TARGETING LIPID RAFTS AS A STRATEGY AGAINST INFECTION AND CANCER

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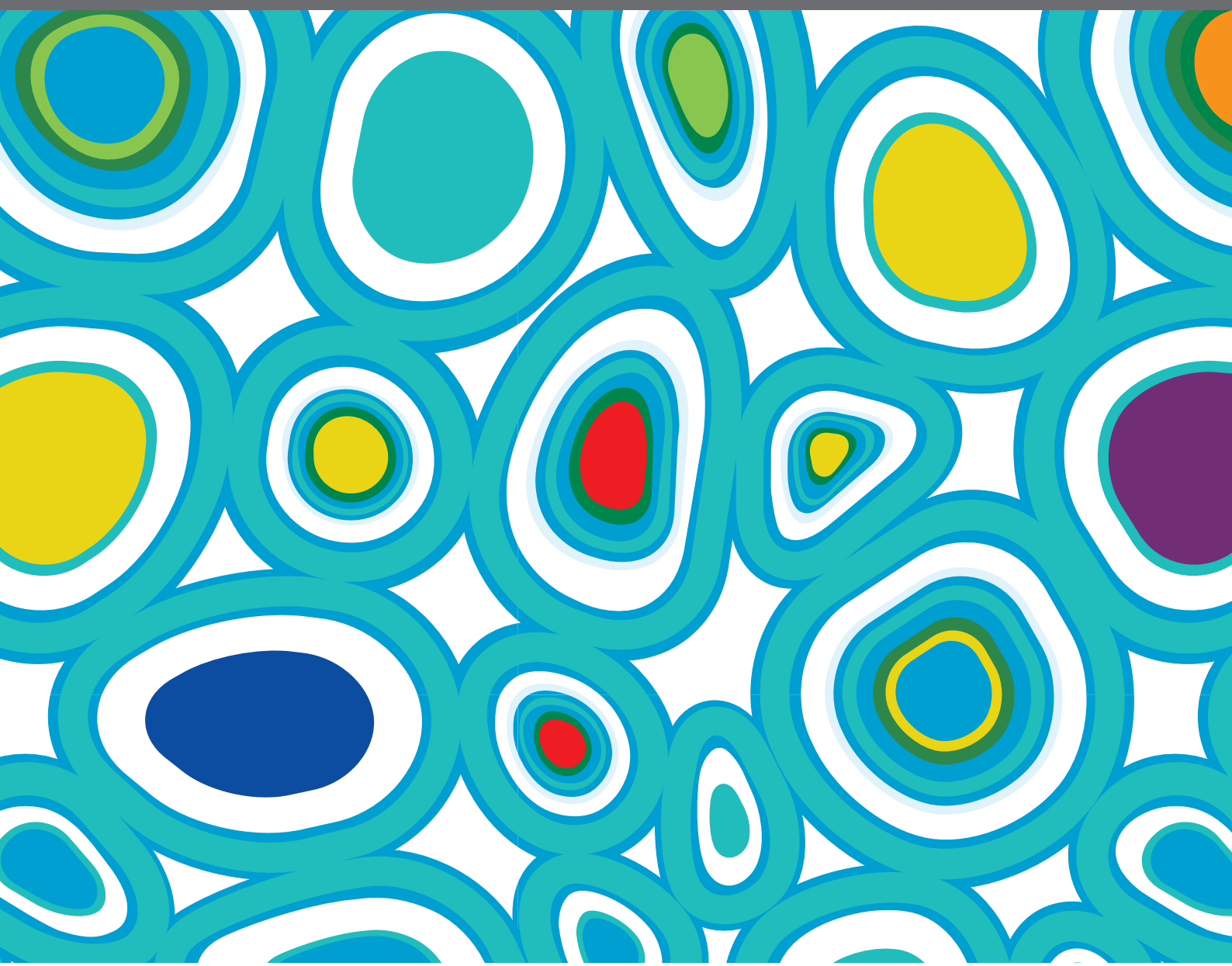

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TARGETING LIPID RAFTS AS A STRATEGY AGAINST INFECTION AND CANCER

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EDITORIAL
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Editorial: Targeting Lipid Rafts as a Strategy Against Infection and Cancer

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Editorial on the Research Topic

Targeting Lipid Rafts as a Strategy Against Infection and Cancer

The concept of specialized "membrane microdomains" (MM), often referred as lipid rafts, has extensively influenced the molecular biology of plasma membrane over the last decades. These cholesterol/sphingolipid-rich domains, play an essential role in the regulation of cellular processes including intracellular signaling, cell death, and redox homeostasis (Simons and Toomre, 2000; Mollinedo and Gajate, 2015). The involvement of MM in the pathogenesis of several conditions has been elucidated over the last years, leading to the development of innovative pharmacological approaches, specifically targeting their components, including both lipids and proteins. The specific interactions between the various classes of molecules give lipid rafts some peculiar properties, both physical and biochemical. Indeed, the physicochemical basis of raft hypothesis was derived by several studies on model membranes, where mixture of lipids, resembling the composition of the outer plasma membrane, segregates in liquid ordered and disordered domains with distinct characteristics (Brown and London, 1998; Simons and Vaz, 2004). The effects of different drugs on membrane properties were unveiled using artificial membranes, putting the basis for new therapeutic strategies based on modification of membrane biophysical properties (Peetla et al., 2009; Knobloch et al., 2015). Statins are the ideal example of how this innovative approach connects with the classical strategy based on membrane cholesterol depletion. Statins, a well-known class of cholesterol lowering agents, possess several pleiotropic effects (i.e., cholesterol-independent), including the ability to influence the organization of artificial and biological membranes (Wang et al., 2008; Redondo-Morata et al., 2016; Galiullina et al., 2019; Penkauskas et al., 2020). For this reason, they are increasingly used to enhance delivery and efficiency of chemotherapeutic drugs (Pinzon-Daza et al., 2012; Di Bello et al., 2020). In his minireview, Preta summarizes cancer therapeutic strategies based on altering membrane cholesterol/sphingolipid content as well on changing cancer membrane bilayer properties, as fluidity or thickness, with the final aim to increase sensitivity to cytotoxic drugs and defeat multidrug resistance. Vona et al. review provides an update on cholesterol-targeting strategies based on inhibition of its synthesis, modulation of its uptake and intracellular transport and on the possibility of therapeutic intervention in the treatment and/or prevention of certain types of cancer.

However, the exclusive properties of lipid rafts and their vital importance for the dynamics of a cell, make them susceptible to pathogens hijacking. Indeed, many steps of pathogen interaction with host cells rely on host lipid rafts and in several cases this interaction lead to microdomains modifications. During bacterial infections, many toxins interact with membrane rafts. Yeh et al. report the ability of *Campylobacter jejuni* cytolethal distending toxin (CDT) in reducing the effect of two other lipid rafts-binding cytotoxins, vacuolating cytotoxin A (VacA), and cytotoxin-associated

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gene A (CagA) involved in *H. pylori* disease progression. The authors demonstrate that CDT is capable to hijack cholesterol, competing with the other two toxins and drastically mitigating *H. pylori* pathogenesis. Mergani et al. outline the modifications of lipid rafts-dependent sorting of sucrose isomaltase during *Staphylococcus aureus* infection, elucidating the molecular mechanisms associated with the bacterial alterations of intestinal functions. Sucrose isomaltase is the major intestinal α-glucosidase responsible for catalyzing the hydrolysis of dietary carbohydrates and its deficiency/malfunction is often associated with gastro-intestinal symptoms.

In their review Sorice et al. describe how lipid rafts provides a multimolecular platform to segregate the angiotensin-converting enzyme (ACE-2) receptor, the main receptor of SARS-CoV-2. If we take into consideration the probable hypothesis that the fundamental contact for the entry of the virus into the host cell occurs at the level of these MM, lipid rafts-targeting drugs, alone or in combination with other compounds may play an antiviral role (Fecchi et al., 2020). Statins (by inhibition of cholesterol synthesis) or cyclodextrins (by depletion of membrane cholesterol), affecting cholesterol levels and disrupting lipid rafts, could effectively inhibit coronavirus adhesion and binding, preventing the progression of the virus (Baglivo et al., 2020). Indeed, according to recent observational studies, statins were effective in reducing the severity or mortality of COVID-19 (Kow and Hasan, 2020; Zhang et al., 2020). In the same way, cyclodextrins have been extensively used to increase stability, solubility and bioavailability of many drugs. From this point of view, one of the most interesting hypothesis is the one formulated in a recent letter which reports the mechanism of cyclodextrin- soluble angiotensin - converting enzyme 2 (CD-sACE2) inclusion compounds in the treatment of SARS-CoV-2 infections (Sun et al., 2020).

In conclusion the progress made in the last years in understanding the biological significance of lipid rafts is indisputable, however few gray areas remain to be further investigated including the interplay between lipids and membrane proteins in regulating membrane organization and how targeting these rafts proteins could provide new therapeutic strategies. Continuous advancement in microscopic techniques could largely contribute to this emerging investigational field and potentially allow a direct observation of membrane domains interactions *in vivo* (Levental et al., 2020). We hope that all the information reported in this Research Topic will be useful to researchers in this exciting field, and will push further studies aimed to test new effective membrane-lipid targeting agents. We want to acknowledge the great work of the authors, co-authors, and reviewers, and to thanks the support constantly received from Frontiers Team members.

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MINI REVIEW
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New Insights Into Targeting Membrane Lipids for Cancer Therapy

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Modulation of membrane lipid composition and organization is currently developing as an effective therapeutic strategy against a wide range of diseases, including cancer. This field, known as membrane-lipid therapy, has risen from new discoveries on the complex organization of lipids and between lipids and proteins in the plasma membranes. Membrane microdomains present in the membrane of all eukaryotic cells, known as lipid rafts, have been recognized as an important concentrating platform for protein receptors involved in the regulation of intracellular signaling, apoptosis, redox balance and immune response. The difference in lipid composition between the cellular membranes of healthy cells and tumor cells allows for the development of novel therapies based on targeting membrane lipids in cancer cells to increase sensitivity to chemotherapeutic agents and consequently defeat multidrug resistance. In the current manuscript strategies based on influencing cholesterol/sphingolipids content will be presented together with innovative ones, more focused in changing biophysical properties of the membrane bilayer without affecting the composition of its constituents.

Keywords: lipid rafts, membrane fluidity, cell signaling, amphiphilic molecules, protein receptors

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INTRODUCTION

Lipid-driven membrane organization is essential for the physiological functions of eukaryotic cells since it regulates a multitude of processes including intracellular signaling, redox balance and cell death (Muro et al., 2014; Santos and Preta, 2018). Behind these regulatory properties, there is the lipids capacity to laterally aggregate, forming highly dynamic and heterogeneous regions, referred to as lipid rafts. Lipid rafts are nanoscale membrane microdomains (<200 nm), particularly enriched in cholesterol and sphingolipids, that selectively recruit certain protein receptors (Simons and Toomre, 2000; Sezgin et al., 2017). Lipid rafts form microscopic domains (>300 nm) upon clustering induced by protein-protein or protein-lipid interaction. The raft model was supported by observation on artificial membrane models, demonstrating that certain lipids specifically tend to interact with others to generate large scale lateral domains (Simons and Vaz, 2004; Kaiser et al., 2009). The presence of plasma membrane specific organization has been observed across different organisms, ranging from bacteria to yeasts, providing further support for their biological significance (Kaiser et al., 2009; Henderson and Block, 2014; Lopez, 2015). Changes in the composition and organization of lipids have several effects on cellular functions, influencing signal transduction, membrane plasticity, and membrane trafficking. Plasma membrane cholesterol is one of the most important regulators of lipid organization, representing the majority (up to 90%) of the total cellular cholesterol and its levels in the cells are tightly regulated (de Duve, 1971; Lange et al., 1989). According to a recent study there are three pools of cholesterol in the plasma

membrane: a labile pool, depleted by cholesterol-targeting agents, a sphingomyelin-bound pool and an essential pool, necessary for cell viability (Das et al., 2014). Only the cholesterol not sequestered by proteins or lipids can be transported in the endoplasmic reticulum (ER) where it binds to specific sensors, shutting down cholesterol synthesis and uptake (Infante and Radhakrishnan, 2017). The pathway between cholesterol removal from plasma membrane and its subsequent transport to the ER represents a field of extensive investigation aimed to identify specific transporters involved in the regulation of cholesterol homeostasis. Recent studies identified Aster/GRAMD1 as essential transporters of cholesterol into ER and regulating the cellular uptake of HDL-derived cholesterol (Sandhu et al., 2018; Naito et al., 2019). ORP2 protein was also identified as a unique transporter of cholesterol from ER to the plasma membrane (Wang et al., 2019). There is no doubt that this recent progress in understanding cholesterol homeostasis and metabolism set the basis for the development of current therapies based on cholesterol and lipids targeting. A decrease in membrane cholesterol has been observed to have beneficial effects against different pathological condition including cancer and neurodegenerative diseases (Simons et al., 1998; Canevari and Clark, 2007; Guardia-Laguarta et al., 2009; Barros et al., 2018; Chen et al., 2018; Gu et al., 2019). Cholesterol-targeting can be achieved via cholesterol depletion, sequestration or inhibition of synthesis. The first effect is observed using cyclodextrins, a group of chemical compounds extracting cholesterol from the plasma membrane and widely used in the biomedical field in different experimental settings (Zidovetzki and Levitan, 2007; Lopez et al., 2013; Mahammad and Parmryd, 2015). Cholesterol sequestration is the mechanism used by different pore forming agents, by the antibiotic filipin, amphotericin, and nystatin (Bittman and Fischkoff, 1972; Silva et al., 2006; Kaminski, 2014). Cholesterol sequestration also effectively reduces the ability of cholesterol to interact with other membrane constituents. Statins, a widely used class of lipid-lowering medications are the best representatives of the inhibitors of cholesterol synthesis (Stancu and Sima, 2001; Kuipers and van den Elsen, 2007). These include other compounds like bisphosphonates or zaragozic acid acting at different levels of the mevalonate pathway (Amin et al., 1992; Griffin et al., 2017).

It is relevant to underline that few chemical compounds can affect the lipid membranes by different mechanisms. The dynamin inhibitor Dynasore has been shown to influence both cholesterol transport on the cell membrane and cholesterol concentration (Girard et al., 2011; Preta et al., 2015a,b). Beyond the cholesterol-lowering effects of statins, cholesterol-independent or pleiotropic effects are reported, including the capacity to modify plasma-membrane organization and structure (Wang et al., 2008; Penkauskas et al., 2020). Studies using artificial model membranes showed that statins alter the nanomechanical stability of the bilayers, intercalating the lipid-water interface and increasing membrane heterogeneity (Redondo-Morata et al., 2016; Galiullina et al., 2019). A better understanding of how therapeutic agents affect the membrane organization and composition, led in the last years to the development of a new field, named membrane-lipid therapy (MLT). MLT involves the identification and optimization of drugs capable to modify membrane lipid structures for pharmaceutical applications (Escriba, 2006; Escriba et al., 2015). Due to the essential role of the plasma membrane in many physiological processes, it is expected that MLT will provide new treatments for a wide range of diseases, including oncological disorders, neurodegenerative diseases, diabetes and stroke (Escriba, 2017).

MLT FOR CANCER THERAPY: A BRIEF OUTLINE

One of the hallmarks of cancer is the resistance to apoptosis and, more in general, the higher rate of proliferation versus death (Hanahan and Weinberg, 2000, 2011). The dynamicity of cell membranes plays an essential role in the regulation of cell surviving, through all the phases of a cell: lipid flexibility contributes to an increase in the mechanical stability during division and to a decrease of shear force during cell separation (Patra, 2008). To adapt rapidly, cancer cells re-organize their plasma membranes to preserve proliferation, escape apoptosis and resist to anticancer drugs treatment (Bernardes and Fialho, 2018). The latter is a crucial problem in anticancer therapy and often leads to multidrug resistance (MDR). Among many others, one of the causes of MDR is the decreased free diffusion of anticancer drugs through the plasma membrane. Therefore, the study and development of anticancer drugs capable to exert therapeutic effect by modulating the properties of tumor membranes, is constantly increasing (Rios-Marco et al., 2017; Zalba and Ten Hagen, 2017; Kopecka et al., 2020). The rationale for MLT is that there are fundamental differences in composition between normal and MDR cancer cells (**Figure 1**). MDR cells possess higher levels of total cholesterol as a result of an increased activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis (Kawata et al., 1990; Harwood et al., 1991). Additional studies reported an increase in mevalonate levels and in the expression of the low-density lipoprotein receptor compared to normal cells (Duncan et al., 2004; Kopecka et al., 2011). The observed higher amount of membrane cholesterol is responsible of a more rigid and less permeable membrane (Peetla et al., 2013; Niero et al., 2014). Moreover, MDR cells keep low ceramide levels by increasing sphingomyelin (SM) synthesis: this is an important anti-apoptotic strategy since implies a decrease in ceramide-enriched lipid rafts involved in the induction of cell death. Furthermore, phosphatidylserine (PS) and phosphatidylethanolamine (PE) which, under physiological conditions exist mainly in the inner leaflet of cell membranes have increased surface expressions on the outer membrane of tumor cells (Tan et al., 2017). The asymmetrical distribution of PS, maintained by a group of amino-phospholipid translocases that use ATP hydrolysis to flip PS from the external to the cytosolic leaflet, is also lost during the apoptotic process. The loss of PS asymmetry in cancer cells may be related to a reduced activity of these ATP-dependent phospholipid translocases or to an elevated activity of phospholipid scramblase, due to high levels of

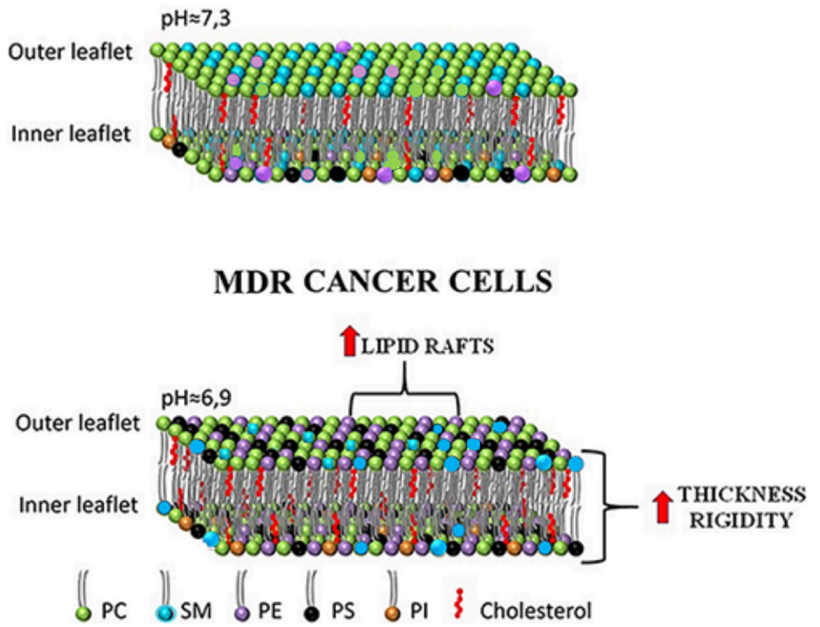

FIGURE 1 | Differences between membrane lipid composition and organization in normal vs MDR cancer cells. In cancer cells PS and PE, mainly confined in the inner leaflet of the membranes, are present in high concentrations in the outer leaflet. Cancer cells have also higher concentrations of cholesterol and consequently an increase in membrane thickness and rigidity is observed. Increased levels of saturated fatty acyl chains in membrane lipids have been associated to the presence of more lipid rafts while low amount of ceramide in MDR cells is a consequence of the low activity of SMase or of the increased SM levels. Changes in lipid composition of the outer membrane of cancer cells are also correlated to a more acidic extracellular pH.

intracellular calcium ($Ca^{2+}{}_i$) (Chen et al., 1999). PS on the outer membrane of tumor cells can be used as an effective target for cancer therapy (Ran et al., 2002; Riedl et al., 2011; Davis et al., 2019). The PS-targeting antibody bavituximab (Chalasani et al., 2015; Gerber et al., 2015; Grilley-Olson et al., 2018) and the PS-binding peptide/peptoid hybrid PPS1D1 (Desai et al., 2016; Desai and Udugamasooriya, 2017) have shown significant cytotoxic effects in cancer cells. Another strategy largely used in anticancer therapy is to entrap the drug in a specific carrier, which held the tumor-targeting property (Dass and Choong, 2006; Fanciullino and Ciccolini, 2009). For example, a cationic liposomal carrier, phosphatidylcholine-stearylamine (PC-SA), strongly binds and kills cancer cells through direct interaction with negatively charged surface-exposed PS (De et al., 2018). The anticancer properties of drugs like camptothecin and doxorubicin entrapped in PC-SA liposomes was demonstrated on cancer cell lines, both *in vitro* and in different mice models (De et al., 2018, 2020). These and other studies showed the potential use in MLT of PS-targeting vesicle alone or in combination with anticancer drugs (Blanco et al., 2014; Ayesa et al., 2017). PE represents another chemotherapeutic target on the membrane surface of cancer cells. Duramycin is a small tetracyclic peptide produced by the bacterium *Streptoverticillium cinnamoneus* and is closely related to cinnamycin produced by *Streptomyces* sp. (Iwamoto et al., 2007; Hullin-Matsuda et al., 2016). Both duramycin and cinnamycin are capable to bind to PE specifically into areas of membrane with high curvature, inducing trans-bilayer phospholipid movements that lead to cell death (Makino et al., 2003; Iwamoto et al., 2007; Hullin-Matsuda et al., 2016). Another group of interesting molecules are cyclotides, cyclic peptides which exert their biological activities by acting on cell membrane, binding to phospholipids containing PE headgroups. This binding is followed by an insertion that subsequently leads to membrane disruption and cell death as a result of pore formation (Wang et al., 2012). The increased levels of exposed PE on the outer membrane of cancer cell allow those membrane-active peptides to exert their cytotoxic effects without harming healthy cells. A third target for MLT is ceramide. Ceramide is present in small amounts in cell membranes, as intermediate in the metabolism of sphingolipids or as a result of sphingomyelinase activity, which produces ceramide from SM (Kartal Yandim et al., 2013; Peetla et al., 2013). Altered ceramide metabolism in cancer has been described as an effective drug resistance mechanism: tumors have low levels of ceramide by increasing SM synthesis or by preventing its degradation (Senchenkov et al., 2001; Lewis et al., 2018). One possible strategy is to increase ceramide membrane levels using short chain ceramide and use lipid rafts as platforms to enhance apoptosis, since in presence of an excess of ceramide, cholesterol is displaced from lipid rafts, inducing activation of Fas/CD95 pathway (Selzner et al., 2001; Stover and Kester, 2003; Stover et al., 2005; Chiantia et al., 2007). Ceramide levels

can also be increased by inhibiting the enzyme ceramidase (using the ceramide analogs B13, LCL-464 and KPB-27) or sphingosine kinase inhibitors (like N,N-dimethylsphingosine) (Bhabak and Arenz, 2012; Bhabak et al., 2013; Chen et al., 2014). Few reviews provide a complete list of compounds used in MLT based on regulation of ceramide levels (Lin et al., 2006; Kartal Yandim et al., 2013; Liu et al., 2013).

