instructions (StemCell Technologies) from 10.00

TABLE 1 | Mouse genotypes.

Mouse ID	Background	F5 TCR	Endogenous L-selectin	CD62L transgene	Thy	Tumor bearing host or T cell donor
C57BL/6 (B6)	B6	No	+/+	No	Thy1.2	Host
Thy1.1	B6	No	+/+	No	Thy1.1	Host
FoxP3^{DTR}	B6	No	+/+	No	Thy1.2	Host
CD62Lwt	B6	No	−/−	Wildtype hemizygous	Thy1.2	Host
CD62LΔP	B6	No	−/−	LΔP hemizygous	Thy1.2	Host
F5B6	B6	Homozygous	+/+	No	Thy1.2	Donor
F5LselKO	B6	Homozygous	−/−	No	Thy1.2	Donor
F5LΔP	B6	Homozygous	−/−	LΔP homozygous	Thy1.2	Donor
F5/B6	B6	Hemizygous	+/+	No	Thy1.2	Donor

to 12.00 GMT on the day of injection. Briefly, spleens and/or lymph nodes were harvested from adult mice and mashed through a 70 μm cell strainer (BD Pharmingen). Red blood cells were lysed using red cell lysis buffer (Biolegend) and lymphocytes washed with ice-cold phosphate buffered saline (PBS) supplemented with 2% fetal calf serum (FCS) prior to magnetic isolation. The enriched CD8a$^+$ cell fraction was counted using a hemocytometer, resuspended in sterile PBS for injection and analyzed for CD8, CD62L, CD44, CD27, and F5 TCR expression.

CTLs were generated as described previously (11). Briefly, CD8$^+$ T cells from naïve mice (2×10^6 cells/well) in 24-well plates (Nunclon) in complete medium (DMEM supplemented with 10% FCS, penicillin-streptomycin, L-glutamine, non-essential amino-acids and 2-mercaptoethanol) were incubated with 6×10^6 NP68-peptide (ASNENMDAM; Peptide Synthetics) pulsed irradiated splenocytes (5 μM NP68 peptide for 1 h at 37°C, irradiated at 3,000 cGy), and plates incubated at 37°C in 5% CO_2. Fresh complete medium supplemented with 360 IU/ml hrIL-2 (Proleukin, Chiron Ltd, UK) was added after 2 days and replaced with fresh medium on day 5. CTLs were harvested at day 7. Media was also supplemented with 25 ng/ml mu IL-15 (Peprotech, UK) for T cell cultures beyond 7 days.

Tumor Cell Lines

The NP68-B16 melanoma cell line was generated as described previously (21, 22) and used to study adoptive T cell immunity to tumors in mice. Cells were validated for NP68 expression and confirmed mycoplasma free. In some experiments, the parental B16F10 melanoma cell line (ATCC number CRL-6475) was used to study endogenous T cell dependent immunity to tumors, as described previously (23).

Solid Tumor Model

Frozen B16F10 or NP68-B16 cells were thawed from liquid nitrogen and cultured until second passage reached 100% confluence. Upon confluence, the cells were dislodged using StemPro Accutase (ThermoFisher Scientific) at 37°C and resuspended at 5×10^5 cells in 200 μl sterile PBS. NP68-B16 cells were injected subcutaneously into the shaven left flank of female B6, Thy1.1, or Foxp3DTR mice. B16F10 cells were injected subcutaneously into the shaven left flank of female B6, CD62Lwt, or CD62LΔP mice. Mice were randomized into treatment groups for each experiment of 8–10 mice per group, tumor cells injected at 13.00–15.00 GMT and mice returned to their home cage. Tumors were measured every 3–4 days using digital calipers and tumor size calculated as the product of two perpendicular diameters.

For adoptive T cell therapy, NP68-B16 tumor bearing mice were sub-lethally irradiated with 597cGy total body irradiation (TBI) on day 6 following tumor cell injections. On day 7, mice were injected subcutaneously with 100 μg NP68 peptide in incomplete Freund's adjuvant (IFA; final volume of 200 μl) into the right flank prior to injection of T cells isolated from female donor mice into the tail vein. T cells and peptide were injected at 14.00–16.00 GMT and mice returned to their home cage. The checkpoint inhibitor, InVivoPlus anti-mouse PD-1, clone RMP1-14 (2BSCIENTIFIC), was injected on Day 10, 13, and 16 and InVivoMab anti-mouse CTLA-4 (CD152), clone 9H10, on Day 9, 12, and 15 (100 μg per injection). Other groups were treated with InVivoMAb rat IgG2a Isotype control (BE0089; 2BSCIENTIFIC) on the same days, times and doses as anti-PD-1 and InVivo MAb polyclonal Syrian Hamster IgG (BE087, 2BSCIENTIFIC) on the same days, times and doses as anti-CTLA-4. Antibodies were administered at 10.00–12.00 or 14.00–16.00 GMT and mice returned to their home cage.

To study the effect of regulatory T cell depletion, non-irradiated, NP68-B16 tumor-bearing FoxP3^{DTR} mice were injected intraperitoneally with 2.5 or 5 μg/kg diphtheria toxin (DT) 3 times a week or 100 or 200 mg/kg cyclophosphamide (CY) in 10% DMSO in PBS once a week starting at day 7 following tumor inoculation. A control group of tumor-bearing FoxP3^{DTR} mice received an equal volume of 10% DMSO in PBS once a week. Treatments were administered at 10.00–12.00 or 14.00–16.00 GMT and mice returned to their home cage.

At the end of the experiment, blood was collected from each mouse by cardiac puncture, serum prepared and stored frozen at −80°C. Tumors were excised, and either fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin wax, or snap-frozen in OCT without fixative, or, in some cases, large tumors were bisected and treated with both preservation methods. Tumor growth rates were calculated as described previously (18). Briefly, tumor growth rate (k, days^{-1}) was determined using a statistical software package Prism 5 (GraphPad) with the

following equation for exponential growth: $Y = Y0 \times \exp(k \times X)$. Tumor diameter (X, mm) was measured every 3–4 days using calipers. On average, measurements were taken for 23 days for both untreated mice and F5 T cell treated mice. On average 5–7 measurements were used to calculate tumor growth rate.

Disseminated Tumor Model

Adult male B6 mice were exposed to 597 cGy TBI on day 1 and randomly assigned to treatment groups of 8 mice per group for each experiment. For the therapeutic model, 5×10^6 NP68-B16 tumor cells were injected intravenously on day 2. On day 4, 3.4×10^4 T cells isolated from male donor mice were injected intravenously, followed by subcutaneous administration of NP68 peptide in IFA (**Figure 3A**). For the prophylactic model, timings of the tumor and T cell injections were reversed (**Figure 3A**). Mice were monitored until day 13 or 14 and then sacrificed. The lungs were excised, weighed, fixed in 10% NBF and embedded in paraffin wax. Five micrometer sections were stained with haematoxylin and eosin and tumor nodules analyzed by microscopy. In some studies, Thy 1.1 mice were injected with NP68-B16 tumor cells and the number and activation status of donor F5 T cells ($Thy1.2^+$ $CD8^+$) in the lungs at end stage (day 14) were determined by flow cytometry following tissue disaggregation.

L-Selectin Dependent T Cell Homing

A short-term homing assay in which the recruitment of L-selectin expressing, and L-selectin deficient T cells are compared directly in individual mice was used as described previously (11). Briefly, Thy1.1 mice were subcutaneously injected with 1.5×10^6 NP68-B16 tumor cells and tumors grown for 7–14 days. Thy1.1 mice served as non-tumor bearing mice. Tumor bearing mice and non-tumor bearing mice received 597cGy TBI. $CD8^+$ T cells isolated from naïve F5LΔP and F5LselKO (both Thy1.2) were mixed 1:1 (numbers determined by haemocytometer, and confirmed by flow cytometry using Cytocount beads) and a total of $6–10 \times 10^6$ T cells injected intravenously into mice at 14.00–16.00 GMT on the day following irradiation. All mice received 100 μg NP68-IFA s.c. prior to T cells. The next morning (10.00–11.00 GMT; 18 h following T cell injections), mice were culled and tissues including blood, spleen, tumor-draining and peptide-draining lymph nodes, non-draining lymph nodes and tumor were collected. Tumors were disaggregated using gentle MACS (Miltenyi). Lymphoprep was used to isolate immune cells from blood and tumor by centrifugation. Following isolation, cells were stained for CD8, Thy1.2, and L-selectin, analyzed by flow cytometry and L-selectin expression on donor $CD8^+$, Thy1.2 cells determined. Cell numbers were determined using Cytocount beads (Dako).

Longitudinal Tracking of Adoptively Transferred T Cells Using Micro PET/CT Imaging

^{89}Zr was produced via proton bombardment of an Yttrium target *via* an adaptation of the methods of Walther et al. (24) and Dabkowski et al. (25). Briefly, a disk of natural abundance ^{89}Y foil (300 μM thick, Goodfellow) in a custom made aluminum holder was loaded into a COSTIS Solid Target System (STS) fitted to an IBA Cyclone (18/9) cyclotron equipped with a 400 μM thick niobium beam degrader. The disk was irradiated for 4 h with a beam energy of 40 μA. The irradiated disk is left in the cyclotron for 12 h to allow any short lived ^{89M}Zr to decay to ^{89}Zr before removal for purification (activity 1.5–2GBq). The disk was dissolved in 2 M HCl with stirring and heat and the ^{89}Zr was isolated by flowing over a hydroxymate functionalized ion exchange resin column (prepared in house freshly for each separation). The column was rinsed with 2 M HCl and water to remove ^{89}Y before the ^{89}Zr was liberated with 1 M oxalic acid in 3 × 1 ml fractions. The most concentrated fraction contained 800–1000MBq.

^{89}Zr Oxine for cell labeling was prepared via an adaptation of the methods of Ferris et al. (26). Freshly prepared ^{89}Zr Oxalate (200 μl, ∼150–200 MBq) was adjusted to pH 7.0 with 0.5 M Na_2CO_3 (∼270–390 μl) and diluted to 2 ml with distilled water in a 15 ml centrifuge tube. To this was added 2 ml of oxine solution in chloroform (1 mg/ml) and the resultant biphasic mixture was shaken at room temperature (RT) at 1,000 rpm for 1 h. The mixture was then allowed to settle and the lower chloroform layer was removed and the activity measured by dose calibrator (typically 1–20MBq). A further 2 ml of oxine chloroform solution was added to the remaining aqueous phase and the mixture was shaken overnight (1,000 rpm, RT). The resultant mixture was allowed to settle and the chloroform layer removed and the activity measured (typically 100–150 MBq). The chloroform was removed by heating and gentle stream of air until a dry dusty yellow crust remained in the vial. This was redissolved in DMSO (20 μl) and then made up to 2 ml with PBS for cell labelling.

$CD8^+$ T cells were isolated from spleens and lymph nodes of naïve F5B6 and F5LΔP mice, resuspended to $13–66 \times 10^6$/ml in PBS and incubated with ^{89}Zr oxine (26–65 MBq) with shaking at 550 rpm for 30 min at RT. Unincorporated ^{89}Zr oxine was removed by repeated washing in 10 ml PBS, cells collected by centrifugation (350 g, 5 min, RT) and supernatant checked for removal of free ^{89}Zr. Uptake of ^{89}Zr oxine by $CD8^+$ T cells was 18–20% efficient, with labelled cell yields of between 30 and 50% and viability >90%. $8–17 \times 10^6$ T cells labeled with 0.7–1.63 MBq ^{89}Zr were resuspended in 200 μl PBS for injection into sub-lethally irradiated, tumor-bearing mice. One hundred microgram NP68-IFA was injected s.c. prior to the T-cell injection. Mice were anesthetized with isofluorothane (5% in O_2 gas 1–2 l/min) and ^{89}Zr labeled T cells injected intravenously. Mice were then injected with iopamidol contrast agent (Niopam 300, Bracco100 μl/mouse ip) and mice were scanned in a Mediso PET/CT Preclinical Imaging System (nanoScan122S PET CT Mediso). Respiration was monitored with a pressure pad connected to differential pressure transducers for low-range pressure monitoring during the entire PET-CT examination and anesthesia was maintained through the nose cone of the bed (1∼2% isoflurane in O_2 gas 1–2 l/min). PET emission data were collected for 60 min. Spatial resolution of PET measurements was 0.4 mm and energy lower/upper limit was 400/600. The CT scan parameters were set as follow: tube voltage was 50 kVp, tube current was 1 mA, exposure time 300 ms and maximum number of projections was 400. Reconstructed resolution of CT was 0.25 mm. PET-CT scans were repeated at 20, 44, 68, 140, 188, 212, and 235 h following T cell injections. Whole

body radioactivity was independently measured immediately after scanning by placing the anesthetized mouse in a Capintec CRC-25 dose calibrator on calibrator setting 465.

PET/CT scans were analyzed using Vivo Quant software (inviCRO). Regions of interest were drawn over tumor, lung and skeleton for each mouse. Total radioactivity in each organ was calculated and expressed as percentage of total measured radioactivity in the mouse at each timepoint.

Flow Cytometry

Cells were stained with fixable LIVE/DEAD^{TM} Aqua dye as per the manufacturer's protocol (Invitrogen), treated with 50 nM dasatinib (Sigma) to prevent TCR downregulation, then stained with PE-conjugated NP68 tetramer at 37°C, followed by surface antibodies at 4°C as follows: anti-TCRVβ11-FITC, anti-CD8-PerCPCy5.5, anti-CD27-BV421, anti-CD44-APCCy7, anti-CD62L-PECy7, and anti-CD69-APC (all BioLegend). Cells were fixed with 10% formalin and data collected using a BD FACSCanto II within 4 days of staining. Between fixing and data collection, cells were stored in PBS 2% FCS at 4°C in the dark. Voltages and compensation were set using OneComp eBeads (eBioscience) and Arc-reactive beads (Invitrogen) and the FACSDiva automatic compensation function. CytoCount beads were used to quantify cell numbers, according to the manufacturer's instructions (Dako). Figures were prepared using FlowJo software (TreeStar Inc.).

Immunohistochemistry

Formalin-fixed paraffin-embedded tumor tissue was cut into 5 μm sections and rehydrated. Antigen retrieval was performed using Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH9). Sections were blocked with 0.5% H_2O_2 in methanol, rinsed in PBS, blocked with horse serum (Vector Laboratories) and stained overnight with rabbit polyclonal anti-CD3 (A0452, Dako) or rabbit polclonal anti-CD8 (BS-0648R, Biosciences). After staining with horse anti-rabbit-HRP (Vector Laboratories), sections were developed for 90–120 s with SG detection solution (Vector Laboratories). Slides were mounted using DPX (Fisher Chemical). Sections were photographed at 20× magnification using an EVOS XL Core Microscope (Thermo Fisher Scientific).

Quantification of CD3 and CD8 Staining

The whole tumor area was photographed at 20× magnification. Five sequentially numbered fields of view (FOV) were analyzed per tumor. Where 5–6 FOV were taken, images 1–5 were analyzed; for 7–8 FOV, images 1, 3–5, 7 were analyzed; for 9 FOV, images 1, 3, 5, 7, and 9 were analyzed. Images were analyzed using the Fiji version of ImageJ. The scale was set to 3.4 pixels = 1 μm (based on image scale bar). Non-specific staining was subtracted using Background Correction, and the images were separated into FastRed, FastBlue, and DAB using Color Deconvolution. The FastBlue image was used to analyse CD3 and CD8. Minimum image threshold was set at 87, and maximum image threshold was set at 173–210. Percent area was analyzed and plotted. In cases where the tumor section did not cover the whole FOV, the blank area was subtracted prior to analysis. Four to twelve tumors were analyzed per treatment group.

Soluble L-Selectin

Serum levels of L-selectin in tumor-bearing mice were quantified using a mouse L-Selectin/CD62L DuoSet ELISA (R&D Systems) and optical densities measured on a CLARIO microplate reader (430-0841).

Statistics and Figures

Statistical analyses were performed in Prism 7 (GraphPad Software Inc.) as detailed in figure legends. Figures were prepared using FlowJo software (Treestar Inc.), Prism 7 (GraphPad Software Inc.), MS Powerpoint, and Adobe Illustrator.

RESULTS

Solid Tumor Model to Study T Cell-Dependent Tumor Immunotherapy

To measure T cell responses in tumor-bearing mice we used a modified B16F10 cell line expressing the H2D^b-restricted internal influenza nucleoprotein epitope NP68 (NP68-B16) as a surrogate tumor antigen (22). The lowest tumor cell dose that would consistently result in tumor growth in host animals was 5×10^5 cells per animal and this dose was used throughout this paper. We used CD8$^+$ T cells transgenic for the F5 TCR, which is specific for NP68 (20), as a source of tumor-specific T cells. Initial studies involved adoptive transfer of *in-vitro* activated CTLs expressing different levels of F5 TCR. CTLs hemizygous for F5 (F5B6/B6; **Table 1**) were not able to control the growth of NP68-B16 tumors (**Figure 1A**). However, F5B6 CTLs expressing increased levels of F5 TCR were able to control tumor growth (**Figure 1A**). We have previously shown that CTLs hemizygous for F5 control pulmonary influenza virus infections (11); the increased level of TCR required to control tumor growth most likely reflects the necessity to overcome tumor-induced immunosuppression. When we compared the ability of CD8$^+$ T cells from naïve mice to CTLs, T cells from naïve mice were considerably more efficacious, with a dose of only 5×10^5 T cells having the equivalent effect to 2×10^7 *in vitro*-stimulated CTLs (**Figure 1A**). All subsequent experiments were conducted with CD8$^+$ T cells freshly isolated from naïve mice, unless detailed otherwise.

To dissect the role of the host's immune system in this model, we transplanted the NP68-B16 tumor cells into FoxP3^{DTR} mice. These mice express the diphtheria toxin receptor on cells positive for the transcription factor FoxP3, which is expressed by regulatory T cells (Tregs). Once the tumors had been allowed to grow for 7 days, tumor-bearing mice were treated with 5 μg/kg diphtheria toxin (DT), to deplete Tregs (**Figure 1B**). This treatment completely halted tumor growth, indicating a very strong role for Tregs in restricting the growth of NP68-B16 melanomas. This also demonstrated that, once relieved of suppression by Tregs, the growth of solid tumors could be controlled by host T cells. The chemotherapeutic agent cyclophosphamide (CY) is thought to preferentially kill Tregs at low doses, due to their rapid division (27). Comparison of the prevalence of circulating Tregs in tumor-bearing hosts following either DT or CY treatment showed that 100 mg/kg CY affords a similar level of Treg depletion as DT treatment in FoxP3^{DTR} mice (**Figure 1C**). Furthermore, treatment with 100 mg/kg CY

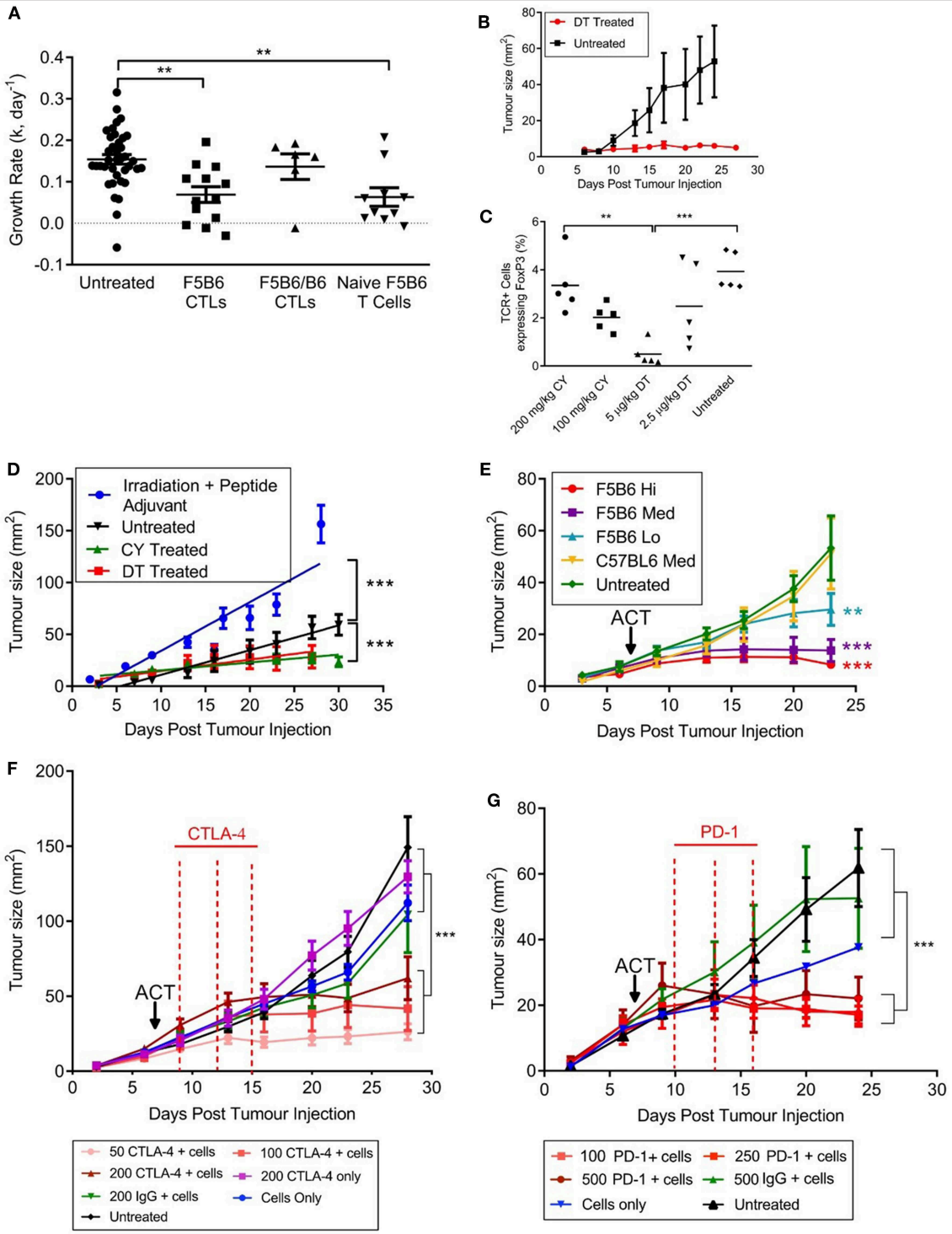

FIGURE 1 | An immunogenic solid tumor model in mice. **(A)** B16 melanoma cells expressing the NP68 antigen were transplanted subcutaneously into immunocompetent mice. Seven days after tumor transfer, mice were treated intravenously with either naïve $CD8^+$ T cells carrying the F5 TCR, or $CD8^+$ T cells activated *in vitro* with NP68 peptide (CTLs). F5B6 cells are homozygous and F5B6/B6 cells are hemizygous for the F5 TCR. Growth rates of tumors over time were measured in two dimensions and a "k" value was calculated. Data from more than one experiment. Scatter plots show individual mice and bars show means and SEM. (Untreated, $n = 41$, F5B6 CTLs, $n = 14$; F5B6/B6 CTLs, $n = 6$; naïve F5B6 T cells, $n = 10$). **(B)** NP68-B16 cells were transplanted into FoxP3DTR mice, and 7 days following tumor transfer, half the mice were treated with 5 µg/kg DT diphtheria toxin (DT) every 3 days for 2 weeks, $n = 6$. **(C)** NP68-B16 cells were transplanted

(Continued)

FIGURE 1 | into FoxP3DTR mice, and 7 days following tumor transfer mice were treated with either 5 μg/kg DT, 2.5 μg/kg DT, 200 mg/kg cyclophosphamide (CY) or 100 mg/kg cyclophosphamide. After 7 days of treatment, peripheral blood was sampled from the tail vein, and the percentage of cells positive for TCR and FoxP3 was enumerated by flow cytometry. Scatter plots show individual mice and bars show means. **(D)** Tumor growth rates in mice receiving different host conditioning regimes. Mice were either untreated or conditioned 7 days following tumor transfer by sublethal irradiation and peptide adjuvant (C57BL/6 hosts), CY or DT treatment (FoxP3DTR hosts), $n = 10$–14. Data from more than one experiment. Differences in tumor growth rate were calculated by linear regression and significances indicate differences in slope. $^{***}P \leq 0.001$. **(E)** C57BL/6 mice with NP68-B16 tumors established for 7 days were treated with sublethal irradiation (597cGy) and peptide adjuvant together with naïve $CD8^+$ T cells carrying the F5 TCR or polyclonal T cells (C57BL6). T cell doses were 5×10^4 = Lo; 2.25×10^5 = Med; 5×10^5 = High; $n = 7$–8. **(F)** C57BL/6 mice with NP68-B16 tumors established for 7 days were treated with sublethal irradiation (597cGy) and peptide adjuvant together with 2.25×10^5 naïve $CD8^+$ T cells carrying the F5 TCR in addition to treatment with anti-CTLA-4 antibody on days 9, 12, and 15 post tumor transfer, $n = 8$. **(G)** C57BL/6 mice with NP68-B16 tumors established for 7 days were treated with sublethal irradiation (597cGy) and peptide adjuvant together with 5×10^4 naïve $CD8^+$ T cells carrying the F5 TCR in addition to treatment with anti-PD-1 antibody at 10, 13, and 16 days post tumor transfer, $n = 5$–9. **(A,C)** Significance was calculated using one-way ANOVA with Tukey's multiple comparisons test. $^{***}P \leq 0.001$, $^{**}P < 0.01$. **(D–G)** Differences in tumor growth rate were calculated by linear regression and significances indicate differences in slope compared to untreated groups. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

gave the same level of tumor growth control as DT treatment (**Figure 1D**). In contrast, treating tumor-bearing hosts receiving a non-lethal dose of radiation, together with a bolus of NP68 peptide in incomplete Freund's adjuvant (peptide-IFA) led to an increase in tumor growth (**Figure 1D**). Increased growth of NP68-B16 tumors following sub-lethal irradiation could reflect a loss of endogenous T cells which partially control tumor growth or a response to locally-induced inflammation which is known to promote tumor growth (28). Non-lethal doses of irradiation are commonly used to create niche space prior to adoptive cell transfer (ACT), and this pre-conditioning regime is employed throughout this paper. It is important to note that despite the clinical use of radiation for cancer treatment, in this model system, irradiation alone does not control tumor growth, and, in fact, accelerates growth. The final aspect of the model to establish is the therapeutic dose of T cells required to control tumor growth. In **Figure 1E**, we demonstrate that differing levels of tumor growth control can be produced by just 5-fold differences in F5 $CD8^+$ T cell dose whereas equivalent doses of polyclonal $CD8^+$ T cells do not control tumor growth.

We have demonstrated that the growth of NP68-B16 melanoma is controlled by the host immune system, including the use of Tregs to escape immune cell attack. However, in fully immunocompetent (non-irradiated) tumor bearing mice, the host immune response to NP68-B16 melanoma is not sufficient to control tumor growth (**Figure 1D**). Given this, we would expect checkpoint inhibitors to improve the control of these tumors, through augmentation of the host immune response. However, when tumor bearing mice are treated with anti-CTLA-4 monoclonal antibodies, mimicking the clinical ipilimumab therapy, improved control of tumor growth is only seen in conjunction with adoptive transfer of F5 T cells (**Figure 1F**). This demonstrates that although there is evidence in **Figure 1D** for a modest host immune response to the tumors, it remains insufficient for tumor control, even in the presence of checkpoint inhibition. This is in line with previously published observations of the poor immunogenicity of the unmodified B16 melanoma cell line (29). However, treatment with even a very low dose of anti-CTLA-4 acts synergistically with low dose ACT to produce striking control of tumor growth, even where ACT alone was not effective. The same powerful synergy is seen with anti-PD-1 monoclonal antibody treatment (**Figure 1G**).

L-Selectin Enhanced T Cells in Adoptive Cell Therapy for Solid Tumors

Subcutaneous B16F10 melanomas show accelerated growth in L-selectin knockout mice (30) but which L-selectin expressing leucocyte population controls B16F10 melanoma growth was not determined. To study the role of L-selectin on T cells, we generated gain-of-function L-selectin transgenic mice in which T cells express either wildtype (CD62Lwt) or a shedding-resistant form of L-selectin (CD62LΔP) expressed under a heterologous promoter which we have shown to abrogate cytokine induced transcriptional silencing in virus-infected mice (11, 13, 17). When parental B16F10 cells are transplanted into CD62Lwt mice, tumor growth was not significantly different than in the background C57BL/6 (B6) strain (**Figure 2A**), indicating that abrogating gene silencing of L-selectin on its own does not boost anti-tumor immunity over and above that seen in wildtype B6 mice expressing endogenous L-selectin. When parental B16F10 cells are transplanted into CD62LΔP mice, tumor growth is significantly slower than in either B6 or CD62Lwt mouse strains with survival of 80% in CD62LΔP mice compared with <40% in B6 and CD62Lwt mice (**Figure 2A**). These data demonstrate that abrogating ectodomain shedding as well as transcriptional silencing of L-selectin boosts T cell-dependent tumor immunity. Interestingly, this finding suggests that abrogation of shedding, rather than gene silencing, plays a dominant in promoting T cell dependent immune responses even against poorly immunogenic unmodified B16 melanoma cell line (29). CD62LΔP mice do not express endogenous L-selectin and expression of transgenic L-selectin is restricted to T cells (16). Together these data indicate that L-selectin expressed by T cells, and not by other leucocytes, regulates the growth of B16F10 melanomas. These data strongly support our hypothesis that L-selectin enhancement by maintaining expression on activated T cells improves T cell dependent immunity to tumors.

To determine whether this effect was due to the action of enhanced expression of L-selectin on antigen-specific T cells, $CD8^+$ T cells from transgenic mice homozygous for both the F5 TCR and CD62LΔP (F5LΔP) were transferred into C57BL/6 mice with established NP68-B16 melanomas (**Figure 2B**). Expression levels of L-selectin and the transgenic F5 TCR were confirmed by flow cytometric analysis of transferred T cells (**Figure 2C**). Whilst $CD8^+$ T cells expressing endogenous,

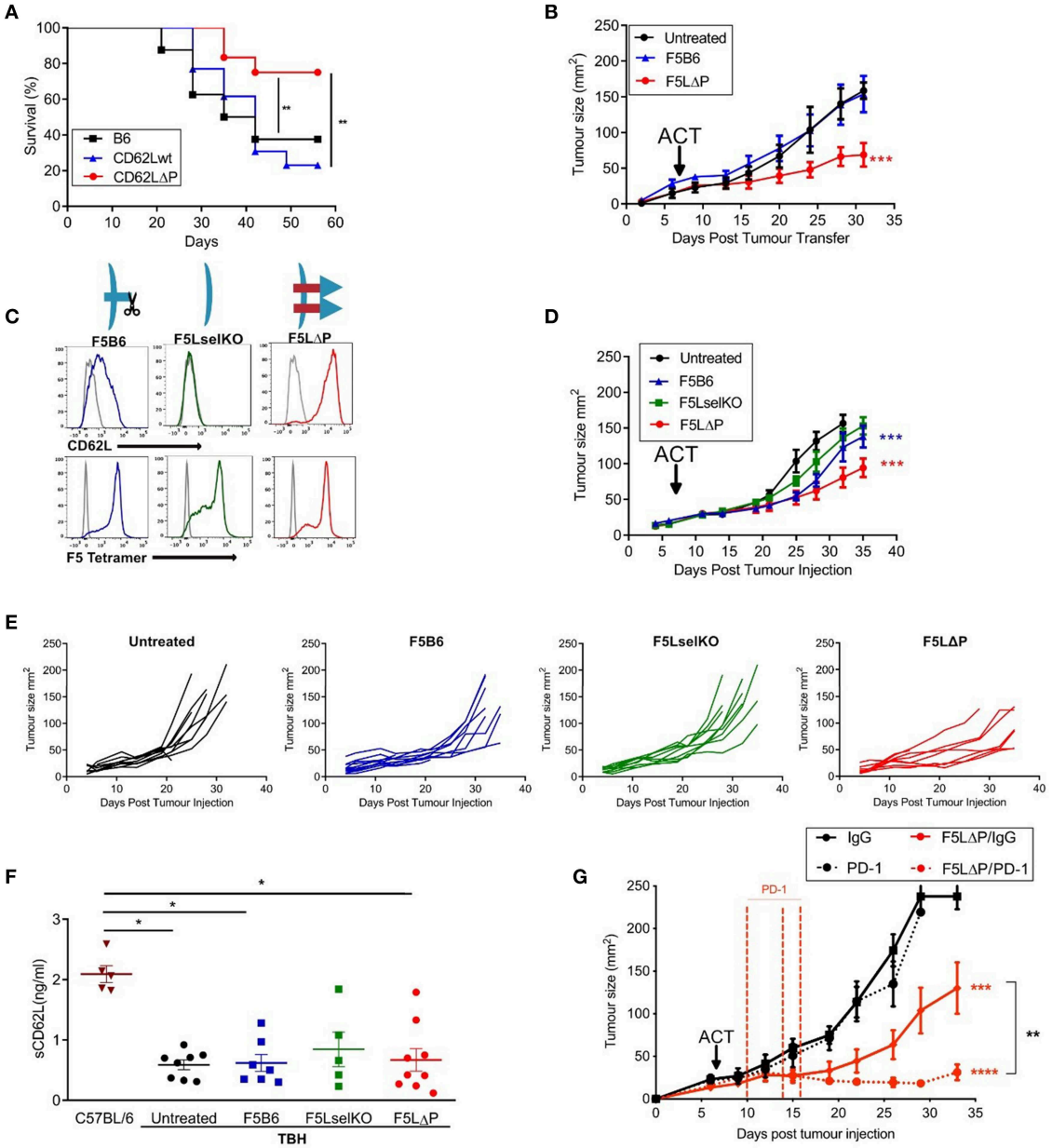

FIGURE 2 | L-selectin enhanced T cells expressing gain-of-function L-selectin exhibit improved control of solid tumor growth. **(A)** B16F10 melanomas were grown subcutaneously in unmanipulated C57BL/6 (B6) mice or transgenic mice expressing either wildtype (CD62Lwt) or mutant (CD62LΔP) L-selectin and tumor growth measured in the absence of host conditioning. Data represent the survival of mice with tumors ≥ 200 mm^3. $n = 11–13$ mice per group. **(B)** C57BL/6 mice with NP68-B16 tumors established for 7 days were treated with sublethal irradiation (597cGy) and peptide adjuvant together with 5×10^5 naïve $CD8^+$ T cells carrying the F5 TCR and either endogenous WT (F5B6) or gain-of-function transgenic L-selectin/CD62L (F5LΔP), $n = 7–8$. The untreated control group for this experiment has been published previously (22). The experiment was a multi-arm study, with a shared control group, for the purposes of Replacement, Refinement and Reduction of Animals in Research in the UK (3Rs). The F5 T cell-treated groups have not been published previously. **(C)** Flow cytometric analysis of $CD8^+$ T cells prior to adoptive transfer, indicating levels of CD62L and transgenic F5 TCR. Gray histograms are background staining. Graphics show L-selectin expression and shedding in different T cell populations. **(D)** Effect of the F5 T cell populations shown in **(C)** on NP68-B16 tumors established for 7 days and conditioned with sublethal irradiation and peptide adjuvant, $n = 10$. **(E)** Tumor growth curves for individual animals in experiment **(D)**. **(F)** Soluble L-selectin in naïve C57BL/6 mice and tumor-bearing hosts (TBH) without (untreated) and with F5 T cell therapy. Symbols are data from individual mice. Results are mean + SEM. Significance tested using one-way ANOVA and Tukey's multiple comparison test. $^*P < 0.05$. **(G)** Synergy between checkpoint blockade inhibition and L-selectin expressing T cells. Effect of F5LΔP T cells with and without anti-PD1 therapy ($n = 8$). Differences in tumor growth rate compared to untreated groups and between treatment groups were calculated by linear regression. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

wildtype L-selectin (F5B6) were unable to control tumor growth, L-selectin enhanced F5LΔP T cells produced a significant reduction in the rate of tumor growth (**Figure 2B**). To expand upon this, we repeated this study with F5 T cells that were either knockout or wildtype for L-selectin, and compared them to F5LΔP T cells (**Figure 2D**). As before, tumors were established for 7 days before ACT, and, as would be expected from the findings of Gattinoni et al. (14), cells that are knockout for L-selectin (F5LselKO) are unable to control tumor growth (**Figure 2D**). In contrast, F5LΔP T cells offer significantly improved control over F5B6 cells (**Figure 2D**). The improved control of tumor growth may be seen in more detail by viewing the individual tumor growth curves for each mouse, displayed as average curves in **Figure 2E**.

Ectodomain shedding of L-selectin on T cells and other leucocytes releases soluble L-selectin into the bloodstream which can compete for ligands on blood vessels and restrict L-selectin dependent leucocyte recruitment (31). Blood levels of soluble L-selectin in F5LΔP and LΔP mice are <5% of those in matched control mice expressing wildtype L-selectin due to lack of homeostatic and activation induced shedding (16, 19). To determine if the increased efficacy of F5LΔP T cells is dependent on altered blood levels of soluble L-selectin, soluble L-selectin in peripheral blood was measured at the time of tumor harvest. As shown in **Figure 2F**, soluble L-selectin was detected in tumor-bearing mice in the complete absence of T cell transfers. Moreover, levels of soluble L-selectin did not change following transfer of F5LselKO T cells, F5B6 T cells, or F5LΔP T cells (**Figure 2F**) showing clearly that soluble L-selectin in tumor-bearing mice is not derived from injected donor T cells but, instead, is derived from host leucocytes. Interestingly, soluble L-selectin levels in tumor-bearing mice were 60% lower than in age- and sex-matched, naive C57BL/6 mice. Although not investigated further, this may be due to reconstitution of the immune system following sublethal irradiation. Together, these findings show that the increased efficacy of F5LΔP over F5B6 and F5LselKO T cells was not dependent on altered blood levels of soluble L-selectin in tumor-bearing mice, but instead depended on increased and maintained expression of L-selectin at the surface of F5LΔP T cells.

To assess the potential for L-selectin enhanced T cells to complement other immunotherapeutic strategies, we determined the efficacy of F5LΔP T cells in the presence and absence of PD-1 blockade. In **Figures 2B,D**, the benefit of maintained expression of L-selectin over wild type in the treatment of solid tumors is clearly shown. When F5LΔP T cells are combined with anti-PD-1 therapy, a striking synergistic effect is seen, with tumor growth being arrested following combinatorial treatment (**Figure 2G**), demonstrating the clear potential of combining F5LΔP T cells with checkpoint blockade inhibition.

L-Selectin Enhanced T Cells in Adoptive Cell Therapy for Disseminated Tumors

To determine if L-selectin enhanced T cells confer protection against disseminated tumors, we used NP68-B16 tumor cells administered via the bloodstream which results in colonization of the lungs in the absence of a primary solid tumor. We tested the impact of enhanced L-selectin on the outcome of T cell therapies delivered either before or after intravenous injection of tumor cells. In a prophylactic model, F5 T cells (Thy1.2) expressing variable levels of L-selectin were injected intravenously into sub-lethally irradiated (Thy1.1) mice and mice vaccinated in the right flank with NP68 peptide in adjuvant. NP68-B16 tumor cells were injected intravenously the following day. In a therapeutic model, NP68-B16 tumor cells were intravenously injected into sub-lethally irradiated mice and allowed to colonize the lungs. Two days later, F5 T cells expressing variable levels of L-selectin were injected intravenously and mice vaccinated in the right flank with NP68 peptide in adjuvant (**Figure 3A**). In both models, mice were harvested 2 weeks after tumor administration, lungs excised, and tumor loads determined by total lung weight and confirmed by histology. F5 T cells deficient in L-selectin or expressing wildtype L-selectin did not affect tumor loads in either the prophylactic or therapeutic models when compared with tumor-bearing mice that did not receive T cells (**Figure 3B**). However, F5LΔP T cells significantly reduced tumor load in both models and average lung weights reduced from 463 mg in untreated mice to 277 mg following F5LΔP T cell therapy which approached that of age- and sex-matched non-tumor bearing mice at 169 mg (**Figure 3B**). The improved efficacy of F5LΔP T cells was also evident by the absence of tumor nodules and normal appearance of the lungs (**Figure 3B**). Prior to injection, the mean level of L-selectin on F5LΔP T cells was 3-fold higher than on F5B6 T cells (**Figure 3C**), as reported previously (19). L-selectin expression was maintained on F5LΔP T cells but completely downregulated on F5B6 T cells within 2 weeks of transfer into tumor bearing mice (**Figure 3D**) demonstrating clearly the ability of the genetic mutant to resist ectodomain shedding as well as transcriptional silencing in tumor-bearing mice. The activation status of injected T cells was not affected by L-selectin expression and injected F5B6, F5LselKO, and F5 LΔP T cells comprised >94% $CD44^{lo}$ naïve T cells (**Figure 3E**). The numbers, frequencies and extent of activation-induced degranulation (measured by CD107a mobilization) of donor derived F5LΔP T cells in the lungs after 14 days were not significantly different from either F5B6 or F5LselKO T cells (**Figures 3F–H**). The frequencies of $CD44^{+}CD27^{+}$ central memory T cells were higher in the F5LΔP population in the lungs, but not in the draining lymph nodes (LN) or spleen (**Figures 3I–K**). Therefore, maintained L-selectin appears to have a role in the activation of tumor-specific T cells, as indicated by CD44 and CD27 expression at the site of the tumor, but not in tumor-draining LN or spleen.

Impact of L-Selectin on T Cell Homing in a Solid Tumor Model

L-selectin is an obligate homing molecule on naïve T cells for recruitment from the bloodstream into subcutaneous (peripheral) lymph nodes via specialized high endothelial venule (HEV) blood vessels (32). Previous studies by Klebanoff et al. (15) showed that adoptive T cell therapy of L-selectin expressing CTLs did not have any anti-tumor effect in lymphotoxin-α deficient mice, which lack lymph nodes. It was concluded that the role

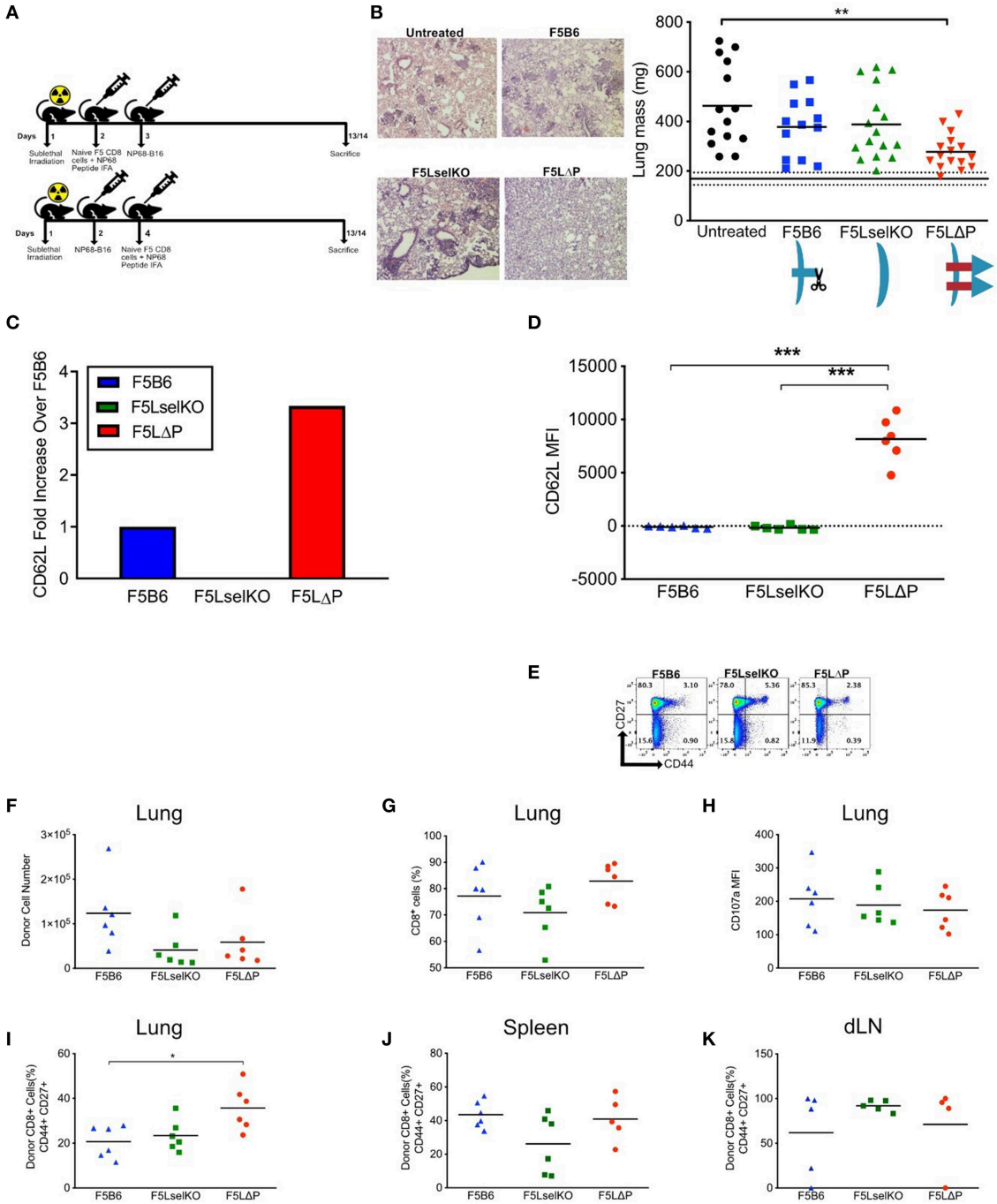

FIGURE 3 | L-selectin enhanced T cells expressing gain-of-function L-selectin exhibit improved control of disseminated tumor growth. **(A)** Experimental protocol schematic. Both NP68-B16 tumor cells and naïve $CD8^+$ cells were introduced via intravenous injection. **(B)** Tumor load measured by representage images of H&E stained lung sections and total lung mass. Data are pooled from more than one experiment. Symbols show individual mice and bars show means. Solid and dashed black lines show mean lung weight ± SD in age- and sex-matched naïve mice[1]. Graphic shows L-selectin expression and shedding in different T cell populations. **(C)** Fold increase in expression of CD62L on F5LΔP naïve $CD8^+$ T cells compared to F5B6 and F5LselKO $CD8^+$ T cells. **(D)** CD62L expression of donor T cells recovered from the lungs of tumor bearing hosts on day 14, measured by mean fluorescence intensity. **(E)** CD44 and CD27 expression on donor $CD8^+$ T cells prior to adoptive transfer. **(F,G)** Donor cells recovered from tumor-bearing lungs, by total number **(F)** and percentage $CD8^+$ cells **(G)**. **(H)** Expression of CD107a on donor T cells recovered from tumor-bearing lungs, measured by mean fluorescence intensity. **(I)** Percentage of donor T cells recovered from tumor-bearing lungs that are positive for CD44 and CD27. **(J)** Percentage of donor T cells recovered from spleen that are positive for CD44 and CD27. **(K)** Percentage of donor T cells recovered from the lung-draining lymph nodes that are positive for CD44 and CD27. **(B,D,F–K)** Significance was calculated using one-way ANOVA with Tukey's multiple comparisons test. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

of L-selectin in adoptive T cell therapy is to direct homing of adoptively transferred CTLs to LN, although T cell homing to lymph nodes was not studied. HEV-like blood vessels expressing peripheral node addressin (PNAd), a ligand for L-selectin, have been reported in B16F10 melanomas and shown to support L-selectin dependent recruitment of T cells (33). This raises the possibility that L-selectin promotes homing of adoptively transferred naïve T cells from the bloodstream into the tumor via PNAd expressing tumor blood vessels, where T cells could be activated to kill tumor cells, without the need for LN entry. To determine the role of L-selectin in T cell homing to LN and tumors in tumor-bearing mice, we used a competitive homing assay that we have previously employed to determine the role of L-selectin in recruitment of CTLs from the bloodstream into virus-infected lungs (11). F5LΔP and F5LselKO T cells (both Thy1.2) are mixed 1:1 and injected into Thy1.1 tumor-bearing mice. This assay avoids the use of cell tracker dyes which may affect T cell trafficking and, unlike cell tracker dyes, the Thy1 marker is not diluted through cell division. It also exploits the fact that L-selectin sufficient T cells (F5LΔP) can be distinguished from L-selectin deficient (F5 LselKO) T cells due to L-selectin expression which is maintained following T cell activation (11) and, as shown here, in tumor-bearing mice (**Figure 3D**). The use of F5LΔP T cells also prevents the loss of L-selectin from T cells after they have been recruited from the bloodstream into cancerous tissues. After 18 h, the ratio of F5LΔP:F5LselKO T cells in tumors, spleen, blood, tumor-draining and peptide-draining inguinal and axillary LN, and brachial LN were analyzed by flow cytometry (**Figure 4A**). In our experience, brachial LN are not involved in draining the tumor in this model, whereas both inguinal and axillary LN have been observed to be tumor draining (data not shown). The brachial LN are therefore included to represent uninvolved, peripheral LN which depend on L-selectin for naïve T cell recruitment from the bloodstream via HEV (32).

L-selectin sufficient and L-selectin deficient T cells were detected in approximately equal numbers in all peripheral LN analyzed (tumor-draining and peptide-draining inguinal and axillary as well as brachial LN) (**Figure 4B**). Although not significant, L-selectin expression conferred slightly improved homing to inguinal LN, but not to other peripheral LN (**Figure 4B**). These findings are in marked contrast to published data including own our studies in naïve and virus-infected mice where peripheral LN are highly enriched in L-selectin positive T cells (11, 16, 32). L-selectin sufficient and L-selectin deficient T cells were both found in the spleen (which lack HEV), as reported previously (32), however, a higher proportion of L-selectin-deficient cells was observed in the blood which fits with previous observations that these cells are unable to traffic from the bloodstream into tissues (32). In contrast to expectations, F5 T cells were found associated with solid tumors 18 h following intravenous administration. Moreover, L-selectin did not improve early homing of transferred T cells to the tumor, with the ratio of F5LΔP:F5LselKO cells harvested from resected tumors remaining similar to the injected population. These results show clearly that the improved control of tumor growth by L-selectin enhanced T cells is not simply explained by increased homing either to LN or to the tumor within the first 18 h following ACT.

Impact of L-Selectin on T Cell Activation in a Solid Tumor Model

The unexpected homing of L-selectin deficient T cells to peptide-draining and tumor-draining peripheral LN of tumor bearing mice, as well as to tumors, suggests that the lack of tumor growth control by F5LselKO T cells is not simply related to an inability to home to sites of tumor antigen-presentation in LN or tumors. In the absence of a clear role for L-selectin in T cell homing, we considered whether L-selectin expression controls T cell activation in lymphoid organs or inside tumors. The expression of early activation marker CD69 on donor cells on the day of transfer in the competitive homing assay was quantified by median fluorescence intensity (MFI). Fold increase in expression of CD69 was subsequently calculated based on the MFI of donor T cells following recovery from tissues at the end of the study, compared to CD69 expression on T cell populations prior to transfer. CD69 expression was upregulated on L-selectin sufficient (F5LΔP) T cells harvested from tumor tissues 18 h following transfer to tumor-bearing mice whereas CD69 on L-selectin deficient T cells (F5Lselko) was not increased (**Figure 4C**). CD69 expression was upregulated on both L-selectin sufficient and L-selectin deficient T cells harvested from spleen of tumor bearing mice, however it was significantly higher on F5LΔP T cells compared to F5LselKO T cells (**Figure 4D**). When L-selectin sufficient and deficient cells were harvested from the spleens of non-tumor bearing mice which had undergone sub-lethal irradiation and peptide vaccination, there was a uniform increase in CD69 expression in both F5LΔP and F5LselKO T cells. These data show that the conditioning regime of irradiation and peptide vaccination increases CD69 equally on injected F5LΔP and F5LselKO T cells but it does not contribute to L-selectin-dependent increases in CD69 expression. Rather, the higher levels of CD69 on L-selectin sufficient T cells in the spleens of tumor-bearing mice are a direct consequence of tumor load (**Figure 4E**). Representative plots showing the extent of CD69 upregulation on injected T cells in the spleens of tumor-bearing mice are shown in **Figure 4F**.