Activation of the Fas pathway is the target of treatment with different anticancer agents including Edelfosine, Miltefosine and Perifosine, lipid clustering agents promoting apoptosis (Gajate and Mollinedo, 2007; Gomide et al., 2013). Resveratrol, a common constituent of red wine, has been shown to have anti-tumor activity for its tendency to accumulate in lipid rafts and is mainly used in combination with death receptor agonists (Delmas et al., 2013). Azurin is a membrane-associated protein from *Pseudomonas aeruginosa*. Azurin and its derived peptide p28 have been intensively studied as an anticancer protein, down-regulating fundamental signaling pathways downstream of membrane receptors and affecting processes such as adhesion and invasiveness (Gao et al., 2017; Bernardes and Fialho, 2018). These effects are dependent on the caveolin 1 and ganglioside 1-mediated uptake of azurin, leading to alteration of lipid rafts; decrease in plasma membrane stiffness and in the number of ordered domains (Bernardes and Fialho, 2018). The increased sensitivity of cancer cells to chemotherapeutic agents like paclitaxel and doxorubicin in combination with azurin confirms that part of the anticancer effect of azurin occurs by altering the membrane properties and increasing the membrane permeability to anticancer drugs (Bernardes et al., 2018). However, the use of these peptides in MLT, has some limitations: they require further optimization to enhance their selectivity toward cancer cells and to decrease toxicity; in few cases their use alone or in combination with chemotherapeutic agents did not show any beneficial effect in clinical trials (Planting et al., 1993; Gills and Dennis, 2009; Cho et al., 2012; Gerber et al., 2018).

AMPHIPHILIC MOLECULES IN CANCER THERAPY

Changing the membrane bilayer properties such as intrinsic curvature, elasticity and fluidity is a characteristic of several amphiphilic molecules. Statins, beyond the classical cholesterol lowering effects have been shown to alter lipid organization of artificial membranes and cell membranes in a cholesterol-independent way (Redondo-Morata et al., 2016; Penkauskas et al., 2020). This property can be included among the pleiotropic effects of statins, which are behind many benefits observed during statin therapy (Banfi et al., 2017; Oesterle et al., 2017). According to an established hypothesis, the biological properties of statins depend on their localization in the cellular membrane due to their amphiphilic properties (Mason et al., 2005; Galiullina et al., 2017). Several recent studies investigated the interactions of different statins with phospholipid membranes and their influence on the membrane structure (Sahu et al., 2019; Sariisik et al., 2019; Penkauskas et al., 2020). Statins seem to bind and influence lipid membranes, possessing different average location into the bilayer (Galiullina et al., 2019). However, a clear connection between a determined statin and the capacity to interact and alter membrane bilayer properties cannot be fully established, mainly due to the different membrane models and experimental settings used. Clinical studies in cancer patients have suggested lower cancer mortality and less side effects with lipophilic statins compared to hydrophilic ones (Ahern et al., 2011; Ahmadi et al., 2018; Beckwitt et al., 2018). In the last years the anti-tumor activity of statins was remarkably improved by using statins formulated in different drug delivery systems (Coimbra et al., 2010; Alupei et al., 2015; Safwat et al., 2017a; Matusewicz et al., 2019). In many cases the drug delivery system includes the statin in combination with a chemotherapeutic agent as doxorubicin (Pinzon-Daza et al., 2012). Indeed, the incorporation of statins in nanoparticulate drug delivery systems not only increased statins cytotoxicity but also overcame the resistance of cancer cells against common chemotherapeutic agents (Safwat et al., 2017b). This field is continuously developing, trying to identify the best carrier capable to enhance drug loading capacity, stability and therapeutic activity. According to this point of view, chitosan nanoparticles (CSNPs) are an optimal choice since they possess low toxicity and immunogenicity and good levels of biodegradability (Prabaharan, 2015). In **Figure 2** are presented the different rationales behind the use of statins in cancer therapy for modulation of membrane lipids. An innovative strategy for treatment of oncological disorders is the use of amphiphilic drug-drug conjugates (ADDC), where an amphiphilic molecule, with high capacity to interact with and penetrate the lipid bilayer, is created by combining an hydrophilic anticancer drug with a hydrophobic one (Huang et al., 2014; Gao et al., 2018). Most of the times, this strategy overcomes the necessity to use a proper delivery system and since these two drugs have different pharmacokinetics, it is possible that these molecules induce synergistic pharmacological effects improving the therapeutic efficacy both *in vitro* and *in vivo*. However, ADDC is another example where the overall effect achieved in cancer therapy should not be only reconnected to the sum of the individual ones. For example, camptothecin-classical anticancer activity is related to binding to the topoisomerase-1 and DNA complex, while floxuridine, a derivative of 5-fluorouracil, is known for its high antitumor activity against cancer metastases. The combination of the hydrophobic camptothecin and hydrophilic floxuridine, used to enhance apoptosis in colon cancer cell lines creates amphiphilic molecules capable to alter the lipid bilayer properties (Hu et al., 2015). Additionally, changes in bilayer physical properties regulate membrane protein functions including the ones involved in the regulation of apoptosis (Lundbaek et al., 2010). Therefore, behind the individual molecular target of each chemotherapeutic agents, the potential effect on membrane bilayers, derived by the creation of amphiphilic molecules should be evaluated (Bruno et al., 2013; Kumar et al., 2015). A better understanding of the biological effects of chemotherapeutic drugs on lipid membranes is essential to overcome MDR since, as mentioned before, cancer cells rearrange lipid composition and organization to avoid apoptosis and resist anticancer drugs (Bernardes and Fialho, 2018; Rivel et al., 2019).

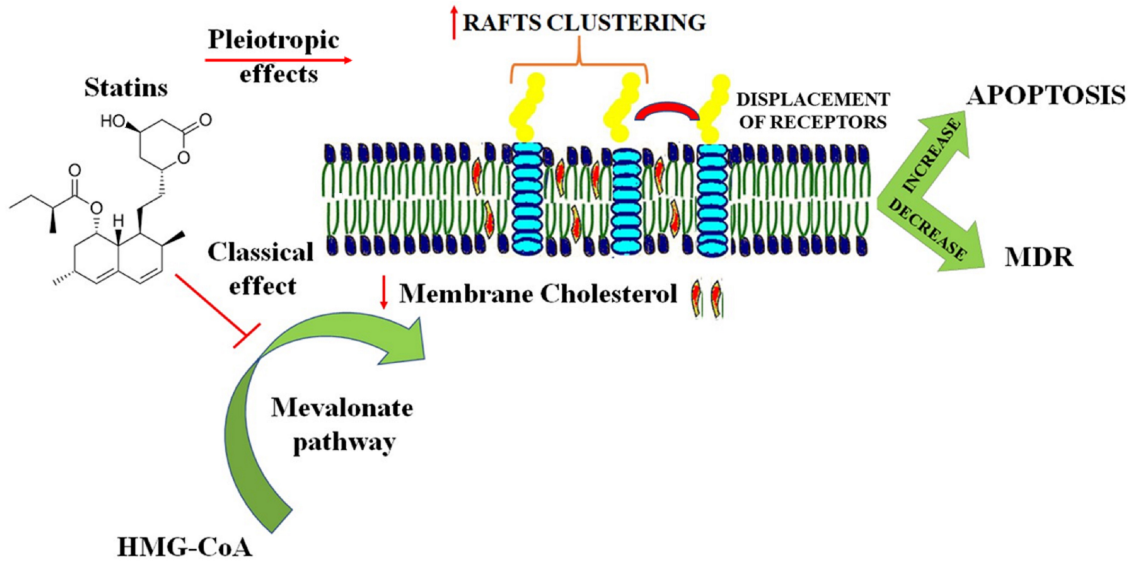

FIGURE 2 | Different rationales behind the use of statins in cancer therapy for modulation of membrane lipids. The beneficial effects of statins in cancer therapy are achieved by a combination of classical and pleiotropic effects. The classical cholesterol lowering activity decreases the cholesterol concentration in the plasma membrane influencing membrane fluidity and thickness. Pleiotropic effects include lipid rafts clustering and displacement of receptors in non-raft domains. All these effects contribute to an increase in apoptosis and in decrease in resistance of cancer cells to chemotherapeutic agents.

TABLE 1 | List of compounds described in this manuscript with the related mechanism of action.

Compound	Mechanism of Action	References
Cyclodextrins	Cholesterol depletion	Zidovetzki and Levitan, 2007; Lopez et al., 2013; Mahammad and Parmryd, 2015
Filipin, Amphotericin, Nystatin	Cholesterol sequestration	Bittman and Fischkoff, 1972; Silva et al., 2006; Kaminski, 2014
Statins	Cholesterol synthesis inhibition/Lipid organization	Stancu and Sima, 2001; Kuipers and van den Elsen, 2007; Wang et al., 2008; Penkauskas et al., 2020
Dynasore	Cholesterol transport inhibition/Cholesterol depletion	Girard et al., 2011; Preta et al., 2015a,b
Bavituximab, PPS1D1, PC-SA	Binding to phosphatidylserine (PS)	Chalasani et al., 2015; Gerber et al., 2015; Desai et al., 2016; Desai and Udugamasooriya, 2017; De et al., 2018; Grilley-Olson et al., 2018
Duramycin, Cinnamycin	Binding to phosphatidylethanolamine (PE)	Iwamoto et al., 2007; Hullin-Matsuda et al., 2016
Cyclotides	Binding to phospholipids containing PE headgroups	Wang et al., 2012
Short chain ceramide	Increase ceramide membrane levels	Selzner et al., 2001; Stover and Kester, 2003; Stover et al., 2005; Chiantia et al., 2007
B13, LCL-464, KPB-27	Inhibition of ceramidase	Bhabak and Arenz, 2012; Bhabak et al., 2013
N,N-dimethylsphingosine	Inhibition of sphingosine kinase	Chen et al., 2014
Edelfosine, Miltefosine	Lipid rafts clustering	Gajate and Mollinedo, 2007; Gomide et al., 2013
Resveratrol, Azurin, p28 peptide	Lipid rafts organization	Delmas et al., 2013; Gao et al., 2017; Bernardes and Fialho, 2018
Amphiphilic drug-drug conjugate	Membrane lipid organization	Huang et al., 2014; Gao et al., 2018
DHA	Lipid rafts clustering/Membrane fluidity, permeability	Shaikh et al., 2009; Turk and Chapkin, 2013; Wassall et al., 2018
EGCG	Lipid rafts clustering	Tsukamoto et al., 2012; Huang et al., 2015

DIETARY MODIFICATION OF MEMBRANE LIPIDS

Several clinical studies have strongly indicated a role for fish oil and polyunsaturated fatty acids (PUFA) in cancer prevention (Caygill et al., 1996; Azrad et al., 2013). One of the main lipids present in fish oil, docosahexaenoic acid (DHA), has been shown to alter plasma membrane properties including membrane fluidity, phase behavior and permeability (Yang et al., 2011; Levental et al., 2016; Bie et al., 2020). Moreover, different studies shown that DHA can influence lipid rafts composition, altering their size or clustering capacities and consequently

affecting lipid raft-regulated signaling (Shaikh et al., 2009; Turk and Chapkin, 2013; Wassall et al., 2018). Studies in mice fed with a PUFA-enriched diet shown that the molecular targets of DHA are cholesterol and sphingomyelin, two essentials building blocks of lipid rafts (Fan et al., 2003, 2004). These properties can have beneficial effects in anti-cancer therapy since behind the modification of membrane lipids, there is also a regulation of the protein receptors enriched in these membrane microdomains. For example, in breast cancer cell lines, DHA was found to influence epidermal growth factors receptors function and to enhance chemotherapy efficacy, by inducing CD95 translocation into lipid rafts, while in colon cancer cell lines it was responsible for an increase in oxidative stress and in TRAIL-induced apoptosis (Ewaschuk et al., 2012; Skender et al., 2014; Pettersen et al., 2016). These and many similar studies demonstrated two bullet-points: (1) lipid rafts play a functional role during tumorigenesis of different types of cancer (2) a therapeutic role for PUFA, since these fatty acids alter lipid raft structure/organization/function. The role for PUFA in prevention and treatment of cancer is wide and well documented, but the real efficacy of PUFA is still debated. Indeed, it is not fully established whether dietary PUFAs are integrated into raft lipids or whether their low affinity to cholesterol causes phase separation from rafts and, consequently, displacement of raft proteins (Yaqoob and Shaikh, 2010). Currently, they are mainly used in combination with different cytotoxic drugs to enhance chemotherapy efficiency (Granci et al., 2013; Newell et al., 2019). A compound reported to induce lipid rafts clustering is epigallocatechin-3-gallate (EGCG) the major polyphenol of green tea with chemo-preventives and chemo-therapeutic activities (Surh, 2003; Yang et al., 2009). However, the overall effect seems to be dependent by the tumor type since this polyphenol was observed to induce apoptosis in multiple myeloma cells (Tsukamoto et al., 2012; Huang et al., 2015), while in colon adenocarcinoma cells it increased cell viability and proliferation (Pajak et al., 2011). This discrepancy is probably related to the fact that EGCG modulates a wide spectrum of molecular targets including epidermal growth factor receptor, mitogen activated protein kinase and cyclin-dependent kinases (Khan et al., 2006; Ma et al., 2014; Fang et al., 2015). Therefore, there is not always a unique pattern of response to disruption of lipid rafts or to depletion of cholesterol from the membrane and each treatment should be evaluated in the context of the particular type of cancer and also of the specific therapeutic strategy adopted. In the last years, many scientists became interested in the evaluation of the synergistic effects of the combination of EGCG and anticancer compounds. For example, Fujiki et al. (2015) showed that the combinations of EGCG or other green tea catechins and 46 anticancer drugs synergistically induced *in vitro* anticancer effects in 58 different human cancer cell lines. Therefore, EGCG is a natural compound with proven beneficial effects both in cancer prevention and cancer therapy in combination with anticancer compounds (Fujiki and Suganuma, 2012; Fujiki et al., 2015). In **Table 1** is presented the list of compounds described in this manuscript with the main mechanism of actions for MLT.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The better understanding of membrane lipid composition and organization gained in the last years, together with the lipidic alterations reported in tumor membranes, provides a big opportunity for cancer prevention and treatment. Nowadays, the strategy to modify membrane cholesterol/sphingolipids content is gradually replaced by a more focused approach on the modulation of membrane bilayer properties, including fluidity and elasticity, by inducing changes in the organization of lipid rafts. Rafts proteins have also an essential role in regulating lipid properties and a future field of study in MLT could be the investigation of how changes in the structural composition of raft proteins influence lipid microdomains organization. The lack of attention toward targeting these proteins as a strategy for MLT is quite surprising and it is related to the consideration of these membrane proteins as merely guests rather than as active components of lipid rafts. Further studies on these protein-lipid interactions may lead to a better understanding of the molecular mechanism of raft domains organization and may provide new strategies for their manipulation. The final aim of this modulation in cancer therapy is to increase the overall efficiency of chemotherapeutic agents, achieving a synergistic effect and defeating MDR. Studying and testing membrane-lipid targeting agents in combination with chemotherapeutic agents is a promising and innovative approach for the development of new therapeutic strategies.

AUTHOR CONTRIBUTIONS

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

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frontiers in Cell and Developmental Biology

REVIEW
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Targeting Lipid Rafts as a Strategy Against Coronavirus

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Lipid rafts are functional membrane microdomains containing sphingolipids, including gangliosides, and cholesterol. These regions are characterized by highly ordered and tightly packed lipid molecules. Several studies revealed that lipid rafts are involved in life cycle of different viruses, including coronaviruses. Among these recently emerged the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The main receptor for SARS-CoV-2 is represented by the angiotensin-converting enzyme-2 (ACE-2), although it also binds to sialic acids linked to host cell surface gangliosides. A new type of ganglioside-binding domain within the N-terminal portion of the SARS-CoV-2 spike protein was identified. Lipid rafts provide a suitable platform able to concentrate ACE-2 receptor on host cell membranes where they may interact with the spike protein on viral envelope. This review is focused on selective targeting lipid rafts components as a strategy against coronavirus. Indeed, cholesterol-binding agents, including statins or methyl-β-cyclodextrin (MβCD), can affect cholesterol, causing disruption of lipid rafts, consequently impairing coronavirus adhesion and binding. Moreover, these compounds can block downstream key molecules in virus infectivity, reducing the levels of proinflammatory molecules [tumor necrosis factor alpha (TNF-α), interleukin (IL)-6], and/or affecting the autophagic process involved in both viral replication and clearance. Furthermore, cyclodextrins can assemble into complexes with various drugs to form host–guest inclusions and may be used as pharmaceutical excipients of antiviral compounds, such as lopinavir and remdesivir, by improving bioavailability and solubility. In conclusion, the role of lipid rafts-affecting drugs in the process of coronavirus entry into the host cells prompts to introduce a new potential task in the pharmacological approach against coronavirus.

Keywords: coronavirus, SARS-CoV-2, lipid rafts, statins, cyclodextrins

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CORONAVIRUSES

Coronaviridae family is morphologically characterized by a crown shape deriving from the presence on the envelope of a 20-nm long protein called "spike" (Cong and Ren, 2014; Tortorici and Veesler, 2019). It is possible to divide the coronaviridae family into four genera: α, β, γ, and δ coronaviruses. To date, there are 46 known coronaviruses species (ICT, 2019) that infect several hosts, including humans, mammals, birds, and other animals; in particular, when considering humans, they are infected mainly by both α- and β-coronaviruses (Geng et al., 2020). β-Coronaviruses can be further

subdivided into five subgenes, namely, Embecovirus, Hibecovirus, Merbecovirus, Nobecovirus, and Sarbecovirus (ICT, 2019; Jeong-Min et al., 2020).

Coronavirus is an RNA virus with positive single-stranded RNA. Regarding the virus structure, this family is characterized by a lipid coating deriving from the host called "envelope" and a nucleocapsid in which the genetic material is contained. The envelope, in addition to the transmembrane glycoprotein named M and the envelope protein (E), contains the spike glycoprotein involved in the process of recognizing the host cell. Spike protein differs for point mutation in coronaviruses (Wang Q. et al., 2020; Yan et al., 2020). Moreover, the nucleocapsid is helical in shape and consists of a positive polarity polyadenylated RNA molecule equipped with CAP and associated with protein N.

The mechanisms of coronaviruses entry are complex and differ among coronavirus species and strains. Coronaviruses entry can occur by direct fusion at the cell surface after binding to the receptor or after internalization via endocytosis with fusion taking place in the endosomal compartment (Belouzard et al., 2012; Wędrowska et al., 2020). The main mechanism of coronaviruses entrance is based on spike protein that is the primary determinant of cell tropism. Spike is a class I transmembrane protein, synthesized as a precursor protein with a typical size ranging from 1,200 to 1,300 amino acids (Wang Q. et al., 2020). The fusion mechanism of viral membranes with host membranes is related to conformational changes of the spike protein (Belouzard et al., 2012; Wędrowska et al., 2020). In particular, several coronaviruses may enter directly from the cell surface, when receptor-bound viruses are treated with proteases activating S proteins. This process generates homotrimers on the virion surface triggering the early fusion pathway. Alternatively, coronavirus may be endocytosed within the endosome where the low pH activates cathepsin L, cleaving S2′ site, triggering the fusion pathway, and releasing the coronaviruses genome (Tang et al., 2020).

In November 2019, a new virus named SARS-CoV-2, belonging to the Coronaviridae family, appeared in Wuhan for the first time. In March 2020, the World Health Organization (WHO) declared pandemic the viral disease caused by this virus. SARS-CoV-2 virus has been isolated from nasopharyngeal and oropharyngeal samples from patients affected with a flu-like disease (Jeong-Min et al., 2020). To date, there are several hypotheses on the SARS-CoV-2 origins; the most accredited hypothesis by scientists regard the transmission from wild animals to humans. In fact, several wild animals serve as a reservoir for new coronaviruses; these include bats, pangolins, and others. In a recent work, Lam et al., by metagenomic sequencing, have identified some new coronaviruses isolated from the pangolin that show a high similarity with SARS-CoV-2 in the receptor binding domain (Lam et al., 2020).

Coronaviruses that infect humans are involved in acute respiratory diseases, including colds, pharyngitis, nasal congestion, as well as, in some cases, headache, cough, muscle pain, and fever.

The clinical courses of infected hosts may be vary, ranging from asymptomatic, mild symptoms, or severe symptoms and cause respiratory, enteric, hepatic, and neurological diseases (Monchatre-Leroy et al., 2017; Cui et al., 2019). At present, seven types of coronavirus are known as inducing infections in humans. In particular, the species HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 usually cause mild symptoms, whereas SARS-CoV-2, SARS-CoV, and Middle East respiratory syndrome coronavirus (MERS-CoV) are able to cause severe respiratory disease like pneumonia and death (de Wit et al., 2016; Corman et al., 2018; Walls et al., 2020).

Infected people can be asymptomatic or present a flu-like disease with an incubation period that can vary from 2 to 14 days during which the individual is able to transmit the virus. From current data, it has been estimated that in 3–15% cases, the virus can lead to a severe respiratory disease as pneumonia and cause death. The large majority of deaths is represented by elderly people over 70 years of age and with comorbidities.

The emergency caused by the SARS-CoV-2 infection in Wuhan has spread to many countries and forced the WHO to declare a pandemic in March 2020. The SARS-CoV-2 infection is currently underway and is exponentially developing especially in USA, Europe, South America, Russia, and India, recording more than 2,000,000 deaths.

LIPID RAFTS

Lipid rafts are highly dynamic structures that can play a key role in pathogens–cell interactions, including coronaviruses–host cell (Carotenuto et al., 2020; Fecchi et al., 2020).

Lipid rafts are functional membrane microdomains that contain sphingolipids and cholesterol. These regions are characterized by a highly ordered and tightly packed lipid molecules compared to the surrounding bilayer (Simons and Ikonen, 1997; Wang and Silvius, 2001). It has been estimated that the size of lipid raft is around 10–200 nm (Pralle et al., 2000) in a dynamic conformation, since they can combine to form larger raft domains.

Domain properties such as composition, size, and lifetimes have been thoroughly investigated (Levental and Veatch, 2016; Sezgin et al., 2017; Levental et al., 2020). The distribution of lipid rafts in cell membranes can vary greatly, from small isolated domains to larger coalescing rafts, depending on a variety of factors, including cell type, specific condition, and type of membrane (e.g., plasma membrane or intracellular membrane). Thus, lipids rafts can be considered like nanodomains enriched in the plasma membrane that can coalesce, forming microdomains platforms for proper cell functioning.