In contrast to the spleen, CD69 expression on F5LΔP T cells and F5LselKO T cells harvested from LN was variable and depended on LN location. In tumor-draining and peptide-draining axillary LN, CD69 was higher on L-selectin sufficient T cells whereas in tumor-draining and peptide-draining inguinal LN, CD69 was upregulated equally (**Figure 4G**). CD69 was upregulated on both L-selectin sufficient and L-selectin deficient T cells in brachial LN (**Figure 4G**). As brachial LN is not a site of tumor antigen presentation, this finding suggests a role for the host conditioning regime in upregulating CD69 on F5LΔP T cells and F5LselKO T cells as found in the

[1]https://www.taconic.com/phenotypic-data/b6-physiological-data-summary.html

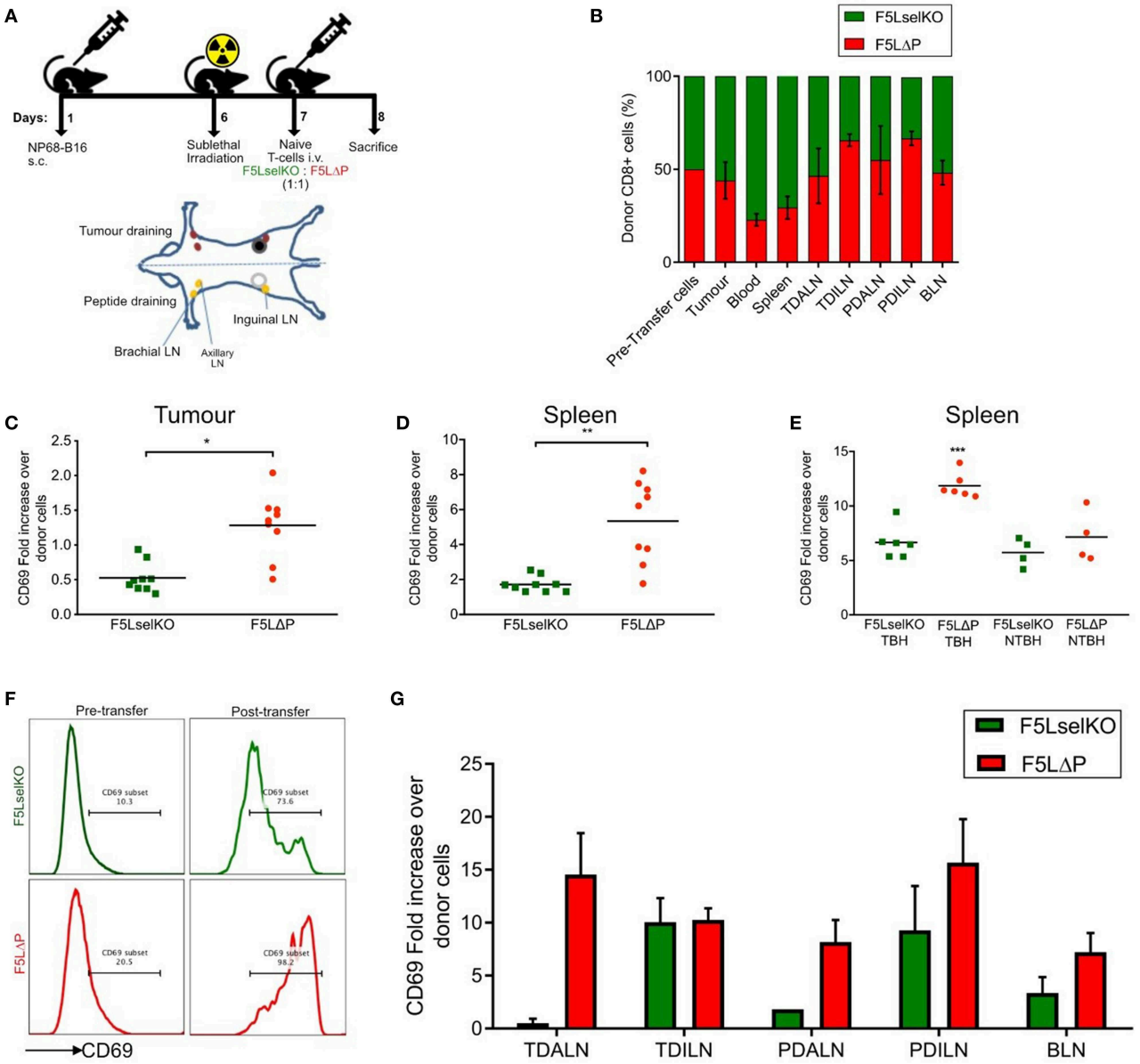

FIGURE 4 | L-selectin dependent T cell homing and activation in tumor bearing mice. **(A)** Schematic of *in vivo* homing study and the location of lymph nodes in relation to tumor and peptide adjuvant injections. Diagram is of ventral view of mice showing various LNs. **(B)** Competitive homing of L-selectin sufficient (F5LΔP) and L-selectin deficient (F5LselKO) naïve T cells. T cells were allowed to home for 18 h before being recovered and analyzed by flow cytometry based on expression of F5 and L-selectin transgenes. Values are normalized to a total donor cell population of 100%. TDALN, tumor draining axillary lymph node; TDILN, tumor draining inguinal lymph node; PDALN, peptide draining axillary lymph node; PDILN, peptide draining inguinal lymph node; BLN, brachial lymph node. **(C–G)** Expression of activation marker CD69 on donor T cells recovered from tumor, spleen and LN calculated as fold increase over pre-transfer expression. Pre-transfer CD69 MFI values were 1,580 for F5LselKO cells and 1,664 for F5LΔP cells. **(C)** CD69 expression levels on donor T cells recovered from tumors of mice 18 h after injection. **(D)** CD69 expression levels on donor T cells recovered from spleens of tumor-bearing hosts after 18 h. **(E)** Comparison of CD69 expression on donor cells recovered from the spleens of tumor-bearing hosts (TBH) and non-TBH after 18 h, in a competitive homing study similar to that described in **(A)**. **(F)** Representative plots showing CD69 expression on donor T cell pre-transfer to tumor bearing mice and following isolation from spleens of tumor-bearing mice (post-transfer). Percentages of CD69 positive donor T cells are shown. **(G)** Comparison of CD69 expression on donor cells recovered from the LN of tumor-bearing hosts after 18 h, in a competitive homing study similar to that described in **(A)**. **(B,G)** Bars show means ± SEM ($n = 5$). **(B–E,G)** Significance was calculated using two-way ANOVA and multiple *t*-test. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

spleens of non-tumor bearing mice (**Figure 4E**). Together, these findings suggest that L-selectin expression on adoptively transferred T cells does not promote homing to tumors or to LN. Instead, L-selectin expression promotes early T cell activation as measured by CD69 expression inside the tumor, spleen and involved LN.

Longitudinal Tracking of ^{89}Zr Labeled T Cells in Tumor Bearing Mice Using PET-CT

The localization of CD8$^+$ T cells isolated from naïve mice to tumors within 18 h following intravenous injection was unexpected. It was predicted that T cells would require prior activation by peptide-MHC complexes on APC in lymphoid organs and subsequent differentiation to effector T cells before being able to enter tumors from the bloodstream. T cell activation takes longer than the 18 h timeframe of the homing study (11, 34). To study the kinetics of T cell recruitment into tumors in individual mice following adoptive T cell therapy, we labeled F5 CD8$^+$ T cells *ex vivo* with ^{89}Zr oxine, as described previously for human T cells (35, 36), and injected ^{89}Zr-labeled T cells to tumor bearing mice following the normal conditioning protocol of sub-lethal irradiation and peptide vaccination. Mice were scanned within 1 h following injection of T cells, rescanned the following day and at 1–2 day intervals thereafter. ^{89}Zr was detectable in tumors, lungs, skeleton, liver, and spleen within 1 h following injection and for the following 5 days, but was not detected in heart, kidney or bladder (**Figure 5A** and data not shown). To determine whether ^{89}Zr remains associated with T cells following injection into mice or is released and excreted as waste, the predicted radioactive decay curve for total injected ^{89}Zr was plotted alongside the actual amount of ^{89}Zr detected in individual mice receiving either F5B6 or F5 LΔP T cells. The total amount of ^{89}Zr detected in a single mouse completely overlaid the predicted radioactive decay curves for both types of T cell indicating that cell-free ^{89}Zr was not excreted during the course of the study (**Figures 5B,C**). We exploited the known kinetics of temporary intravascular trapping and release of T cells from the lungs (37) over the first 24–48 h following intravenous injection to assess whether ^{89}Zr remains associated with T cells. The fraction of total ^{89}Zr in the lungs of mice receiving F5B6 T cells fell from 3 to 5% at 1 h to ∼1% at 20 h after which it gradually increased over the following 5 days (**Figure 5C**). Lung associated ^{89}Zr in F5LΔP recipients showed similar kinetics decreasing from 2% at 1 h to <1% at 20 h (**Figure 5C**) suggesting that ^{89}Zr is T cell associated, at least for the first 20 h following injection. The fraction of total ^{89}Zr in the skeletons of mice receiving F5B6 T cells was ∼4% 1 h after injection, increased gradually and stabilized at 6–8% at 44 h after injection (**Figure 5D**). Skeletal localization was slightly lower in mice receiving F5LΔP T cells at 2% after 1 h and gradually increased to ∼4% over the next 6 days (**Figure 5D**). Skeletal localization of ^{89}Zr did not exceed 8% of total ^{89}Zr, even after 140 h, which supports the hypothesis that the bulk of ^{89}Zr is not being released as cell-free ^{89}Zr during the course of the experiment which would be expected to localize to the skeleton (38). ^{89}Zr was detectable in tumors within 1 h following intravenous injection of either F5B6 or F5LΔP T cells and ^{89}Zr levels increased as tumors grew (**Figures 5E,F**). ^{89}Zr in tumors fell below the level of detection after 188 h, which is before the effects of ACT are detectable. Although these data suggest that ^{89}Zr remains T cell associated for 7 days, we cannot formally exclude localization of ^{89}Zr in macrophages following death of T cells at time points beyond 20 h. These studies did not allow a direct comparison between F5B6 and F5LΔP T cells in the same mouse, however, they do show clearly that both T cell populations localize to tumors within 1 h of injection into the bloodstream. Taken together with the 18 h homing data, this shows that there is extremely rapid recruitment of T cells from the bloodstream and entry into solid tumors that is not dependent on L-selectin, followed by ongoing T cell accumulation over more than a week.

End Stage Analysis of TILs and T Cell Proliferation

To determine if maintained L-selectin alters the number of tumor infiltrating lymphocytes (TILs), the total numbers of CD3$^+$ and CD8$^+$ T cells were enumerated in tumor sections from solid tumors harvested at the end of the study (this could represent either an individual mouse culled due to tumor size, or animals sacrificed as a group at the end of the entire study). Mice were pooled from more than one experiment where experimental conditions were the same. As shown in **Figure 6A**, host-derived CD3$^+$ T cells were readily detectable in tumors of mice which did not receive T cells (untreated). The total numbers of CD3$^+$ TILs (donor and host) were not significantly increased following transfer of either F5B6 or F5LselKO T cells. The total number of CD3$^+$ TILs in the F5LΔP treatment groups was significantly increased by approximately 2-fold. Similarly, the total number of CD8$^+$ TILs in F5LΔP treated mice was significantly higher than in either F5B6 treated or untreated mice which had similar numbers CD8$^+$ TILs (**Figure 6B**). The classical ligand for L-selectin, PNAd, was not detectable on tumor blood vessels using MECA79 antibody, although MECA79 positive HEV were detected in peripheral LN of naïve mice using the same staining procedure used for tumors (data not shown).

These data show that the increased number of CD3$^+$ TILs in F5LΔP recipients is not simply related to increased L-selectin dependent recruitment from PNAd expressing blood vessels inside the tumor either at the start of or during the course of ACT. This is supported by the disseminated tumor model, which does not rely on disordered tumor vasculature for T cell entry, but instead utilizes the established and extensive lung vasculature. No increase in injected T cells was observed in the lungs of tumor-bearing mice, despite improved tumor control by F5LΔP T cells. Instead, it could reflect increased retention due to higher CD69 expression and possibly increased frequency of central memory CD44$^+$ CD27$^+$ T cells at the tumor site which are known to be more efficacious in mouse models of ACT (15), as found in the disseminated tumor model (**Figure 3I**). We also considered whether the level of L-selectin on T cells determines other T cell properties that could contribute to the control of tumor growth. Cross-linking of L-selectin costimulates antigen-induced T cell proliferation (39) and controls important effector functions such as superoxide production (40), colony-stimulating factor 1 release (41) and lytic activity (42), all of which may contribute to tumor cell killing. Our previous studies of T cell dependent virus immunity have demonstrated that ectodomain shedding of L-selectin controls the clonal expansion of CD8$^+$ T cells (43) so we started by analyzing the impact of L-selectin on T cell proliferation. In the mouse tumor model, F5 T cells are exposed to NP68 cognate

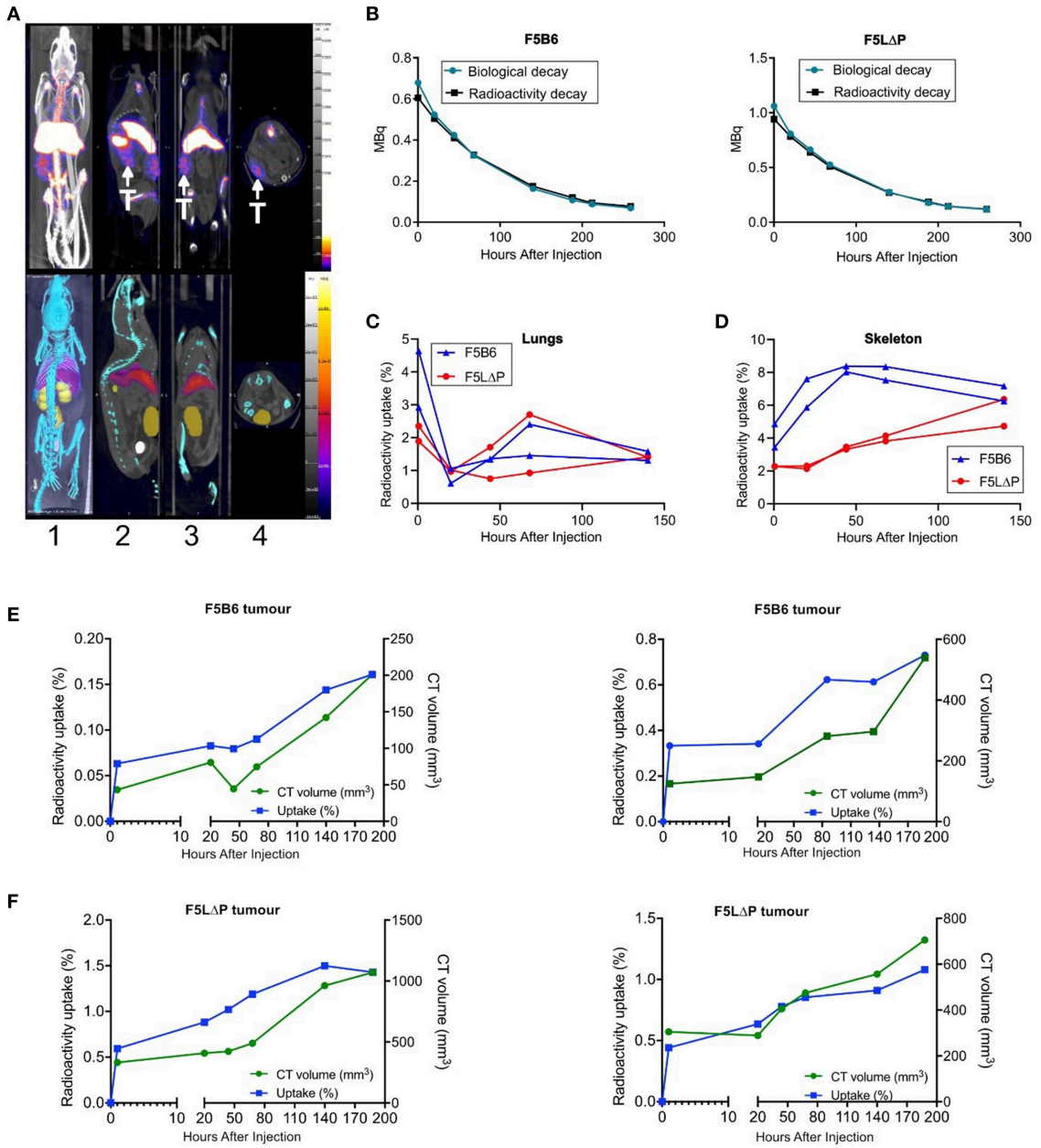

FIGURE 5 | Longitudinal tracking of ^{89}Zr labelled T cells in tumor bearing mice using PET-CT. **(A)** Representative PET-CT images of NP68-B16 tumor bearing B6 mice 5 days after intravenous injection of ^{89}Zr labeled T cells. T, tumor. Image orientations: 1, Maximum intensity projection; 2, Sagittal; 3, Coronal; 4, Transverse. **(B)** Representative time-activity curves from dosimeter quantification of ^{89}Zr in the whole mouse overlaid with predicted radioactive decay of ^{89}Zr in recipients of F5B6 (left) and F5LΔP (right) T cells. **(C,D)** Time-activity curves from image-based quantification of ^{89}Zr in the lungs **(C)** and skeletons **(D)** of 2 mice receiving F5B6 (blue) or F5LΔP (red) T cells expressed as percentage of total ^{89}Zr in the mouse at each timepoint. The earliest time point in **(B–D)** is 1 h after injection. **(E,F)** Time-activity curves from image-based quantification of ^{89}Zr in tumors expressed as percentage of total ^{89}Zr in the mouse (blue line) overlaid with tumor size measured by CT (volume; green line). Graphs show overlaid curves from each of two mice receiving either F5B6 **(E)** or F5LΔP **(F)** T cells.

peptide expressed on tumor cells and to exogenously injected or tumor derived peptide on APC in spleen and LN, as evidenced by CD69 upregulation at these sites (**Figures 4E–G**). To simulate continuous exposure of T cells to tumor antigen and measure T cell proliferation we exposed isolated T cells to peptide-pulsed APC *in vitro* and measured T cell proliferation and expression of the proliferation marker Ki67. Peptide stimulation was repeated every 7 days. As shown in **Figure 6C**, L-selectin expression is downregulated on F5B6 T cells over the first 7 days of stimulation and remains low following subsequent restimulations whereas

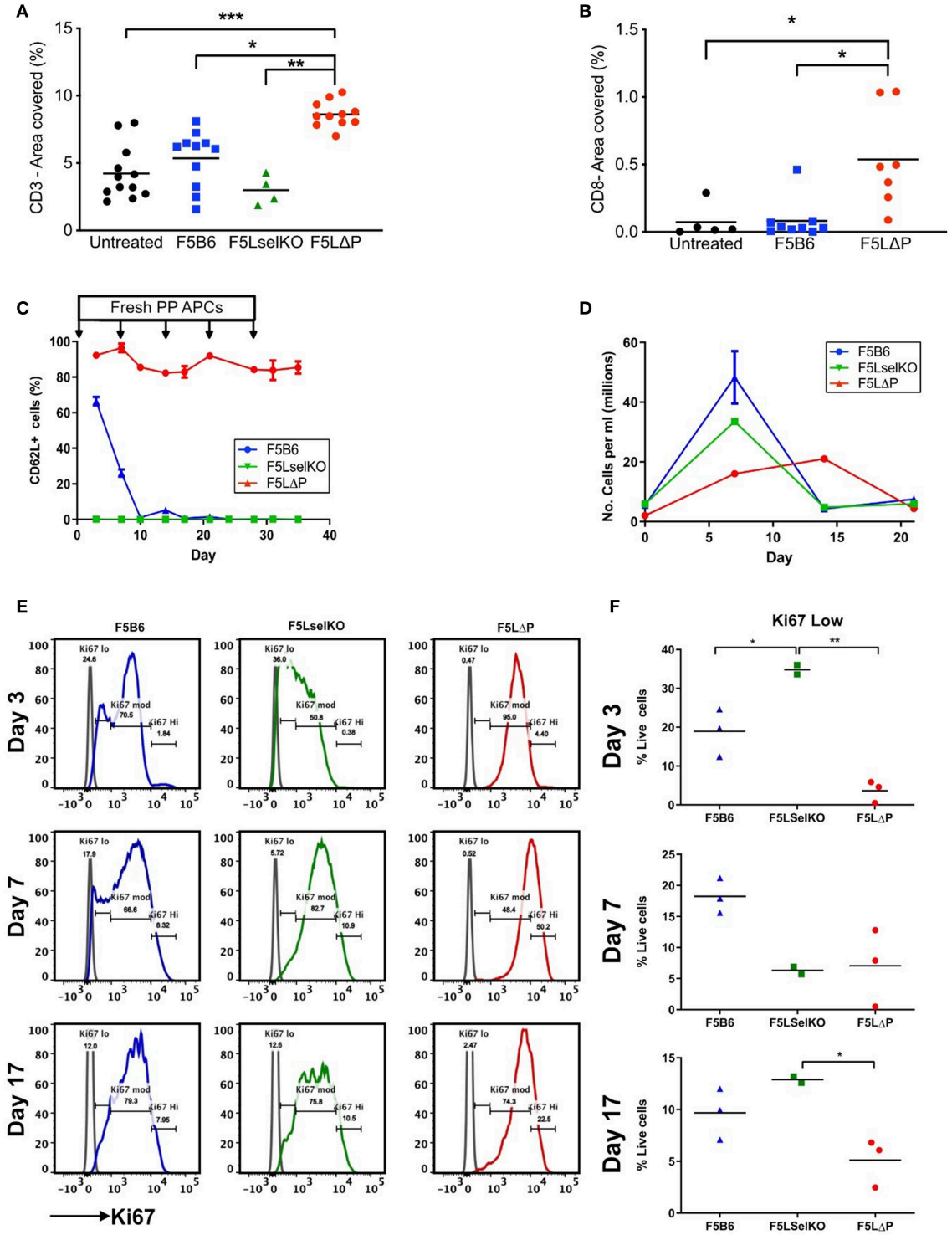

FIGURE 6 | Impact of L-selectin on TILs and T cell proliferation *ex vivo*. **(A,B)** Subcutaneous tumors were fixed in formalin and embedded in paraffin. Five micrometer sections were stained for CD3 **(A)** and CD8 **(B)** and images analyzed using Fuji Image J software. Each symbol represents % area stained in tumors from single mice receiving no T cells (untreated), T cells expressing endogenous L-selectin (F5B6), no L-selectin (F5 LselKO), or enhanced L-selectin (F5LΔP). Data are pooled from more than one experiment conducted under identical conditions. Bars show means (Untreated, $n = 5$–12, F5B6, $n = 9$–11; F5LselKO, $n = 4$; F5LΔP, $n = 7$–10). **(C)** $CD8^+$ T cells from F5B6, F5LΔP, and F5LselKO mice were stimulated *in vitro* on NP68-peptide pulsed APCs, with restimulation every 7 days. Every 3 days, a sample of cells was collected, and analyzed by flow cytometry for a range of markers, including CD62L, $n = 2$–3. **(D)** Total live cells were enumerated by haemocytometer at each timepoint. **(E)** The proliferation marker Ki67 was compared on the same cell populations at days 3, 7, and 17. **(F)** The frequency of $Ki67^{lo}$ cells at each time point. **(A,B,F)** Significance was calculated using one-way ANOVA with Tukey's multiple comparisons test**.** $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

F5LΔP cells maintain high levels of L-selectin throughout primary and subsequent re-stimulations. F5B6 undergo rapid clonal expansion during primary stimulation and T cell numbers peak after 7 days at 50×10^6/ml and gradually decline following restimulation (**Figure 6D**). Proliferation of F5LselKO T cells followed similar kinetics as F5B6 cells showing early clonal expansion and subsequent contraction after 7 days. However, F5LΔP T cells showed a lag in proliferation which peaked later at 14 days. Moreover, the extent of clonal expansion of F5LΔP T cells was smaller resulting in a 10-fold increase in T cell numbers at day 14, compared to F5B6 and F5LselKO T cells which increased by ∼20-fold by day 7. Analysis of Ki67 expression revealed differential patterns of expression in all three T cell populations. Expression levels were categorized as low, medium or high (**Figure 6E**) and showed different frequencies at each level between F5B6, F5LΔP, and F5LselKO T cells. Statistically significant differences in the frequencies of Ki67 low cells were seen at day 3 and day 17 but not at later times (**Figure 6F**). The decrease in the number of F5LΔP cells that are Ki67 low suggests that, despite the slower and overall lower level of proliferation, expression of the proliferation marker Ki67 was higher in F5LΔP cells. Whether this assay replicates the stimulation and expansion of F5LΔP T cells in tumor bearing mice is not known. However, it demonstrates that L-selectin enhanced T cells behave differently to cells expressing either wildtype endogenous L-selectin or no L-selectin in response to immune stimulation in a model system where cell trafficking is not relevant.

DISCUSSION

L-selectin (also known as CD62L) is an important homing molecule on the surface of T cells for their recruitment from the bloodstream into lymph nodes where T cells first become activated by peptide-MHC complexes on dendritic cells. L-selectin (CD62L), is commonly used as a marker of T cell activation, due to the well-documented observation that expression of L-selectin is downregulated when T cells are activated to CTL status. In mouse models of ACT, studies have shown that even when T cells are matched for activation status, L-selectin expressing CTLs confer an advantage over L-selectin knockout CTLs in controlling tumor growth (14). Based on these findings, we investigated the hypothesis that CTLs expressing a "gain-of-function" form of L-selectin that is never downregulated, even following T cell receptor engagement (LΔP; L-selectin enhanced), are better able to control the growth of solid cancers than wild type T cells which downregulate L-selectin expression. We report here that L-selectin enhanced T cells show improved control of tumor growth. Previous studies have linked L-selectin to T cell function due to the differential homing of L-selectin expressing naïve/central memory T cells to lymph nodes and L-selectin negative effector/effector memory T cells to non-lymphoid/ inflamed tissues. In this study, we show clearly that the effect of L-selectin on tumor growth is unrelated to L-selectin dependent homing but instead correlates with early activation of therapeutic T cells inside tumors.

In two different tumor models, T cells expressing LΔP-selectin were better able to control the growth of transplanted tumors than wild type T cells. These findings support the hypothesis of the study and show clearly that maintaining L-selectin expression is beneficial for adoptive T cell tumor therapy. L-selectin enhanced T cells controlled the growth of solid, vascularized subcutaneous tumors as well as disseminated tumors which had colonized the lungs showing that the effect of L-selectin enhanced T cells is not restricted by tumor location or tumor size at the start of therapy. Clinically, the most marked effects of cancer immunotherapy have been achieved through the use of checkpoint blockade inhibitors. In the solid tumor model, checkpoint blockade inhibitors did not control tumor growth in the absence of T cell transfers. However, anti-CTL-4 and anti-PD-1 monoclonal antibody therapy showed marked synergy with the transfer of T cells expressing wild type L-selectin resulting in arrest of growing tumors. This powerful synergy is also demonstrated by the combination of anti-PD-1 with F5LΔP $CD8^+$ T cell transfer.

The obvious mechanism for control of tumor growth by F5 LΔP T cells would be improved trafficking into the tumor. Indeed, the motivation behind applying this molecule to ACT is based on our own findings of improved anti-viral immunity due to homing of LΔP T cells to virus-infected lungs (11). Increased numbers of $CD3^+$ and $CD8^+$ cells were identified in solid tumors in mice treated with F5LΔP cells compared to either wild type or L-selectin knockout T cells at experimental endpoints. However, increased TILs in solid tumors up to 21 days after transfer could be due to a range of scenarios, including improved homing, increased proliferation, increased retention or prolonged survival. To determine the kinetics of T cell recruitment from the bloodstream into solid tumors, we tracked ^{89}Zr labeled T cells following transfer to tumor-bearing mice by PET/CT. Since therapeutic T cells were not pre-activated prior to transfer, we expected a lag between T cell injection and localization of CTLs inside tumors which would reflect activation in secondary lymphoid organs of tumor-bearing mice. However, tumor-specific T cells were detected inside tumors within 1 h of injection into the bloodstream. ^{89}Zr label steadily increased inside solid tumors over the subsequent 8 days of tumor growth which is before the therapy took effect. Wild type T cells and L-selectin enhanced T cells were tracked in the tumor at every time point including 1 h after transfer. Although direct comparison of different T cell populations in the same tumor-bearing mouse was not possible using this method, it did demonstrate that F5B6 (unmodified) T cells are able to enter the tumor, and therefore the benefit of maintaining L-selectin is not simply a case of licensing T cells to enter the tumor stroma. When L-selectin positive and L-selectin knockout T cells were compared directly in a competitive homing assay, no evidence of L-selectin dependent homing to tumors was observed. A single report describes induction of PNAd on tumor blood vessels in B16 melanomas expressing the surrogate tumor antigen OVA in peritoneal, but not subcutaneously, growing tumors which supports recruitment of naïve L-selectin expressing T cells (33). The absence of PNAd expressing tumor blood vessels and L-selectin dependent homing of T cells in subcutaneously growing

NP68-B16 tumors agrees with this published report. Most studies of T cell homing to tumors have focussed on the recruitment of effector T cells into B16 melanomas and shown important roles for P/E-selectin and ICAM-1 on tumor blood vessels as well as CXCR3 ligands (44, 45). Further studies will be required to determine which adhesion molecules and chemokines control the L-selectin independent recruitment of unactivated, therapeutic T cells into solid tumors that our studies have revealed.

An alternative explanation for the beneficial effects of LΔP-selectin expressing T cells is to sustain trafficking to lymph nodes where T cells are licensed by activating, tolerogenic or homeostatic signals which could affect the differentiation and/or survival of F5 LΔP T cells. However, F5 LΔP T cells and F5 L-selectin knockout T cells homed equally well to peripheral lymph nodes in tumor bearing mice at the start of therapy. The loss of L-selectin dependent homing of T cells to peripheral lymph nodes in tumor-bearing mice contrasts sharply with our own findings and published studies using similar competitive homing assays where T cell homing to peripheral LN in both naïve and virus infected mice is exquisitely L-selectin dependent (11, 16, 32, 46). Although not explored further, a likely explanation is induction of MAdCAM-1 on HEV in peripheral LN of tumor bearing mice, which occurs in immunized mice (47). Co-expression of PNAd and MAdCAM-1, which binds both α4β7 integrin and L-selectin on T cells (48, 49), would allow entry of L-selectin expressing and L-selectin knockout T cells into peripheral LN, as we have found. Previous studies have shown that B16F10 melanoma regulate CCL21 dependent T cell recruitment from HEV blood vessels in tumor draining LN but the role of L-selectin was not explored (50).

Although equal numbers of F5LΔP and F5 L-selectin knockout T cells reached solid tumors, the activation of T cells was different. Prior to transfer, unactivated T cells had equally low expression of CD69. However, 18 h after transfer, CD69 expression was upregulated on F5LΔP cells but not on F5L-selectin knockout T cells inside tumors. Although CD69 was upregulated on both F5LΔP cells and F5 L-selectin knockout T cells inside lymph nodes and spleen, CD69 was significantly higher on F5LΔP cells, particularly in the spleen. In non-tumor bearing animals which had been irradiated and vaccinated with peptide, CD69 was equally upregulated on F5LΔP cells and F5 L-selectin knockout T cells in the spleen. Therefore, L-selectin enhanced T cells respond to the presence of a tumor differently to L-selectin knockout T cells and upregulate CD69 both inside the tumor and at sites of antigen presentation and priming in secondary lymphoid organs. This is the first report, to our knowledge, that the combination of tumor load and L-selectin status changes the activation of T cells *in vivo* independently of effects on homing. The steady increase in ^{89}Zr in tumors following transfer of L-selectin expressing T cells suggests that T cells continue to be recruited over the first 8 days of therapy, but where incoming T cells were before entering the tumor and whether they are already activated is not possible to determine by PET scanning. Whether CD69 expression is maintained on activated F5LΔP tumor infiltrating T cells throughout the course of the therapy and increases the number of $CD8^+$ tumor infiltrating T cells due to retention will be interesting to address. L-selectin does not control the differentiation of CTLs in tumor bearing mice, at least in the lungs, where CD107a expression on transferred T cells was unrelated to L-selectin expression. However, L-selectin controls other aspects of T cell activation as shown by increased numbers of $CD44^+$ $CD27^+$ "central memory" F5LΔP cells in the lungs of tumor bearing mice as well as Ki67 expression and altered proliferation *ex vivo*. Further studies will be required to fully dissect the mechanism(s) underlying L-selectin dependent control of T cell activation, differentiation and proliferation. Which of these pathways underlies the beneficial effects of F5LΔP T cells in adoptive cell tumor therapies and whether they differ for solid and disseminated tumors will be important to address. Since L-selectin knockout T cells do not control tumor growth yet CD69 is upregulated in secondary lymphoid organs, it is unlikely to be related to events in lymph nodes or spleen. The striking finding that CD69 is not upregulated on L-selectin knockout T cells in solid tumors suggests that the beneficial role of L-selectin is linked to activation of therapeutic T cells inside tumors.

The dominant role of L-selectin in controlling T cell homing has focused attention on its adhesive function. The impact of F5LΔP T cells on tumor growth correlated with increased cell surface expression of L-selectin rather than altered levels of soluble L-selectin. Maintained expression of L-selectin at the T cell surface could prolong binding to accessory cells expressing non-PNAd ligands for L-selectin, such as PSGL-1 (51) or MAdCAM-1 (48), and alter transmission of co-stimulatory signals to T cells resulting in increased expression of CD69. L-selectin dependent upregulation of CD69 was seen in tumors and lymphoid organs suggesting that a ligand-expressing accessory cell is unlikely to be tumor cells but an antigen-presenting cell that matures in the tumor microenvironment and re-locates to lymphoid organs. Although other mechanisms are possible, it is striking that the increased efficacy of F5LΔP T cells against pulmonary influenza infection differs fundamentally from the findings reported here because it is not related to altered T cell activation, differentiation or cytotoxicity but instead depends on increased homing of CTLs to virus-infected lungs (11).

Despite remarkable successes in the field of cancer immunotherapy, many solid tumors remain beyond the reach of this approach. One factor that is believed to be problematic is the recruitment of immune cells (endogenous or exogenous) into the tumor stroma, due to the atypical nature of tumor vasculature (7). In clinical and experimental cancers, the development of PNAd expressing HEV correlates with improved patient outcome (52, 53) and the control of tumor growth (33, 54, 55). L-selectin enhanced T cells might be reasonably hypothesized to have an even greater effect where PNAd-expressing tumor vasculature develop, such as in response to immunotherapies (33, 54, 55). The fact that L-selectin is both shed and gene-silenced in the course of normal T cell activation poses potential challenges for translation of this approach. However, the advent of 2nd generation CAR-T cell therapies which involve multiple modifications, means that the gain-of-function modification used in these studies could be applied to CAR-T cells to broaden their clinical scope. However, monitoring

endogenous L-selectin expression on TILs or CAR-T cells may be of immediate clinical benefit in the selection of T cell subsets for administration to cancer patients. The fact that we observed improved control of tumor growth independently of homing, which is associated with alterations in early activation, and works in combination with checkpoint blockade inhibitors makes enhancing L-selectin expression a high-value modification that is likely to improve several aspects of T cell function in the fight against cancer.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All mouse experiments conformed to the British Home Office Regulations [Animal (Scientific Procedures) Act, 1986 (Project Licenses PPL30/3188 and 30/2635 to AA)] and the protocol was approved by the Animal Welfare and Ethical Review Body at Cardiff University.

AUTHOR CONTRIBUTIONS

HAW and AA conceived the study. HAW, RRPD, JO, and MA performed experiments with assistance from RNM, MV, and SGR. CM developed methods for, and supervised, ^{89}Zr production. SJP, MA, and HA performed ^{89}Zr cell labeling, PET/CT imaging, and image analysis. AA supervised the study with critical input from AG. HAW and AA wrote the manuscript with contributions from all authors.

FUNDING

HAW was funded by Wellcome Trust grant 094511/Z/10/Z and Medical Research Council UK grant MR/L008742/1, RRPD by Cancer Research UK grant C42412/A24416, JO and MA by Health and Care Research Wales grants to AA. AG is funded by Cancer Research UK grant C16731/A21200.

ACKNOWLEDGMENTS

We thank Victoria O'Leary for support, Dr. Garry Dolton and Dr. James Matthews for NP68-B16 cell line, Owen Moon for help with the figures, staff in Joint Biological Services at Cardiff University for mouse care and Dr. Vera Knäuper for critical appraisal of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 21 June 2019
doi: 10.3389/fonc.2019.00492

Matrix Hyaluronan-CD44 Interaction Activates MicroRNA and LncRNA Signaling Associated With Chemoresistance, Invasion, and Tumor Progression

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Tumor malignancies involve cancer cell growth, issue invasion, metastasis and often drug resistance. A great deal of effort has been placed on searching for unique molecule(s) overexpressed in cancer cells that correlate(s) with tumor cell-specific behaviors. Hyaluronan (HA), one of the major ECM (extracellular matrix) components have been identified as a physiological ligand for surface CD44 isoforms which are frequently overexpressed in malignant tumor cells during cancer progression. The binding interaction between HA and CD44 isoforms often stimulates aberrant cellular signaling processes and appears to be responsible for the induction of multiple oncogenic events required for cancer-specific phenotypes and behaviors. In recent years, both microRNAs (miRNAs) (with ∼20–25 nucleotides) and long non-coding RNAs (lncRNAs) (with ∼200 nucleotides) have been found to be abnormally expressed in cancer cells and actively participate in numerous oncogenic signaling events needed for tumor cell-specific functions. In this review, I plan to place a special emphasis on HA/CD44-induced signaling pathways and the presence of several novel miRNAs (e.g., miR-10b/miR-302/miR-21) and lncRNAs (e.g., UCA1) together with their target functions (e.g., tumor cell migration, invasion, and chemoresistance) during cancer development and progression. I believe that important information can be obtained from these studies on HA/CD44-activated miRNAs and lncRNA that may be very valuable for the future development of innovative therapeutic drugs for the treatment of matrix HA/CD44-mediated cancers.

Keywords: hyaluronan (HA), CD44, miRNAs, LncRNA UCA1, chemoresistance, invasion, tumor progression

OPEN ACCESS

Edited by:
Toshiyuki Murai, Osaka University, Japan

Reviewed by:
Thomas N. Wight, Benaroya Research Institute, United States
Paul Laszlo Bollyky, Stanford University, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *14 March 2019*
Accepted: *24 May 2019*
Published: *21 June 2019*

Citation:
Bourguignon LYW (2019) Matrix Hyaluronan-CD44 Interaction Activates MicroRNA and LncRNA Signaling Associated With Chemoresistance, Invasion, and Tumor Progression. Front. Oncol. 9:492. doi: 10.3389/fonc.2019.00492

INTRODUCTION

Cancer cells are known to display dysregulated signaling pathways which are responsible for abnormal cellular functions (1–3). Myriad studies have attempted to understand the cellular and molecular mechanisms involved in the onset of tumor cell-specific behaviors (e.g., tumor cell migration, invasion, survival, and chemoresistance). Interactions between matrix hyaluronan (HA), the major glycosaminoglycan component of extracellular matrix (ECM), and variant

isoforms of CD44 (HA receptor) have been shown to be tightly linked to the development of aberrant signaling events in a variety of cancers (4, 5, 5–30). It is known that HA binding to certain isoforms of CD44 selectively activates multiple oncogenic signaling pathways leading to tumor cell-specific phenotypes (4, 5, 5–30). HA is also present in different sizes (e.g., large vs. small sizes) (4, 5, 5–28). The binding interaction between large size HA-CD44 and small size HA-CD44 may cause selective activation of downstream effector functions in cancer stem cells (31–35). Furthermore, recent studies indicate that HA-CD44 interaction stimulates the expression of specific microRNAs (miRNAs) and coordinates downstream, intracellular signaling pathways that influence multiple tumor cell-specific functions (31–35). This review focuses first on matrix HA interaction with CD44 in regulating cancer cell signaling pathways, and then describes downstream target functions of these signaling events that contribute to tumor initiation, migration, invasion, chemoresistance, and tumor progression. We believe that this new information could establish the ground work for developing novel therapeutic agents that would effectively target HA/CD44-activated signaling events and specific downstream target molecules/functions in tumor cells-thus providing important new cancer therapies.

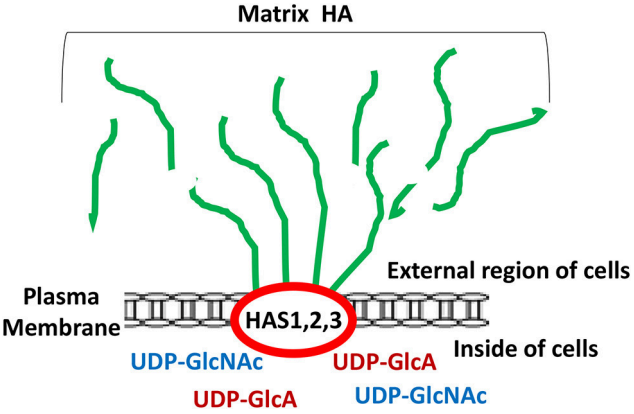

FIGURE 1 | Illustration of matrix hyaluronan (HA) structure regulated by HA synthases (HAS1, HAS2, and HAS3). Green lines represent matrix HA which is made by HAS1, HAS2, and HAS3 followed by being secreted into the external region of the cells.

MATRIX HYALURONAN (HA) IN CANCERS

It has been well accepted fact that unique oncogenesis-induced migration, invasion and metastasis of tumor cells play key roles in causing morbidity in patients (1–3). Many studies have searched for unique molecules which are frequently expressed by cancer cells which correlate with tumor-specific properties. Matrix hyaluronan (HA) known to consist of both D-glucuronic acid and N-acetyl-D-glucosamine in a form of repeating disaccharide units in the extracellular matrix (ECM) (4–7) has been recognized as one of the important contributors in causing tumor development and progression (5, 8–11). It is well-documented that HA is first made by two precursor molecules, uridine diphosphate-glucuronic acid (UDP-GlcA) and uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) through the regulation of HAS1, HAS2, and HAS3 (also known as HA synthase enzymes) inside of the cells and then becomes secreted into the external environment (outside of the cells) as one of the major ECM components in both normal and malignant cells (**Figure 1**). Generation of large sizes of HA polymers (>2–3 $\times$ 10^6 Daltons) often requires HAS1 and HAS2, whereas the production of smaller-size of HA (<1–2 $\times$ 10^5 Daltons) appears to rely on HAS3 (8–10). A few oncogenic signaling events have been shown to be involved in the unusual activities of HAS1, HAS2, and HAS3 and cause aberrant synthesis and production of HA which then promotes changes of cellular functions and onset of malignant transformation and cancer development (8–10). Large sizes of HA often can be degraded into many biologically active mid-sized and/or small-sized fragments by hyaluronidases such as Hyal-1, Hyal-2, or PH20/Spam1 (11). Most importantly, the level of HA appears to be elevated at the contact region between cancer cells and extracellular matrix (ECM) which may be responsible for the induction of cancer cell-associated properties (5). Thus, over production of HA may be used as a predictor of cancer development.

It has been well documented that HA promotes a variety of oncogenic signaling pathways and causes abnormal physiological changes in cancer cells. For example, HA activates PI3K-AKT signaling pathway which is known to be responsible for tumor cell proliferation, glucose metabolism, cytokine production, angiogenesis and survival (36, 37). Overproduction of HA often induces certain metabolic changes such as accelerating the hexosamine biosynthetic pathway and glycolysis process in breast cancer cells (38). There is also growing evidence that treatment of cancer cells with HA upregulates the expression of the multidrug transporter, *MDR1* (P-glycoprotein), and ABC drug transporters (ABCB3, ABCC1, ABCC2, and ABCC3) leading to aberrant drug fluxes and chemoresistance in breast and ovarian cancer cells (39, 40). Most importantly, HA activates cytoskeleton regulators such as RhoGTPases (e.g., Rho, Rac, and Cdc42) which are known to regulate tumor cell migration, and invasion (41). Additionally, HA is capable of upregulating Rho-kinase activities which in turn stimulates 1,4,5-triphosphate (IP3)-mediated Ca2+ fluxes and endothelial cell migration-a required step for angiogenesis (42, 43). Moreover, certain sizes of low molecular weight hyaluronan appears to induce angiogenesis involving Cdc42 signaling (44). Thus, these findings suggest that abnormal HA-mediated signaling processes may play a critical role in regulating tumor cell-specific properties. To further dissect the cellular and molecular mechanisms involved in HA-mediated oncogenesis, we decided to focus on the interaction between HA and its binding receptor, CD44, in a variety of cancer cells as described below.

CD44 IN CANCERS

HA binding receptor, CD44 is a transmembrane glycoprotein and has been detected in both normal and tumor cells (12–16).

Importantly, upregulation of CD44 is often closely associated with abnormal tumor cell behaviors (e.g., proliferation, survival, migration/invasion, and chemoresistance) (13–15). Based on the results from nucleotide sequence analyses, CD44 appears to be encoded by a single gene with 19 exons and exhibits in many different isoforms (16, 17). For example, CD44s (so-called CD44 standard form), contains exons 1–5 at the N-terminal region (with HA binding sites), exons 15–16 at the membrane proximal area and exon 17 at the transmembrane region, as well as exons 18–19 at C-terminal region (with signaling regulation capacity) (**Figure 2**). CD44 is also known to undergo alternative spicing processes (16, 17). Potentially, the alternative splicing events can occur at 12 exons (out of the 19 exons). Frequently, it has been observed that different exons become inserted at the external region near the membrane proximal domain (between exon 6-14 or v1-v10) of CD44 (16, 17) (**Figure 2**). For example, exons 12 (v8), 13 (v9), and 14 (v10) are inserted into the CD44s transcripts in epithelial cells (18, 19). Additional exon 7-14 (v3-v10) and exon 14 (v10) have been found to be inserted into the CD44s transcript in keratinocytes and endothelial cells, respectively (20, 21) and these isoforms have been designated as CD44v10 and CD44v3-10 (20, 21) (**Figure 2**). Most of these CD44 variant (CD44v) isoforms share similar HA binding capacity at the N-terminal region of CD44 (exon 1-5) and a transmembrane domain (exon 17) as well as a signaling interactive region at the cytoplasmic site (exon 18–19). The differences of CD44v isoforms appear to occur at the membrane proximal region (exon 6–14) of the CD44 molecules. A variety of unique CD44 isoforms have been detected in cancer cells and tumor samples (18, 22–28). Thus, selective expression of CD44v isoforms may be considered as a useful bio-marker for the detection of a variety of cancers (18, 22–28).

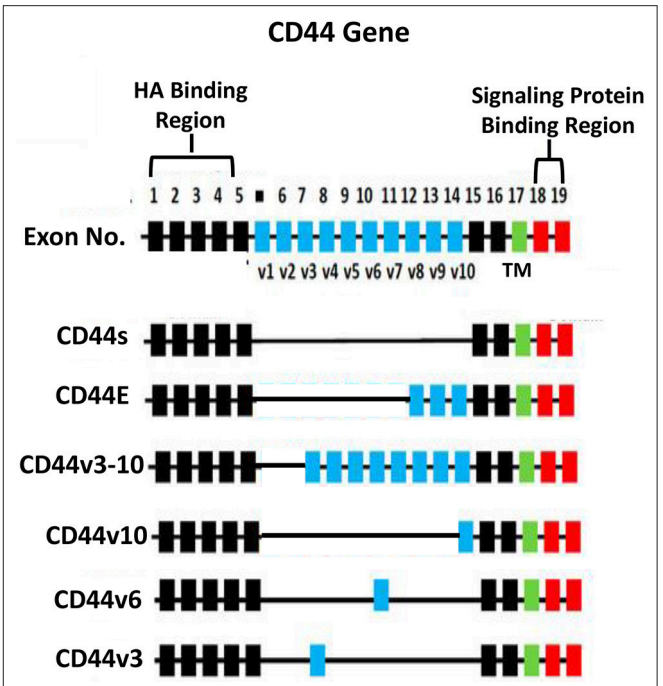

FIGURE 2 | Illustration of CD44 gene, CD44s (the standard form) and alternative spliced variants (CD44E, CD44v3-10, CD44v10, CD44v6, and CD44v3 isoforms). The HA binding domain is located at the external (N-terminal exon 1–5) region of all CD44 isoforms and the signaling protein binding sites are located at the cytoplasmic domain (exon 18–19) of CD44 isoforms. All isoforms contain a transmembrane domain (TM) (exon 17).

CD44 isoforms have also been detected in cancer stem cells (CSCs) which appear to display unique ability to initiate tumor cell-specific properties (29–33). For example, tumor cells with high expression of CD44 (but not cells with low CD44 expression) have been shown to induce the formation of tumors in animals with a small numbers of tumor cell injection (29, 30). In head and neck cancer, tumors also contain a cell subpopulation characterized by a high level of CD44v3 expression (29, 30). Furthermore, injection of cells with a high level of CD44v3 expression into immunodeficient mice has been shown to induce multiple types of phenotypically distinct cells, resulting in heterogeneous tumors (31–33). Thus, CD44 isoforms may be used as an important tumor marker for the detection of CSCs. Most importantly, HA-CD44 interaction stimulates CSC downstream signaling processes leading to cancer cell properties and tumor progression (14, 15, 19, 31–33, 35, 36).

A previous study found that CD44 is frequently located in specialized microdomains in the plasma membrane, so-called lipid rafts of cancer cells (45). The binding of HA to CD44 recruits Na+-H+ exchanger (NHE1) and Hyal-2 into CD44-containing lipid rafts, leading to both intracellular and extracellular acidification, HA modification, cathepsin B activation, and breast tumor cell invasion (i). In endothelial cells CD44v10 also interacts with the membrane-associated cytoskeletal protein, ankyrin and an intracellular calcium channel IP3 receptor in the lipid raft (43). These events result in endothelial cell adhesion and proliferation (43). Another study indicated that HA binding to CD44 promotes recruitment of adaptor and/or linker molecules with CD44 in cancer cells. For example, HA induces CD44v3-Vav2 (a guanine nucleotide exchange factor) and Grb2-p185(HER2) complex formation which then causes the co-activation of both Rac1 and Ras signaling leading to the concomitant onset of tumor cell growth and migration required for ovarian tumor progression (46). In addition, HA induces CD44 interaction with a RhoA-specific guanine nucleotide exchange factor (leukemia-associated RhoGEF (LARG) in human head and neck squamous carcinoma cells (47). This event results in Rho/Ras co-activation leading to PLC epsilon-Ca2+ signaling, and Raf/ERK up-regulation required for CaMKII-mediated cytoskeleton function in head and neck squamous cell carcinoma progression (47). Moreover, HA stimulates CD44 interaction with the transforming growth factor beta (TGF-beta) receptors (a family of serine/threonine kinase membrane receptors) in human metastatic breast tumor cells (MDA-MB-231 cell line). This interaction promotes activation of multiple signaling pathways leading to membrane-cytoskeleton interaction, tumor cell migration, and important oncogenic events (e.g., Smad2/Smad3 phosphor and PTH-rP production) during HA and TGF-beta-mediated metastatic breast tumor progression (48). Additionally, it has been observed that HA induces CD44 interaction with RHAMM (receptor of

HA-mediated motility) and causes cell motility, increased wound healing, and modification of signal transduction of the Ras signaling cascade (49–51). Furthermore, there is a report showing low molecular weight HA induces CD44 interaction with toll-like receptors. This signaling event then promotes the actin filament-associated protein 110-actin binding and MyD88-NFκB signaling resulting in proinflammatory cytokine/chemokine production and breast tumor invasion (52). Together these findings strongly suggest that the interaction between HA/CD44 and a variety of different membrane proteins and/or regulatory molecules plays a pivotal role in regulating solid tumor cancer progression.

HA-CD44 INTERACTION IN PROMOTING MICRORNA SIGNALING AND TUMOR PROGRESSION

A class of 21–25 nucleotide length small RNAs, so called microRNAs (or miRNAs) have been shown to be involved in gene regulation (53). Overall, the impact of miRNA-regulated gene expression appears to be significant since specific miRNAs may influence the downstream effector gene expression and functions (53). For example, at least four miRNA clusters, such as let-7a-d, let-7i, miR-15b-16-2, and miR-106b-25, have been identified as being involved in G1-S transition (54) during cell cycle progression and tumor progression (55, 56). Moreover, dysregulation of certain miRNAs appears to be associated with a variety of cancers (57, 58). For example, miR-21 was first discovered as an oncomiRNA due to its universal overexpression in a variety of cancers (57, 58). Aberrant biosynthetic process of miRNAs (e.g., miR-21) has also been shown to be involved in the production of oncomiRNAs (58, 59). In addition, miRNA genes are frequently subjected to epigenetic changes in cancer leading to tumor progression (56). Furthermore, using a systematic miRNA inhibitor treatment technique on cancer cells, Ma et al discovered miR-10b overexpression which is required for tumor migration and invasion in metastatic breast cancer cells (60). Interestingly, unique miRNA such as miR-302 appears to play a key role in the maintenance of stemness properties in normal stem cells and in cancer stem cells (61). Aside from the abnormal biosynthetic processes and epigenetic modifications of miRNA genes, it has become apparent recently that tight interactions between certain miRNAs and transcription factor-mediated regulatory circuits may also influence important biological outcomes and drives cellular transformation (57, 58). Nevertheless, the abnormal signaling pathways responsible for the onset of oncogenic miRNAs during cancer development and progression remains poorly understood. In this review article, I plan to focus on several HA-CD44 interaction-induced oncogenic signaling pathways that regulate several miRNAs and downstream effector functions in a variety of cancer cells during tumor progression.

Regulation of miR-21 Signaling by HA-CD44 Interaction in Cancers

Upregulation of miR-21 has been detected in tumors and to a lesser extent in normal tissues (58, 59). In recent years, miR-21 has received a great of attention due to the discovery of its specific targets and functional involvement in cancer progression (62, 63). For example, the gene expression of program cell death (PDCD4, a tumor suppressor protein) can be blocked by miR-21 (62, 63). This miR-21-medited downregulation of PDCD4 results in tumor progression (62–64). Therefore, miR-21 can be viewed as a cancer cell activator. During HA/CD44 signaling, miR-21 has also been suggested to regulate tumor cell proliferation, invasion, survival, chemoresistance and tumor progression (19, 62–67). The oncogenic signaling pathways involved in the regulation of miR-21 and its function by HA-CD44 interaction in solid tumor cancers are described below:

The Expression of miR-21 and Nanog-DROSHA-p68 Signaling

Several reports showed that the interaction between RNase III DROSHA/RNA helicase p68 (DROSHA/p68) and other regulatory molecules plays an important role in regulating the biogenesis of miRNAs (68). It has been shown that p53 and the DROSHA form complexes with the RNA helicases (p68/p72) during miRNA production in HCT116 cells (68). It is also documented that TGFβ-mediated SMAD-2 signaling promotes miR-21 expression (69). Specifically, DROSHA-p68 complex promotes the biogenesis of miR-21 by converting pri-miR-21 into pre-miR-21 during TGFβ-specific SMAD signaling events (69). Thus, it is apparent that the production of miR-21 is closely regulated by the DROSHA/p68 microprocessor complex during cellular signaling.

HA/CD44 activated stem cell marker (Nanog) signaling pathways are also involved in regulating miR-21 expression in both breast and head and neck cancer cell lines (19, 65–67). For example, HA binding to CD44 promotes Nanog association with DROSHA/p68 microprocessor complex resulting in the upregulation of miR-21 and downregulation of PDCD4 (a tumor suppressor protein) in cancer cells. Consequently, several inhibitors of apoptosis proteins (IAPs) (e.g., c-IAP-1, cIAP-2, and XIAP) are also upregulated resulting in anti-apoptosis and chemotherapy resistance (**Figure 3A**). The knowledge obtained from this biogenesis study of miR-21 regulated by Nanog-DROSHA-p68 complexes may provide useful foundation for designing new drug target to downregulate miR-21 and increase tumor cell death and enhance chemosensitivity for the treatment of HA/CD44-mediated cancer.