The advancement of technology made it possible to exploit some crucial characteristics of lipid rafts. In fact, since lipid rafts are relatively resistant to non-ionic detergents, such as Triton X-100 (Brown and London, 1998, Raggi et al., 2019), and they are present in low-density fractions after density centrifugation, many authors refer to lipid rafts also as glycolipid enriched and insoluble or detergent-resistant membrane complexes (DRMs) (Simons and Ikonen, 1997).

These characteristics of lipid domains are mainly related to their cholesterol content. In fact, it has been shown that cholesterol sequestering agents selectively destroy rafts. Thus,

the use of cholesterol sequestering molecules is a useful tool for identifying proteins as components of the lipid raft or simply copurified contaminants (Foster, 2009), as well as for determining the role of rafts in modulating cellular processes (Mattei et al., 2015; Martellucci et al., 2019).

Noteworthy, these lipid domains show a peculiar fluidity, which allow lateral assembly and rapid reorganization upon diverse biological stimuli. Some molecules associate/dissociate from rafts in a regulated manner depending on their state of activation. These clusters allow the formation of highly efficient lipid–protein molecular associations that operate in several important cellular processes, including membrane trafficking, cell signaling, cell migration, and axonal guidance (Lingwood and Simons, 2010; Sezgin et al., 2017). This structure can concentrate membrane-associated proteins as receptors and molecules involved in signaling pathways (Levental et al., 2010; Martellucci et al., 2018; Mattei et al., 2020; Riitano et al., 2020). Of interest, in polarized cells, lipid rafts show a characteristic sorting on apical surface able to segregate distinct functional proteins, whereas in non-polarized cells, they are distributed randomly on the cell surface.

Lipid rafts play important roles in innate and adaptive immunity; in T lymphocytes, rafts are enriched in many receptors and signaling molecules and participate in T-cell receptor (TCR) triggering and T-cell activation (Varshney et al., 2016; Robinson et al., 2017; Nakayama et al., 2018).

Thus, lipid rafts are thought to function as platforms that recruit specific proteins or concentrate some specific components and exclude others (Wang and Silvius, 2001; Pizzo and Viola, 2004; Pizzo et al., 2004), thus initiating and controlling cell signaling (Simons and Ikonen, 1997; Barbat et al., 2007). Lipid rafts have been proposed to mediate multiple stages of apoptosis (Sorice et al., 2012), including the recruitment of the different key molecules involved in the process, including Fas and the tumor necrosis factor receptor (TNF-α-R) (Garcia-Ruiz et al., 2002; Legler et al., 2003), as well as protein recruitment of the Bcl-2 proapoptotic family, including truncated Bid, t-Bid, and Bax, following the trigger of Fas (Scheel-Toellner et al., 2002).

Lipid rafts are not merely confined to the plasma membrane. In fact, as reported by numerous studies, lipid microdomains are formed similarly in the subcellular organelles, such as Golgi, ER, or mitochondria, termed as *raft-like microdomains* (Garofalo et al., 2005). In particular, functional studies suggest that mitochondrial lipid microdomains participate in the mitochondrial network of fusion and fission during remodeling, as well as in the regulation of cell fate, i.e., survival or death through activation of intracellular signaling (Ciarlo et al., 2010, 2018; Matarrese et al., 2014; Garofalo et al., 2018). Interesting emerging data establish that the interaction of the ER with the mitochondria occurs through endoplasmic reticulum (ER)–mitochondria-associated membrane (MAM) subdomains, and this interaction allows the membrane scrambling, contributing to the multiple functions of ER (Raturi and Simmen, 2013). Since some components of lipid microdomains are present within MAM subdomains (Sano et al., 2009; Garofalo et al., 2016), several authors assume a key role of these subdomains in regulating and influencing a variety of cellular activities (Annunziata et al., 2018), including the early stages of autophagosome formation in mammalian cells (Hamasaki et al., 2013; Garofalo et al., 2016). They are also enriched in caveolin-1 (Sala-Vila et al., 2016), lipid synthesis enzymes (Vance, 1990; Vance et al., 1997), and cholesterol (Area-Gomez et al., 2012; Fujimoto et al., 2012). This particularity suggests that these areas act as non-vesicular lipid transfer sites between ER and mitochondria. In recent years, it has become evident that a complex network of lipid–lipid and lipid–protein interactions contributes to protein sorting and intracellular transport. The hypothesis that the Golgi system sorts the proteins and sends them to the plasma membrane through preferential membrane sites such as lipid rafts, dates back to 1988 (van Meer and Simons, 1988). Moreover, host lipid rafts have been reported to be critically involved in apical targeting, assembly, and virus budding. In this case, the subcellular distribution of lipid raft on internal membranes, including the Golgi apparatus or the ER, has a significant impact in the sorting of proteins and in the trafficking and overall exocytosis of viral proteins, which constitute fundamental steps to support viral infection (Takeda et al., 2003; Von Blume and Hausser, 2019; Stalder and Gershlick, 2020).

Furthermore, at the cellular level, rafts and related membrane microdomains, such as caveolae, characterized by a high expression of caveolin-1, have been proposed to play important roles in the sorting of membrane and non-membrane molecules (Browne and London, 2000; Parton and Richards, 2003). In fact, the study of caveolar platform has been proposed as a potential target to inhibit the entry of SARS-CoV-2 (Filippini and D'Alessio, 2020).

Functionally, lipid rafts host exo-/endocytosis molecular apparatuses that form the functional communication platforms inside and outside the cell (Manes et al., 2003). Thermodynamically, it would be energetically challenging due to the stiff and efficiently packed nature of lipid rafts owing to the fact that fusion mechanism involves processes like membrane bending and non-bilayer lipid intermediates, requiring substantial flexibility of membrane structures (Dadhich and Kapoor, 2020). Thus, Yang et al. (2015) proposed the role of the edges of raft domains, rather than the bulk region, as the preferred sites for fusion. Later on, they verified the mechanisms of fusion to be driven by the effect of hydrophobic mismatch at the edges of raft and not raft (liquid ordered–liquid disordered) domains. Although we cannot refer to the lipid raft as an area dedicated to endocytosis, however, many endocytic (and exocytic) mechanisms involve the lipid rafts to some extent (Pelkmans and Helenius, 2002). Many viruses, including SARS-Cov-2, can enter into the host cells by receptor-dependent endocytosis. One of the best characterized pathways is the clathrin-dependent one, based on viral entry and translocation into endosomes where they are degraded or recycled (Wang et al., 2019). Alternatively, a caveolae-dependent pathway may be used. Caveolae are small invaginations of the plasma membrane that are composed of cholesterol, glycosphingolipids, and caveolin (Filippini and D'Alessio, 2020). Caveolin is able to oligomerize, leading to the formation of caveolin-rich microdomains in the plasma membrane, and subsequently, the caveolar vesicles

may fuse with the early endosomal compartment. For instance, coronavirus infection may employ distinct endocytic pathways in the upper and lower respiratory tract related to different signaling molecules. Indeed, a large GTPase, dynamin, which is required for endocytosis, is abundant in the nasal epithelium but undetectable in pneumocytes (Glebov, 2020).

Lipid rafts have been shown to be exploited by intracellular pathogens at different times of the infectious process, as a gateway to the cell. Indeed, many steps of pathogen interaction with host cells, and sometimes all steps within the entire lifecycle of various pathogens, rely on host lipid rafts (Bukrinsky et al., 2020). In addition, the activation of the innate and acquired immune responses by the hosts is regulated by the rafts in many crucial steps; in this regard, some pathogens have the ability to shut down the immunological response by altering the cholesterol content of the lipid raft (immune evasion) (Mackenzie and Khromykh, 2007 and Sen et al., 2011). Possibly, a similar strategy could be shared by SARS-CoV2.

ROLE OF LIPID RAFTS IN THE PROCESS OF CORONAVIRUS ENTRY INTO THE CELLS

Several studies pointed out the key role of lipid rafts during viral infection. Indeed, lipid rafts are involved in different stages of the life cycle of different viruses, including dengue and hepatitis C viruses (Aizaki et al., 2004). Moreover, lipid rafts contribute to the binding and entry of several viruses to host cells, such as human immunodeficiency virus (HIV) (Viard et al., 2002), human herpes virus 6 (Huang et al., 2006), poliovirus (Danthi and Chow, 2004), West Nile virus (Medigeshi et al., 2008), foot-and-mouth disease virus (Martin-Acebes et al., 2007), and simian virus 40 (Parton and Lindsay, 1999). Coronaviruses also interact with lipid rafts for cellular entry (Nomura et al., 2004; Choi et al., 2005; Liao et al., 2006; Li et al., 2007; Pratelli and Colao, 2015; Hu et al., 2016) (**Figure 1**). The functional role of lipid rafts in this process was supported by the observation that cholesterol depletion prevented coronavirus entry into host cells (Thorp and Gallagher, 2004). Lu et al. reported that lipid rafts are crucial for SARS-CoV entry into cells (Lu et al., 2008). Virus envelope contains the major attachment spike protein (S), the membrane protein (M), and the minor envelope protein (E). Spikes are composed of S protein trimers, which are involved in viral attachment, as well as in the subsequent fusion of viral with cellular membranes (Yang et al., 2012). The S protein comprises two subunits: S1 and S2. Subsequently, the S protein is cleaved by receptor transmembrane serine protease 2 (TMPRSS2) (Hoffmann et al., 2020), a predominantly raft-resident protein (Ballout et al., 2020), with the help of FURIN precleavage, which facilitates the entry of the virus into the cell after binding (Tay et al., 2020). Furin has been found in small fraction on the cell surface, while the predominant amount is in Golgi network (Coutard et al., 2020). Once spike activation has been promoted, virus enters host cells

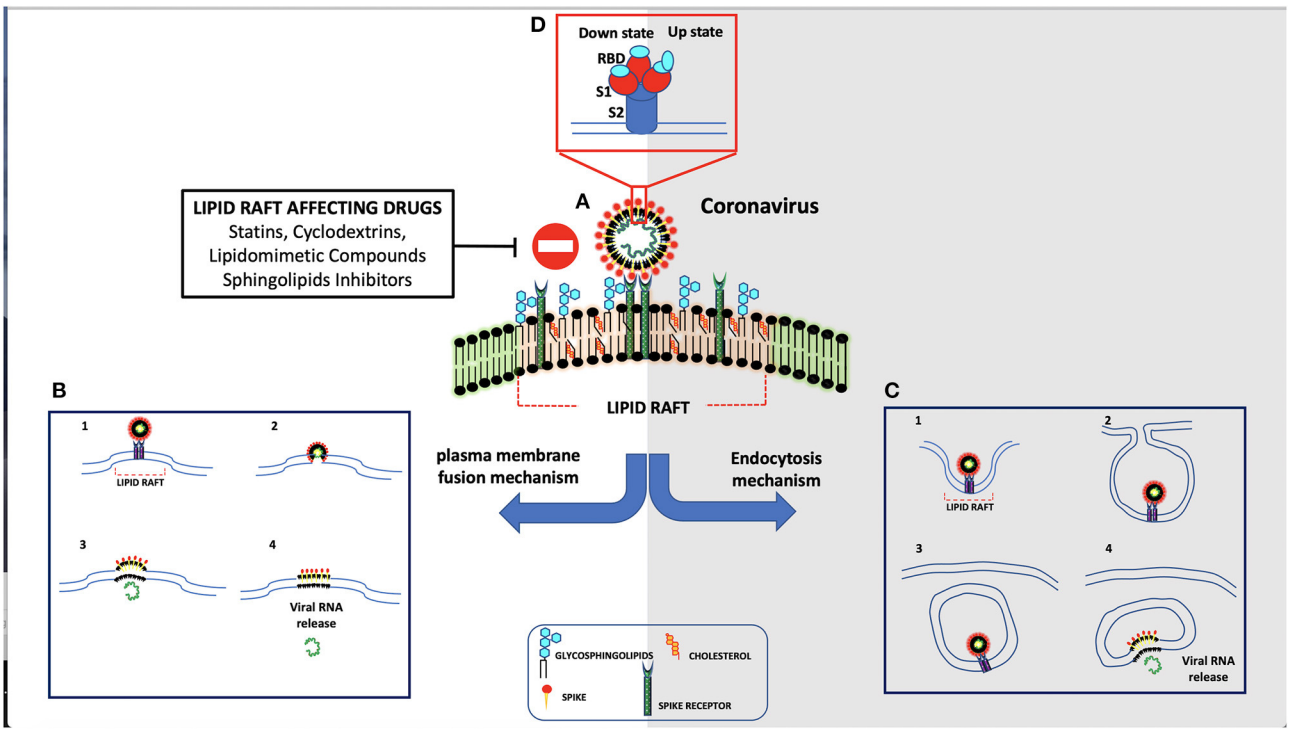

FIGURE 1 | Schematic representation of coronavirus entry mechanism. **(A)** Spike protein interacts through its receptor-binding domain (RBD) with ACE-2 receptor. **(B)** As result of their interaction, spike is activated by human proteases and is internalized by direct fusion with plasma membrane. **(C)** In the absence of proteases, the virus is endocytosed.

through specific interactions involving cellular surface receptors and viral structural proteins, the viral interactome.

The main receptor for SARS-CoV-2 is represented by the angiotensin-converting enzyme-2 (ACE-2) (Mathewson et al., 2008), a type I transmembrane metallocarboxypeptidase, with its enzymatically active domain exposed on the cell surface. The binding with ACE-2 receptor may facilitate virus surface S1 subunit proteolysis by plasma-membrane-bound serine protease TMPRSS2 and Cathepsine L (CatL), which may be associated with caveolae (Gopal et al., 2006). Once the SARS-CoV-2 reaches intracellular endosomes, CatL becomes the major protease that cleaves the virus S1 subunit (Liu C. et al., 2020). ACE-2 is present on non-immune cells, including endothelial cells, respiratory and intestinal epithelial cells, kidney cells, cerebral neurons, and alveolar monocytes/macrophages. In particular, the ability of SARS-CoV-2 to infect human cells seems to depend by its interaction with human ACE-2 by gln493 residue. However, the S protein uses not only the ACE-2 receptor for entry but also sialic acids linked to host cell surface gangliosides. Indeed, a new type of ganglioside-binding domain within the N-terminal domain of the SARS-CoV-2 S protein has been identified. This domain (111–158), which is a highly conserved sequence, may be responsible for attachment of the virus to lipid rafts, thus facilitating contact with the ACE-2 receptor (Fantini et al., 2020). In particular, ACE-2 is largely colocalized both with raft markers GM1 and caveolin-1. Coronaviruses may enter the host cells either by direct membrane fusion with the plasma membrane or by receptor-mediated endocytosis (Manes et al., 2003). In both processes, lipid rafts play a key role, since they concentrate components of the membrane docking and fusion machinery for endocytosis, such as actin polymerization, which is important for the membrane fusion and endocytosis. When these proteins are concentrated within lipid rafts, their intermolecular interactions are highly facilitated (Nicolau et al., 2006), since partitioning of protein into lipid rafts increases specific interprotein collision rates. Thus, lipid rafts may represent plasma membrane "chambers," able to increase protein interactions on the plasma membrane and, in turn, increase the collision rate and consequently the efficiency of membrane reactions. In particular, lipid rafts may provide suitable platforms able to concentrate ACE-2 receptor on host cell membranes where they may interact with the S protein on viral envelope. Only in the "open" S conformation, RBD engages PD of ACE-2, and the complex may involve a dimeric ACE-2 that accommodates two S protein trimers (Yan et al., 2020). A clustering of ACE-2 in certain areas of the membrane may allow multivalent binding of virus particles to the cell surface. In this way, microdomains may increase the efficiency of infection but are not an absolute requirement for the entry process. This explanation is in agreement with the finding that cholesterol depletion reduces the susceptibility to infection but does not abolish it (Glende et al., 2008).

In addition, methyl-β-cyclodextrin (MβCD) and mevastatin-induced disruption of lipid rafts inhibited infectious bronchitis virus infection, suggesting that lipid rafts are involved in viral attachment (Guo et al., 2017; Wang et al., 2019). These results indicated that lipid rafts on cell plasma membrane may mediate viral adhesion to facilitate virus endocytosis. It is likely that pathogen–host interactions promote lipid raft clustering and focal adhesion formation during endocytosis.

Thus, we can conclude that lipid rafts may represent attachment factors during the early stages of coronavirus infection.

EFFECT OF LIPID RAFTS-AFFECTING DRUGS ON CORONAVIRUS INFECTION

Lipid rafts affecting drugs, alone or in combination with other compounds, may play a role in antiviral activity. Indeed, as reported above, lipid rafts are crucial components of the viral envelope (Scheiffele et al., 1999), where cholesterol is a known critical structural component. Barman and Nayak (2007) demonstrated that lipid rafts disruption by MβCD-mediated cholesterol depletion is able to reduce influenza virus infectivity. Indeed, it leads to reduced infectivity of virus particles, holes on the viral envelope with consequent effects on particle structure, and altered release of viral proteins. In addition, depletion of cholesterol on host plasma membrane makes it less vulnerable to influenza virus infection. Several authors reported the importance of cholesterol for viral entry into host cells and suggested a role for cholesterol-lowering therapies in reducing SARS-CoV-2 infectivity (Bailly and Vergoten, 2020; Fecchi et al., 2020; Radenkovic et al., 2020; Tang et al., 2020). Drugs such as lovastatin or squalestatin induce cholesterol depletion by inhibiting biosynthesis; as a result, different steps of the virus life cycle can be disrupted. Other drugs, such as filipin, digitonin, nystatin, saponin, or MβCD, cause disruption of lipid rafts in a short period of time, directly removing cholesterol (Barman and Nayak, 2007), although their effects are different at the level of the membrane bilayer (Awasthi-Kalia et al., 2001). For instance, filipin leads to the dispersion of glycosylphosphatidylinositol (GPI)-anchored proteins at the cell surface favoring their release from lipid rafts and decreases the number of caveolae (Robinson and Karnovsky, 1980). Important factors involved in virus infectivity could be afflicted by statins, and some of them are able to reduce the levels of proinflammatory molecules, such as interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α, affecting the autophagic process involved in both viral replication and clearance (Mehrbod et al., 2014). On the basis of these findings, the possibility to undertake studies on patients with severe SARS-CoV-2 infection has been suggested (Fedson et al., 2020). Although there are still controversial theories about the benefits of using statins in patients with SARS-CoV-2, large-scale observational or randomized studies supported this hypothesis (Shu, 2015; Rodrigues-Diez et al., 2020; Subir et al., 2020).

Differently, cyclodextrins have always been considered as excipients with stabilizing and solubilizing properties. At the end of the twentieth century, cyclodextrins have been used as medicinal compounds. The first isolation of cyclodextrins was made by Antoine Villiers in 1981 from starch. Typical cyclodextrins contain three common ring types: (α-CD) alpha-cyclodextrin, (β-CD) beta-cyclodextrin, and (γ-CD) gamma-cyclodextrin. Cyclodextrins can assemble into

TABLE 1 | Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) clinical trials.

Compounds	Action Method	References
Convalescent Plasma	Isolation of IgG and IgM vs. SARS-CoV-2 in order to scale up polyclonal antibody manufacturing to produce treatment cocktails directed against the betacoronavirus causing COVID-19	Shen et al., 2020
Chloroquine/Hydroxychloroquine	Interfering with the glycosylation of angiotensin-converting enzyme 2 (ACE2) and blocking SARS-CoV-2 fusion with the host cell. Impaired terminal glycosylation of ACE2 may reduce the binding efficiency between ACE2 on host cells and the SARS-CoV-2 spike protein	Golden et al., 2015; Torres et al., 2019; Gao et al., 2020
Favipiravir	A guanine analog that inhibit the RNA-dependent RNA polymerase of RNA virus. It has been approved for some other viruses like Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and influenza	Li and De Clercq, 2020; Liu T. et al., 2020
Remdesivir	A monophosphoramidate prodrug of an adenosine analog with a chemical structure similar to that of tenofovir alafenamide, an approved HIV reverse transcriptase inhibitor. Remdesivir has broad-spectrum activities against RNA viruses such as MERS and SARS in cell cultures and animal models and has been tested in a clinical trial for Ebola	de Wit et al., 2020; Li and De Clercq, 2020
Galidesivir	An adenosine analog that was originally developed for HCV, is currently in early-stage clinical studies evaluating its safety in healthy subjects and its efficacy against yellow fever, and has shown antiviral activities in preclinical studies against many RNA viruses, including SARS and MERS2	Li and De Clercq, 2020
Ribavirin	A guanine derivative approved for treating HCV and respiratory syncytial virus (RSV) that has been evaluated in patients with SARS and MERS, but its side effects such as anemia may be severe at high doses and whether it offers sufficient potency against 2019-nCoV is uncertain	ClinicalTrials.gov, 2020; Li and De Clercq, 2020
human mAb 47D11	A human monoclonal antibody that neutralizes SARS-CoV-2 (and SARS-CoV) in cell culture. This cross-neutralizing antibody targets a communal epitope on these viruses and may offer potential for prevention and treatment of COVID-19	Jiang et al., 2020; Wang C. et al., 2020
Cyclodextrins	The cyclodextrin structure can be modified and used for containment of infections or as virucidal agents. The use of a mouth rinses and/or nasal applications that contain cyclodextrins combined with other drugs could provide a valuable adjunct treatment. Both are locally administered delivery systems that could lower the SARS-CoV-2 viral load	Serno et al., 2010; Lembo et al., 2018; Torres et al., 2019

complexes with various drugs to form host–guest inclusions and have therefore been accepted as pharmaceutical excipients. Cyclodextrins were found attractive for a variety of applications because they could protect sensitive organic guest molecules from oxidation and from volatilization and could make more soluble apolar guests, too. The synthetic derivatives of native cyclodextrins are divided into three groups: ionizable, such as sulfobutylether β-CD (SBE-β-CD); hydrophobic, such as 2,6-di-O-ethyl-β-CD; and hydrophilic, such as 2-hydroxypropyl-β-CD (HP-β-CD). Modified beta-cyclodextrin owns antiviral activities (Braga, 2019). For example, biocompatible sulfonated MβCD mimics some features of heparan sulfate (Jones et al., 2020); in fact, it can act as a broad-spectrum antiviral agent, since it has been proven to reduce influenza A and coronavirus infectivity through depletion of cholesterol. Moreover, drug delivery systems of cyclodextrins can overcome formulation challenges of antiviral drugs improving solubility and bioavailability (Jones et al., 2020).

However, the use of cyclodextrins or statins as active drugs against coronaviruses has some limitations. Indeed, they have a pleiotropic effect in cultured cells by affecting many different signaling pathways. Moreover, in addition to cholesterol, MβCD also extracts other lipids, such as fatty acids and ceramides from cell membranes. Finally, MβCD depolymerizes the actin meshwork, drastically affecting whole cellular architecture.

Antiviral drugs targeting Ebola and HIV have demonstrated encouraging results in SARS-CoV-2 patients, and cyclodextrin seems to be the best excipient to enhance the properties of these drugs, including the antiviral drug Kaletra, a combination of lopinavir and ritonavir, a protease inhibitor for HIV, which demonstrates a benefit in treatment of viral pneumonia (Lim et al., 2020; Wan et al., 2020); the anti-HIV combination lopinavir–ritonavir, currently employed in clinical trials (ClinicalTrials.gov, 2020); and the purine nucleoside Favipiravir, which has recently been authorized for a clinical trial (Liu T. et al., 2020). An additional strategy to disrupt lipid rafts is to use lipidomimetic antiviral agents that alter either viral or host cell membrane blocking viral infection (Nieto-Garai et al., 2018). Therefore, a new antiviral strategy could be assumed based on a rafts-like lipid scaffold.

Remdesivir, an antiviral developed by Gilead Sciences Inc. and previously approved on patients with Ebola, has shown promising results in animal models for MERS and SARS. A formulation with cyclodextrin and remdesivir has been recently proposed (de Wit et al., 2020). In addition, a combination of chloroquine and remdesivir was found to effectively inhibit SARS-CoV-2 *in vitro*. Chloroquine phosphate is an old antimalarial drug and has been effective in inhibiting the exacerbation of SARS-COV-2 pneumonia (Gao et al., 2020). Furthermore, chloroquine displays an immunomodulatory effect by inhibiting TNF-α and IL-6. It also exhibits autophagy inhibitory properties by the elevation of endosomal pH, which may interfere with viral infection and replication (Golden et al., 2015). Hydroxychloroquine presents a terminal hydroxyl

group in molecular structure, and several studies have shown the capacity of chloroquine and hydroxychloroquine to bind the sialic acids and gangliosides of the host cells lipid rafts, destabilizing the order, that SARS-COV-2 uses to enter besides the receptor ACE-2 (Fantini et al., 2020; Yuan et al., 2020). This fact suggested a possible additional role for cyclodextrins. Indeed, it was shown that complexation with cyclodextrins lead to an increase in the activity of the antimalarial drug (Torres et al., 2019).

In addition, losartan, a generic blood pressure medication able to block ACE-2 receptor, could be associated with cyclodextrins. Other drugs, such as selective estrogen receptor modulators (SERMs), offer alternative candidate drugs for SARS-CoV-2 (Zhou et al., 2020). Indeed, an overexpression of estrogen receptor, which is localized within lipid rafts (Marin et al., 2012), has been proven to interfere in viral replication through the non-classical pathways associated with estrogen receptor (Lasso et al., 2019). A reasonable solubility is essential to induce bioavailability, and in the case of parenteral therapy, where intravenous solutions must be buffered to physiological pH and be particulate-free, drug solubility is critical, and cyclodextrin represents the best candidate to improve complex therapies. Although researchers are searching for preventive intervention strategies, including interferon therapies, peptides, vaccines, small-molecule drugs, and monoclonal antibodies to treat SARS-CoV-2 infection, these may require several months to test, and all depends on the results of the clinical trials (Shanmugaraj et al., 2020). A few companies are developing actions to accelerate the formation of their neutralizing antibodies, driven by previous successes in the treatment of other diseases (Jiang et al., 2020; Wang C. et al., 2020). Adequate excipients are crucial during shipment and storage to maintain antibody and drugs stability. The protective properties of cyclodextrin, such as the inhibition of proteins aggregation under various stress conditions, have been shown by many case studies (Serno et al., 2010). Finally, researchers have been racing to find possible vaccines for future prevention. Cyclodextrin, as an adjuvant, stabilizes therapeutic monoclonal antibodies, preserves longer immune response, increases antigen (vaccine)-specific antibody titers, and induces type 2 T-helper (Th2) cell response (Onishi et al., 2015) by affecting key signal transduction pathway(s) triggered by lipid rafts.

Further applications for the use of lipid raft affecting drugs are derived from the observations of Zhou and Simmons (2012), who pointed out novel broad-spectrum antiviral compounds to target different stages of the viral life cycle. Certain molecules prove able to be able interfere with the infectivity of some coronaviruses, possibly by viral lipid-dependent attachment to cells (Baglivo et al., 2020). The main pharmacological approaches against coronaviruses are summarized in **Table 1**.

CONCLUSION

It is conceivable that the first contact between coronavirus and host cells occurs into lipid rafts, specialized regions of cell plasma membrane, which provide a suitable platform able of concentrating ACE-2 receptor, thus representing a port of cell entry for viruses.

This review is focused on targeting lipid rafts as a strategy against coronavirus. We report that agents, such as statins or cyclodextrins, can deplete cholesterol and cause disruption of lipid rafts, consequently affecting coronavirus adhesion and binding. Furthermore, these compounds can assemble into complexes with various drugs to form host–guest inclusions and may be used as pharmaceutical excipients of antiviral drugs, such as lopinavir and remdesivir, by improving bioavailability and solubility. Thus, the possible use of drugs affecting lipid rafts in the process of coronavirus entry into the cells introduces a potential new task in the pharmacological strategy against coronavirus.

AUTHOR CONTRIBUTIONS

MS, RM, GR, VMan, SM, AL, TG, and VMat wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Campylobacter jejuni Cytolethal Distending Toxin C Exploits Lipid Rafts to Mitigate *Helicobacter pylori*-Induced Pathogenesis

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Helicobacter pylori infection is associated with several gastrointestinal diseases, including gastritis, peptic ulcer, and gastrointestinal adenocarcinoma. Two major cytotoxins, vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA), interact closely with lipid rafts, contributing to *H. pylori*-associated disease progression. The *Campylobacter jejuni* cytolethal distending toxin consists of three subunits: CdtA, CdtB, and CdtC. Among them, CdtA and CdtC bind to membrane lipid rafts, which is crucial for CdtB entry into cells. In this study, we employed recombinant CdtC (rCdtC) to antagonize the functions of *H. pylori* cytotoxin in cells. Our results showed that rCdtC alleviates cell vacuolation induced by *H. pylori* VacA. Furthermore, rCdtC reduces *H. pylori* CagA translocation, which decreases nuclear factor kappa-B activation and interleukin-8 production, resulting in the mitigation of gastric epithelial cell inflammation. These results reveal that CdtC hijacks cholesterol to compete for *H. pylori* cytotoxin actions *via* lipid rafts, ameliorating *H. pylori*-induced pathogenesis.

Keywords: *Helicobacter pylori*, cytolethal distending toxin, lipid rafts, cytotoxin-associated gene A, vacuolating cytotoxin A

INTRODUCTION

Helicobacter pylori commonly colonizes the human stomach and infects approximately 50% of humans worldwide (Amieva and Peek, 2016). Persistent *H. pylori* infection may be associated with a high risk of gastrointestinal diseases, including chronic gastritis, peptic ulcers, and gastric cancer (Crowe, 2019). Combination therapies containing a proton pump inhibitor (PPI) and several antibiotics have been used to eradicate *H. pylori* for decades (Hentschel et al., 1993). Given

the widespread use of antibiotics, *H. pylori* antimicrobial resistance rates have increased annually and is currently the main cause of treatment failure (Alba et al., 2017).

Several virulence factors are involved in *H. pylori*-induced pathogenesis and sustain infection in a host organism (Lu et al., 2005). Vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) are two major bacterial cytotoxins that contribute to *H. pylori*-related disease progression (Salama et al., 2013). VacA secreted by *H. pylori* binds to its receptors to form vesicles containing anion-selective channels, resulting in increased osmotic swelling, and vacuolation (Cover and Blanke, 2005). CagA, another crucial virulence factor, is injected into host cells *via* the *H. pylori* type IV secretion system (TFSS) (Hatakeyama, 2008; Herrera and Parsonnet, 2009). Translocated CagA is then phosphorylated, leading to NF-κB activation, IL-8 production (Brandt et al., 2005), and cell scattering (also referred to as the hummingbird phenotype) (Segal et al., 1999).

Lipid rafts are rigid microdomains located on the cell membrane, which comprise large proportions of phospholipids, sphingolipids, and cholesterol (Ikonen, 2001). Several studies have demonstrated that *H. pylori* VacA and CagA exploit lipid rafts as toxin receptors for their intoxication (Schraw et al., 2002; Lai et al., 2008; Murata-Kamiya et al., 2010). In addition, *H. pylori* cholesterol-α-glucosyltransferase catalyzes the conversion of cholesterol into cholesterol α-glucosides, which are crucial for bacterial evasion of the immune response (Wunder et al., 2006; Lai et al., 2018; Morey et al., 2018). *Campylobacter jejuni* cytolethal distending toxin (CDT), a genotoxin, contains three subunits: CdtA, CdtB, and CdtC (Lara-Tejero and Galan, 2000). Similar to many bacterial toxins, CdtA and CdtC serve as binding moieties and attach to cholesterol-rich microdomains that enhance CdtB delivery into cells (Lin et al., 2011).

Usurping or depleting cholesterol disrupts lipid rafts, which ameliorates *H. pylori*-induced inflammation and pathogenesis (Lin C. J. et al., 2016; Lin et al., 2017). The development of effective agents against VacA and CagA that interact with lipid rafts might be an ideal strategy for alleviating *H. pylori*-induced pathogenesis. Given the notion that CdtC contains a cholesterol recognition/interaction amino acid consensus (CRAC) domain that specifically binds to cholesterol in the membrane rafts (Lai et al., 2013), it is worth investigating whether CdtC has an inhibitory effect on *H. pylori* cytotoxin-induced pathogenesis. In this study, the biological functions of CdtC antagonizing VacA/CagA toxin actions in cells were extensively investigated. Our results reveal that *C. jejuni* CdtC hijacks cholesterol to compete with cytotoxin functions. Thus, *C. jejuni* CdtC can be potentially developed as a preventive agent against *H. pylori*-induced pathogenesis.

MATERIALS AND METHODS

Cell and Bacterial Culture

Human gastric epithelial cells (AGS cells, ATCC CRL-1739) were cultured in F12 medium (Hyclone, Logan, UT, United States) containing 10% fetal bovine serum (Hyclone). The cells were maintained at 37°C containing 5% CO_2. *H. pylori* strain 26695 (ATCC 700392) and CagA-EGFP *H. pylori* (Lin et al., 2013) were routinely cultured on blood agar plates (Brucella agar with 10% defibrinated sheep blood) and incubated at 37°C in a microaerophilic environment (5% O_2, 10% CO_2, and 85% N_2). *Escherichia coli* strain BL21-DE3 (pET21d-*cdtC*) was cultured on LB agar plate containing 100 μg/ml ampicillin and incubated at 37°C as described previously (Lin et al., 2011).

Preparation of Recombinant CdtC

Recombinant CdtC was constructed and characterized as described previously (Lin et al., 2019). Briefly, *E. coli* BL21-DE3 containing pET21d-*cdtC* was cultured and induced by isopropyl β-D-thiogalactopyranoside (1 mM) at 16°C for 4 h. The bacterial cells were lysed, and rCdtC protein was purified by metal affinity chromatography (Clontech, Palo-Alto, CA, United States). The purified rCdtC was characterized by SDS-PAGE and western blot assay (**Supplementary Figure 1**).

Cell Survival Assay

AGS cells (2×10^4) were cultured in 96-well plates for 16 h and incubated with rCdtC (200, 500, and 1,000 nM) at 37°C for 1, 7, and 11 h. The cells were then treated with 100 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich, St. Louis, MO, United States) at 37°C for 2 h. The ability of viable cells to reduce MTT to formazan was measured (Chen Y. W. et al., 2020). Cell viability was expressed as fold changes compared to the untreated group.

Bacterial Viability Assay

Helicobacter pylori was cultured for 30 h to reach OD_{600} of 1.0. The bacteria were treated with rCdtC (200, 500, and 1,000 nM) at 37°C for 6 h. The bacteria were then plated by serial dilution on blood agar plates. Colony-forming units (CFUs) were enumerated to determine the viable bacteria (Lien et al., 2019).

Vacuolation Activity Assay

Helicobacter pylori VacA-induced cell vacuolation was assessed using neutral red uptake assay as described previously (Cover et al., 1991). Briefly, AGS cells (1×10^5) were plated in 24-well plates and incubated with 200 nM rCdtC for 1 h, followed by *H. pylori* infection at a multiplicity of infection (MOI) of 100 for 6 h. The cells were washed with phosphate-buffered saline (PBS) and incubated with 0.05% neutral red (Sigma-Aldrich) for 4 min with gentle shaking. Afterward, acidified alcohol (1% 12 N HCl in 75% ethanol) was added to elute the solution, which was determined at OD_{540} by using a spectrophotometer (Molecular Devices, San Jose, CA, United States).

Immunofluorescence Staining and Confocal Microscopic Analysis

AGS cells (3×10^5) were seeded in six-well plates and cultured for 16 h. rCdtC (200 nM) was treated for 1 h prior to infection with wild-type or CagA-EGFP *H. pylori* at a MOI of 20 for 2 h. The cells were fixed in 4% paraformaldehyde (Alfa

Aesar, Haverhill, MA, United States) for 1 h, probed with anti-CdtC antibody and anti-VacA antibody (Santa Cruz, Dallas, TX, United States), and then stained with Alexa Fluor 488-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, United States), Cy5-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, United States), or anti-goat IgG-conjugated CruzFluor 555 (Santa Cruz). The plasma membrane was probed with wheat germ agglutinin (WGA)-conjugated Alexa Flour 594 (Thermo Fisher Scientific, Waltham, MA United States). The nuclei were stained with Hoechst 33342 for 20 min. The stained cells were visualized using a confocal laser scanning microscope (LSM780, Carl Zeiss, Germany) and analyzed by the software ZEN (Carl Zeiss). VacA and CagA presented in the cytoplasm were quantified by green fluorescence intensity. Green puncta representing VacA and CagA existing in the bacteria were excluded from the fluorescence intensity analysis. The quantification was the mean pixel intensity of the VacA and CagA signal shown in the cytoplasm of each cell (50 cells were analyzed per sample).

Western Blot Assay

The rCdtC-treated and *H. pylori*-infected cells were prepared and analyzed by 12% SDS-PAGE, followed by transferring them onto polyvinylidene difluoride membrane (Millipore, Burlington, MA, United States). The membrane was incubated with primary antibody and then incubated with horseradish peroxidase-conjugated secondary antibody (Millipore, Temecula, CA, United States). The protein expression level was detected using ECL western blotting detection reagents (GE Healthcare, Chicago, IL, United States) and analyzed by Azure 400 (Azure Biosystems, Dublin, CA, United States).

Quantitation of Cells With Hummingbird Phenotype

AGS cells (3×10^5) were seeded in six-well plates and cultured for 16 h. The cells were treated with rCdtC (200 and 500 nM) or 0.1 μM of bafilomycin A1 (BafA1) (InvivoGen, San Diego, CA, United States) for 1 h prior to *H. pylori* infection at a synchronized MOI of 100 for 6 h. The elongated cells (hummingbird phenotype) were defined as cells that showed thin needle-like protrusions of more than 20 μm in length and a typical elongated shape, as reported previously (Wang et al., 2012). The percentage of elongated cells was determined as the number of cells having the hummingbird phenotype.

Luciferase Reporter Assay

AGS cells were transfected with NF-κB-luciferase reporter by jetPEI (Polyplus-transfection, France) as described previously (Lin H. J. et al., 2016). The cells were treated with 200 nM rCdtC for 1 h, followed by *H. pylori* infection at a MOI of 100 for 6 h. The cells were lysed, and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, MA, United States) with a microplate luminometer (Biotek, Winooski, VT, United States). Luciferase activity was normalized for transfection efficiency by the co-transfected β-galactosidase expression vector (Promega).

IL-8 Measurement

AGS cells (1×10^5) were seeded in 24-well plates and cultured for 16 h. The cells were treated with 200 nM rCdtC for 1 h and then infected with *H. pylori* at a MOI of 100 for 6 h. The supernatant was prepared, and the IL-8 concentration was measured by using sandwich enzyme-linked immunosorbent (ELISA) assay according to the manufacturer's instructions (Thermo Fisher Scientific).

Gentamicin Protection Assay

AGS cells were treated with 200 nM rCdtC for 1 h prior to *H. pylori* infection at a MOI of 100 for 6 h. The treated cells were washed with PBS three times, followed by incubation with 100 μg/ml gentamicin (Sigma-Aldrich) for 90 min. The cells were lysed with sterile H_2O for 10 min, and cell lysate was plated by serial dilution on blood agar plates. Viable CFUs were enumerated to determine the activity of *H. pylori* invasion of cells, as described previously (Chen et al., 2019).

Statistical Analysis

The data were presented as mean ± standard deviation of triplicate independent experiments. Student's *t*-test was performed to evaluate the statistical significance of the experimental results between two groups by using SPSS program (version 12.0 for Windows, SPSS Inc., Chicago, IL, United States). A *p*-value less than 0.05 was considered as statistically significant.

RESULTS

Purification and Characterization of *C. jejuni* rCdtC

Recombinant CdtC was first purified and validated by SDS-PAGE and western blot analysis (**Supplementary Figure 1**). We then examined the influence of rCdtC on cell viability by treating AGS cells with various concentrations of rCdtC (200, 500, and 1,000 nM) for 1, 7, and 11 h. As shown in **Supplementary Figure 2A**, cell viability was barely affected by the treatment doses. In addition, incubation of *H. pylori* with rCdtC for 6 h only resulted in a marginal influence on bacterial survival at the highest dose (1,000 nM) (**Supplementary Figure 2B**). These results indicate that rCdtC neither influences cell viability nor affects *H. pylori* survival.

rCdtC Reduces *H. pylori*-Induced Vacuolation in Gastric Epithelial Cells

Helicobacter pylori VacA assembles on the cell membrane and is delivered intracellularly by exploiting cholesterol-rich lipid rafts (Schraw et al., 2002; Gupta et al., 2008). Binding of *C. jejuni* CdtC to membrane cholesterol is a crucial step for CDT entry into cells (Lai et al., 2013, 2015). Considering that membrane cholesterol is essential for CdtC binding and VacA delivery, we investigated whether rCdtC hijacks cholesterol to compete with VacA actions in cells. AGS cells were untreated (**Figure 1A**) or pretreated with rCdtC (**Figures 1B,D,E**) or bafilomycin A1

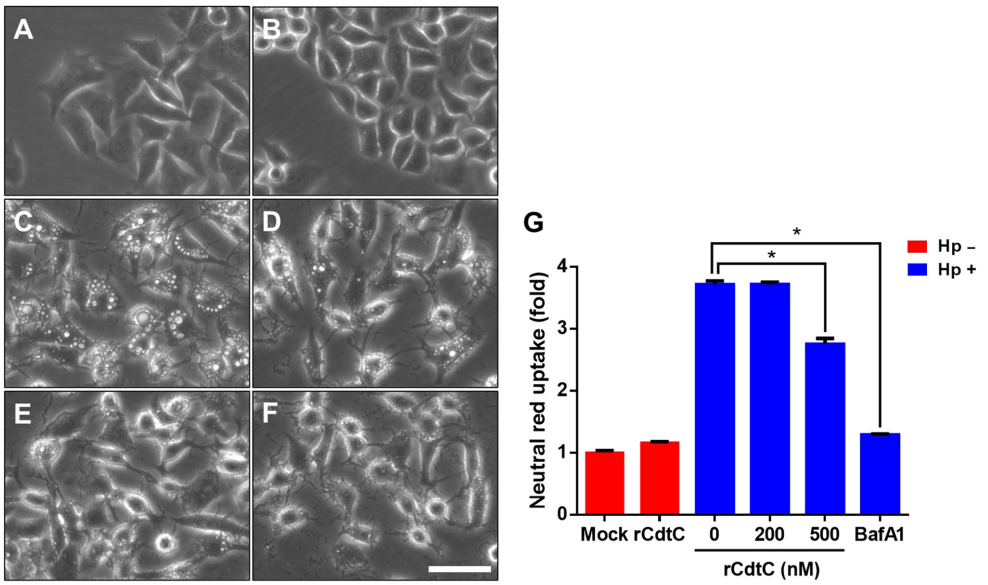

FIGURE 1 | rCdtC inhibits *Helicobacter pylori*-induced vacuolation in gastric epithelial cells. AGS cells were **(A)** untreated with rCdtC, **(B)** treated with 200 nM rCdtC alone, **(C)** infected with *H. pylori* for 6 h, **(D)** pretreated with 200 nM rCdtC prior to *H. pylori* infection for 6 h, **(E)** pretreated with 500 nM rCdtC followed by *H. pylori* infection for 6 h, and **(F)** pretreated with 0.1 μM BafA1 then infected with *H. pylori* for 6 h. Cell vacuolation was observed by using a phase-contrast microscope. Scale bar, 100 μm. **(G)** Neutral red uptake assay was employed to analyze the vacuolating activity. Each group was performed in three independent experiments (*$P < 0.05$).

(BafA1) (**Figure 1F**) for 1 h, followed by *H. pylori* infection for 6 h. As shown in **Figure 1C**, vacuole formation in *H. pylori*-infected cells was noticeable when compared to the mock control. Treatment of cells with rCdtC (500 nM) or BafA1 (0.1 μM) markedly inhibited *H. pylori*-induced vacuolation in cells (**Figure 1G**). The intracellular delivery efficiency of VacA was then investigated using confocal microscopy. As shown in **Figure 2**, *H. pylori*-infected cells exhibited VacA in the cytoplasm. Pretreatment of cells with rCdtC significantly reduced intracellular VacA delivery. These results demonstrate that rCdtC possesses inhibitory activity against *H. pylori* VacA actions in gastric epithelial cells.

rCdtC Suppresses *H. pylori* CagA-Induced Pathogenesis in Gastric Epithelial Cells

Helicobacter pylori CagA translocation is important for cytoskeleton rearrangement, leading to cell elongation and scattering (hummingbird phenotype) (Segal et al., 1999). We then evaluated whether rCdtC influences cell scattering in *H. pylori*-infected cells. As shown in **Figure 3C**, the hummingbird phenotype appeared in *H. pylori*-infected cells, but not shown in cells untreated with rCdtC (**Figure 3A**) and treated with rCdtC along (**Figure 3B**). However, the *H. pylori*-induced elongated phenotype was significantly inhibited in the cells pretreated with rCdtC (**Figures 3D,E**). We further analyzed the inhibition of CagA translocation by rCdtC using confocal microscopy. As shown in **Figure 4A**, WGA was used to label the plasma membrane. Infection of cells with CagA-EGFP *H. pylori* led to prominent CagA translocation to the cytoplasm (**Figures 4A,B**). Noticeably, less CagA translocation was observed in cells treated with rCdtC, followed by *H. pylori* infection (**Figure 4C**). We then investigated the distribution of rCdtC and CagA in cells by confocal microscopy. As shown in **Supplementary Figure 3**, the area across the cell membrane is represented by the peak of WGA fluorescence intensity. In the absence of rCdtC treatment, CagA-EGFP showed cytoplasmic distribution. In contrast, cells pretreated with rCdtC exhibited low CagA-EGFP signal in the cytoplasm. It can be noted from **Supplementary Figure 3** that the rCdtC fluorescence intensity overlaps with the WGA signal, indicating the colocalization of rCdtC to the cell membrane. These results demonstrate that rCdtC binds to the cell membrane and inhibits CagA translocation, leading to the alleviation of CagA-induced pathogenesis.

rCdtC Attenuates *H. pylori*-Induced Inflammation in Gastric Epithelial Cells

Since CagA translocation leads to NF-κB activation and IL-8 production enhancement in AGS cells (Brandt et al., 2005), we sought to investigate whether rCdtC affects IL-8 production in *H. pylori*-infected cells. As shown in **Figure 5A**, *H. pylori* infection induced higher NF-κB luciferase activity than mock or rCdtC treatment alone. However, in cells pretreated with rCdtC, *H. pylori*-induced NF-κB activation was markedly diminished compared to the rCdtC-untreated group. Similar to the results of NF-κB activation, we observed an inhibitory effect on IL-8 production (**Figure 5B**). These results demonstrate that rCdtC inhibits CagA-associated NF-κB activation and IL-8 secretion, and it subsequently ameliorates *H. pylori*-induced inflammation.