The Expression of miR-21 and Nanog-Stat-3 Signaling

Abnormal Stat-3 signaling are well-known to play important roles in oncogenesis (70). Constitutively activated Stat-3 has been closely associated with human malignancies (46). It has been shown that Nanog and Stat-3 are functionally coupled in many cancer cells (39) (**Figure 3A**). For example, HA binding to CD44 induces a physical association between Nanog and Stat-3 in head and neck cancer cells leading to miRNA-21 gene expression and production (66). Most importantly, treatments of cancer cells with several signaling perturbation agents such as Nanog siRNA or Stat-3siRNA or an anti-miR-21 inhibitor

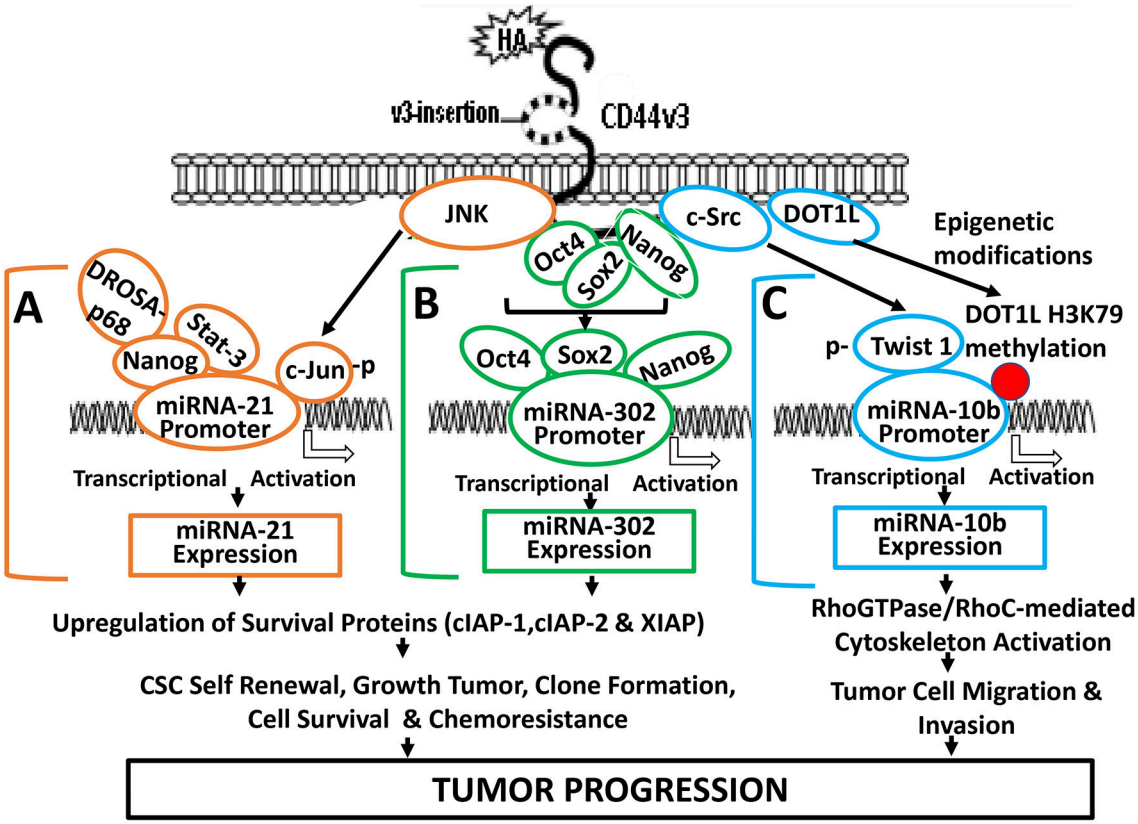

FIGURE 3 | A proposed model for HA-CD44-mediated signaling activation in the regulation of miRNA-21, miR-302, and miR-10b production and oncogenesis in tumor cells. **(A)** The binding of HA to CD44 promotes JNK activity which, in turn, causes phosphorylation of c-Jun. Phosphorylated c-Jun then binds to the miR-21 promoter and induces miR-21 expression. HA binding to CD44 also causes Nanog interaction with Stat-3 and the microprocessor complex containing the RNase III (DROSHA) and the RNA helicase (p68). These Nanog-associated signaling complexes (containing Stat-3 and/or DROSHA and p68) then bind to miR-21 promoter region, resulting in miR-21 production leading to upregulation of IAP protein expression, tumor cell survival, and chemoresistance. **(B)** HA-CD44 interaction promotes miRNA-302 expression and chemoresistance: HA binding to CD44 promotes an association between CD44v3 and OCT4/SOX2/Nanog. Subsequently, OCT4/SOX2/Nanog complexes interact with the promoter region (containing OCT4-, SOX2-, and Nanog-binding sites) of the miR-302 cluster resulting in miR-302 cluster gene expression and mature miR-302 production. The resultant miR-302 then functions to induce IAP (cIAP-1, cIAP-2, and XIAP) expression, tumor cell growth, self-renewal, clone formation, tumor cell survival, and chemoresistance in tumor cells. **(C)** HA-CD44 interaction promotes miRNA-10b expression and tumor migration/invasion: HA binding to CD44 promotes c-Src phosphorylation (kinase activation), which, in turn, causes phosphorylation of Twist. Phosphorylated Twist then interacts with the E-box elements of mR-10b promoter, resulting in miR-10b gene expression, and mature miR-10b production. The binding of HA to CD44 also enhances DOT1L upregulation and DOT1L/H3K79 methylation-mediated epigenetic changes, resulting in methyl-H3K79 binding to miR-10b promoter, and miR-10b gene expression/production. The expressed miR-10b then promotes upregulation of RhoGTPase-mediated cytoskeleton activation leading to tumor cell migration and invasion. Red dot represents DOT1L/H3K79-mediated histone modifications (via epigenetic changes).

result in downregulation of survival proteins (e.g., cIAP-1, cIAP-2, and XIAP) and upregulation of PDCD4 leading to tumor cell apoptosis/death and chemosensitivity in head and neck cancer (19, 65–67). Thus, this newly-discovered Nanog-Stat-3-regulated miR-21 signaling pathways during HA-CD44 interaction may be considered as another new drug target to treat cancers.

The Expression of miR-21 and JNK/c-Jun Signaling

Induction of oncogenic signaling frequently involves abnormal JNK-regulated c-Jun activities (71, 72). The transcription factor, c-Jun belongs to the AP-1 family which has been shown to play an important role in regulating cell transformation (73). Specifically, c-Jun has been shown to regulate the expression of p53 and cyclin D1 (73, 74) and has also been shown to accelerate leukemogenesis by activating cell cycle-related genes in cancer cells (73). JNK-regulated c-Jun often functions as a "bodyguard" which prevents certain gene modification(s) during cancer-related process (73–76). A previous study showed that c-Jun involves transcriptional activation of miR-21 at the miR-21 promoter region located at AP-binding sites (76). HA-CD44 binding has also been shown to cause miR-21 production in a JNK/c-Jun-dependent manner in breast tumor cells (67) (**Figure 3A**). We have found that inhibition of JNK/c-Jun-induced miR-21 signaling by various signaling perturbation agents such as JNK inhibitor or c-Jun siRNA or anti-miR-21 inhibitor effectively downregulates the expression of survival proteins such as Bcl2 and IAP family of proteins leading to apoptosis/cell death and chemosensitivity. These findings strongly suggest that JNK/c-Jun-regulated miR-21 activated by HA-CD44 interaction plays a pivotal role in tumorigenesis and

drug resistance. Consequently, it is possible to design therapeutic drugs to target JNK/c-Jun-regulated miR-21 for the treatment of HA/CD44-mediated cancer.

HA-CD44-Mediated miR-10b Signaling in the Regulation of Tumor Cell Migration and Invasion

Tumor-specific phenotypes (e.g., tumor cell migration, invasion and metastasis) are often regulated by oncogenic signaling processed and/or cytoskeleton functions (1). Overexpression of miRNA-10b has been shown to be closely associated with upregulation of RhoC during glioma invasion and migration (77). In addition, the expression of a zinc finger protein, KLF4 (Kruppel-like factor 4) was found to be regulated by miR-10b in certain cancer cell lines (78). Moreover, it has been reported that miR-10b is responsible for activating both tumor cell invasion and metastasis (79). HA appears to interact with CD44 and induces miR-10b expression in head and neck cancer cells (32, 33). Interestingly, 200 kDa-HA fragments (to a lesser extent 5 kDa, 20 kDa, or 700 kDa) appears to preferentially enhances miR-10b expression in CSCs from head and neck cancer cells (32). The level of miR-10b expression is significantly higher (at least 5–10-fold increase) than other miRNAs (e.g., miR-373, 27b,181-miRNA, miR-34b, and miR-145) detected in 200 kDa-HA-treated head and neck cancer cells (32). Here, several HA/CD44-mediated miR-10b signaling events and functions in various cancers will be described below:

c-Src and Twist Signaling in the Regulation of miR-10b Expression

Src kinase family members (e.g., Lck, Yes, and Fyn) have been shown to participate in CD44-mediated cellular signaling processes (80–82). For example, during T-cell activation Lck is found to be tightly linked to CD44 (80). Both Lck and Fyn have also been shown to be closely complexed with CD44 in a specialized plasma membrane domain enriched in glycosphingolipid in lymphoid cells (81). Moreover, CD44 has been shown to form a tight association with other Src kinase family of proteins (e.g., c-Src, Yes, and Fyn) during abnormal prostate cancer cell proliferation and growing processes (82). These findings strongly support the notion that CD44 and certain c-Src kinases family members are physically linked and functionally coupled.

Twist (one of c-Src substrates) has been shown to promote a variety of tumor cell-specific functions (e.g., EMT transition, invasion and drug resistance) (83–85). Twist is also considered as a putative oncogene for its role in regulating CD44-expressing breast cancer stem cells (CSCs) (86). Several Twist-regulated oncogenic events have been reported to be regulated by the binding of Twist to the promoters (containing the E-boxes) of specific genes (e.g., E-cadherin) required for tumor cell survival and invasiveness (87) as well as transcriptionally repression of E-cadherin gene expression in breast cancer (88). Previous studies showed that c-Src-activated Twist promotes miR-10b expression in breast tumor cells (79, 89). During HA/CD44-mediated signaling process, Twist phosphorylated by c-Src is also able to interact with miR-10 promoter (with E-box domain) and activates the onset of miR-10b gene expression/production and tumor cell-specific activities in cancer cells (89). Treatment of cancer cells with c-Src inhibitor, PP2, or Twist siRNA significantly blocks the production of HA/CD44-mediated miR-10 expression and downstream RhoGTPase (RhoC)-ROK effector functions (89). These observations strongly suggest that HA-CD44 interaction promotes miR-10b expression required for tumor cell-specific functions (e.g., cytoskeleton-associated metastasis, invasion, and metastasis) in a c-Src/Twist-dependent manner.

Role of Epigenetic Modifications in Regulating miR-10b Expression

Epigenetic regulation via histone methylation participates in modifying chromatin organization together with reprogramming gene expression during cancer progression (90). The histone methyltransferase, DOT1 is known to be solely responsible for catalyzing methylation of histone at lysine 79 residues in three different ways such as H3K79me1/H3K79me2/ H3K79me3 in budding yeast, *Saccharomyces cerevisiae* (91, 92). Mammalian DOT1 (so-called DOT1L) has also been documented to display an ability to conduct histone methylation at lysine 79 residues as methyltransferases involved in modifications of gene expression (93). Both histone methyltransferases play an important role in H3K79 methylation involved in transcriptional regulation for the DNA damage checkpoint, meiotic checkpoint and cell cycle progression (94). Abnormal DOT1L-mediated H3K79 methylation has also been detected in mixed lineage leukemia (MLL) (95). In addition, suppression of DOT1L expression causes a reduction of tumor cell growth (96). These findings all indicate that histone methyltransferase (e.g., DOT1L) is closely involved in the development of cancer. DOT1L-mediated methylation of histone H3 at lysine 79 (H3K79) is also involved in the development of embryonic stem (ES) cells (97). Recent studies indicate that the activity of histone methyltransferase (e.g., DOT1L) can be detected in HA-activated head and neck cancer stem cells (CSCs) (89). Specifically, HA promotes DOT1L-regulated H3K79 methylation of miR-10b promoter binding sites leading to miR-10 production resulting in CSC-specific functions in head and neck cancer (**Figure 3C**). Silencing of DOT1L with DOT1LsiRNA and/or miR-10b with antagomirs (an anti-miR-10 inhibitor) significantly decreases the amount of miR-10b production resulting in downregulation of RhoC expression and tumor cell migration/invasion (66, 74). These findings may provide ground work for the development of new therapeutic drugs to target either DOT1L or miR-10 for the treatment of HA/CD44-activated cancer.

Nanog/Oct4/Sox2 Signaling in Regulating miR-302 Expression and Cancer Stem Cell (CSC) Activation and Chemotherapy Resistance

The miR-302 family which encodes a cluster of eight miRNAs has been shown to be important in the "stemness" properties of either normal and abnormal stem cells (98–100). These observations

strongly suggest that there is a close involvement of miR-302 in the regulation of pluripotency of stem cells. The transcription factors such as Nanog, Oct4, and Sox2 often interact with each other during transcriptional events (61, 100, 101). Oct4, Sox2, and Nanog have also been detected to co-occupy the promoter sites of miR-302 for the activation of target genes required for development and oncogenesis (61, 100, 101). In addition, miR-302 family plays key roles in regulating cell proliferation and cell fate determination during differentiation at the post-translational level (61, 100, 101). A previous study showed that HA binding to CD44 promotes the expression of miR-302 in a Nanog/Oct4/Sox2-dependent manner in head and neck cancer stem cells (CSCs) (31) (**Figure 3B**).

Several miR-302 downstream targets such as AOF1 and AOF2 known as lysine-specific histone demethylases have been shown to play a role in demethylating H3K4 and inhibiting transcription of genes (31, 102, 103). Suppression of AOF1 and AOF2 is known to induce DNA (cytosine-5)-methyltransferase 1 (e.g., DNMT1) degradation and global demethylation leading to reprogramming of somatic cells into induced pluripotent stem cells (31). HA-CD44-activated miR-302 has also been shown to cause DNMT1 reduction and DNA demethylation in CD44v3-expressing cancer stem cells (CSCs) (31). Moreover, this DNA demethylation process regulated by HA-CD44-activated miR-302 can activate the expression of several Inhibitor of Apoptosis Protein (IAP) family of proteins such as c-IAP1, c-IAP2, and XIAP which appear to be closely linked to several important activities unique for cancer stem cells (CSCs) isolated from head and neck caner (31) (**Figure 3B**). Most importantly, treatments of CSCs with anti-miR-302 inhibitors readily upregulate lysine-specific histone demethylases and reduces DNA global demethylation as well as impairs HA/CD44-activated CSC functions (79). It is likely that miR-302 signaling pathway regulated by stem cell markers such as Nanog/Oct4/Sox2 during HA-CD44 interaction may be used as a novel therapeutic drug target to downregulate cancer stem cell (CSC) functions and to overcome chemotherapy resistance in cancer cells.

HA-CD44 INTERACTION IN STIMULATING LNCRNA (UCA1) SIGNALING AND TUMOR PROGRESSION

The evolutionarily conserved long non-coding RNAs (so-called lncRNAs >200 nucleotides) are now recognized as a major component of the human transcriptome (104, 105). Most of these molecules remain to be functionally unknown (104, 105). Dysregulation of lncRNAs frequently involves alterations of transcriptional and post-transcriptional activities of gene regulation in many cancers (106–111). For example, downregulation of PTCSC3 was detected in thyroid cancers (112). Malfunction of HULC and XIST is also reported in

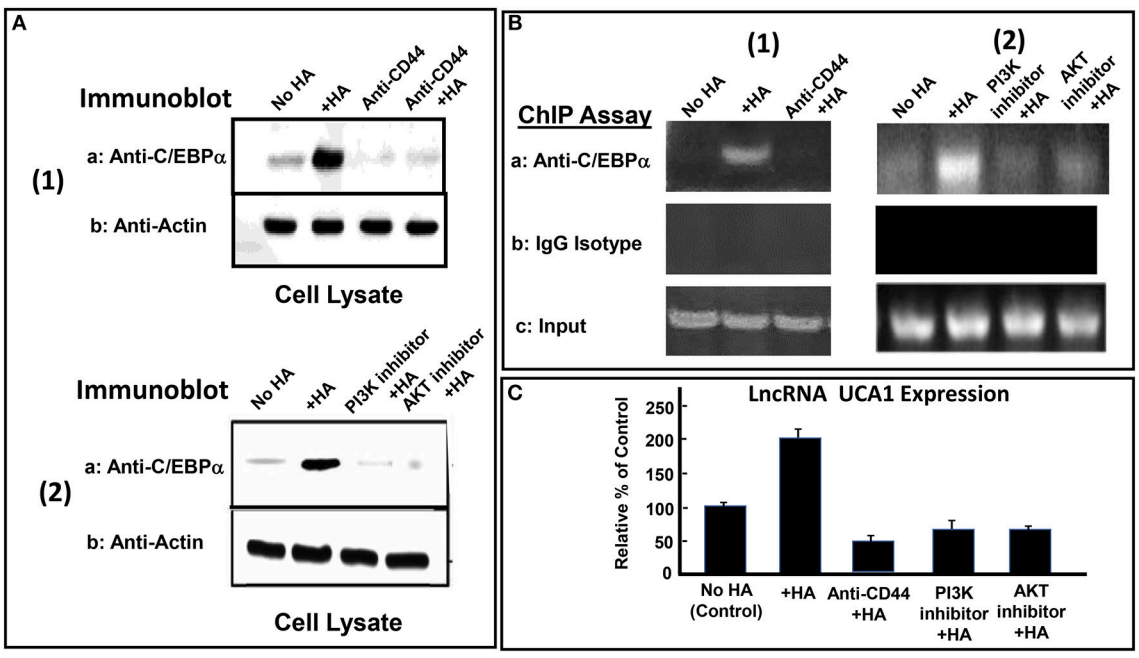

FIGURE 4 | HA-CD44-mediated LncRNA UCA1 expression in tumor cells. **(A-1)** Detection of C/EBPα phosphorylation in CD44v3high HSC-3 cells treated with no HA, (lane 1); or with HA (lane 2); or with anti-CD44 antibody with no HA (lane 3); or with anti-CD44 antibody + HA (lane 4). **(A-2)** Detection of C/EBPα phosphorylation in CD44v3high HSC-3 cells treated with no HA, (lane 1); or with HA (lane 2); or with PI3K inhibitor (GDC-0941) plus HA, (lane 3); or with AKT inhibitor (GSK795) plus HA (lane 4). **(B)** ChIP assay of p-C/EBPα binding to lncRNA UCA1 promoter in CD44v3high HSC-3 cells treated with no HA (lane 1) or with HA (lane 2) or with PI3K inhibitor (GDC-0941) plus HA (lane 3) or with AKT inhibitor (GSK795) plus HA (lane 4) using anti-p-C/EBPα or IgG control. Co-immunoprecipitated DNA was amplified by PCR with primers specific for the lncRNA *UCA1* promoter. **(C)** The expression of lncRNA *UCA1* by qRT-PCR in CD44v3high head and neck cancer cells (HSC-3 cells) treated with no HA (bar 1) or with HA (bar 2) or with anti-CD44 antibody plus HA (lane 3) or with PI3K inhibitor (GDC-0941) plus HA (bar 4) or with AKT inhibitor (GSK795) plus HA (bar 5) using lncRNA *UCA1*-specific primers and Q-PCR assay.

various cancers (113–118). Furthermore, both GAPLINC and MALAT1 have been used as unfavorable predictors for a few solid tumor cancers (119–123). Overexpression of HOTAIR is linked to metastasis in colorectal, liver, pancreatic, breast and gastric cancers (124–131) whereas ANRIL and PRNCR1 upregulation is detected in prostate cancer (132, 133). High levels of KCNQ1OT1 and H19 expression were also detected in colorectal cancer (132) and hepatocellular carcinoma (133), respectively. Therefore, aberrant expression of certain lncRNAs appears to be closely linked to various tumor progression.

Another important member of lncRNA family, urothelial carcinoma associated 1 (lncRNA UCA1) has been shown to be correlated with tumor growth, progression and recurrence (108–111). Several studies focusing on the transcriptional regulation of lncRNA UCA1 show that many transcription factors (e.g., C/EBPα, Ets-2, TAZ/YAP/TEAD, HIF-1α, SATB1, CAPERα/TBX3, etc.) may participate in the regulation of lncRNA UCA1 by binding to the promoter sites of lncRNA UCA1 (134–139). A specific example for the regulation of lncRNA UCA1 expression by certain transcription factor during HA-CD44 interaction in head and neck cancer cells is described as follows:

Role of C/EBPα in Regulating HA-CD44-mediated lncRNA UCA1 Expression

Many transcription factors have been shown to be involved in the regulation of lncRNA UCA1 expression (135). For example, the interaction between the transcription factor, C/EBPα and the promoter of lncRNA UCA1 often promotes an upregulation of lncRNA UCA1 production leading to anti-cell death and cell survival (135). Recent study indicates that a cross-talk between PI3K-AKT pathway and lncRNA UCA1 expression also occurs during breast cancer cell invasion (140). We have found that HA induces C/EBPα phosphorylation in CD44v3high head and neck cancer cells (HSC-3 cells) in a CD44-dependent manner (**Figure 4A-1**). Downregulation of AKT by treating CD44v3high HSC-3 cells with AKT inhibitor (GSK795) or PI3K inhibitor (GDC-0941) blocks HA-mediated C/EBPα phosphorylation (**Figure 4A-2**). These findings suggest that C/EBPα phosphorylation is PI3K-AKT signaling-dependent in HA-treated CD44v3high head and neck cancer cells (HSC-3 cells) (**Figures 4A-1, 2**). To examine whether phosphorylated C/EBPα (induced by HA-mediated CD44v3-mediated PI3K-AKT activation) directly interacts with the promoter region of lncRNA UCA1, chromatin immunoprecipitation (ChIP) assays were performed in head and neck cancer cells with HA (or without HA). Preliminary data indicate that phosphorylated C/EBPa is directly recruited into the promoter region of lncRNA UCA1 in HA-treated CD44v3high head and neck cancer cells, resulting in lncRNA UCA1 expression (**Figure 4B–1**). However, HA-mediated recruitment of phosphorylated C/EBPa into LncRNA UCA1 promoter sites appears to be blocked in cells treated with anti-CD44 antibody (**Figure 4B-1**). Consequently, lncRNA UCA1 expression is also inhibited (**Figure 4C**). Downregulation of PI3K or AKT by treating cells with either PI3K inhibitor (GDC-0941) or AKT inhibitor (GSK795) effectively inhibits the complex formation between phospho-C/EBPa and the promoter region of lncRNA UCA1 in HA-treated CD44v3high head and neck cancer cells, as well as lncRNA UCA1 production (**Figure 4B-2** and **Figure 4C**). These findings suggest that the binding of phosphorylated C/EBPa to the lncRNA UCA1 promoter and lncRNA UCA1 expression is CD44/PI3/AKT-dependent and GDC-0941/GSK795-sensitive in HA-treated CD44v3high head and neck cancer cells. Therefore, we believe that the regulation of lncRNA UCA1 expression in head and neck cancer cells is HA-dependent and CD44-specific.

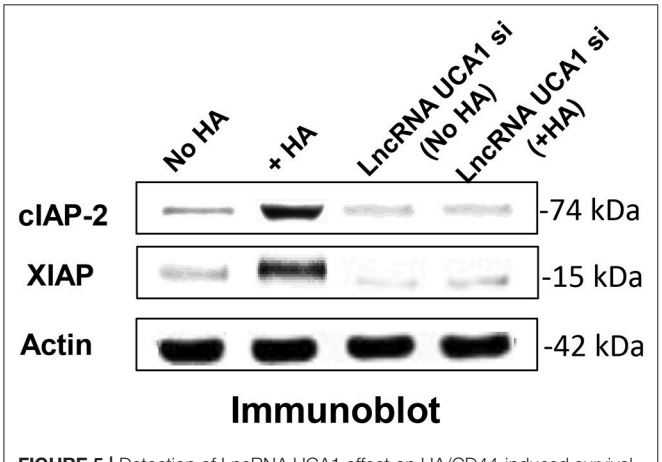

FIGURE 5 | Detection of LncRNA UCA1 effect on HA/CD44-induced survival protein expression in cancer cells (HSC-3 cells) with no HA or with HA or treated with lncRNA UCA1 si with no HA or with HA.

Role of lncRNA UCA1 in Regulating Tumor Cell Survival and Chemoresistance

Several regulatory small and long non-coding RNAs have been well-documented in many cancers resistant to therapeutic drug (e.g., cisplatin) treatment (141–144). The ability of cisplatin to induce tumor cell death is often counteracted by the presence of anti-apoptotic regulators and/or survival proteins leading to chemoresistance (142–144). The IAP family (e.g., cIAP-2 and XIAP) is well-documented to play critical roles in promoting tumorigenesis through the action of both anti-apoptosis and anti-cell death (145). These proteins also

TABLE 1 | Chemosensitivity assay treatment.

Chemosensitivity assay treatment	Growth inhibition IC50 (μM)*
No HA (Control)	1.00 ± 0.05
+HA	2.69 ± 0.11
LncRNA UCA1si-treated cells (+HA)	0.50 ± 0.07
cIAP-2 siRNA-treated cells (+HA)	0.42 ± 0.04
XIAP siRNA-treated cells (+HA)	0.45 ± 0.02

** The procedures for measuring cisplatin-induced tumor cell growth inhibition (IC 50) is the same as described previously (31–33).*

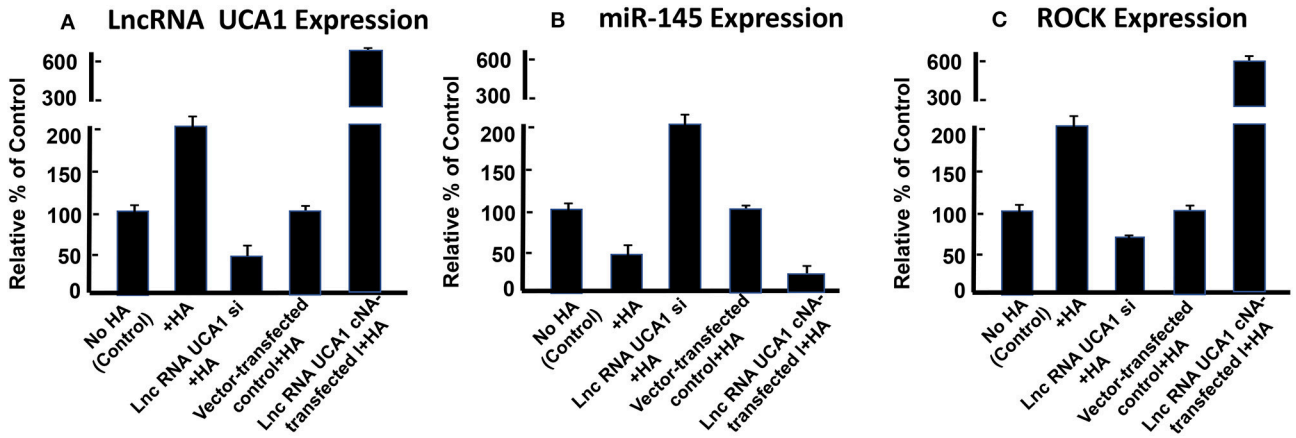

FIGURE 6 | Detection of HA/CD44-mediated expression of lncRNA UCA1 **(A)**, miR-145 **(B),** and ROCK **(C)** by qRT-PCR in CD44v3high head and neck cells (HSC-3 cells) treated with no HA (bar 1) or with HA (bar 2) or transfected with si lncRNA UCA1 plus HA (bar 3) or transfected with pcDNA 3.1 plus HA (bar 4) or transfected with pcDNA 3.1-lncRNA UCA1 plus HA (bar 5).

participate in chemoresistance by reducing tumor cell death or apoptosis caused by chemotherapeutic drugs (146). Although several other survival proteins such as Bcl2 and BclxL are known to play roles in regulating tumor cell survival and chemoresistance in cancer cells during HA-CD44 binding (147), the involvement of cIAP-1, cIAP-2, and XIAP in promoting HA/CD44-mediated tumor cell survival and drug resistance has only recently received some attentions.

LncRNA UCA1 has been reported to induce drug resistance in bladder cancer and many other cancers (108, 109, 148–163), thereby greatly reducing the efficacy of cancer therapy. Recently, we found that the expression of both cIAP-2 and XIAP appear to be downregulated in head and neck cancer cells treated with anti-lncRNA UCA1 inhibitor (**Figure 5**). The suppression of these survival proteins leads to tumor cell death and effective chemotherapeutic drug treatment (**Table 1**). The fact that reduction of anti-apoptosis proteins (cIAP-2 or XIAP) by treating head and neck cancer cells with specific inhibitory siRNAs (e.g., cIAP-2 siRNA or XIAP siRNA) during HA/CD44 interaction appears to increase chemosensitivity suggests that downregulation of lncRNA UCA1 together with blockage of survival protein pathways may provide a new therapeutic strategy in cancer therapy, especially dealing with drug resistance.

The Role of lncRNA UCA1 in Regulating miR-145-ROCK1 Pathway and Tumor Cell Migration and Invasion

During the regulation of miRNA expression, lncRNA can compete the common response elements of miRNAs (161). LncRNAs can also bind DNAs, RNAs and proteins by acting as decoy, guide or scaffold (161). LncRNA has been reported to bind to many different miRNAs including miR-145 in a variety of cancer cells resulting in carcinogenesis, tumor cell migration, invasion or drug resistance (152, 153, 162–167, 167–169). Recent observations indicate that lncRNA UCA1 promotes migration and invasion in bladder cancer cells (164). Accumulating evidence indicates that miR-145 known as a tumor suppressor is frequently downregulated in various cancers (170). It has been postulated that a signaling pathway may be involved in the formation of a regulatory loop between lncRNA UCA1 and miR-145 via a reciprocal repression process required for tumor cell-specific activities (e.g., migration and/or invasion) (164). There is evidence that upregulation of miR-145 impairs cancer cell motility by downregulating the expression of its target genes including ROCK (a Rho-associated protein kinase), a key regulator of actin cytoskeleton reorganization required for cancer cell migration and invasion (164, 171). Thus, both up- or down-regulation of miR-145 appear to play important roles in cancer cell-related activities (e.g., cell migration and invasion).

Furthermore, it has been reported that lncRNA UCA1 suppresses the tumor suppressor miR-145 for tumor cell invasion/migration through the expression of miR-145 target proteins such as ROCK1 in glioma cancer cells (109). These findings are consistent with our observations showing that HA-CD44v3 interaction stimulates lncRNA UCA1 expression in CD44v3high tumor cells (**Figure 6A**). Moreover, upregulation of lncRNA UCA1 (by transfecting cells with lncRNA UCA1 cDNA, but not vector control cDNA) significantly suppresses miR-145 expression (**Figure 6B**) leading to an increase of miR-145 target gene, ROCK1 expression (**Figure 6C**). Conversely, when CD44v3high head and neck cancer cells (HSC-3 cells) were transfected with lncRNA UCA RNAi inhibitor, the expression level of miR-145 is significantly up-regulated (**Figure 6B**). Consequently, ROCK1 (a miR-145 target) gene/protein is downregulated (**Figure 6C**). These findings suggest that the miR-145-ROCK1 pathway serves as a possible downstream functional target for lncRNA UCA1 in CD44v3high head and neck cancer cells. Furthermore, our recent data show that upregulation of lncRNA UCA1 by HA-CD44v3 binding in CD44v3high head

TABLE 2 | Cell migration and invasion treatments.

Cell migration and invasion treatments	Tumor cell migration (% of control)	Tumor cell invasion (% of control)
No HA (Control)	100 ± 2	100 ± 5
+HA	223 ± 12	245 ± 10
LncRNA UCA1 si-treated (+HA)	65 ± 3	63 ± 2
miR-145 mimic-treated cells (+HA)	65 ± 2	62 ± 2
ROCK inhibitor (Y-27632)-treated cells (+HA)	60 ± 2	66 ± 3

The measurements of in vitro tumor cell migration and invasion were performed using twenty-four transwell units as described previously (66, 131, 154).

and neck cancer cells significantly enhances ROCK-mediated head and neck cancer cell migration and invasion (**Table 2**). Treatment of cells with lncRNA UCA1 RNAi inhibitor or miR-145 mimic or a ROCK inhibitor, Y-27632 significantly reduces tumor cell invasion (**Table 2**). These observations suggest that the miR-145-ROCK1 pathway serves as a new downstream functional target for lncRNA UCA1 in regulating CD44v3high head and neck cancer cell activities including cell migration and invasion. The results of these studies strongly indicate that lncRNA LncRNA UCA1 is one of the important regulatory molecules in controlling miR-145-ROCK pathway and tumor cell migration and invasion.

In summary, we would like to propose that HA binding CD44 stimulates PI3K and AKT signaling which in turn causes c/EBPα phosphorylation. Phosphorylated c/EBPα then binds to the site of LncRNA UCA1 promoter and induces transcription activity for the expression of LncRNA UCA1 which then upregulates survival proteins (IAPs) and chemoresistance. HA/CD44-activated lncRNA UCA1 also downregulates miR-145 expression and stimulates ROCK expression required for cytoskeletal activation and tumor cell motility (e.g., migration and invasion) (**Figure 7**). Therefore, it is feasible to use either LncRNA UCA1 si treatment and/or ROCK inhibitor to limit tumor cell migration and invasion (**Table 2**) and to reduce HA/CD44-induced tumor metastasis and progression.

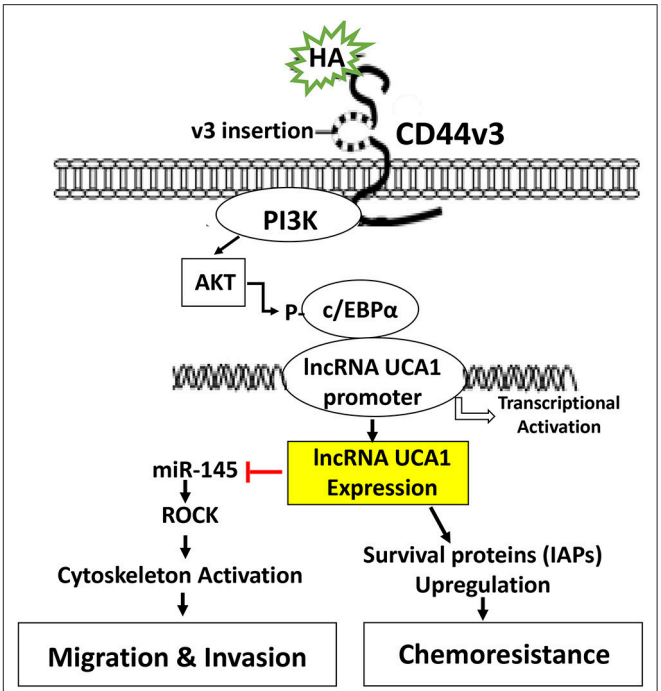

FIGURE 7 | A proposed model for HA-CD44-mediated signaling activation in the regulation of LncRNA UCA1 production and oncogenesis in tumor cells. The binding of HA to CD44 stimulates PI3K and AKT signaling which in turn causes c/EBPα phosphorylation. Phosphorylated c/EBPα then binds to the promoter of LncRNA UCA1 and induces transcriptional activation for LncRNA UCA1 expression. The production of LncRNA UCA1 upregulates survival proteins (IAPs) and chemoresistance. LncRNA UCA1 also downregulatemiR-145 expression and stimulates ROCK expression and cytoskeletal activation leading to tumor cell migration and invasion.

CONCLUSION

The newly discovered signaling events regulated by HA-CD44 interaction may be very useful for a better understanding of cancer cell-specific behaviors including transcriptional activation, tumor cell growth, inflammatory cytokine/chemokine production, migration/invasion and survival as well as chemoresistance as summarized in **Figure 8**. Consequently, targeting CD44 using anti-CD44 and/or CD44 variant-specific antibody and/or anti-sense strategies to downregulate CD44 and/or CD44 variants may be a possible choice for the development of new cancer cell-based therapies. Furthermore, HA-based nanoparticles containing therapeutic drugs (e.g., cisplatin or doxorubicin) may be used to accurately deliver therapeutic drugs into CD44v isoform-expressing cancer cells to enhance chemo-sensitivity and downregulate CD44v isoform-mediated oncogenic signaling. It is also feasible to design signaling perturbation strategies to downregulate the expression of HA/CD44-regulated Nanog/Oct4/Sox2 and c-Jun as well as certain miRNAs (e.g., miR-21, miR-10b, and miR-302) using specific inhibitors such as siRNA and shRNA and anti-miRNA inhibitor approaches to simultaneously suppress both oncogenic behaviors and cancer progression. Since many LncRNAs have been shown to be closely associated with tumor cell-specific properties including cell survival, chemoresistance, tumor cell migration and invasion, it will also be possible to develop novel signaling perturbation techniques to simultaneously inhibit both oncogenic miRNAs and LncRNA UCA1 using miRNA-21/miRNA-10b/miR-302 RNAi inhibitor and/or lncRNA UCA1 RNAi inhibitor treatments. These strategies could synergistically cause apoptotic responses and chemosensitivity. These new approaches could indicate that the impairment of specific signaling pathways together with suppression of miRNAs (miR-21/miR-10b/miR-302) and/or lncRNA UCA1 in HA-CD44-activated cancer cells may be more effective than chemotherapy alone. Novel therapeutic strategies described in this review may offer helpful information for understanding the initiation and development mechanisms of different cancers

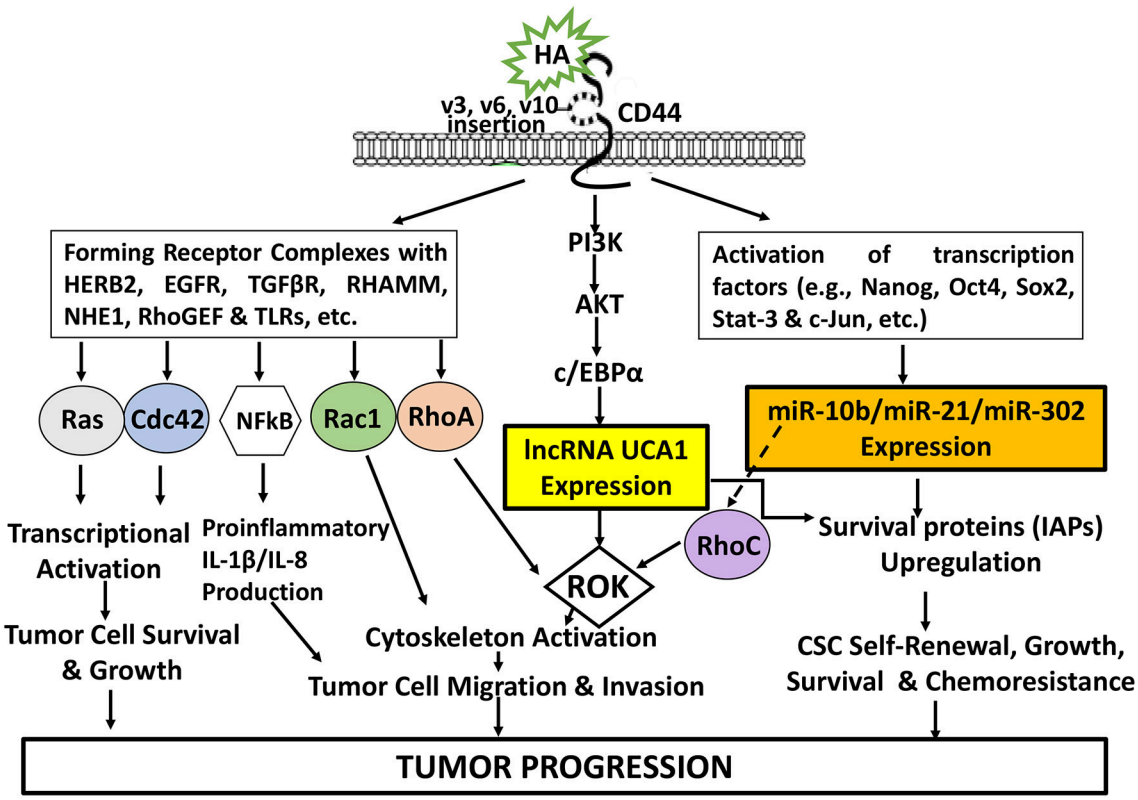

FIGURE 8 | An Illustration of HA-CD44 interaction-induced oncogenic signaling events in cancer. The binding of HA (large vs. small HA) to CD44v isoforms interaction stimulates CD44-other receptor (HERB2, EGFR, TGFβR, RHAMM, NHE1, RhoGEF, and TLRs, etc.) complex formation and Ras/Cdc42/Rac1/RhoA/NFkB activation for transcriptional activation and proinflammatory cytokine/chemokine production as well as tumor cell survival, growth, invasion and migration. HA-CD44 interaction also promotes PI3K/AKT activation and LncRNA (UCA1) production resulting in ROK-mediated cytoskeleton function required for tumor cell migration and invasion. Moreover, HA/CD44 activates transcriptional factor-induced miRNA (miR-10b/miRNA-21/miR-302) expression leading to CSC self-renewal, growth, survival and chemoresistance. Furthermore, the induction of miR-21 by HA-CD44 interaction also stimulates RhoC upregulation and ROK-regulated tumor cell migration and invasion. All these events contribute to HA-CD44 interaction-mediated tumor progression.

comprehensively and suggest new therapeutic targets for clinical treatment of HA/CD44-activated cancer development and tumor progression.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

We would like to thank both Dr. Gerard J. Bourguignon and Ms. Christine Earle for their assistance with manuscript preparation and LncRNA UCA1 studies. LB is a VA Senior Research Career Scientist. This work was sponsored by United States Public Health Grants (R01 CA66163) and by Veterans Affairs Merit Review Awards.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
published: 04 September 2019
doi: 10.3389/fonc.2019.00866

The Association Between the Incidence Risk of Peripheral Neuropathy and PD-1/PD-L1 Inhibitors in the Treatment for Solid Tumor Patients: A Systematic Review and Meta-Analysis

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Purpose: We conducted this study to determine the relationship between PD-1/PD-L1 inhibitors and the incidence risk of peripheral neuropathy in patients with solid tumors.

Method: The process of the meta-analysis was performed by us according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines. Incidence of all-grade and grade 3–5 treatment-related peripheral neuropathy in patients with solid tumors were taken into account.

Results: After screening and eligibility assessment, a total of 17 clinical trials involving 10,500 patients were selected for the final meta-analysis. The incidence risk of peripheral neuropathy for all grade was significantly lower in the PD-1/PD-L1 inhibitor group than that of the control group, either monotherapy (OR = 0.08, 95%CI:[0.03, 0.19]) or chemotherapy (OR = 0.05, 95%CI:[0.03, 0.11]). Similar incidence trend could also be seen for the incidence risk of grade 3–5 peripheral neuropathy. When PD-1/PD-L1 inhibitors were used in combination with chemotherapy, the incidence risk of peripheral neuropathy was higher than in the control chemotherapy group, whether it was all-grade (OR = 1.22, 95%CI:[1.00, 1.49]) or grade 3–5 degree (OR = 1.74, 95%CI:[1.03, 2.92]).

Conclusion: Compared with chemotherapy, incidence risk of peripheral neuropathy related to PD-1/PD-L1 inhibitor was significantly lower than that of the chemotherapy group, while PD-1/PD-L1 inhibitor increased the incidence risk of peripheral neuropathy when it was combined with chemotherapy.

Keywords: incidence risk, peripheral neuropathy, PD-1/PD-L1, solid tumor, meta-analysis

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Edited by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *20 July 2019*
Accepted: *21 August 2019*
Published: *04 September 2019*

Citation:
Si Z, Zhang S, Yang X, Ding N, Xiang M, Zhu Q, Mao Y, Lv Y, Yu L, Shang H, Xie J and Tian Y (2019) The Association Between the Incidence Risk of Peripheral Neuropathy and PD-1/PD-L1 Inhibitors in the Treatment for Solid Tumor Patients: A Systematic Review and Meta-Analysis. Front. Oncol. 9:866. doi: 10.3389/fonc.2019.00866

INTRODUCTION

Peripheral neuropathy is a syndrome characterized by loss of sensation, muscle weakness and atrophy, loss of tendon reflexes, and/or abnormal vascular motion as a clinical manifestation, either alone or in any combination. Drugs, especially for anti-tumor drugs, are one of the common pathogenic factors for the disease (1–10). During the course of anti-tumor therapy, whether it is chemotherapy or targeted therapy drugs (1–5), peripheral neuropathy is often reported as a drug side effect (1–10). Although reports of death due to peripheral neuropathy were rare, it seriously affected the quality of life for patients with malignant tumors (8–10). Therefore, peripheral neuropathy caused by anti-tumor drugs had increasingly attracted the attention of clinical doctors (11–13).

As a new targeted anti-tumor drug, PD-1/PD-L1 inhibitors have achieved satisfactory clinical efficacy for the treatment of solid tumors, either alone or in combination (14–29). With the increasing clinical applications, more and more drug-related side toxicity effects had been reported, and peripheral neuropathy was one of them (14–29). Because of the low incidence of peripheral neuropathy, we were unable to determine the association between its incidence risk and PD-1/PD-L1 inhibitors. Some chemotherapeutic drugs, such as paclitaxel, might cause delayed peripheral neuropathy (12, 13). It was impossible for us to identify the association between the incidence risk of peripheral neuropathy and PD-1/PD-L1 inhibitors when they were used in combination with other anti-tumor drugs or prescribed as a second-line treatment after chemotherapy (14–30).

For drug-induced peripheral neuropathy, stopping the drug remained to be the primary treatment method (1–10). However, for patients with malignant tumors, when severe drug side effects were encountered (12, 13), careful consideration for stopping the drug should be taken into account. Because of the sudden stop of anti-tumor treatment, it was very likely to cause rapid progression of the tumor. When PD-1/PD-L1 inhibitors were used in combination with chemotherapy, it was particularly important to determine the cause of peripheral neuropathy and then decide which drug to be discontinued (15–18).

To solve the above problems and clarify the association between incidence risk of peripheral neuropathy and PD-1/PD-L1 inhibitors, we designed this meta-analysis.

METHODS

The process of the meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (31).

Abbreviations: CI, confidence interval; CIPN, Chemotherapy-induced peripheral neuropathy; DIPN, drug induced peripheral neuropathy; FE, fixed effect; HR, hazard ratios; OR, odds ratio; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand 1; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RD, risk difference; RE, random effect; RR, risk ratio.

Types of Enrolled Studies

According to the research design, the selected clinical studies must meet the following criteria: (1) Randomized controlled clinical trials would be prioritized, (2) PD-1/PD-L1 inhibitor was prescribed for at least one group of participants, (3)The control group was an anti-tumor drug or PD-1/PD-L1 in combination with an anti-tumor drug rather than a placebo, (4) Participants were diagnosed with solid malignant tumors rather than hematological malignancy, (5) Data on peripheral neuropathy were reported in the study, (6) the enrolled study was published in English.

Search Strategy

Original articles including PD1/PD-L1 inhibitor regimens for solid tumor patients were verified by a systematic search of PubMed. The reported date of the results was limited from Jan 22, 2013 to May 31, 2019. The following subject terms would be used in the literature search process: "cancer," "tumor," "PD1/PD-L1," "nivolumab," "Opdivo," "pembrolizumab," "Keytruda," "Imfinzi,","MK-3475," "atezolizumab," "Tecentriq," "MPDL3280A," "avelumab," "Bavencio," "durvalumab," "BMS-963558." Studies limited in human beings, shown in full text, abstract, or poster form, were selected three investigators (Shuisheng Zhang, Yi Zhao, Qingshan Zhu) were appointed to check eligibility and duplicate independently by screening titles and abstracts of relevant studies. If data on peripheral neuropathy had not been reported, we would contact the corresponding author of the article to verify it again, or it would be precluded from the meta-analysis. The basic characteristics information included in the study would be summarized in **Table 1**.

Assessment of Study Quality and Publication Bias

Funnel plot, Egger's test and Newcastle-Ottawa scale, proposed by the Cochrane Collaboration, were taken to evaluate the bias (31–35). Three investigators (Shuisheng Zhang, Yi Zhao, Qingshan Zhu) were appointed to check the quality of all studies. The results, including random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, and selective outcome reporting, would be summarized in a figure together.

Outcome and Exposure of Interest

The study name, year, phase, tumor type, PD-1 and PD-L1 inhibitor regimen, previous therapy regimen, number of evaluable cases, and number of peripheral neuropathy events were extracted from every enrolled study. Both all-grade and grade 3–5 peripheral neuropathy data were taken into account for the final comprehensive meta-analysis.

Assessment of Heterogeneity and Statistical Analysis

Cochrane's Q statistic and the I^2 statistic were taken into account for evaluating the heterogeneity among enrolled studies just as suggested by Moher et al. (31) and Higgins et al. (36). The grade of heterogeneity was calculated by the range of I^2 values.

TABLE 1 | Basic characteristics of the included studies.

No.	Study name	Drug name	Drug type	Treatment regimen	Number of evaluable patients	Peripheral neuropathy	Previous therapy	Phase	Randomized controlled trial (RCT)	Tumor type
1	Cohen et al. (14)	Pembrolizumab	PD-1	Pembrolizumab vs. (Methotrexate, Docetaxel, or Cetuximab)	480	7	Platinum-based	III	RCT	Head-and-neck squamous cell carcinoma
2	Schmid et al. (15)	Atezolizumab	PD-L1	Atezolizumab + Nab-paclitaxel vs. Placebo + Nab-paclitaxel	890	195	NO	III	RCT	Advanced Triple-Negative Breast Cancer (BC)
3	Horn et al. (16)	Atezolizumab	PD-L1	Atezolizumab + Carboplatin + Etoposide vs. Placebo + Carboplatin + Etoposide	394	6	NO	III	RCT	SCLC
4	Socinski et al. (17)	Atezolizumab	PD-L1	Atezolizumab + BCP vs. BCP	787	274	NO	III	RCT	Metastatic non squamous NSCLC
5	Paz-Ares et al. (18)	Pembrolizumab	PD-1	Pembrolizumab + Carboplatin + Paclitaxel vs. Placebo + Carboplatin + Paclitaxel	558	102	NO	III	RCT	Metastatic squamous NSCLC
6	Shitara et al. (19)	Pembrolizumab	PD-1	Pembrolizumab vs. Paclitaxel	570	41	YES	III	RCT	Advanced gastric or gastro-oesophageal junction cancer
7	Powles et al. (20)	Atezolizumab	PD-L1	Atezolizumab vs. Chemotherapy	1,128	53	Platinum-based	III	RCT	Locally advanced or metastatic urothelial carcinoma (UC)
8	Hida et al. (21)	Atezolizumab	PD-L1	Atezolizumab vs. Docetaxel	101	14	Platinum-based	III	RCT	Locally advanced/ metastatic NSCLC
9	Bellmunt et al. (22)	Pembrolizumab	PD-1	Pembrolizumab vs. Chemotherapy	521	28	Platinum-based	III	RCT	Advanced Urothelial Carcinoma (UC)
10	Rittmeyer et al. (23)	Atezolizumab	PD-L1	Atezolizumab vs. Docetaxel	1187	89	Platinum based	III	RCT	Squamous or non squamous NSCLC
11	Ferris et al. (24)	Nivolumab	PD-1	Nivolumab vs. (Methotrexate, Docetaxel, or Cetuximab)	347	8	Platinum-based	III	RCT	Recurrent Squamous-Cell Carcinoma of the Head and Neck
12	Antonia et al. (25)	Nivolumab	PD-1	Nivolumab vs. Nivolumab + Ipilimumab	213	1	Platinum-based	I/II	N/A	Recurrent SCLC
13	Fehrenbacher et al. (26)	Atezolizumab	PD-L1	Atezolizumab vs. Doctaxel	277	16	Platinum-based	II	RCT	NSCLC
14	Herbst et al. (27)	Pembrolizumab	PD-1	Pembrolizumab vs. Docetaxel	991	33	Platinum-containing	II/III	RCT	Advanced NSCLC
15	Borghaei et al. (28)	Nivolumab	PD-1	Nivolumab vs. Docetaxel	555	28	Platinum-based	III	RCT	Non-squamous NSCLC
16	Brahmer et al. (29)	Nivolumab	PD-1	Nivolumab vs. Docetaxel	260	16	Platinum-based	III	RCT	Squamous NSCLC
17	Mok et al. (30)	Pembrolizumab	PD-1	Pembrolizumab vs. Chemotherapy	1,241	51	NO	III	RCT	NSCLC

RCT, Randomized controlled trial; BCP, Bevacizumab plus Carboplatin plus Paclitaxel; NSCLC, Non-Small Cell Lung Cancer; PD-1, Programmed Cell Death 1; PD-L1, Programmed Cell Death Ligand 1; SCLC, Small Cell Lung Cancer; CP, Carboplatin plus Paclitaxel; PC, Pemetrexed plus a platinum-based drug; Chemotherapy, Carboplatin plus Pemetrexed, Cisplatin plus Pemetrexed, Carboplatin plus Gemcitabine, Cisplatin plus Gemcitabine, or Carboplatin plus Paclitaxel; N/A, No Available.

Heterogeneity was considered low, moderate or high according to I^2 values <25%, 25–50%, and >50%, respectively.

Odds ratio (OR) value was reported to be a much more conservative evaluation parameter and might be more inclined to reveal a safety signal, as the method by which an OR is calculated provided a point estimate farther from unity than that provided by a HR. Odds ratio (OR), and 95% confidence interval (CI) would be calculated by random effect (RE) (37). Risk Ratio (RR) and Risk Difference (RD) were also calculated as secondary reference indicators for a more detailed interpretation of the results. $P < 0.05$ was considered to be of statistically significance. In order to clarify the correlation between peripheral neuropathy and PD-1/PD-L1 inhibitors, we performed a large number of subgroup analyses based on the type of tumor, the treatment regimen and the specific drug. The software of Review Manager 5.3 was used for data consolidation and analysis. Statistical tests were all two-sided.

RESULTS

Literature Search Results

According to the searching principle set by our team, 505 related documents were retrieved on the PubMed website, and 58 related documents were found in other websites or published documents.

After screening and eligibility assessment, a total of 17 clinical trials involving 10,500 patients were selected for the final meta-analysis. The flow diagram of the meta-analysis was shown in **Figure 1**, while the risk of bias summary was shown in **Supplemental Figure 1**. All clinical trials enrolled in the meta-analysis included at least one experimental group and one control group (14–30).