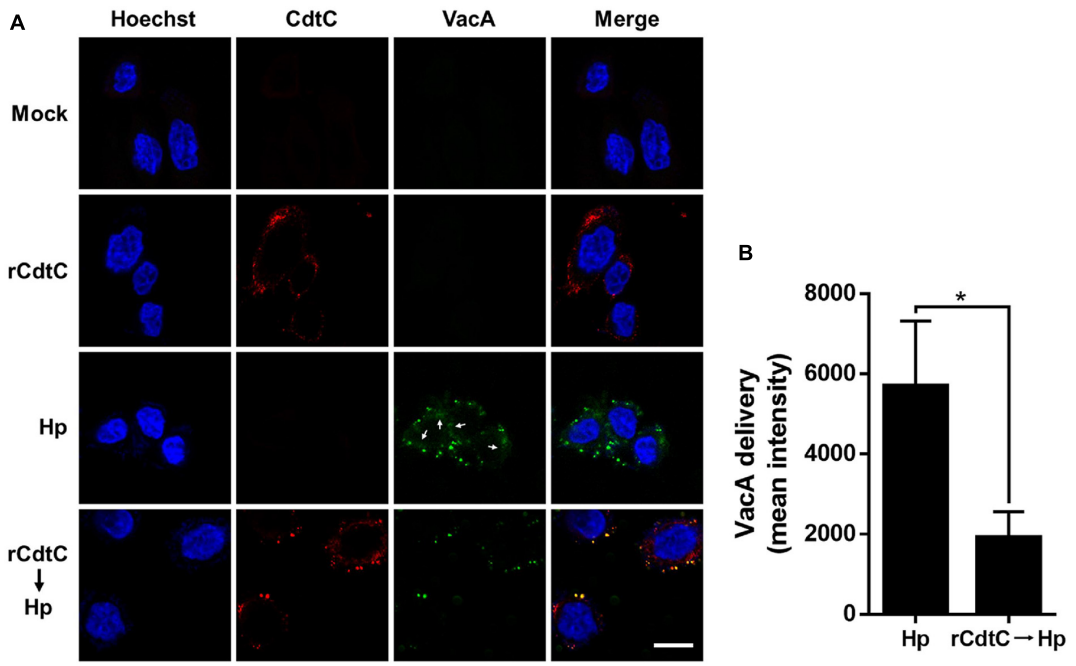

FIGURE 2 | rCdtC declines *Helicobacter pylori* VacA delivery in cells. AGS cells **(A)** pre-incubated with rCdtC (200 nM) were infected with or without *H. pylori* at a multiplicity of infection of 100 for 6 h. The cells were stained with anti-CdtC (red) and anti-VacA (green), and the nuclei were probed with Hoechst 33342 (blue). The image was observed under a confocal microscope with a 63× oil immersion objective and processed by software ZEN (Carl Zeiss, Germany). Arrows indicate VacA delivered into the cytoplasm. Scale bar, 10 μm. **(B)** The delivery of VacA into the cytoplasm was quantified. Green puncta representing VacA localized on the bacterial surface were excluded from the fluorescence intensity analysis. The quantification represented in the figures reflected the mean pixel intensity of the VacA signal in the cytoplasm of each cell (50 cells were analyzed per sample). *$P < 0.05$.

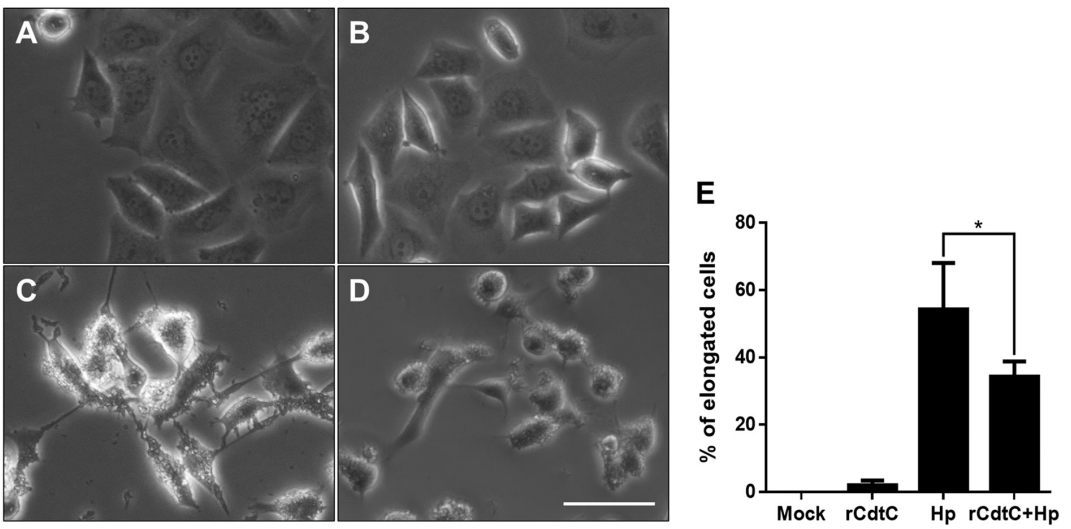

FIGURE 3 | rCdtC attenuates *Helicobacter pylori*-induced cell scattering. AGS cells were **(A)** untreated with rCdtC, **(B)** treated with 200 nM rCdtC alone, **(C)** infected with *H. pylori* for 6 h, and **(D)** pretreated with rCdtC then infected with *H. pylori* for 6 h. Scale bar, 100 μm. **(E)** The elongated cells were counted and expressed as percentage compared to the untreated cells. The results were performed in three independent experiments and represented as mean ± SD (*$P < 0.05$).

rCdtC Reduces *H. pylori* Invasion of Cells

Helicobacter pylori internalized by gastric epithelial cells requires membrane rafts, whereas adhesion does not (Lai et al., 2008). Our results showed that rCdtC did not inhibit *H. pylori* adhesion in AGS cells (**Supplementary Figure 4A**). We then examined whether rCdtC affects *H. pylori* invasion in cells using the gentamicin protection assay. As expected, rCdtC effectively reduced *H. pylori* invasion in cells (**Supplementary Figure 4B**).

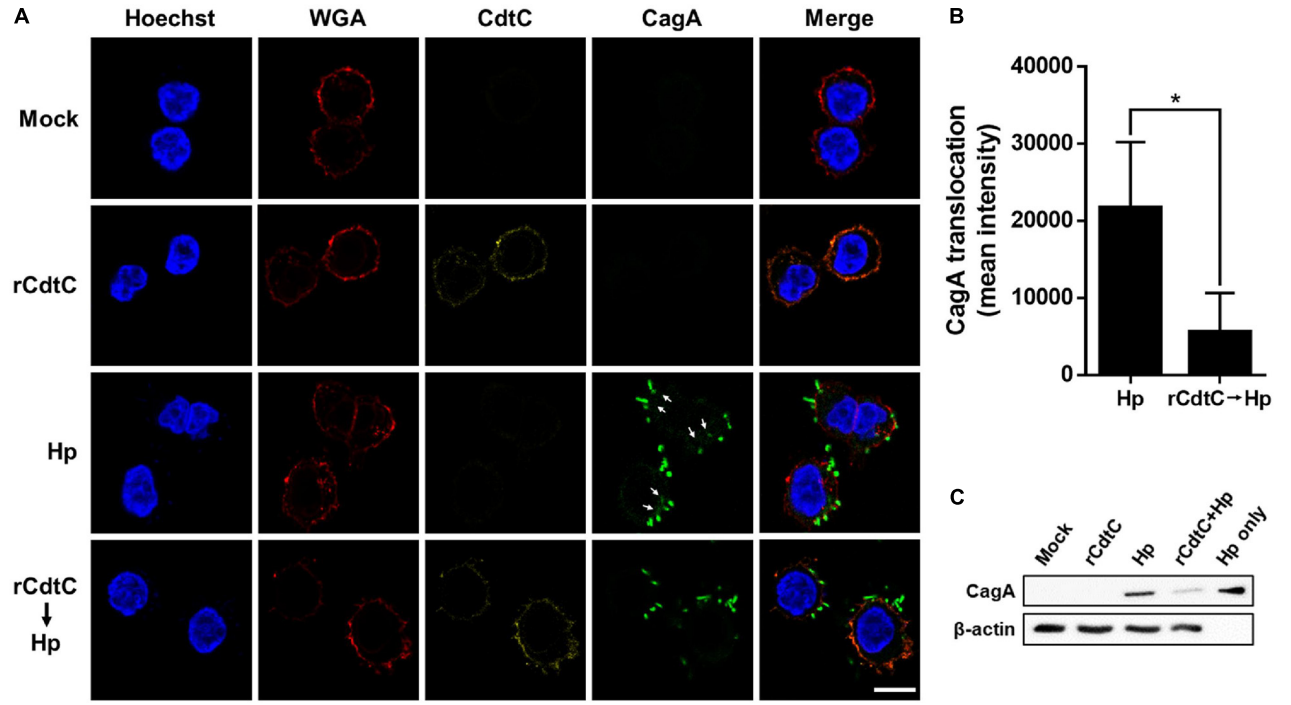

FIGURE 4 | rCdtC decreases CagA translocation in *Helicobacter pylori*-infected cells. **(A)** AGS cells pre-incubated with rCdtC (200 nM) were infected with or without CagA-EGFP *H. pylori* (green) at a multiplicity of infection of 20 for 2 h. The cells were probed with anti-CdtC (yellow), the plasma membrane was stained with WGA-conjugated Alexa Flour 594 (red), and the nuclei were stained with Hoechst 33342 (blue). Arrows indicate CagA translocated into the cytoplasm. The image was analyzed by a confocal microscope with 63× oil immersion objective and processed by software ZEN. Scale bar, 10 μm. **(B)** CagA-EGFP translocation was quantified by green fluorescence intensity. Green puncta representing CagA that existed in the bacteria were excluded from the fluorescence intensity analysis. The quantification indicated the mean pixel intensity of the CagA signal in the cytoplasm of each cell (50 cells were analyzed per sample). *$P < 0.05$. **(C)** The level of CagA translocation was determined by using western blot assay. β-Actin expression was used to represent an internal control for equal loading.

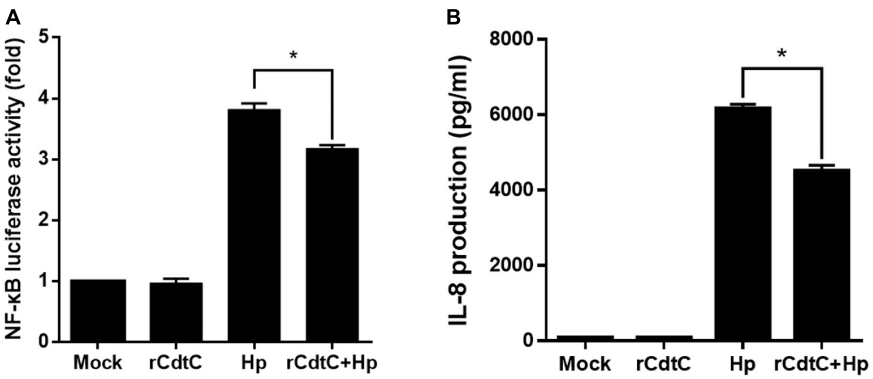

FIGURE 5 | rCdtC suppresses NF-κB activation and IL-8 production in *Helicobacter pylori*-infected cells. AGS cells were untreated with rCdtC (mock), treated with 200 nM rCdtC, infected with *H. pylori* at a multiplicity of infection of 100 for 6 h, or pretreated with rCdtC then infected with *H. pylori*. **(A)** The level of NF-κB activation was determined by luciferase assay. **(B)** IL-8 production was assessed using ELISA. Each group was performed in triplicate experiments (*$P < 0.05$).

These results indicate that rCdtC decreases *H. pylori* invasion of cells but has no impact on *H. pylori* adhesion to cells. Collectively, our results reveal that rCdtC binding to membrane rafts competes with the interactions between cholesterol-rich microdomains and bacterial actions, including VacA delivery, CagA translocation, and bacterial internalization in gastric epithelial cells, alleviating *H. pylori*-induced pathogenesis (**Figure 6**).

DISCUSSION

The gold-standard method for treating *H. pylori*-infected patients consists of PPI and several antibiotics, and it has been used as a first-line regimen for over two decades (Malfertheiner et al., 2017). Although antibiotic therapy showed marked antimicrobial efficacy in the beginning, the regimen is gradually becoming

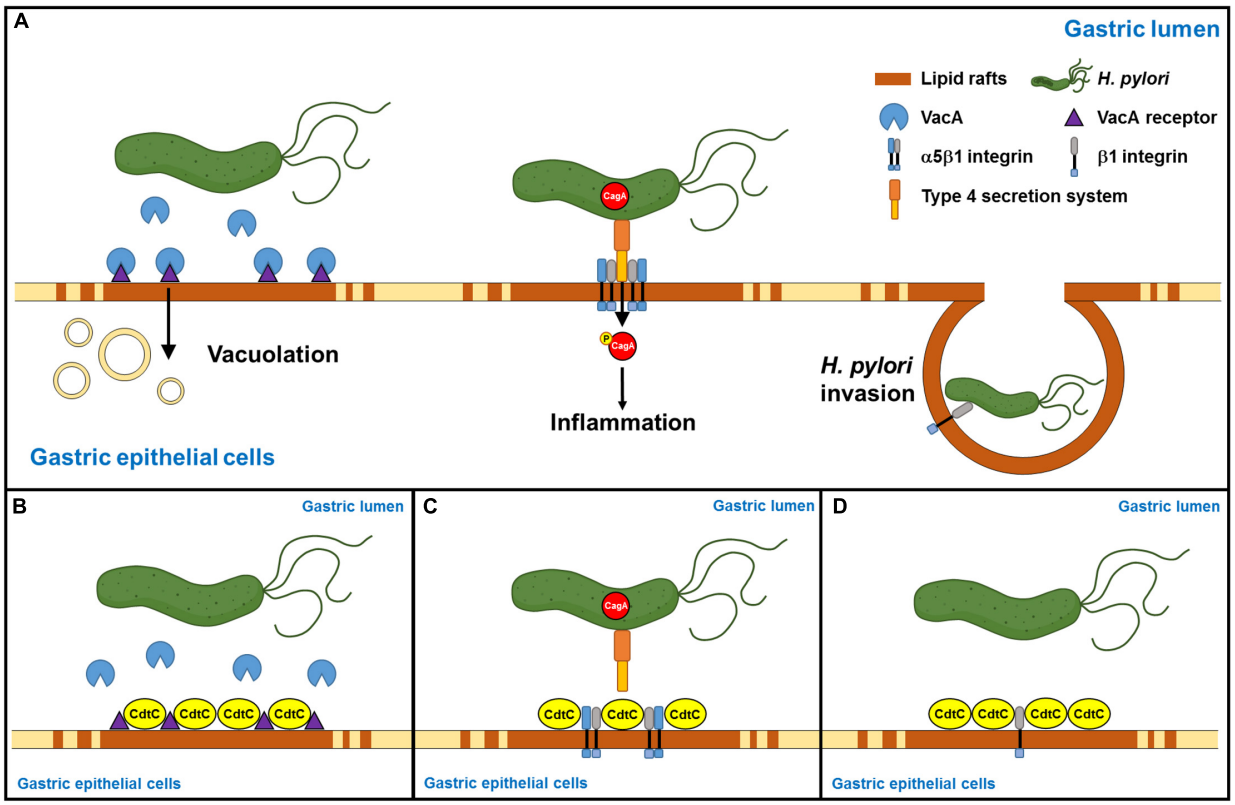

FIGURE 6 | Schematic representation of this study to summarize *Campylobacter jejuni* CdtC action and its potential application in the alleviation of *Helicobacter pylori*-induced pathogenesis. **(A)** *H. pylori*-secreted VacA binds its receptors in the lipid rafts, followed by inducing vacuolation formation in the cells. Furthermore, *H. pylori* directly injects CagA into the host cells through the interaction of TFSS and cellular α5β1 integrin in the lipid rafts. Then, CagA is phosphorylated, which caused an inflammatory response. Subsequently, *H. pylori* invasion depends on *H. pylori* interacting with β1 integrin. **(B)** *C. jejuni* CdtC inhibits VacA-induced cell vacuolation, **(C)** suppresses CagA translocation, and **(D)** declines *H. pylori* invasion in the gastric epithelial cells through the binding of lipid rafts.

ineffective due to the rising antibiotic resistance of *H. pylori* (Alba et al., 2017). Particularly, antibiotic treatment may alter gut microbiota composition, which plays important roles in multiple human physiological processes, where dysbiosis may lead to disease development (Vangay et al., 2015). Considering the many adverse effects of antibiotics, a new therapeutic modality that differs from antibiotics is urgently needed to combat *H. pylori*-associated diseases.

Pathogens and their virulence factors exploiting lipid rafts to gain entry into host cells have been reported elsewhere (Wang and Hajishengallis, 2008; Frisan, 2016; Chen Y. et al., 2020). Previous studies have used cholesterol-depleting agents to reduce pathogen infections by inhibiting their entry into target cells. For example, depletion of membrane cholesterol by methyl-β-cyclodextrin impaired pathogen attachment to the cell surface (Guo et al., 2017; Owczarek et al., 2018; Chen Y. et al., 2020). Statins, inhibitors of HMG-CoA reductase, are cholesterol-lowering agents that have been employed to reduce microbial infectivity (Boyd et al., 2012; Motzkus-Feagans et al., 2012; Skerry et al., 2014). Consistent with the previous reports, our recent studies also revealed that statin use decreased *H. pylori* infection and reduced the incidence of *H. pylori*-associated diseases (Lin C. J. et al., 2016; Lin et al., 2017; Liao et al., 2017). These lines of evidence indicated that pharmaceutical usurping or depletion of cholesterol agents that disrupt lipid rafts may pave the way for treating microbial infections.

We previously reported that CdtA and CdtC coalesce in lipid rafts, which is essential for CdtB delivery in cells (Lin et al., 2011). We then demonstrated that the CRAC motif in CdtC is required for cholesterol binding (Lai et al., 2013). However, the actual actions of CdtC binding to cholesterol-rich microdomains, which prevent lipid raft-mediated toxin functions, have never been studied. The present study showed that rCdtC did not affect cell viability, indicating that the rCdtC that we used in mammalian cells was reliable. Therefore, it is reasonable to investigate whether rCdtC can gain the potency to compete with lipid raft-mediated toxin actions, particularly in the initial step of bacterial toxin binding to the cell membrane.

Vacuolating cytotoxin A, one of the most crucial virulence factors produced by *H. pylori*, has been extensively explored. Previous epidemiological studies have indicated that *H. pylori*-containing toxigenic *vacA* alleles are closely associated with a high risk of severe gastrointestinal diseases, such as peptic ulcer disease and gastric adenocarcinoma (Kidd et al., 2001). In addition, VacA is recognized as a multifunctional toxin that targets various cell types, including gastric epithelial cells,

parietal cells, and immune cells (Cover and Blanke, 2005). VacA possesses pore-forming activity, which is associated with *H. pylori*-induced disease severity (Palframan et al., 2012). Deletion of the membrane-associated region of VacA inhibits vacuole formation in cells, which subsequently prevents toxin-induced pathogenesis (Foo et al., 2010). In line with previous findings, our results demonstrated that rCdtC has an inhibitory effect on *H. pylori*–VacA functions, including VacA delivery and intracellular vacuolation. The molecular mechanism for rCdtC diminishing *H. pylori*-induced vacuolation in gastric epithelial cells is through the competition for VacA binding to membrane rafts at the initial step of toxin entry.

Cytotoxin-associated gene A translocation is mediated by TFSS, which is located in cholesterol-rich microdomains (Lai et al., 2008, 2011; Murata-Kamiya et al., 2010; Lin C. J. et al., 2016). The translocated CagA is then phosphorylated, inducing a scattering phenotype and elevating IL-8 secretion from gastric epithelial cells (Lai et al., 2008). Membrane lipid phosphatidylserine plays a key role in the delivery of CagA in cells (Murata-Kamiya et al., 2010; Tohidpour et al., 2017). Interestingly, the reduction of cellular cholesterol by statins decreases CagA translocation/phosphorylation, which reduces the risk of *H. pylori*-associated gastric cancer (Lin C. J. et al., 2016). Our current study shows that rCdtC binding to membrane rafts avoids CagA action in cells by prohibiting CagA translocation into cells, attenuating NF-κB activation, and decreasing IL-8 production. These lines of evidence indicate that raft therapeutics may be a feasible approach to prevent *H. pylori* CagA-related pathogenesis.

Lipid rafts can be used as a platform to efficiently deliver *H. pylori* virulence factors, including VacA and CagA. In addition, lipid rafts also serve as a gateway for *H. pylori* internalization and multiplication in cells (Sit et al., 2020). Therefore, it is worth developing therapeutic agents against lipid rafts that impede *H. pylori* infection in its initial steps. Although rCdtC exerts inhibitory effects on VacA/CagA functions as studied using cell-based models, the detailed mechanism underlying pharmacological signaling remains to be clarified. It is therefore crucial to examine rCdtC activity using animal or *in vivo* studies, which may provide substantial evidence for combating *H. pylori* infection.

CONCLUSION

In summary, this study provides a cell-based platform to determine whether rCdtC antagonizes *H. pylori*-induced pathogenesis by binding to lipid rafts. Our results demonstrate that rCdtC inhibits cholesterol-mediated VacA delivery and vacuolation in the cytoplasm. Furthermore, binding of rCdtC to membrane rafts significantly restricted CagA translocation, followed by attenuated CagA-mediated pathogenesis. The interaction of CdtC with cholesterol-rich microdomains is likely to contribute to interference with *H. pylori* cytotoxin actions, thereby decreasing their toxicity in cells. These results suggest that the inhibition of membrane raft-mediated toxin functions might be a rational target for the development of novel agents to alleviate *H. pylori*-induced pathogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

C-HsC and C-HL contributed to the conception or design of this work. J-YY, H-JL, C-JK, C-LF, and C-HuC conducted the experimental study. C-DL, H-YW, and C-YL contributed to data analysis and interpretation. J-YY, H-JL, C-JK, C-HsC, and C-HL contributed to the writing of the manuscript. All authors gave final approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.617419/full#supplementary-material

Supplementary Figure 1 | Purification and characterization of *Campylobacter jejuni* rCdtC. rCdtC was purified and analyzed by **(A)** SDS-PAGE and **(B)** western blot assay. Marker of molecular weight in kDa is shown on the left.

Supplementary Figure 2 | rCdtC neither affects cell survival nor influences bacterial viability. **(A)** AGS cells were treated with rCdtC (200, 500, and 1,000 nM) for 1, 7, and 11 h, and cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide assay. **(B)** *Helicobacter pylori* was exposed to rCdtC (200, 500, and 1,000 nM) for 6 h. Bacteria grown on the blood agar plates were counted and defined as *H. pylori* viability.

Supplementary Figure 3 | rCdtC suppresses CagA translocation of *Helicobacter pylori*-infected cells. **(A)** AGS cells pre-incubated with rCdtC (200 nM) were infected with CagA-EGFP *H. pylori* (green) at a multiplicity of infection of 20 for

2 h. The cells were probed with anti-CdtC (yellow), the plasma membrane was stained with WGA-conjugated Alexa Flour 594 (red), and the nuclei were stained with Hoechst 33342 (blue). The image was observed under a confocal microscope with × 63 oil immersion objective and processed by software ZEN. Arrows indicate the CagA translocated into the cytoplasm. Scale bar, 5 μm. **(B)** The distribution of fluorescence intensity for CdtC (yellow line), CagA (green line), and plasma membrane (red) signals across the white lines was calculated and presented as line intensity histograms in the right panels. The area within the cell membrane is represented by the peak of wheat germ agglutinin fluorescence intensity, which is located between the two dotted lines.

Supplementary Figure 4 | rCdtC decreases *Helicobacter pylori* invasion in gastric epithelial cells. AGS cells were untreated with rCdtC (mock), treated with 200 nM rCdtC, infected with *H. pylori* for 6 h, and pretreated with rCdtC then infected with *H. pylori* for 6 h. *H. pylori* **(A)** adhesion and **(B)** invasion to cells were analyzed. *$P < 0.05$.

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REVIEW
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Role of Cholesterol and Lipid Rafts in Cancer Signaling: A Promising Therapeutic Opportunity?

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Cholesterol is a lipid molecule that plays an essential role in a number of biological processes, both physiological and pathological. It is an essential structural constituent of cell membranes, and it is fundamental for biosynthesis, integrity, and functions of biological membranes, including membrane trafficking and signaling. Moreover, cholesterol is the major lipid component of lipid rafts, a sort of lipid-based structures that regulate the assembly and functioning of numerous cell signaling pathways, including those related to cancer, such as tumor cell growth, adhesion, migration, invasion, and apoptosis. Considering the importance of cholesterol metabolism, its homeostasis is strictly regulated at every stage: import, synthesis, export, metabolism, and storage. The alterations of this homeostatic balance are known to be associated with cardiovascular diseases and atherosclerosis, but mounting evidence also connects these behaviors to increased cancer risks. Although there is conflicting evidence on the role of cholesterol in cancer development, most of the studies consistently suggest that a dysregulation of cholesterol homeostasis could lead to cancer development. This review aims to discuss the current understanding of cholesterol homeostasis in normal and cancerous cells, summarizing key findings from recent preclinical and clinical studies that have investigated the role of major players in cholesterol regulation and the organization of lipid rafts, which could represent promising therapeutic targets.

Keywords: cholesterol, cancer, lipid rafts, cell death, invasion, metastases, therapy

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INTRODUCTION

Cholesterol is a primary lipid molecule that plays an essential role in a number of biological processes, both at physiological and pathological level (Maxfield and Tabas, 2005).

Abbreviations: ABCA1, ATP-binding cassette subfamily A member 1; ABCG1, ATP-binding cassette subfamily G member 1; ACAT1, acyl-CoA:cholesteryl acyltransferase 1; AMPK, 5′adenosine monophosphate-activated protein kinase; AMPK, AMP-activated protein kinase; ApoA-I, lipid-poor apolipoprotein A-I; CE, cholesteryl ester; ER, endoplasmic reticulum; FPP, farnesyl pyrophosphate; FPP, farnesyl pyrophosphate; FTase, farnesyl transferase; GGPP, geranylgeranyl pyrophosphate. GGTase, geranylgeranyl transferases; HDL, high-density lipoprotein; HMG-CoA, 3-hydroxy-3-methyl-glutaryl CoA; HMGR, HMG reductase; IGF, insulin-like growth factor; INSIGs, insulin-induced gene; LDL, low-density lipoprotein; LDLR, LDL receptor; LXR, liver X receptor; MβCD, methyl-β-cyclodextrin; miRNA, microRNA; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NPC1L1, Niemann–Pick type C1-like 1; PI3K, phosphatidylinositol 3-kinase; SCAP, SREBP cleavage activating protein; sGTPase, small GTP-binding protein; SOAT, sterol-o-acyltransferase; SQL, squalene; SQLE, squalene epoxidase; SRE, sterol regulatory element; SREBP2, sterol regulatory element-binding protein 2; VLDL, very-low-density lipoprotein.

Cholesterol, in addition to being an important constituent of cell membranes, is fundamental for their biogenesis, and is indispensable for maintaining integrity and functions of biological membranes, including endocytosis, membrane trafficking, and signaling (Maxfield and Tabas, 2005; Yamauchi and Rogers, 2018). Inside the cell, cholesterol, heterogeneously distributed among the organelles, modulates the immune system, and represents a precursor of hormones such as sexual hormones and vitamin D (Mollinedo and Gajate, 2020; **Figure 1**).

Recently, cholesterol has played a key role in cancer research because of its potential therapeutic implications both in prevention and in treatment. However, the role of cholesterol in oncogenesis is still debated (DuBroff and de Lorgeril, 2015). Literature data reported a contradictory role of cholesterol depending on the type of tumor (Ding et al., 2019). Excess of cholesterol is related to breast, colon, rectal, prostatic, and testicular cancers (Llaverias et al., 2011; Pelton et al., 2012; Murai, 2015; Radisauskas et al., 2016), while some prospective cohort studies showed an inverse association in gastric and prostate cancers (Asano et al., 2008; Heir et al., 2016). This review aims to discuss the current knowledge of cholesterol homeostasis, critically analyzing the most recent preclinical and clinical studies investigating the role of the principal players of the cholesterol biosynthetic pathway, and of the cholesterol-based membrane structure's lipid rafts in the field of cancer.

CHOLESTEROL METABOLISM

Cholesterol is produced through a cascade of enzymatic reactions, namely, mevalonate pathway, which requires the participation of different enzymes localized on the membranes of the endoplasmic reticulum (ER). Briefly, the combination of three acetyl-CoA molecules leads to the formation of one 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) molecule. The latter, by the action of HMG-CoA reductase (HMGCR), is converted into mevalonate, in turn transformed into squalene (SQL), and ultimately into cholesterol by means of a number of reactions (**Figure 2**). Food can be a source of cholesterol too. In fact, the Niemann–Pick type C1-like 1 (NPC1L1) protein, present on the membrane of the intestinal enterocytes, is responsible for the absorption of cholesterol, which is released as chylomicrons, triglyceride-rich lipid particles, and is taken up by the liver (Altmann et al., 2004; Luo et al., 2020).

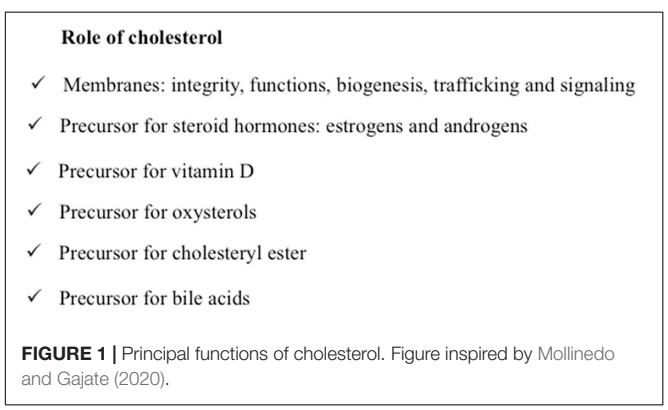

FIGURE 1 | Principal functions of cholesterol. Figure inspired by Mollinedo and Gajate (2020).

Cholesterol is mainly synthesized in the liver and free into the bloodstream as very-low-density lipoproteins (VLDLs). In the bloodstream, the VLDLs are transformed to produce low-density lipoproteins (LDLs), transported to peripheral cells by the bloodstream (Ikonen, 2008; Goldstein and Brown, 2009). LDLs enter cells via receptor (LDLR)-mediated endocytosis and are transported to lysosomes where they are hydrolyzed into free cholesterol molecules, which are transported to cellular membranes to carry out its multiple functions (Brown and Goldstein, 1986; Ikonen, 2008; Maxfield and van Meer, 2010; Kuzu et al., 2016).

Mevalonate pathway is tightly regulated by transcriptional and translational mechanisms capable of responding to physiological signals. Cholesterol biosynthesis is regulated by four principal players: (1) sterol regulatory element-binding protein 2 (SREBP2), which acts through a negative feedback mechanism (Sato, 2010), (2) liver X Receptors (LXRs), (3) HMGCR, and (4) squalene epoxidase (SQLE). HMGCR and SQLE are rate-limiting enzymes, which can regulate cholesterol biosynthesis, the reactions they catalyze being energetically expensive. When intracellular ATP levels are low, 5′adenosine monophosphate-activated protein kinase (AMPK) phosphorylates HMGCR inhibiting its function (Loh et al., 2019). Moreover, HMGCR is influenced by the presence of LDL in the medium; in fact, upon LDL starvation, HMGCR activity enhances, while it highly decreases when LDL is added back. On the other hand, once cholesterol has exhausted its function, its surplus is exported via ATP-binding cassette (ABC) subfamily A member 1 (ABCA1) or ABC subfamily G member 1 (ABCG1) to lipid-poor apolipoprotein A-I (ApoA-I), thus generating high-density lipoproteins (HDLs) (Gelissen et al., 2006; Lorenzi et al., 2008; Daniil et al., 2013; Phillips, 2014). The transcription of ABCA1 is upregulated by nuclear LXRs when the intracellular cholesterol level is high (Wang et al., 2008; Ouvrier et al., 2009; Kuzu et al., 2016). The CoA:cholesteryl acyltransferase 1 (ACAT1) transforms excess cholesterol into less toxic compounds, such as cholesteryl esters (CEs), which are stored as lipid droplets and used for the production of the main plasma lipoproteins (chylomicrons, VLDLs, LDLs, and HDLs). HDLs are then transported from peripheral tissues back to the liver and intestine, to recycle or eliminate cholesterol, and to steroidogenic organs, where cholesterol is used to generate steroid hormone (Chang et al., 2009; Luo et al., 2020).

Recent studies on microRNAs (miRNAs), a class of non-coding RNAs, highlighted their role in the cholesterol homeostasis by adjusting some important components of the system (Wagschal et al., 2015). For instance, under low sterol concentration conditions, higher transcription of miR-33a is required to control cholesterol export and HDL metabolism through ABCA1 inhibition (Wagschal et al., 2015). Conversely, miR-223 regulates cholesterol amount by inhibiting its production and ameliorating cholesterol efflux by enhancing ABCA1 levels (Vickers et al., 2014). miRNA-122, present mainly in hepatocytes, when inhibited significantly suppresses blood cholesterol level (Rotllan and Fernandez-Hernando, 2012).

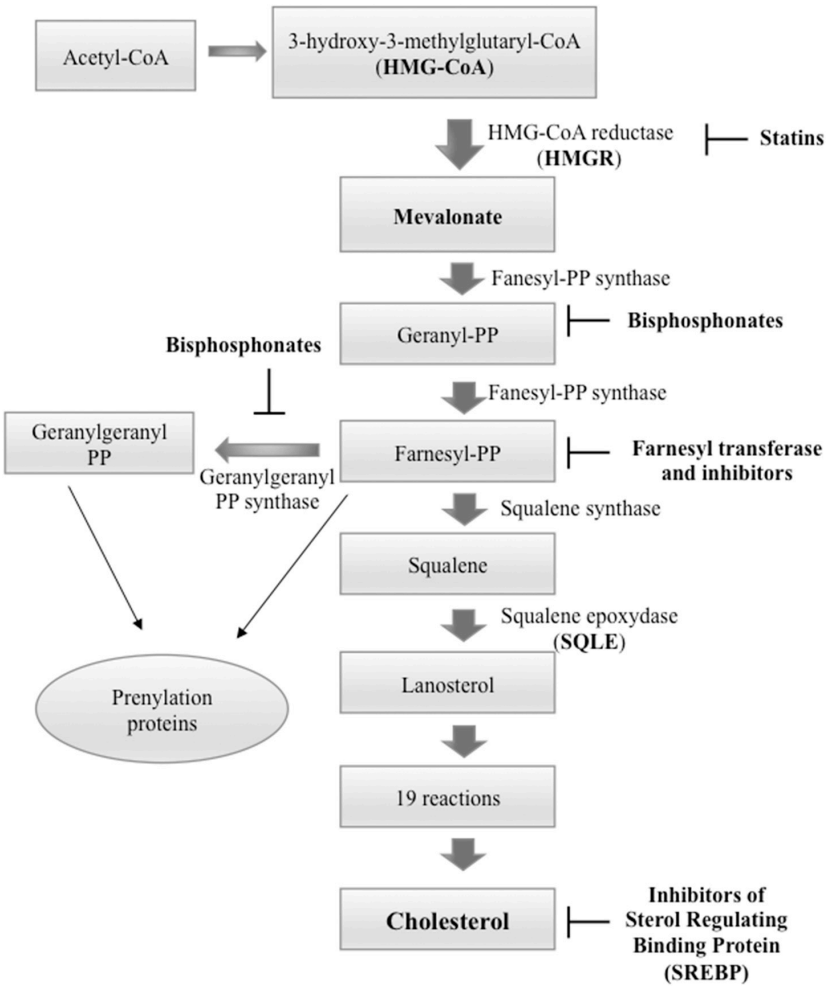

FIGURE 2 | Cholesterol biosynthesis pathway and principal inhibitors. Starting from three molecules of acetyl-coenzyme A (CoA), cholesterol is synthesized in more than 20 enzymatic steps. 3-Hydroxy-3-methylglutaryl-CoAreductase (HMGCR) and squalene epoxidase (SQLE) act as rate-limiting enzymes. The principal inhibitors of cholesterol biosynthesis are statins that inhibit HMGCR, inhibitors of sterol regulating binding protein (SREBP) that inactivate the transcription of cholesterol biosynthesis genes; and bisphosphonates that act downstream of statins and inhibit farnesyl pyrophosphate synthase with consecutive decrease of the farnesyl pyrophosphate and geranylgeranyl pyrophosphate. This step of the cholesterol biosynthesis is also targeted by farnesyl transferase inhibitors.

miR-27a has been shown to control HMGCR level either by a posttranslational block or by mRNA degradation (Khan et al., 2020). Strikingly, other miRNAs have been discovered by meta-analyses to be associated with cholesterol–lipoprotein trafficking alterations, such as miR-128-1, miR-148a, miR-130b, and miR-301b. These miRNAs were able to increase circulating cholesterol by controlling the expression level of LDLR and ABCA1 (Wagschal et al., 2015).

These data suggest the participation of miRNAs in the control of cholesterol metabolism, underlining how they could contribute to the alteration of cholesterol levels when dysregulated.

Cholesterol Homeostasis in Normal and Cancer Cells

Considering the importance of cholesterol metabolism, its cellular homeostasis is strictly regulated at every stage: import, synthesis, export, transport, and esterification (Ikonen, 2008). Sterol regulatory element-binding protein 2 (SREBF2) and LXRs act as key regulators of cholesterol homeostasis (Ikonen, 2008). In the ER, cholesterol itself regulates its homeostasis. Low cholesterol levels induce translocation of SREBP2 to the nucleus where it promotes activation of genes implicated in the biosynthesis (e.g., HMGCR) and uptake (e.g., LDLR) of cholesterol (Ikonen, 2008). High cholesterol levels inhibit the cholesterol synthesis and facilitate its export through the activation of LXRs by oxysterols, oxidized derivatives of cholesterol (Wang et al., 2008; Kuzu et al., 2016). Recent studies on LNCaP prostate cancer cells highlighted an important protective role of LXRs (Pommier et al., 2010; Fu et al., 2014). In fact, the activation of these transcription factors regulating cholesterol homeostasis was able to induce cell cycle arrest and to promote apoptosis (Pommier et al., 2010). The relationship among LXRs, cholesterol, and prostate cancer

highlights that LXRs have good chance to be targeted one day in this tumor.

During cholesterol biosynthesis, depending on the type of tissue, different intermediate sterols are formed, such as cholesteryl ester, oxysterols, bile acids, cholecalciferol/vitamin D, and various steroid hormones. All these sterols have important physiological functions in cells and in tissues (Simons and Ikonen, 2000). Some cholesterol metabolites may also contribute to the progression and metastatization of some types of cancer (Lin et al., 2013; McDonnell et al., 2014; Baek et al., 2017).

Under healthy conditions, the amount of cholesterol is the result of a balance between synthesis, uptake from extracellular milieu, removal of the cholesterol surplus from peripheral tissues, and metabolic conversion (Simons and Ikonen, 2000; **Figure 3**). The alterations of this balance, often caused by unbalanced diets and unhealthy lifestyles (Hu et al., 2012), are known to be associated with cardiovascular diseases and atherosclerosis, but mounting evidence also connects these behaviors to increased cancer risks (Luo et al., 2020).

Over the past few decades, literature data have demonstrated that metabolic alterations represent a hallmark of cancer.

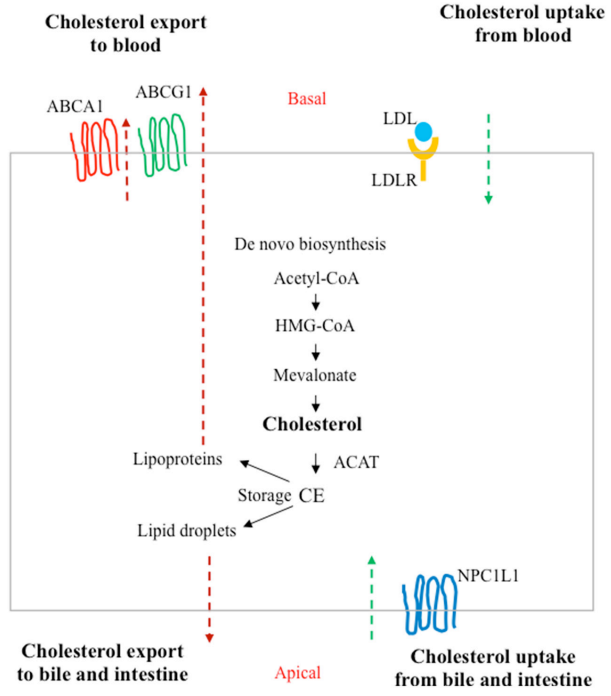

FIGURE 3 | Homeostasis of cholesterol in a polarized cell. In addition to *de novo* biosynthesis, cholesterol carried by low-density lipoprotein (LDLs) in the blood can be taken up by LDL receptors (LDLRs) at the basal surface of the polarized cells (such as enterocytes or hepatocytes). Niemann–Pick type C1-like 1 (NPC1L1) can absorb free cholesterol from dietary sources by enterocytes in the intestine and from bile in the biliary ducts by hepatocytes in the liver. Excess cholesterol is exported to the blood by ATP-binding cassette subfamily A member 1 (ABCA1) or the subfamily G member 1 (ABCG1). Cholesterol can also be converted to cholesteryl ester (CE) by acyl coenzyme A:cholesterol acyltransferase (ACAT) for storage in lipid droplets or for secretion as lipoproteins.

Oncogenesis is an intricate process that involves, in addition to reprogramming energy metabolism, also reprogramming of genetic information, signaling mechanisms, and structural components, which are critical for the survival and growth of cancer cells (Hanahan and Weinberg, 2000, 2011). Reprogramming energy metabolism had already been described by Otto Warburg, which highlighted that cancer cells, even in the presence of abundant oxygen, preferably produce ATP (adenosine triphosphate) through the less efficient glycolytic pathway, rather than through oxidative phosphorylation (OXPHOS), thus producing more lactate than normal cells (Warburg, 1925). This metabolic switch of cancer cells, called Warburg effect, is also known as aerobic glycolysis. More recently, attention has been paid to metabolic heterogeneity within the tumor. Particular emphasis has been given to the cells present in the tumor stroma, which would also have the role of metabolically supporting the cancer cells. In this context fits the theory of the reverse Warburg effect according to which the aerobic glycolysis in the cells of the tumor stroma would have the role of providing, in addition to ATP, metabolites for the generation of further ATP through OXPHOS in cancer cells. Moreover, the metabolic coupling between stromal and tumor cell, with the exchange of useful metabolites, would also allow the tumor cell to increase its proliferation and reduce cell death (Xu et al., 2015; Wilde et al., 2017; Benny et al., 2020).

Alteration of energy metabolism is due to enhanced metabolic needs of cancer cells to support the proliferation, migration, and metastatic cancer activities (Warburg, 1956; Silvente-Poirot and Poirot, 2012). However, there are conflicting epidemiological evidence on the cholesterol function in cancer development, while important preclinical data suggested that a dysregulation of cholesterol homeostasis could lead to cancer progression. In particular, new data suggested that cholesterol amount in cells may have a greater role in cancer development than serum cholesterol (Kuzu et al., 2016). Some oncogenic pathways such as PI3K/AKT/mTOR, RTK/RAS, YAP/TAZ, and p53 are also able to regulate cholesterol production in cancer cells (**Figure 4**).