Characteristics of Identified Trials

The basic characteristics of all the enrolled clinical trials were summarized in **Table 1** (14–30). The involving PD-1/PD-L1 inhibitors were nivolumab ($n = 4$) (24, 25, 28, 29), pembrolizumab ($n = 6$) (14, 18, 19, 22, 27, 30), and atezolizumab ($n = 7$) (15–17, 20, 21, 23, 26). Of all the clinical trials included, 14 were phase III (14–24, 28–30), 1 was phase II (26), 1 was phase II/III (27), and 1 was phase I/II (25). The tumors involved in 17 clinical trials included lung cancer ($n = 11$) (16–18, 21, 23, 25–30), urothelial cancer ($n = 2$) (20, 22), breast cancer ($n = 1$) (15), head and neck carcinoma ($n = 2$) (14, 24), and advanced gastric or gastro-esophageal junction cancer ($n = 1$) (19). Of the 11 lung cancer-related clinical trials, nine were limited to non-small cell lung cancer (NSCLC) and two were limited to small cell lung cancer (SCLC) (16, 25). 16 clinical trials were reported to be randomized controlled trial (RCT) (14–24, 26–30), while the information of one clinical trial was unavailable (25). Twelve trials underwent previous platinum-based treatments before PD-1/PD-L1 inhibitors (14, 19–29), while PD-1/PD-L1 inhibitors were prescribed as the first line therapy regimens for the other five trials (15–18, 30). The drugs used in 10 clinical trials were PD-1 inhibitors (14, 18, 19, 22, 24, 25, 28–30), while PD-L1 inhibitors were just given for the other seven clinical trials (15–17, 20, 21, 23, 26).

Risk of Bias

Study quality and risk of bias among enrolled studies were checked by Newcastle-Ottawa scale (35). Random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), and selective reporting (reporting bias) were assessed by three members of our team independently and summarized in **Supplemental Figure 1**. Publication bias, evaluated by Harbord's test (31), was displayed by funnel plots (**Supplemental Figures 2, 3, 5, 7, 9, 11**).

Incidence Risk of All-Grade Peripheral Neuropathy

All included clinical trials were divided into four groups according to different treatment options, and the specific groups were as follows: Group A (PD-1/PD-L1 vs. Mono-therapy) (19, 21, 23, 26–29), Group B (PD-1/PD-L1 vs. Chemotherapy) (14, 20, 22, 24, 30), Group C (PD-1/PD-L1+ Chemotherapy vs. Chemotherapy) (15–18), Group D (PD-1 vs. PD-1+ CTLA-4) (25). Each group was further divided into two subgroups depending on the respective specific drug and tumor type. Meta-analysis was not performed in group D, because only one group of clinical trials was enrolled, and only one patient in both subgroups was reported with peripheral neuropathy (25).

We first performed a meta-analysis on the data of Group A, and the results of the analysis were summarized at the bottom of **Figure 2A** [OR = 0.08, 95%CI:[0.03, 0.19], I^2 = 69%, Z = 5.64 ($P < 0.00001$)] (19, 21, 23, 26–29). Subgroup analysis was performed according to the different drug types in the control group and the experimental group, and the results were shown in **Figures 2A1,A2**, respectively. Moderate heterogeneity was found in Group A ($I^2 = 69\%$). Subgroup analysis results suggested that the source of heterogeneity was the PD-L1 subgroup [**Figure 2A2**; (21, 23, 26)]. The funnel plots of OR for Group A could be seen in **Supplemental Figures 2A1,A2**. Similar to the results of OR, RR and RD of Group A were displayed in **Supplemental Figures 4A, 6A**.The corresponding funnel plots were gathered in **Supplemental Figures 5A, 7A**.

When PD-1/PD-L1 drugs were compared with chemotherapy (Group B), the incidence of peripheral neuropathy was significantly lower than that of the control group, and the OR results are summarized in **Figure 2B** [OR = 0.05, 95%CI:[0.03, 0.11], I^2 = 0%, Z = 7.68 ($P < 0.00001$)] (14, 20, 22, 24, 30). The funnel plots of OR for Group B could be seen in **Supplemental Figures 2B1,B2**. The subgroup analysis results were also similar to the subgroup analysis results of group A. RR and RD of Group B were displayed in **Supplemental Figures 4B, 6B**.The corresponding funnel plots were gathered in **Supplemental Figures 5B, 7B**. No obvious heterogeneity was found among Group B ($I^2 = 0\%$).

Different from the met-analysis results of group A and group B, we found that the analysis results of OR were not statistically significant when performing meta-analysis on Group C (**Figure 2C**) [OR = 1.22, 95%CI:[1.00, 1.49], I^2 = 4%, $Z = 1.99$ ($P = 0.05$)] (15–18). The same trend could

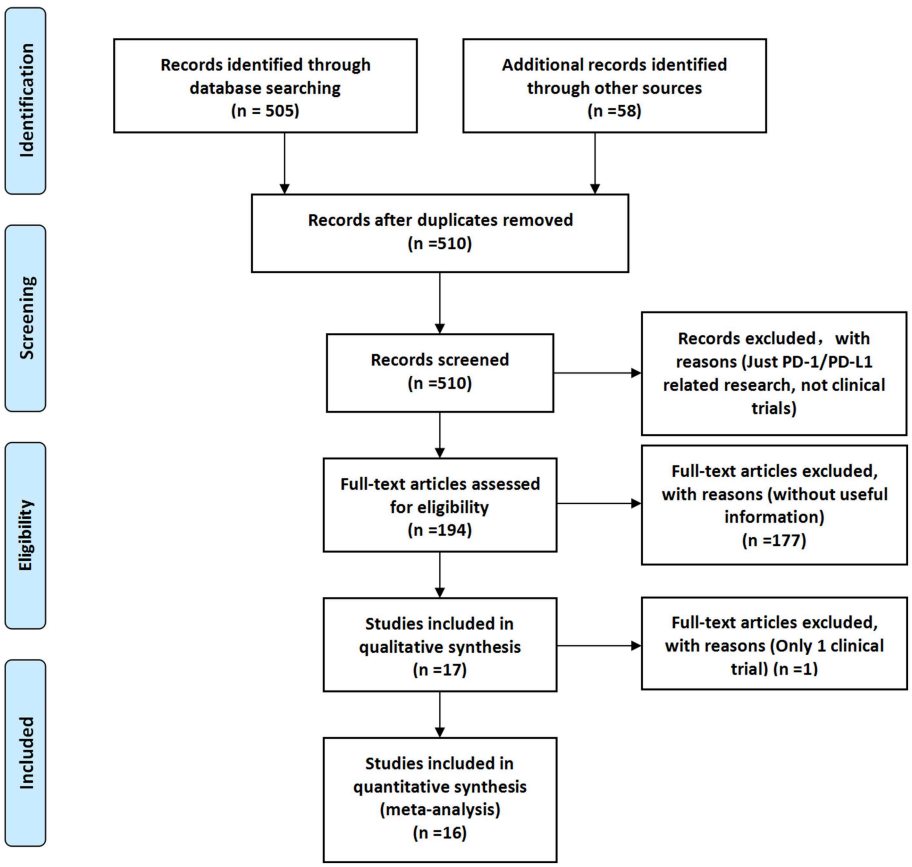

FIGURE 1 | Study flow diagram of inclusion.

be seen in the results of RD (**Supplemental Figure 6C**) [RD = 0.03, 95%CI:[0.01, 0.06], I^2 = 47%, Z = 1.42(P = 0.16)] (15–18). The corresponding funnel plots of them were gathered in **Supplemental Figures 2C, 7C**. The RR of Group C showed that the incidence risk of peripheral neuropathy in the PD-1/PD-L1 combined chemotherapy subgroup was significantly higher than that in the chemotherapy subgroup, and the P-value was statistically significant (**Supplemental Figure 4C**) [RR = 1.16, 95%CI:[1.01, 1.34], I^2 = 0%, Z = 2.13(P = 0.03)] (15–18). The corresponding funnel plots of RR were gathered in **Supplemental Figure 5C**. No obvious heterogeneity was found among Group C (I^2 = 0%).

Incidence Risk of Grade 3–5 Peripheral Neuropathy

Twelve clinical trials with the information of grade 3–5 peripheral neuropathy were taken into account for further meta-analysis (15–20, 22, 23, 27–30). The same grouping and subgroup approach as before were taken for dealing with them. In the experimental subgroup of Group A and Group B, using PD-1/PD-L1 inhibitors alone, the incidence rate of peripheral neuropathy was 0% (19, 20, 22, 23, 27–30). In other words, in patients with solid tumors treated with PD-1/PD-L1 alone, the incidence rate of grade 3–5 peripheral neuropathy was 0% (19, 20, 22, 23, 25, 27–30).

In Group A, the incidence risk of PD-1/PD-L1 subgroup was obvious lower than the control group [OR = 0.13, 95%CI:[0.04, 0.45], I^2 = 0%, Z = 3.24 (p = 0.001); **Figure 3A**; (19, 23, 27–29)]. Different grouping methods for subgroup analysis were adopted for dealing with all the data, no statistically significant difference was found among them (**Figures 3A1,A2**). No heterogeneity was found in Group A (I^2 = 0%). Similar to the results of OR, RR, and RD of Group A were displayed in **Supplemental Figures 8A, 10A**. The corresponding funnel plots were summarized in **Supplemental Figures 9A, 11A**.

When PD-1/PD-L1 drugs were compared with chemotherapy (Group B), the incidence risk of peripheral neuropathy limited to grade 3–5 was significantly lower than that of the control group, and the OR results are summarized in **Figure 3B** [OR = 0.11, 95%CI:[0.03, 0.49], I^2 = 0%, Z = 2.92 (P = 0.004)] (20, 22, 30). The funnel plots of OR for Group B could be seen in **Supplemental Figures 3B1,B2**. Similar to the results of OR, RR, and RD of Group B were displayed in **Supplemental Figures 8B, 10B**.The corresponding funnel plots were gathered in **Supplemental Figures 9B, 11B**. No heterogeneity was found in Group B (I^2 = 0%) (20, 22, 30).

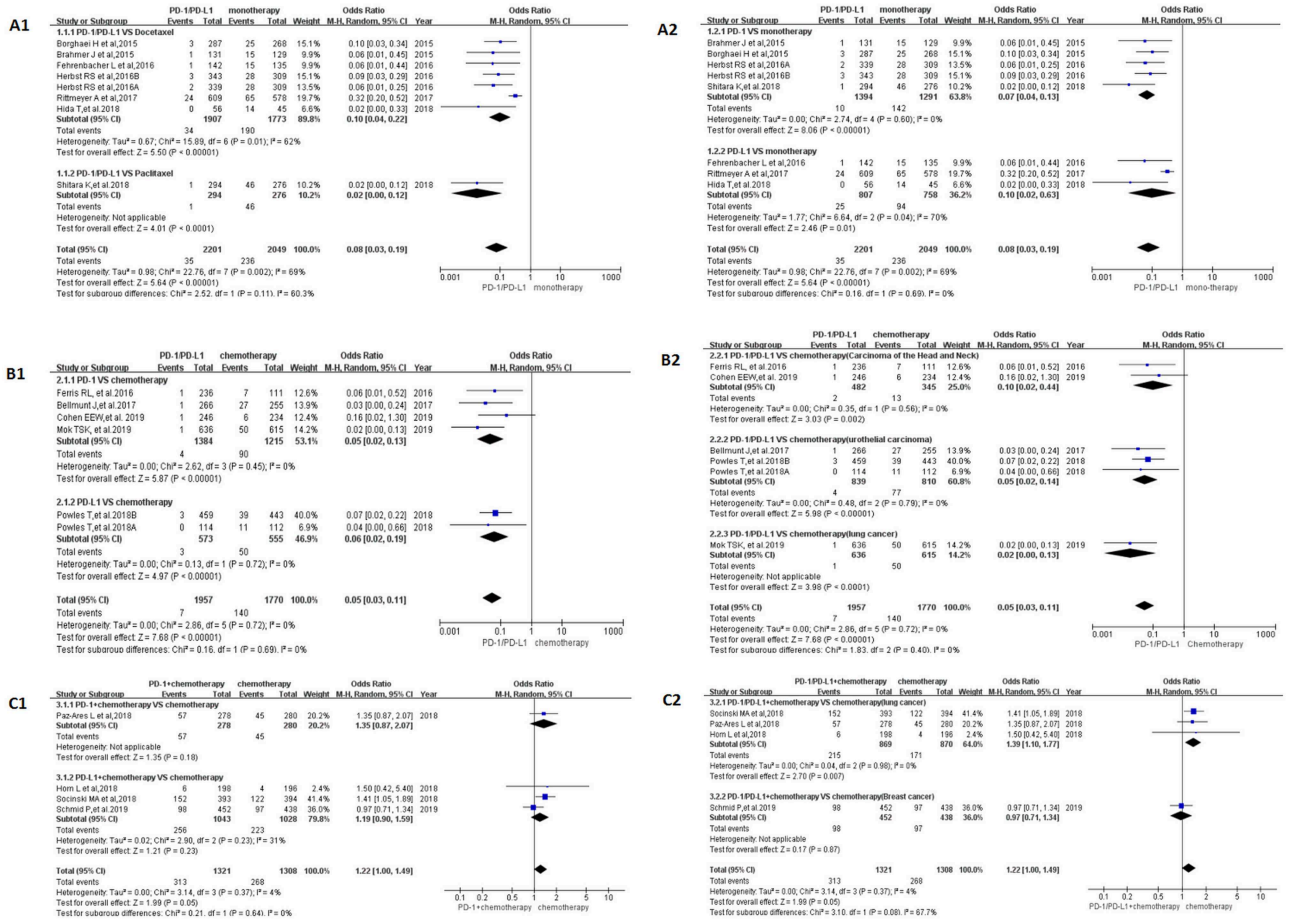

FIGURE 2 | Forest plots for the odds ratio of treatment related peripheral neuropathy for all grade. **(A1)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 vs. Docetaxel/Paclitaxel). Subgroup analysis was performed according to the type of chemotherapy drug in the control group. **(A2)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 vs. monotherapy). Subgroup analysis was performed based on the drug type (PD-1 or PD-L1) of the experimental group. **(B1)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 vs. Chemotherapy). Subgroup analysis was performed based on the drug type (PD-1 or PD-L1) of the experimental group. **(B2)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 vs. Chemotherapy). Subgroup analysis was performed based on the specific types of tumors in the experimental and control groups. **(C1)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 + Chemotherapy vs. Chemotherapy). Subgroup analysis was performed based on the drug type (PD-1 or PD-L1) of the experimental group. **(C2)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 + Chemotherapy vs. Chemotherapy). Subgroup analysis was performed based on the specific types of tumors in the experimental and control groups.

The OR of Group C showed that the incidence risk of peripheral neuropathy in the PD-1/PD-L1 combined chemotherapy subgroup was significantly higher than that in the chemotherapy subgroup, and the *P*-value was statistically significant (**Figure 3C**) [OR = 1.74, 95%CI:[1.03, 2.92], I^2 = 0%, Z = 2.09 (P = 0.04)] (15–18). The corresponding funnel plots of OR were gathered in **Supplemental Figure 3C**. No heterogeneity was found in Group C (I^2 = 0%). Similar analysis results could also be seen in **Supplemental Figure 8C**, when the data of Group C was evaluated by RR [RR = 1.71, 95%CI:[1.03, 2.83], I^2 = 0%, Z = 2.09 (P = 0.04)]. Different from OR and RR, the meta-analysis result was of no statistical significance (**Supplemental Figure 10**), when it was calculated by RD [RD=0.01, 95%CI:[0.00, 0.02], I^2 = 10%, Z = 1.82 (P = 0.07)]. The corresponding funnel plots of RD were gathered in **Supplemental Figure 11C**. Low heterogeneity related to RD was found in Group C (I^2 = 10%). Subgroup analysis revealed that the source of heterogeneity might be related to the inclusion of this clinical trial (15).

DISCUSSION

Peripheral neuropathy is a painful condition deriving from many and varied etiologies (38, 39). Certain medications have been implicated in the iatrogenic development of drug induced peripheral neuropathy (DIPN) and include chemotherapeutic agents, antimicrobials, cardiovascular drugs, psychotropic, anticonvulsants, among others (39). Chemotherapy-induced peripheral neuropathy (CIPN), reported in several studies, especially for paclitaxel induced peripheral neuropathy, was

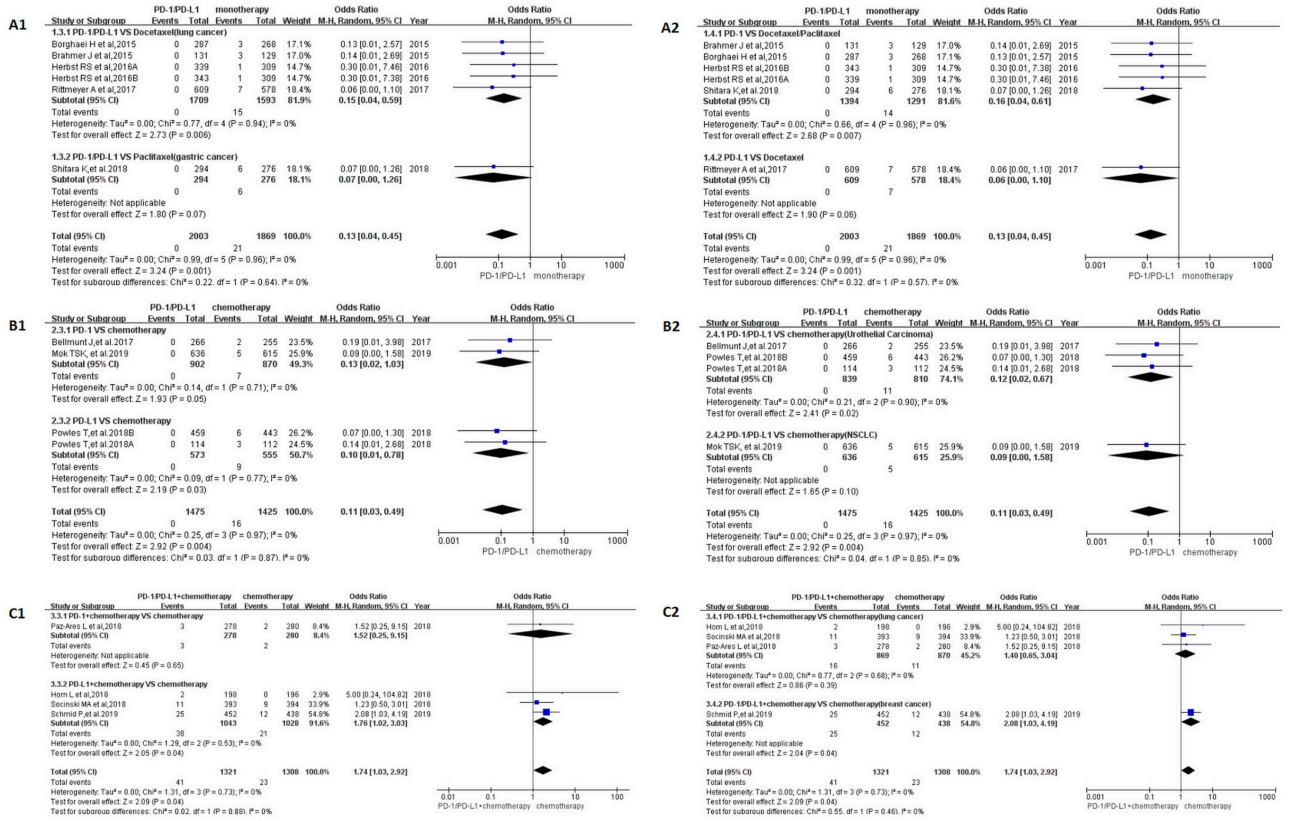

FIGURE 3 | Forest plots for the odds ratio of treatment related peripheral neuropathy for grade 3–5. **(A1)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 vs. Docetaxel/Paclitaxel). Subgroup analysis was performed based on the specific types of tumors in the experimental and control groups. **(A2)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 vs. monotherapy). Subgroup analysis was performed based on the drug type (PD-1 or PD-L1) of the experimental group. **(B1)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 vs. Chemotherapy). Subgroup analysis was performed based on the drug type (PD-1 or PD-L1) of the experimental group. **(B2)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 vs. Chemotherapy). Subgroup analysis was performed based on the specific types of tumors in the experimental and control groups. **(C1)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 + Chemotherapy vs. Chemotherapy). Subgroup analysis was performed based on the drug type (PD-1 or PD-L1) of the experimental group. **(C2)** Forest plots for the odds ratio of treatment related peripheral neuropathy (PD-1/PD-L1 + Chemotherapy vs. Chemotherapy). Subgroup analysis was performed based on the specific types of tumors in the experimental and control groups.

common for cancer patients (40, 41). CIPN was a dose limiting toxicity, negatively impacting both quality of life and disease outcomes (42). However, during the process of anti-tumor treatment, combinations of drugs that were unknown to cause CIPN were prescribed for cancer patients, and sequential treatment for recurrence with additional CIPN-inducing drugs would also be suggested (43). Therefore, it would be difficult for us to determine which specific drug was responsible for the occurrence of peripheral neuropathy, especially for some newly marketed targeted anti-tumor drugs without fully understanding of toxicities, such as PD-1/PD-L1 inhibitors and Brentuximab vedotin (3, 14–30). To clarify the association between incidence risk of peripheral neuropathy and PD-1/PD-L1 inhibitors, we designed this meta-analysis.

After screening and eligibility assessment, a total of 17 clinical trials involving 10,500 patients were selected for the final meta-analysis. The flow diagram of the meta-analysis was shown in **Figure 1**, while the risk of bias summary was shown in **Supplemental Figure 1**. All clinical trials enrolled in the meta-analysis included at least one experimental group and one control group (14–30). Study quality and risk of bias among enrolled studies were checked by Newcastle-Ottawa scale (35). All clinical trials included were considered to be of higher quality. Therefore, the analytical conclusions based on the data of these clinical trials could represent certain reliability, authenticity, and credibility (14–30). In this study, we tried as many subgroup analysis methods as possible, and conducted a systematic and comprehensive analysis of the results, so the analysis results obtained were much more accurate (**Figures 2**, **3** and **Supplemental Figures 4, 6, 8, 10**) than that was analyzed just by one model.

The incidence of peripheral neuropathy for all grade was significantly lower in the PD-1/PD-L1 inhibitor group than that of the control group, either monotherapy (OR = 0.08, 95%CI:[0.03, 0.19], **Figure 2A**) or chemotherapy (OR = 0.05, 95%CI:[0.03, 0.11], **Figure 2B**) (14, 19–24, 26–30). Moderate

heterogeneity was found in Group A ($I^2 = 69\%$) but Group B ($I^2 = 0\%$). Subgroup analysis results suggested that the source of heterogeneity was the PD-L1 subgroup (**Figure 2A2**) (21, 23, 26). The funnel plots of OR for Group A could be seen in **Supplemental Figures 2A1,A2**. Similar to the results of OR, Forest plots of RR and RD for Group A were displayed in **Supplemental Figures 4A, 6A**. The corresponding funnel plots were summarized in **Supplemental Figures 5A, 7A**. We found the existence of asymmetry of the funnel plot of Group A analysis (19, 21, 23, 26–29), so we concluded that there might be publication bias, but we could not rule out the possibility of asymmetry caused by other factors. Similar incidence risk of peripheral neuropathy for grade 3–5 could also be seen in **Figure 3A** (OR = 0.13, 95%CI:[0.04, 0.45]) (19, 23, 27–29). However, the heterogeneity ($I^2 = 0\%$) and the asymmetry of the funnel chart were not found [**Supplemental Figure 3A**; (19, 23, 27–29)]. Based on the above analysis results, we concluded that the heterogeneity and the asymmetry of the funnel plot were mainly derived from those two clinical trials (21, 26).

When PD-1/PD-L1 inhibitors were used in combination with chemotherapy (Group C), the risk of peripheral neuropathy was higher than in the control chemotherapy group, whether it was all-grade (OR = 1.22, 95%CI:[1.00, 1.49], **Figure 2C**) or grade 3–5 degree (OR = 1.74, 95%CI:[1.03, 2.92], **Figure 3C**) (15–18). Similar incidence trend could also be obtained when they were evaluated by RR (**Supplemental Figures 4C, 8C**). No obviously statistical significant results of RD were only seen in **Supplemental Figures 6C, 8C**. Obvious heterogeneity and the asymmetry of the funnel chart were not found in Group C (**Supplemental Figures 2C, 3C, 5C, 7C, 9C, 11C**). It proved that the analytical conclusions we had obtained were credible.

A lot of clinical trials had reported that PD-1/PD-L1 inhibitors had better safety and satisfactory clinical efficacy in the process of anti-tumor therapy (14–30, 44). In the experimental subgroup of Group A and Group B, using PD-1/PD-L1 inhibitors alone, the incidence rate of peripheral neuropathy for grade 3–5 was 0% (19, 20, 22, 23, 27–30). In other words, if we encounter peripheral neuropathy of grade 3–5 in the course of anti-tumor therapy, the possibility caused by the PD-1/PD-L1 inhibitor was firstly excluded. Chemotherapy-induced peripheral neuropathy (CIPN), reported in several studies, especially for paclitaxel induced peripheral neuropathy, was common for cancer patients (40, 41). Stopping the use of related drugs remained to be the primary principle for the treatment of drug-related peripheral neuropathy. However, stopping all anti-tumor treatment for cancer patients, especially for advanced cancer patients, might lead to rapid progression of the tumor, and even endanger the patient's life. Based on the results of our analysis, we found that PD-1/PD-L1 inhibitors often played a secondary role for patients suffering from severe drug-related peripheral neuropathy [**Figures 2**, **3**; (14–30)]. Therefore, when it was necessary to stop anti-tumor therapy to alleviate severe peripheral neuropathy in patients, chemotherapy drugs other than PD-1/PD-L1 would be considered first (**Figures 2C**, **3C**) (15–18). This finding had an important clinical guiding significance in clinical work.

CONCLUSIONS

Compared with chemotherapy, incidence risk of peripheral neuropathy related to PD-1/PD-L1 inhibitor was significantly lower than that of the chemotherapy group, while PD-1/PD-L1 inhibitor increased the incidence risk of peripheral neuropathy when it was combined with chemotherapy.

ETHICS STATEMENT

This study belongs to the type of data analysis and rearrangement, and does not involve human or animal related ethical issues.

AUTHOR CONTRIBUTIONS

YT had full access to all data in the study and all authors had final responsibility for the decision to submit for publication. ZS, SZ, XY, ND, and YT had the full data of the paper. MX, QZ, YL, LY, HS, JX, and YM were responsible for the collection of clinical data. ZS helped to gather online data and write the report.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00866/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 06 September 2019
doi: 10.3389/fimmu.2019.02120

Altered Cell Adhesion and Glycosylation Promote Cancer Immune Suppression and Metastasis

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Cell-cell interactions and cell adhesion are key mediators of cancer progression and facilitate hallmarks of cancer including immune evasion and metastatic dissemination. Many cell adhesion molecules within the tumor microenvironment are changed and significant alterations of glycosylation are observed. These changes in cell adhesion molecules alter the ability of tumor cells to interact with other cells and extracellular matrix proteins. Three families of cell-cell interaction molecules selectins, Siglecs, and integrins have been associated with cancer progression in many pre-clinical studies, yet inhibition of cell adhesion as a therapeutic target is just beginning to be explored. We review how cell-cell interactions mediated by integrins and the glycan-binding receptors selectins and Siglec receptors support cancer progression. The discussion focuses on mechanisms during immune evasion and metastasis that can be therapeutically targeted by blocking these cell-cell interactions.