CHOLESTEROL AS COMPONENT OF LIPID RAFTS

Besides the classical role of cholesterol in determining the correct structure, fluidity, and functioning of eukaryotic cell membranes, cholesterol represents the prevalent lipid component of specific plasma membrane microdomains, known as lipid rafts, also contributing to their organization. Indeed, cholesterol accumulates in specialized region of the membrane and, by interacting with sphingolipids, forms lipid rafts (Brown and London, 2000; Pike, 2003, 2006). These membrane regions are heterogeneous and highly dynamic structures, ranging from 10 to 200 nm in size, that selectively recruit and concentrate different membrane proteins (i.e., receptors, adhesion molecules, etc.) and signaling molecules, forming a sort of platform for signal transduction (Simons and Toomre, 2000). Nitric oxide synthase, GPI-anchored proteins, Src-family tyrosine kinases, G-protein-α subunit, protein kinase C, and protein kinase A are typically

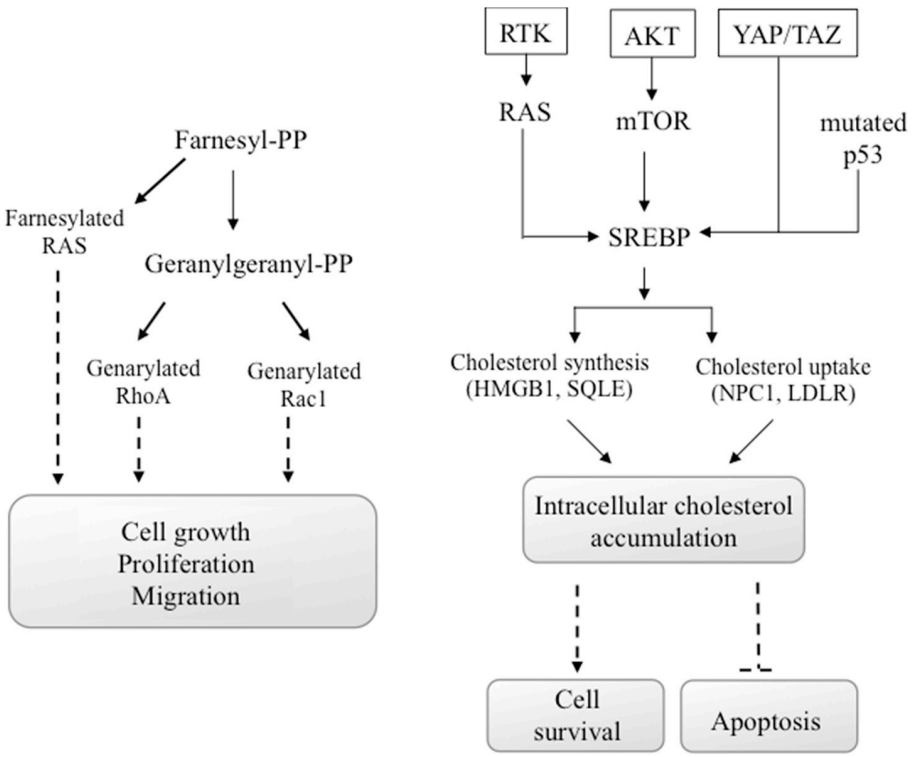

FIGURE 4 | Oncogenic signals involving cholesterol. Some oncogenic signals initiated from AKT/mTOR, RTK/RAS, YAP/TAZ, or mutated p53 induce the activity of SREBP transcription factor, the major regulator of genes encoding cholesterol synthesis and uptake. Also, the activation of the GTP-binding proteins Ras, Rho, and Rac, which functions are involved in carcinogenesis, depends on the availability of cholesterol. In particular, Ras family proteins are prenylated by farnesyl-PP, whereas geranylgeranyl-PP prenylates proteins of the Rho and Rac families. These chemical modifications are mandatory for their activity.

localized within lipid rafts (Song et al., 1997; Galbiati et al., 1999; Razani et al., 1999; Patel and Insel, 2009).

Membrane and signaling proteins reside transiently within lipid rafts. Thus, depending on whether the signal transduction needs to be enhanced or damped, they can be recruited or excluded from lipid rafts in a reversible manner. The mechanisms at the base of reversible raft–protein associations are not yet completely understood. Characteristic lipid post-translational modifications have been described by researchers to be responsible for the recruitment of proteins within lipid rafts. One of the major raft-targeting signals is represented by S-palmitoylation of transmembrane proteins, consisting in palmitate moieties added to cysteine residues through thioester linkage (Resh, 1999, 2004; Petrova et al., 2013). For instance, death receptors such as Fas/CD95 (Chakrabandhu et al., 2007; Feig et al., 2007) and TRAIL receptor 1 (Rossin et al., 2009) are palmitoylated on specific cysteine residues, and this event is essential either for their recruitment within lipid rafts or for an efficient transmission of death signals. In human breast cancer cells, palmitoylation of the surface adhesion receptor CD44 relies on two cysteine residues. This event enhances CD44 affinity for cholesterol-rich lipid rafts, inducing CD44 recruitment into lipid microdomains (Babina et al., 2014), limiting breast cancer cell migration (Donatello et al., 2012). Moreover, proteins can be anchored to plasma membranes through a glycosylphosphatidylinositol (GPI) anchor or they can present a lysine-rich region within their amino acid sequence, which represents an additional signal beside palmitoylation targeting proteins within lipid rafts (Rossin et al., 2009).

Therefore, membrane lipid rafts represent a sort of lipid-based structures that regulate the assembly and functioning of numerous cell signaling, including those related to cancer (Staubach and Hanisch, 2011; Donatello et al., 2012), such as tumor cell growth, adhesion, migration, invasion, and apoptosis (Yang et al., 2014, 2018).

Lipid rafts were also found in mitochondria, ER, in the nuclear membrane, and phagosomes, respectively, where they form a sort of "raft-like microdomain" (Garofalo et al., 2005, 2016; Brunham et al., 2006; Cascianelli et al., 2008; Boslem et al., 2013; Matarrese et al., 2014). These regions, rich in gangliosides, present a content of cholesterol lower as compared with plasma membrane (Garofalo et al., 2015). They are involved in triggering specific events during apoptosis execution, i.e., mitochondria hyperpolarization and depolarization, with consequent release of apoptogenic factors (Garofalo et al., 2005; Scorrano, 2008; Sorice et al., 2009), and regulation of fusion–fission processes (Garofalo et al., 2007; Scorrano, 2008; Sorice et al., 2009; Ciarlo et al., 2010, 2018; Annunziata et al., 2018).

Taking into account all these considerations, the interest in targeting membrane raft cholesterol in cancer cells is

increasing. Specific studies might be helpful to better understand the potential use of lipid rafts as innovative target in anti-cancer therapy.

Raft-Associated Cholesterol Involvement in Migration, Invasion, and Metastatic Processes of Cancer Cells

Mounting evidence of the literature supports a role for lipid rafts in several signaling transduction pathway related to malignancy. Therefore, the interest in studying lipid rafts and the modulation of lipid raft organization is growing. Approaches aimed at disorganizing membrane raft domains were reported by researchers to affect cancer cell proliferation, adhesion, migration, invasiveness, metastatic spread, and apoptosis (Simons and Toomre, 2000; Simons and Ehehalt, 2002; Zidovetzki and Levitan, 2007).

A critical feature of malignancy is represented by the acquisition of a more aggressive phenotype by cancer cells. Because of their malignancy, cancer cells became able to adhere to the extracellular matrix and therefore to migrate and to invade other tissues, leading to metastasis. The plasma membrane certainly represents the first structure of the tumor cell to be involved in the processes of invasion and metastasis. Invadopodia are highly specialized plasma membrane structures of tumor cells, corresponding to podosomes of untransformed cells, able to favor the adhesion and penetration of the tumor cell into the underlying extracellular matrix, stroma, and basement membrane. The formation of invadopodia, structures rich in cytoskeletal elements, adhesion molecules, and degradative enzymes, requires the presence of lipid rafts, which are decisive for the establishment of this invasive and dynamic structure (Nicolson, 2015). Yamaguchi et al. (2009) found that Cav-1, a typical component of caveolar membrane lipid raft structures, represents an essential regulator of invadopodia-mediated human breast cancer cell invasion (Yamaguchi et al., 2009). Disruption of lipid rafts by depletion of membrane cholesterol using methyl-β-cyclodextrin (MβCD) suppressed either invadopodia formation and tumor invasion, thus providing evidence that lipid raft formation was mandatory for invadopodia functioning, at least in human breast cancer (Yang et al., 2016). In bladder cancer cells, Cav-1 overexpression induced epithelial-to-mesenchymal transition that was mediated by PI3K/AKT activation (Liang et al., 2014).

Very interestingly, a reduced expression of Cav-1 was found frequently in human cancer-associated fibroblasts (Martinez-Outschoorn et al., 2014), where it was accompanied by a reduced mitochondrial function and an increased expression of glycolytic enzymes (Asterholm et al., 2012). Some studies have shown that tumor cells, inducing oxidative stress, were responsible for the autophagic degradation of Cav-1 in stromal fibroblast, thus favoring tumor–stroma co-evolution underlying the theory of the reverse Warburg effect (Martinez-Outschoorn et al., 2010).

The dysregulation of flotillin-2, a protein marker of non-caveolar plasma membrane microdomains, has been reported in a variety of tumors. In particular, a recent study related a high expression of flotillin-2 to the incidence of lymph node metastasis and TNM stage in intrahepatic cholangiocarcinoma cell lines (Xu et al., 2020), as also previously observed in a variety of human solid tumors (Liu et al., 2017). High expression of flotillins was also observed in drug-resistant strains of colorectal cancer cells (Ye et al., 2019). Data from Ye and coworkers clearly demonstrated that flotillin silencing using lentivirus-mediated RNAi approach, by inducing destruction of lipid rafts, inhibited the drug resistance of the colorectal cancer cell line HCT-15 (Ye et al., 2019).

Additional findings in prostate cancer cells suggested a potential positive role of membrane cholesterol in modulating the IL-6/STAT3 pathway and chemoattraction (Nakashima et al., 2000; Kim et al., 2004; Akashi et al., 2008; Chinni et al., 2008). The IL6–JAK–STAT3 pathway, known to be an important signaling pathway in prostate cancer, requires intact lipid rafts for sustaining pro-oncogenic signaling. Also in this case, disruption of lipid rafts by MβCD inhibited this pathway thus controlling prostate cancer growth (Dambal et al., 2020). In addition, it has also been reported that hypoxia, a prognostic predictor of poor clinical outcome (Vaupel et al., 2001), was able to increase cholesterol level and recruitment of Notch3 into lipid rafts. Notch3 activity sustained cell proliferation of different hormone-dependent and independent prostate cancer cell lines, and positively correlated with tumor aggressiveness (Danza et al., 2013). In prostate cancer cells, it has also been reported that the interaction of the tetraspanin CD82 with cholesterol in lipid rafts inhibited tumor cell movement and invasion by acting on plasma membrane/cytoskeleton linker ezrin, thus disengaging the plasma membrane–cytoskeleton connection and consequently repressing cell movement (Huang et al., 2020).

Several proteins responsible for development of a more aggressive and metastatic phenotype have been found associated with lipid rafts, including integrins and cell adhesion molecules, ion channels, potassium channels, and MUC1 (Staubach et al., 2009; Haddon and Hugh, 2015), strongly supporting a positive role for lipid rafts in tumor progression.

Several findings reported the localization within lipid rafts of the highly expressed cell surface adhesion receptor CD44, whose lipid raft localization was related to invasion and metastatic processes of cancer cells (Murai, 2015; Mollinedo and Gajate, 2020). In this context, it was reported that MβCD-mediated depletion of cholesterol induced CD44 shedding (Adler, 2011; Murai et al., 2011). Like MβCD, simvastatin, an agent that is able to target cholesterol with consequent disorganization of membrane rafts, also demonstrated to be effective in increasing shedding of CD44 and concomitantly in inhibiting tumor cell migration (Murai et al., 2004, 2006, 2009; Murai, 2015). In the same vein, it has also been shown that other statins induced disruption of lipid rafts, leading to impairment of tumor cell adhesion and migration (Murai et al., 2011; Murai, 2012).

Like other receptors, CD44 has been found into lipid rafts (Murai, 2015) or associated with the actin cytoskeleton through the ezrin protein in non-raft compartments (Martin et al., 2003; Yang et al., 2018). Once CD44 binds ezrin in non-raft compartments, it promotes cancer cell adhesion and migration in hepatocellular carcinoma cells (Yang et al., 2003). Similar results were also reported for breast cancer cells (Donatello et al., 2012).

Thus, CD44–ezrin interaction in non-raft compartments leads to cell migration (Martin et al., 2003; Mollinedo and Gajate, 2020). Taken together, these data point out the importance of lipid rafts in CD44-mediated migration and invasion processes, but the molecular mechanism involved remains to be fully elucidated. Taking into account all these evidences, lipid rafts might therefore represent an important modulator of the invasive and metastatic ability of cancer cells. Accordingly, it has demonstrated that suppressing lipid raft formation using the bioactive natural compound daphnane-type diterpenes from *Daphne genkwa* (GD) strongly impaired human hepatocellular carcinoma cell invasion and migration (Wu et al., 2020).

Raft-Associated Cholesterol in Cancer Cell Survival and Apoptosis

Many studies in the literature evidenced cholesterol-rich rafts as important players regarding cancer cell survival signaling and proliferative pathway. Indeed, it has been reported that survival signal pathways are compartmentalized into lipid rafts where membrane receptors can become in close contact with downstream signaling molecules, inducing the transmission of proliferative signals into the cells. This is the case of the activation of PI3K/AKT-mediated survival signals induced by insulin-like growth factor (Gao and Zhang, 2008, 2009; Gao et al., 2011; Reis-Sobreiro et al., 2013; Mollinedo and Gajate, 2020). In fact, it has been reported that activation of IGF receptors induced the recruitment of several signaling molecules involved in the PI3K/AKT pathway into lipid rafts, favoring activation by phosphorylation of AKT, with subsequent transmission of survival signals into the cells. Disruption of lipid rafts using MβCD or filipin III had demonstrated efficacy in inhibiting the activation of AKT and other survival signaling pathways in human lung adenocarcinoma and human T-cell leukemia, in favor of apoptosis activation (Motoyama et al., 2009).

Data obtained in prostate cancer highlighted the importance of the epidermal growth factor (EGF) receptor recruitment into lipid rafts for an efficient transmission of survival signal within the cells (Zhuang et al., 2002; Zidovetzki and Levitan, 2007). Indeed, it has been reported that disruption of membrane rafts using statins, known cholesterol-lowering agents, impaired EGF receptor pathway, favoring apoptosis (Zhuang et al., 2005; Oh et al., 2007; Hryniewicz-Jankowska et al., 2019).

Particular attention has also been paid by researchers on the role of lipid rafts regarding the apoptotic signaling pathway, which could occur following one of the two classical pathways, the extrinsic membrane receptor-mediated or the intrinsic mitochondria-involving pathway. Interestingly, cholesterol has emerged as key modulator of both apoptotic pathways. Like for cancer survival signaling, lipid rafts have been described as scaffold plasma membrane regions, in which death receptors and downstream signaling molecules (FADD, pro-caspase-8/-10) aggregate and cluster in, favoring apoptosis induction and execution (Gajate and Mollinedo, 2001; Scheel-Toellner et al., 2002; Garofalo et al., 2003; Gajate et al., 2004; Malorni et al., 2007; Mollinedo and Gajate, 2010, 2020; Iessi et al., 2020). Findings from the last two decades evidenced that the localization of death receptors (e.g., CD95/FAS and TRAIL) into these specialized regions is mandatory either for susceptibility to receptor-mediated death signal or for a correct and efficient initiation of the apoptotic signaling (**Figure 5**; Gajate and Mollinedo, 2001, 2007; Mollinedo et al., 2004; Mollinedo and Gajate, 2006, 2020; Marconi et al., 2013). Indeed, recruitment of the death receptor Fas/CD95 into lipid rafts has been described in several studies (Gajate and Mollinedo, 2001, 2015a; Gajate et al., 2004; Mollinedo and Gajate, 2020). Similarly, TRAIL death receptor 1, but not TRAIL death receptor 2, localized constitutively within the lipid rafts in human cancer B-cell lines, and this event was depicted as mandatory for an efficient death receptor-mediated signaling transduction (Marconi et al., 2013). Actin cytoskeleton plays a peculiar role in ensuring a correct lipid localization of membrane proteins and an efficient cell death signal transmission (Algeciras-Schimnich et al., 2002; Gajate and Mollinedo, 2005). The ERM protein ezrin, known linker between membrane proteins and the actin cytoskeleton, was described as a positive regulator of early steps of Fas receptor signaling in lymphoid T cells (Parlato et al., 2000; Lozupone et al., 2004). Moreover, aggregation of death receptors within lipid rafts, by promoting apoptosis induction, was favored by several agents, i.e., oxaliplatin (Xu et al., 2009), epirubicin (Xu et al., 2011), β-elemene (Xu et al., 2018), bufalin (Yan et al., 2014), resveratrol (Delmas et al., 2004; Reis-Sobreiro et al., 2009), ursodeoxycholic acid (Lim et al., 2011), edelfosine (Gajate and Mollinedo, 2001; Gajate et al., 2004; Lim et al., 2016; Mollinedo and Gajate, 2020), and perifosine (Gajate and Mollinedo, 2007; Mollinedo and Gajate, 2020). On the contrary, agents able to disaggregate lipid rafts through membrane cholesterol depletion induced resistance to apoptotic cell death (Delmas et al., 2004; Gajate and Mollinedo, 2007, 2014, 2015b; Gajate et al., 2009; Marconi et al., 2013). For instance, resistance to TRAIL may be promoted by MβCD (Delmas et al., 2004; Marconi et al., 2013), whereas enhancement of TRAIL-induced apoptosis could be observed following perifosine treatment, which favored the recruitment of DRs into lipid rafts (Gajate and Mollinedo, 2007; Marconi et al., 2013). Edelfosine-mediated upregulation of TRAIL-R2, and its subsequent increased recruitment within lipid rafts, turned resistant gastric cancer cells back to a TRAIL-sensitive phenotype (Lim et al., 2016). Enhanced Fas/CD95 recruitment and clustering within lipid rafts, and consequent increased Fas/CD95-mediated apoptosis, was observed after edelfosine treatment in several hematological malignancies (Gajate and Mollinedo, 2001, 2007; Gajate et al., 2004, 2009; Mollinedo and Gajate, 2006, 2015; Mollinedo et al., 2010a,b). As a consequence, depletion of membrane cholesterol by leading to disruption of lipid rafts strongly impaired Fas/CD95 death signaling (Gajate and Mollinedo, 2007, 2014, 2015b; Gajate et al., 2009).

TARGETING CHOLESTEROL PATHWAYS AS CANCER THERAPY

Cancer has often been coupled with modifications in lipid metabolism, in particular in cholesterol metabolism. Alterations in cholesterol homeostasis have been identified in a large number

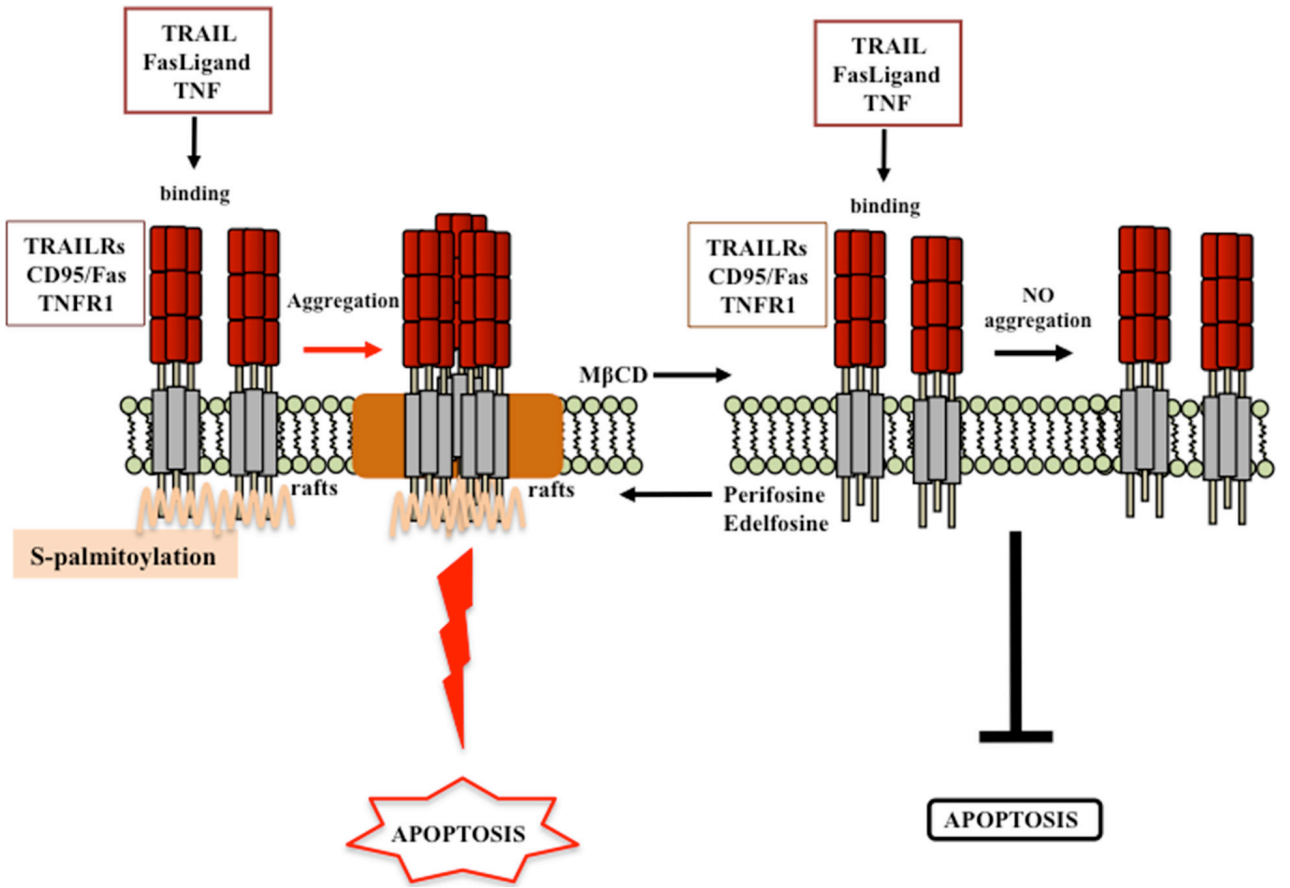

FIGURE 5 | Proposed model for lipid rafts involvement in receptor-mediated cell death. According to the model proposed by Mollinedo and Gajate (2020), engagement of membrane death receptors within lipid rafts promotes their aggregation and the subsequent activation of cell death signaling pathway. Destruction of lipid rafts by cholesterol lowering (e.g., MβCD treatment) inhibits cancer cell death. By contrast, favoring the recruitment of death receptor within lipid rafts (e.g., perifosine or edelfosine treatment), the activation of death signals is favored. Modified from Gajate and Mollinedo (2015a,b); Mollinedo and Gajate (2020).

of cancers. Both circulating and membrane associated cholesterol would appear to play a key role in the regulation of all cancer signaling pathways.