Keywords: selectin, Siglec, integrin, immunity, sialic acid, tumor microenvironment

OPEN ACCESS

Edited by:
Toshiyuki Murai,
Osaka University, Japan

Reviewed by:
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University of Ljubljana, Slovenia
Fabrice Cognasse,
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *14 July 2019*
Accepted: *23 August 2019*
Published: *06 September 2019*

Citation:
Läubli H and Borsig L (2019) Altered Cell Adhesion and Glycosylation Promote Cancer Immune Suppression and Metastasis. Front. Immunol. 10:2120. doi: 10.3389/fimmu.2019.02120

INTRODUCTION

Cancer progression induces immune evasion and eventually metastasis, a process consisting of several steps enabling tumor cells to leave the primary tumor, to intravasate and survive in the circulation, to extravasate and seed in distant organs and to initiate growth of metastatic lesions. Tumor cell interactions with other cells in the environment contribute to immune evasion and metastasis at every step of this process. Adhesion molecules, on any cell, mediate interactions with other cells and the extracellular matrix in the microenvironment (1, 2). Since cell adhesion receptors are connected to signal-transduction pathways, these cell-cell and cell-matrix interactions modulate cell phenotype, proliferation, differentiation, survival, and migration. Consequently, changes in expression of cell adhesion molecules and their ligands directly affect immune evasion and metastasis.

Malignant transformation changes not only the expression of cell adhesion molecules but also causes profound changes in cell surface glycosylation (3, 4). Cancer-associated glycosylation promotes the interaction of tumor cells within a microenvironment through glycan-binding receptors–lectins (5). Glycans are oligosaccharide structures presented on protein and lipids. Endogenous lectins expressed on immune cells and other cells in the stroma, facilitate cell-interactions, -adhesions, thereby contributing to homeostasis. During malignancy, glycans on tumor cells are involved in invasiveness, metastasis, and immune suppression (6–8).

Several families of cell adhesion molecules including cadherins, integrins, junctional-adhesion molecules, and selectins are altered during tumorigenesis. This mini review addresses the role of

cell adhesion and glycan-mediated interactions during metastasis and tumor-induced immune suppression in the context of altered glycosylation as a ubiquitous characteristic of cancer progression with a focus on integrins, selectins, and siglecs.

SELECTINS CONTRIBUTE TO CANCER PROGRESSION

Selectins are vascular cell adhesion receptors present on leukocytes, endothelial cells, and platelets that bind to glycans. The physiological function of selectins is to facilitate the initial tethering of leukocytes at inflammatory sites or secondary lymphoid organs or hemostasis (9, 10). There is accumulating evidence for the involvement of selectins in pathophysiological processes, including cancer metastasis (11, 12). There are three members of the selectin family: P-selectin expressed on activated platelets and endothelial cells, L-selectin present on leukocytes, and E-selectin expressed on activated endothelial cells (10). Upon activation, P-selectin is rapidly presented on the surface of activated endothelial cells or platelets through exocytosis of storage granules. E-selectin is present only on activated endothelial cells and its expression is regulated on a transcription level. L-selectin is constitutively expressed on most subsets of leukocytes.

Selectins are C-type lectins that bind to properly modified glycan ligands, carrying terminal sLe^x or sLe^a structures. Selectin binding to glycans usually requires a protein scaffold that presents selectin ligands in clusters (13). The best characterized selectin ligand is P-selectin glycoprotein ligand 1 (PSGL-1) (9, 10). All three selectins bind to PSGL-1 that is mostly expressed in leukocytes. In addition, selectins binds to these glycan moieties carried on several cell surface proteins, such as CD44, E-selectin ligand-1, CD43, CD34, or addressins with a variable specificity (14).

There is compelling experimental and clinical evidence for the enhanced expression of sLe^x and sLe^a to correlate with poor prognosis due to enhanced metastasis in tumors of gastric, pancreatic, colon, prostate, renal, lung, and melanoma cancers (4, 15). Enhanced expression of selectin ligands is linked to increased activities of glycosyltransferases, responsible for the terminal synthesis, sialyltransferases, and fucosyltransferases. Major carriers of selectin ligands are mucins that are heavily O-glycosylated (16). MUC1, MUC2, MUC4, and MUC16 are mucins associated with cancer progression, whereas MUC16 is also used for cancer diagnostics. However, the spectrum of selectin ligands on tumor cells is rather broad, encompassing glycolipids, proteins, and glycosaminoglycans (4).

During the hematogenous phase of metastasis, tumor cells carrying selectin ligands (**Figure 1**) enter the blood circulation

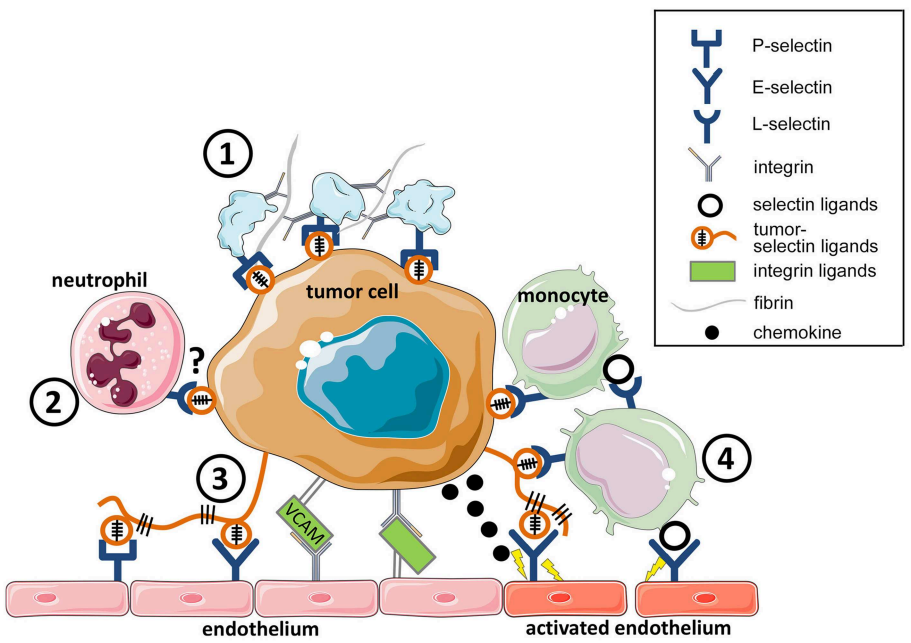

FIGURE 1 | Cell adhesion facilitates tumor cell survival in the circulation and tumor cell extravasation. Tumor cells in the circulation interact through selectins and integrins with blood constituents (platelets, leukocytes, and endothelial cells). **(1)** platelet-tumor cell aggregate formation is mediated by both P-selectin and integrins through fibrin and fibrinogen. **(2)** The survival of circulating tumor cells is further enhanced by aggregation with neutrophils that promote tumor cell proliferation (17). Whether L-selectin or integrins mediate these interactions remains to be determined. **(3)** Tumor cell interaction with the endothelium, leading to adherence, is mediated by P-and E-selectins, and tumor cell firm adhesion is facilitated by integrins, and their interaction, for example with VCAM-1 on tumor cells. **(4)** Tumor cell-endothelial interaction directly or facilitated by monocytes, contribute to the initiation of tumor cell extravasation. This process is dependent both on E-selectin and integrin engagement.

and encounter selectins on platelets, leukocytes, and on the endothelium (14). Tumor cells in the circulation are often associated with platelets that protects them from the immune system (18), and enables tumor cell seeding in distant organs (19). The absence of P-selectin abrogates platelet-tumor cell aggregation and consequently attenuates metastasis (20). In addition to P-selectin, platelets also express CD40 on their surfaces, which upon binding to the CD40 ligand accelerate endothelial inflammation and atherosclerosis (21). Platelet-leukocyte aggregate formation resulted in a release of IL-1β by leukocytes. CD40 deficiency in the blood compartment attenuated experimental lung metastasis (22), indicating a potential involvement of platelet CD40 in cancer progression. Tumor cell arrest in the vasculature induces local activation of the endothelium and results in expression of E-selectin and chemokines (12, 23). Chemokine-driven recruitment of inflammatory monocytes to metastasizing cells was shown to promote metastasis in different cancers [reviewed in (24)]. E-selectin has been shown to promote tumor cell adhesion and thereby metastatic dissemination (14, 25, 26). Enhanced expression of E-selectin ligands on human breast cancer cells, such as CD44, promotes homing to the microvascular endothelium and metastasis (27).

Selectins not only mediate tumor cell adhesion but actively contribute to the formation of a metastasis niche (28, 29). Effective tumor cell extravasation in the lungs requires engagement of E-selectin on activated endothelial cells, which is essential for the loosening of endothelial VE-cadherin junctions (28). E-selectin promotes the recruitment of inflammatory monocytes, Ly6C^{hi} cells that facilitate transendothelial migration of tumor cells (**Figure 1**). The opening of endothelial junctions was shown to be dependent on the Src kinase pathway that induces E-selectin expression (30). Recently, E-selectin in the bone marrow vascular niche was shown to promote metastasis by inducing mesenchymal-epithelial transition of breast cancer cells through the activation of Wnt signaling (29). Tumor cell expression of fucosyltransferase-7, required for E-selectin ligand formation, is essential for the formation of bone metastasis.

L-selectin-mediated recruitment of leukocytes promotes both tumor cell extravasation and the formation of a metastatic niche (28, 31, 32). Tumor-induced endothelial activation is associated with selectin ligand accumulation required for the L-selectin dependent recruitment of myeloid cells (31), which were later identified to be inflammatory monocyte Ly6C^{hi} cells (33, 34). Interestingly, the presence of selectin ligands on leukocytes is also required for their effective recruitment to the metastatic sites (34). L-selectin facilitates the recruitment of T-cells to the lymph nodes. An engagement of a T cell receptor leads to the shedding of L-selectin from the cell surface of T cells (35, 36). Notably, cytotoxic/memory T cells, with L-selectin expression, better controlled tumor growth (37, 38). Sustained L-selectin expression on NK cells was shown to control tumor progression (39). Recently, the role of L-selectin on T cells was investigated in a transgenic mouse model that expressed non-cleavable L-selectin (40). T cells with constitutive expression of L-selectin suppressed lung metastasis. However, how L-selectin on T and NK cells contributes to the immune suppressive activity during metastasis remains to be defined.

SIGLEC-MEDIATED IMMUNE SUPPRESSION IN CANCER

The cell surface glycans of mammalian cells commonly terminate with sialic acid (41). These sialylated structures, also called sialoglycan can engage various endogenous receptors including sialic acid-binding immunoglobulin-like lectins (Siglecs) (7, 42–44). Siglecs are mostly inhibitory receptors with an extracellular part that contains an N-terminal carbohydrate recognition domain (CRD) and a variable number of C2 domains (44). The intracellular part of inhibitory Siglecs contains ITIM or ITIM-like structures mediating immune inhibition (7, 42–44). Activating Siglecs have a positively charged amino acid in the transmembrane domain that mediates interaction with DAP12 upon Siglec engagement (7, 42–44). In humans, 14 different, functionally active Siglecs were identified. The conserved Siglecs Siglec-1 (sialoadhesin), CD22 (Siglec-2), Siglec-4 (MAG), and Siglec-15 have orthologs across different mammalian species (45, 46). CD33-related Siglecs, however, have undergone rapid evolutionary adaptation (45, 46). This subfamily includes CD33 (Siglec-3), Siglec-5, Siglec-6, Siglec-7, Siglec-8, Siglec-9, Siglec-10, and Siglec-11 (45, 46). In mice, no direct orthologs of human CD33-related Siglecs can be found, but functional paralogues with similar expression patterns can be defined (47).

Siglec receptors are predominantly expressed on immune cells (7, 42–44). Inhibitory Siglec receptors can modulate immune cell activation by recruitment of SHP1 and SHP2 phosphatase upon binding to sialoglycans (7, 42–44). Binding-specificity varies between different Siglecs. While Siglec-9 has quite a broad binding spectrum (47, 48), Siglec-8 binds a rather restricted set of sialoglycans which also contain sulfate groups (49, 50). The binding spectrum and the expression patterns determine the function of Siglecs.

Recent evidence has shown that tumor cells can also engage the sialoglycan-Siglec axis to evade immune control (7, 47, 51–56). In many cancer types, the glycocalyx and also the tumor microenvironment are characterized by an enhanced presence of sialoglycans due to changes in sialic acid-modifying enzymes including sialic acid synthesis genes, transporters, sialyltransferases, and sialidases (57–59). Moreover, enzymes such as O-acetylases can directly modify the sialic acid residues (57–59). This upregulation of sialoglycans in some cancers is termed hypersialylation, which is quite heterogenous between different cancer types but also within a specific cancer type. In lung cancer, we have observed considerable heterogeneity of sialoglycan ligands for Siglec-7 and Siglec-9 (51). Similar observations were made in melanoma samples (52). How sialylation differs within a single cancer patient and how hypersialylation evolves during different treatments and during cancer progression over time remains to be determined.

The increased density of sialoglycans can lead to engagement of inhibitory Siglec receptors on immune cells and modulate the immune response to cancer (**Figure 2**). Both innate and

adaptive immune cells can be regulated by the sialoglycan-Siglec checkpoint. Human NK cells express inhibitory Siglec-7 and some subpopulations of NK cells also express Siglec-9 (54, 55). Engagement of Siglec-7 and/or Siglec-9 can inhibit NK cell-mediated tumor cell killing *in vitro* (54, 55). The introduction of a synthetic sialoglycan polymer into the glycocalyx of target cells led to a significant decrease in the NK cell-mediated killing of cells lacking MHC I expression and a reduced antibody-dependent cellular cytotoxicity (54). Antibodies blocking Siglec-7 or Siglec-9 resulted in increased tumor cell killing (55). In addition, sialic acid-dependent NK cell inhibition was also observed in a humanized mouse model (55). Macrophage polarization is also influenced by a sialoglycan-Siglec pathway (47, 56). Alternative M2 polarized macrophages produce cytokines suppressing anti-cancer immunity, secrete pro-angiogenic factors, enhance tumor cell invasion, and thereby promote cancer progression (60, 61). Binding of sialylated, cancer-associated MUC1 to Siglec-9 led to a polarization to M2 macrophages *in vitro* (56). However, studies in Siglec-E deficient mice showed a propensity of Siglec-E deficient macrophages to polarize to M2 macrophages (47). Macrophages express various Siglecs including Siglec-3, Siglec-5/-14, Siglec-7, Siglec-9, and Siglec-10 with some overlapping binding spectra (7, 42–44). The exact function of sialoglycan-Siglec interactions on the influence of pro- and anti-tumorigenic effects of tumor-associated macrophages certainly require further studies. For example, Siglec receptors could also act as potential "don't eat me" signals that inhibit macrophage-mediated phagocytosis (62). Conserved Siglec-15 was identified in a screening of surface markers on antigen-presenting cells that could inhibit T cell activation (63). Antibodies against Siglec-15 tested in a murine tumor model led to enhanced anti-cancer immunity (63). Antibodies were humanized and early clinical trials are being planned.

Recent work provided evidence that Siglec receptors are expressed on platelets in both humans and mice (64, 65). Engagement of Siglec-9 or Siglec-E on platelets increased the infectivity of group B streptococci by modulation of platelet activation (64). One could hypothesize that interactions of tumor cell-sialoglycans could also modulate platelet activation and influence metastatic progression.

Two recent studies have found that the sialoglycan-Siglec glyco-immune checkpoint influences activation of tumor-infiltrating lymphocytes (TILs), particularly cytotoxic CD8+ T cells (51, 52). We have found that TILs upregulate different inhibitory CD33-related Siglecs, predominantly Siglec-9 in patients with non-small cell lung cancer, colorectal cancer, epithelial ovarian cancer and melanoma (51, 52). Healthy peripheral blood T cells, however, were not expressing these inhibitory receptors, as described earlier (51, 52). Siglec-E was upregulated on tumor-infiltrating T cells in murine tumor models (51). Inhibition of the sialoglycan-Siglec axis with blocking antibodies or genetic models enhances T cell-mediated anti-cancer immunity *in vitro* and *in vivo* (51, 66, 67). These results directly implicate that Siglec-9 is a new target that can improve anti-tumoral T cell activation.

Targeting the sialoglycan-Siglec glyco-immune checkpoint can be achieved by using Siglec-blocking antibodies. Another approach is the reduction of the ligand-density by targeting sialoglycans. Using a sialic acid mimetic that inhibits intratumoral sialoglycan production led to enhanced T cell-mediated anti-tumor immunity (68). Similar findings were observed with tumor cell lines with defects in sialic acid biosynthesis (51, 69). An elegant therapeutic approach is the

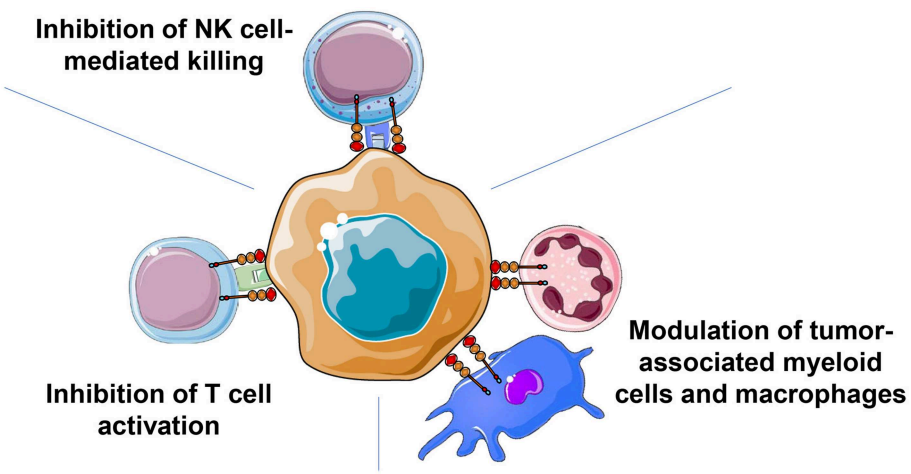

FIGURE 2 | The sialoglycan-Siglec glyco-immune checkpoint involves cells of the innate and the adaptive immune response. Cancer-associated sialoglycans on the surface of tumor cells but also within the tumor microenvironment can mediate immune evasion by engaging Siglec receptors on cells of the innate (NK cells, myeloid cells, and macrophages) and the adaptive (T cells) immune system. Inhibitory Siglec receptors, for example Siglec-9, can inhibit T cell activation by modulating signaling of the T cell receptor. Similarly, NK cell activation and tumor cell killing can be reduced by inhibitory Siglecs such as Siglec-7 and Siglec-9. Interactions of cancer-associated sialoglycans can also regulate myeloid cells and tumor-associated macrophages by influencing the polarization of TAMs and potentially influencing macrophage-mediated phagocytosis via inhibitory Siglec receptors.

use of sialidases fused to tumor-targeting antibodies that, upon systemic application, mediate hyposialylation of the tumor microenvironment. Xiao et al. have used the anti-HER2 antibody trastuzumab fused with a bacterial sialidase which was shown to increase tumor cell killing *in vitro* (70) and is currently being tested in pre-clinical mouse models.

INTEGRINS DURING TUMOR CELL DISSEMINATION AND METASTATIC COLONIZATION

Integrin binding to the components of extracellular matrix (ECM) enables the cell "to sense" the environment and to activate intracellular signaling, which modulates cellular behaviors including survival, proliferation, and migration; thereby sustaining homeostasis. During malignancy, altered expression of integrins together with the loss of cell polarity profoundly changes the cell signaling, which alters oncogenic activity, cell stemness, epithelial plasticity, and angiogenesis [reviewed elsewhere (2, 71, 72)].

The integrins comprise a family of heterodimeric α/β integrin receptors, which facilitates contacts with components of the extracellular matrix (ECM) and in some cases with adhesion receptors on other cells. A particular integrin receptor with a preference for a specific ligand defines a cell based on the recognition of ECM (e.g., fibronectin, laminin, or collagen). Two receptors, vascular cell adhesion molecule 1 (VCAM1), and intracellular cell adhesion molecules (ICAMs) serve as cell surface receptors for integrins. Integrin-based adhesion of a cell facilitates intracellular adaptor proteins that recruit kinases, for example focal adhesion kinase (FAK) or Src family kinases; and induces signal transduction (2, 72, 73). Conversely, external signals (growth factors or cytokines) may change the intracellular recruitment of integrins resulting in the modulation of integrin affinity. Integrins can initiate pro-survival but also pro-apoptotic signals (74–76).

A variety of integrins expressed on tumors that originate from epithelial cells, typically facilitate cell adhesion to the basement membrane. Tumor cell surface expression of integrins can vary widely, but is generally associated with the enhanced presence of αvβ3, αvβ6, α5β1, α6β4 which correlates with the metastatic progression in melanoma, prostate, pancreatic, colon, lung, and breast cancers [reviewed in (73)]. Importantly, integrins within the tumor microenvironment present on endothelial cells, leukocytes, platelets, and other cells of the stroma significantly modulate tumor progression and particularly metastasis.

During the hematogenous phase of metastasis tumor induced platelet activation and the formation of platelet-and fibrin-rich tumor cell thrombi (**Figure 1**), are mediated both by integrins and selectins (18, 77–80). In particular, susceptibility to metastasis is associated with tumor cell-derived deposition of certain ECM proteins, such as tenascin C (TNC) in the metastatic niche. TNC is a ligand for β1- and β3-integrin and its accumulation in the lungs promotes tumor cell outgrowth and metastasis (81). Osteosarcoma metastasis to the lungs is dependent on TNC expression and the respective expression a receptor on tumor cell α9β1 integrin (82). The trabecular bone rich in TNC was shown to promote prostate homing of cancer metastasis through α9β1 integrin (83). Metastatic lung colonization was associated with an induction of stromal periostin expression that is recognized by β1 and β5 integrins on tumor cells (84).

Integrins also contribute to the formation of a tumor microenvironment. The systemic absence of β4 integrin resulted in the attenuation of tumor growth due to impaired angiogenesis (85). Tumor-induced lymphangiogenesis promotes metastasis to the lymph node through the activation of α4β1 integrin on lymphatic endothelium, which binds to VCAM1-positive tumor cells (86). In addition, myeloid cells expressing α4β1 integrins accumulate on the tumor-activated endothelium and promote metastasis (87). Antagonist of α4β1 integrin blocked the recruitment of myeloid cells and thereby angiogenesis and tumor growth. The aberrant expression of VCAM-1 on dormant tumor cells in bone marrow was shown to recruit α4β1-expressing osteoclast progenitors during bone metastasis (88). It has recently been demonstrated that chemoresistant disseminated tumors occupying the perivascular niche, interacts through β1-integrin with VCAM-1 on endothelial cells (89). The inhibition of β1-integrin or VCAM-1 sensitizes tumor cells to chemotherapy, making integrin inhibition a viable therapeutic approach to prevent metastasis.

Tumor cell expression of αvβ3 of α4β1 integrins is linked to bone metastasis, where they support tumor cell adhesion to ECM proteins such as osteopontin or type I collagen (90). Melanoma cells expressing α4β1 integrins metastasized to lymph nodes (**Figure 1**) by binding to VCAM-1 on lymphatic endothelial cells (91). Tumor cell $\alpha_3\beta_1$ integrin facilitates metastasis by binding to the exposed basement membrane protein laminin-5 in the lungs, thereby promoting tumor cell arrest and the onset of outgrowth (92, 93). Recently, integrins on tumor-derived exosomes were shown to drive the organotropism of tumor metastasis (94). Tumor-derived exosomes carrying α6β4 integrin, target a laminin-rich lung microenvironment, where they induce the accumulation of pro-inflammatory factors required for the promotion of tumor cell seeding and metastasis. While tumor cells do not express leukocyte-specific β2 integrins, for example LFA-1, tumor cells that express ICAM-1 facilitate their adherence to leukocytes, particularly neutrophils, through β2 integrins, which in turn bind to the endothelium, thereby promoting metastasis (95, 96). The role of integrins during cancer is dependent on cues in a tissue context-dependent manner.

Altered glycosylation of integrins on tumor cells modulate the intracellular signaling and cell adhesion (97, 98). An overexpression of branched β1,6-N-acetylglucosamine (GlcNAc) on N-glycans, which is catalyzed by a GnT-V enzyme, is associated with poor prognosis (99), while a knock-down of GnT-V in breast carcinoma cells result in reduced invasiveness (100). Increased branched N-glycans on α3β1 integrins in B16 mouse melanoma correlated with lung metastasis (101). Galectins are a family of β-galactoside-binding soluble lectins expressed by tumor cells. The N-glycan on integrin induces the complex formation of α6β4 integrin/EGFR/ galectin-3 which

promotes integrin clustering and cell migration and proliferation (102, 103). Galectin-3 induced αvβ3 integrin-mediated clustering was shown to cause tumor growth and drug resistance (104). Mechanistically, unbound αvβ3 integrin on tumor cells recruits KRAS to the cell membrane and activates downstream signaling through the NF-κB pathway and promotes pancreatic carcinoma. In hepatocellular carcinoma metastasis, O-glycosylation of β1 integrin influences tumor migration (105). Terminal sialylation on N-glycans and O-glycans of glycoproteins is frequently observed in cancer (44, 59, 106). Hypersialylation detected in colon, stomach, and ovarian cancers has been linked to an enhanced expression of α2,6-sialyltransferase (ST6Gal-I) and is identified as a marker of poor prognosis (106). In colon carcinoma, enhanced sialylation of β1 integrin facilitates adhesion to collagen I and the migration of tumor cells (107). Accordingly, inhibition of ST6Gal-I expression blocks collagen binding and tumor cell migration. Desialylation of O-glycans by sialidase NEU1 suppresses colon-tumor cell adhesion to laminin, tyrosine phosphorylation of integrin β4 and metastasis (108). However, in breast cancer cells the α2,6 hypersialylation of β1 integrin decreased the adhesion but did not affect invasiveness of these cells (109). These data indicate that glycosylation of integrins modulates adhesion, migration, and signaling of metastatic cells.

CONCLUSIONS AND FUTURE DIRECTIONS

Alterations in cell adhesion and cell-cell interactions of tumor cells are inherently linked to many processes associated with immune evasion and metastasis that go beyond the scope of this review. The very nature of adhesion receptors on cells to interrogate signals from outside makes the tumor microenvironment a crucial factor during immune evasion and metastasis. The biology of cell adhesion during cancer progression remains complex mainly due to: (a) several receptors likely act in parallel during any metastasis; (b) cell surface changes are linked to tumor heterogeneity; (c) diverse tumor glycosylation affect both receptors and ligands. Nevertheless, the potential to target cell adhesion mechanisms for tumor therapies is continuously being explored. For instance, fusion of a IL-2 cytokine with an Fc part of an antibody targeting RGD sequence of an integrin demonstrated promising results in mouse models when applied in combination with a PD-1 checkpoint blockade inhibitor (110). Another study has demonstrated that fucosylated nanoparticles can target irradiation-activated vasculature of the tumor, associated with enhanced P-selectin expression (111). Altered tumor glycosylation is a common culprit that contributes to tumor cell dissemination and immune suppression. Thus, further efforts to "edit" the tumor glycosylation landscape using sialidase and thereby changing the Siglec immune responsiveness or metastasis holds great potential in clinical applications (70). Nevertheless, further understanding of the tumor microenvironment is a prerequisite for designing an intervention on cell adhesion mechanisms that will be likely used in combination either with standard- or immune-therapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the SNF grant #310030-173076 to LB and #310030-184720 to HL.

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Conflict of Interest Statement: HL received travel grants and consultant fees from Bristol-Myers Squibb (BMS) and Merck, Sharp, and Dohme (MSD), and Roche. HL received research support from BMS and Palleon Pharmaceuticals.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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