According with this, it has been suggested that several anticancer drugs may have anti-proliferative function, limiting the content or production of cholesterol. For instance, it has been shown that doxorubicin caused the death of cancer cells by promoting a reduction in HMGCR levels and causing a decrease in cholesterol content (Yun et al., 2019). Other data displayed that tamoxifen modulated cholesterol metabolism in breast cancer cells (Segala et al., 2013). Many natural compounds, including terpenoids, green tea, garlic extract, and curcumin, effective in cancer prevention and therapy, were identified to target cholesterol homeostasis in cancer cells (Mok and Lee, 2020). Thus, these natural compounds might be useful in regulating cholesterol homeostasis as adjuvants to complement current anticancer therapies.

The cholesterol synthesis pathway consists of a number of enzymes, each of which can represent a possible target to disturb the mevalonate pathway in cancer cells. Many studies suggested that pharmacological modulation of intracellular cholesterol balance could be effective in controlling cancer progression (**Figure 6**).

Targeting Cholesterol Synthesis and Uptake

Cellular cholesterol levels are the result of a balance among the processes of production, transport, and storage. The biochemical events and the main players that in cells are underlying synthesis and trafficking cholesterol, and which, therefore, can represent potential therapeutic targets, are briefly mentioned in the following section.

HMGCR

The rate-limiting reduction of HMG-CoA to mevalonate is an important regulatory step in cholesterol synthesis. HMG-CoA is highly regulated at transcriptional, translational, and post-translational planes (Goldstein and Brown, 1990). The HMGCR is an ER-localized glycoprotein that converts HMG-CoA to mevalonate. HMGCR is genetically activated by nSREBP2 when sterol concentrations are low. Sterols, mainly oxysterols, and two members of the vitamin E family can induce its

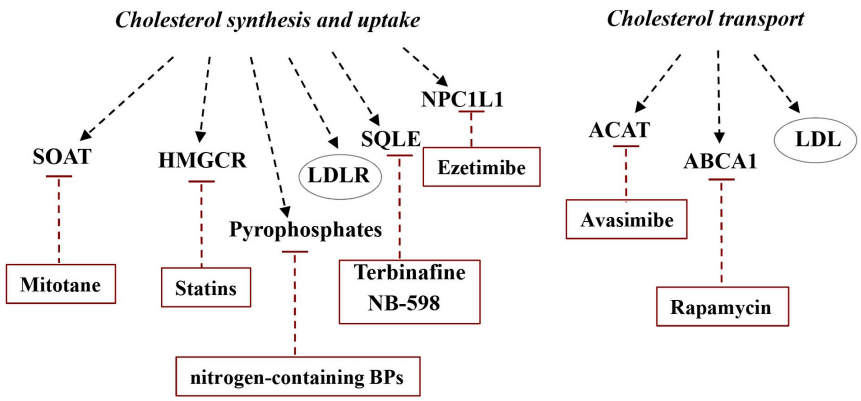

FIGURE 6 | Targeting cholesterol as cancer therapy. Altering the balance between *de novo* synthesis of cholesterol and its transport could be useful for modulating cancer development. Some drugs or molecules are able to specifically inhibit some of the major players in cholesterol synthesis, uptake, and transport and can therefore be considered potentially useful in cancer therapy.

degradation (Song et al., 2005; Chen et al., 2019). Moreover, ubiquitination and proteasome-mediated degradation are also known mechanisms regulating HMGCR. HMGCR changes from a phosphorylated to a dephosphorylated form. Phosphorylation inhibits HMGCR activity. AMP-activated protein kinase (AMPK) is the main actor for hepatic HMGCR phosphorylation, which in turn results in hampering of cholesterol production when low intracellular ATP levels are perceived. Conversely, HMGCR-mediated cholesterol biosynthesis is triggered when AMPK suppression occurs (Zhang et al., 2015; Luo et al., 2017; Soto-Acosta et al., 2017). The interaction between HMGCR and the insulin-induced gene protein (INSIG-1 or -2) at ER membrane is pivotal to HMGCR ubiquitination and degradation (Gong et al., 2006). Overexpression of HMGCR has been well documented in gastric cancer cells, when it promoted growth and migration, while HMGCR knockdown inhibited growth, migration, and tumorigenesis (Chushi et al., 2016; Yang et al., 2020). An upregulation of HMGCR was also observed in glioblastoma and prostate cancer (Qiu et al., 2016; Longo et al., 2019).

Statins were first used in the treatment of dyslipidemic disorders as therapeutic target to limiting production of mevalonate. In fact, statins are competitive inhibitors of HMGCR, and act by inhibiting the mevalonate pathway and reducing the end-product amounts such as cholesterol, isoprenoids, ubiquinone, and isopentenyladenine (Clendening et al., 2010; Thurnher et al., 2012; Cruz et al., 2013). Recently, a role in anti-cancer treatments has been proposed for statins (Farwell et al., 2008). Literature data showed how the use of statins, alone or in combination with chemotherapeutic agents, could have anti-tumor effects and reduce the onset of multidrug resistance (Mollinedo et al., 2010a; Cruz et al., 2013; Warita et al., 2014; Kuzu et al., 2016). As they have been shown very effective against mesenchymal-like cancer cells, Warita et al. have hypothesized their possible use during the transition from epithelial to mesenchymal phenotype of metastatic cells (Warita et al., 2014). It was reported that statin supply is effective in reducing the occurrence of a wide range of cancers (e.g., liver, gastric, colorectal, pancreatic, and prostate) and cancer-related mortality ratios (Gbelcova et al., 2008; Ginestier et al., 2012; Nielsen et al., 2013; Benakanakere et al., 2014; Mok and Lee, 2020).

SREBP

Lipid homeostasis is controlled by membrane-bound transcription factors. These include the family of sterol regulatory element-binding proteins (SREBPs), which consist of three isoforms (SREBP1a, SREBP1c, and SREBP2). Among them, SREBP2 modulates more than 30 genes specific to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids (Horton et al., 2002; Luo et al., 2020). SREBPs are inactive when high cholesterol amounts are sensed and bound to SREBP cleavage activating protein (SCAP) and insulin-induced genes (INSIGs). Under cholesterol depletion conditions, feedback responses determine the dissociation of INSIGs from the SREBP2/SCAP complex, which move from the ER toward the Golgi apparatus. Subsequently, SREBP2 is processed and addressed to the nucleus. Nuclear SREBP2 (nSREBP2) induces the transcription of target genes, such as HMGCR and SQLE, by the direct interaction with the sterol regulatory element (SRE) motifs in their promoters (Brown et al., 2018). The upregulation of SREBP-activated downstream genes has been observed in a plethora of cancers, including glioblastoma (Guo et al., 2009; Yamauchi and Rogers, 2018), prostate cancer (Ettinger et al., 2004), breast cancer (Pitroda et al., 2009), and melanoma (Yamauchi et al., 2011; Kuzu et al., 2016), and was often found associated with poor prognosis/survival. The increase of cholesterol synthesis is mediated by the activation of PI3K–AKT–mTORC1 signaling (Porstmann et al., 2008; Duvel et al., 2010). In fact, PI3K–AKT pathway induced expression of SREBP genes and increased the stability of nuclear SREBPs

by preventing their proteasomal degradation. Similarly, the transcription of nSREBP2 can also be induced by the mammalian target of rapamycin complex 1 (mTORC1) via inhibition of lipin-1 (Peterson et al., 2011; Mok and Lee, 2020). Results from preclinical studies showed that the AKT/mTORC1/SREBP pathway takes part in cancer cell growth by inducing biosynthesis of cholesterol (Porstmann et al., 2008). Finally, direct inhibition of SREBP2 (e.g., using fatostatin), resulting in a decrease in cholesterol content, may represent an interesting target in the treatment of a wide range of cancers (Gholkar et al., 2016; Gao et al., 2018).

SQLE

Squalene epoxidase catalyzes the conversion of squalene to (S)-2,3-epoxysqualene (SQLE) and shares with HMGCR the low rates of catalysis in the cholesterol biosynthesis. Like HMGCR, SQLE abundance and activity are regulated at gene and protein levels. The *SQLE* gene responds to sterols via SREBP2 (Nagai et al., 2002; Howe et al., 2017; Luo et al., 2020) and the protein can be degraded in the presence of cholesterol by ubiquitination. HMGCR and SQLE are both transcriptionally controlled by nSREBP2, but they can lead to cholesterol biosynthesis independently of each other. In the presence of an excess of cholesterol, squalene epoxidase is degraded by the E3 ubiquitin ligase MARCH6 (Yoshioka et al., 2020).

Robust scientific evidence demonstrated that SQLE can be considered an important oncogene and, consequently, may represent a possible target in cancer therapy (Cirmena et al., 2018). In particular, *in vitro* and *in vivo* studies have shown that SQLE could promote tumor cell proliferation and migration, and that treatment with its inhibitors (i.e., terbinafine or NB-598) could induce cancer cell demise (Chua et al., 2018). Moreover, a further study on nasopharyngeal carcinoma (NPC) linked the oncogenic effect of SQLE to the cholesteryl ester accumulation, which promoted NPC cell proliferation by activating the PI3K/AKT pathway (Li et al., 2020). In the same vein, in hepatocellular carcinoma (HCC), the overexpression of SQLE was demonstrated to promote cell proliferation and migration by ERK signaling, while downregulation of SQLE was able to inhibit the tumorigenicity of HCC cells (Sui et al., 2015). In addition, SQLE overexpression seem to represent a negative prognostic factor both in breast and prostate cancers (Stopsack et al., 2016; Brown et al., 2019).

NPC1L1

Niemann–Pick type C1-like 1 is the principal responsible for cholesterol absorption from dietary sources. It is a membrane protein expressed on the apical surface of enterocytes and on the membrane of bile canaliculi of human hepatocytes (Altmann et al., 2004). Under normal growth conditions, NPC1L1 is present primarily in the endocytic recycling compartment and translocate rapidly to the plasma membrane upon cholesterol depletion (Yu et al., 2006; Ge et al., 2008). Supplying of cholesterol triggers the inward transport of NPC1L1 together with cholesterol from the plasma membrane to the endocytic recycling compartment (Ge et al., 2008). NPC1L1 can be sent back to the plasma membrane for recycling. This process requires, in addition to other proteins, the small GTPase CDC42 and actin filaments (Zang et al., 2001; Xie et al., 2011; Luo et al., 2020). The human NPC1L1 gene is activated by SREBP2. Moreover, NPC1L1 protein amount can be modulated by degradation (Malhotra et al., 2019).

Recent literature data obtained both *in vivo* and *in vitro* indicated that NPC1L1 could represent a highly effective therapeutic target for treating pancreatic ductal adenocarcinoma (PDAC) because aberrant cholesterol uptake has been suggested to play a role in the proliferation and survival of pancreatic cancer cells (Guillaumond et al., 2015). Accordingly, the use of ezetimibe, an inhibitor of NPC1L1 clinically used for the hypercholesterolemia treatment, significantly reduced the survival capacity of PDAC cells (Nicolle et al., 2017) and tumor-associated blood vessel development (Solomon et al., 2009).

LDLR

Low-density lipoprotein receptor is a cell surface glycoprotein, which causes cellular absorption of cholesterol carried by the bloodstream. LDLR is the transcriptional target of SREBP2 and thyroid hormones (Lopez et al., 2007). LDLR binds circulating LDLs to clathrin-coated vesicles, which then accede the endocytic pathway (Goldstein and Brown, 2009). The endosome acidity induces conformational modifications that allow detaching LDLR from LDLs (Rudenko et al., 2002). LDLR is then redirected to the cell surface for further endocytic cycles (Bartuzi et al., 2016; Fedoseienko et al., 2018) or it is addressed to lysosomes for degradation. After endocytosis, lysosomal lipase hydrolyzes LDL-carried CE and produces cholesterol, which is finally exported by the coordinate actions of NPC2, NPC1, and LAMP2 (Kwon et al., 2009; Li and Pfeffer, 2016). Inhibition of the LDLR at any level (e.g., biosynthesis, membrane localization, internalization, recycling, and degradation) can affect its availability or functionality and, consequently, LDL removal. LXRs bind to promoter of a LDLR inhibitor (IDOL) and upregulate its expression (Zelcer et al., 2009). Accordingly, using synthetic LXR agonists decreased LDLR abundance and limited LDL uptake in cultured cells and livers of non-human primates (Zelcer et al., 2009; Luo et al., 2020). The increased PCSK9 binding with LDLR prevents LDLR recycling and promotes its degradation in lysosomes (Zhang et al., 2008; Chang et al., 2009; Luo et al., 2020). Several studies performed by using LXR agonists showed that cholesterol levels of glioblastoma cells decreased after stimulation of LXRs. The cholesterol decrement is due to enhanced cholesterol export and reduction of cholesterol import (Guo et al., 2011; Villa et al., 2016). LXR agonists also suppressed metastases of melanoma and prolonged animal survival in several *in vivo* models (Pencheva et al., 2014; Gabitova et al., 2015; Yamauchi and Rogers, 2018). In human cancers, the upregulation of PI3K–AKT–mTORC1 signaling promoted cellular cholesterol accumulation trough stimulation of LDLR-mediated cholesterol import. In glioblastoma, the overexpression of LDLR was caused by AKT, and pharmacological inhibition of LDLR actually favored cancer cell death (Guo et al., 2011; Kuzu et al., 2016). As mentioned previously, the increase of oxysterol, a by-product of cholesterol biosynthesis, was able to activate LXRs, which have been

proposed as therapeutic targets in multiple cancer types (Lin et al., 2016; Komati et al., 2017).

SOAT

Sterol-*o*-acyltransferase (SOAT) is a protein of the rough surface ER responsible for cholesteryl ester (CE) production from cholesterol and long-chain fatty acids. SOAT plays an important function by regulating cellular cholesterol storage and free cholesterol content (Pramfalk et al., 2005). Because SOAT has a key role in regulating cellular cholesterol metabolism, it may represent a potential target for treatment of different sickness, including cancer (Rogers et al., 2015; Yang et al., 2020). For instance, it is well known that cholesterol esterification and lipid droplet formation are typical features of glioblastoma (GBM). In fact, accumulation of lipid droplets in the tumor in patients with glioma correlated with GBM progression and poor survival. Genetic inhibition of SOAT1 to block cholesterol esterification was observed to suppress GBM growth and prolong the survival in xenograft models (Geng et al., 2016). In addition, literature data also demonstrated that treating adrenocortical carcinoma (ACC) with mitotane reduced cancer cell survival by inhibiting SOAT1 activity (Sbiera et al., 2015). Very recently, Jiang and colleagues also highlighted that the high expression of SOAT1 was associated with a poor prognosis in hepatocellular carcinoma, whereas its inhibition suppressed cell proliferation and migration (Jiang et al., 2019).

Pyrophosphates

Different enzymes involved in the biosynthesis of cholesterol could be targeted by anticancer drugs. Farnesyl transferase (FTase) and geranylgeranyl transferases (GGTase) are enzymes that catalyzed the production of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), both involved in cellular protein prenylation (Berndt et al., 2011; Sorrentino et al., 2014; Mok and Lee, 2020). The prenylation method consists in covalent bonds of hydrophobic molecules (both the C-15 isoprene farnesyl and the C-20 isoprene geranylgeranyl groups) to the C-terminal portion of different proteins, including the small GTP-binding proteins (sGTPases). sGTPases represent the greater and better described class of prenylated proteins. Each member of the Ras, Rho, and Rab GTPase families, many of which are involved in tumorigenesis, requires the structural alteration given by prenylation to bind to cell surface. Such posttranslational modifications and activation of GTP-binding proteins play a key function in most significant cellular processes. Inhibition of the mevalonate pathway reducing the isoprenylation of sGTPases could bring tumor cell death (Szajnman et al., 2005). Because the administration of GGPP and FPP prevented cancer cell death, it has been hypothesized that GGPP and FPP were necessary for the survival of cancer cells. It has been shown that bisphosphonates (BP) were able to block FPP synthase during the production of mevalonate, thus decreasing the formation of FPP and GGPP (Szajnman et al., 2005). Although BPs blocking osteoclast-mediated bone resorption are currently considered the most significant pharmaceutical treatment for metabolic bone disease, recently nitrogen-containing BPs have aroused interest in their antitumor properties (Nooh et al., 2017).

Steroid Hormones

Cholesterol is the precursor molecule of five different groups of steroid hormones synthesized by specific organs, the adrenal cortex and the gonads. Steroid hormones are widely known to control growth and differentiation. Moreover, steroid hormones are closely linked with the progression of ovarian, breast, and prostate cancers (Lukanova and Kaaks, 2005; Llaverias et al., 2011; Pelton et al., 2012; Murai, 2015; Radisauskas et al., 2016). According with this, inhibition of estrogen or androgen signaling represents the most used strategy in the treatment of these hormone-dependent tumors (Huang and Tindall, 2002; Lu-Yao et al., 2014; Fan et al., 2015; Watson et al., 2015; Simigdala et al., 2016; Shah et al., 2020). With regard to prostate cancer, although almost all patients with metastatic disease initially respond to androgen-ablation therapies, unfortunately the majority of patients progress to a castrate-resistant stage (Huang and Tindall, 2002; Lu-Yao et al., 2014; Watson et al., 2015; Shah et al., 2020). There is accumulating evidence suggesting that cholesterol, as a precursor of steroid hormones, effects on breast and prostate cancers. In this regard, it was observed that *in vitro* treatment of prostate and breast cancer cells with mevalonate was able to induce a high proliferation rate (Mokarram et al., 2017).

Targeting Cholesterol Transport

To keep the cellular cholesterol levels constant, it is necessary that the excess cholesterol is carried out of the cell, and this can take place through active export and passive diffusion. Active export requires energy and involves several ABC transporter proteins using ATP. Passive diffusion is caused by a difference in cholesterol content between the cell membrane and cholesterol acceptors such as HDL.

LDLs/HDLs

Cholesterol is a highly insoluble molecule that is transported in the bloodstream via lipoproteins LDLs and HDLs. Cells can acquire exogenous cholesterol from LDLs that carry abundant cholesterol esters. LDL initially binds to the LDLR receptor on the plasma membrane and is subsequently internalized by clathrin vesicles (Brown and Goldstein, 1986). In LDL, the lipase activity degrades esterified cholesterol producing free cholesterol, which is internalized by the plasma membrane and reaches the endosome/lysosome (Sugii et al., 2003; Yamauchi et al., 2007; Yamauchi and Rogers, 2018). ApoA-I is one of the best known HDL apolipoproteins, capable of binding cholesterol and phospholipids. HDLs bind and carry excess cholesterol from peripheral tissues to the liver where cholesterol is transformed into bile acids for excretion (reverse cholesterol transport) (Yamauchi and Rogers, 2018). Lipoproteins are involved in the elaboration and transport of dietary cholesterol to tissues and aid in keeping a constant level of cholesterol by removing its excess to carry it to the liver. Therefore, lipoproteins could be involved in tumor progression by promoting the availability of cholesterol to tumor cells (Silvente-Poirot and Poirot, 2012; Cruz et al., 2013).

ABCA1

ATP-binding cassette transporters are drug efflux pumps that can cause multidrug resistance and tumor treatment failure (Deeley et al., 2006; Borel et al., 2012). Regarding ABCA1, it is responsible for the bidirectional transport of cholesterol across the cell surface, regulating its availability within the cell. In addition, it mediates the export of cholesterol and phospholipids in ApoA-I for HDL assembly (Oram and Heinecke, 2005). Some research works with *Abca1* knockout mice have shown that both hepatic and intestinal ABCA1 concur to the production of total plasma HDL, although to varying degrees (Timmins et al., 2005; Brunham et al., 2006). ABCA1 localizes both at the plasma membrane, where it mediates the assembly of nascent HDLs (Denis et al., 2008), and at the endocytic compartments, where it is degraded or recycled from the plasmatic membrane (Lu et al., 2008). An excess of ABCA1 generates deformation sites in the plasma membrane, where apoA-I binds (Vedhachalam et al., 2007). This could be a result of the disturbing action of ABCA1 on lipid raft organization (Landry et al., 2006). In contrast, ABCA1 deficiency has been found to increase lipid rafts and cholesterol contents in the plasma membrane (Koseki et al., 2007; Zhu et al., 2008; Yamauchi et al., 2015). ABCA1 is closely regulated both at gene and protein levels. LXRs are mainly responsible for activating the *ABCA1* gene (Costet et al., 2000; Venkateswaran et al., 2000). Accordingly, data obtained *in vitro* by using LXR agonists revealed that the activation of LXR reduced intracellular cholesterol amount by increasing its exportation and decreasing its absorption therefore determining a growth inhibition of glioblastoma cells (Guo et al., 2011; Villa et al., 2016). Moreover, LXR agonists suppressed melanoma metastases and prolonged animal survival in several *in vivo* models (Pencheva et al., 2014; Gabitova et al., 2015; Yamauchi and Rogers, 2018).

On the other hand, it was also reported the PI3K/AKT signaling, through the inhibition of ABCA1 mTORC1-dependent pathway, resulted in an increase of intracellular level of cholesterol (Porstmann et al., 2008; Dong et al., 2014). Rapamycin, the mTORC1 inhibitor, repressed the cholesterol export due to the action of ABCA1 (Dong et al., 2014).

Finally, the regulation of *ABCA1* gene was also modulated by some miRNAs, including miR-33a/b and miR-148 (Najafi-Shoushtari et al., 2010; Rayner et al., 2011; Feinberg and Moore, 2016). These miRNAs suppressed *ABCA1* gene expression and reduced plasma HDL levels in mice and non-human primates (Najafi-Shoushtari et al., 2010; Yamauchi and Rogers, 2018).

ACAT

Acetyl-CoA acetyltransferase 1 and 2 (ACAT1 and ACAT2, respectively) play an important role in lipid metabolism. After esterification into CEs by ACAT1, surplus cholesterol can be stored as lipid droplets (Chang et al., 2009). ACAT1 overexpression and CE accumulation were found to play pro-tumor functions. In fact, it was observed that ACAT1 induced an excessive storage of cholesterol in lipid droplets in prostate (Yue et al., 2014), pancreatic cancers (Li et al., 2016), and glioblastoma (Geng et al., 2016). According with this, ACAT1 inhibition was found to reduce migration of breast cancer cells (Antalis et al., 2010) and decrease prostate cancer progression (Saraon et al., 2014). Moreover, ACAT1 inactivation determined abundant ER cholesterol content, which is acting on SREBP-1 reduced cholesterol synthesis and uptake (Yue et al., 2014). Recently, pharmacological or genetic ACAT1 inhibition has been associated with attenuated cancer cell proliferation (Yang et al., 2020).

In light of all this, ACAT1 can be considered a possible therapeutic target for the treatment of some neoplasms linked to the abundant presence of cholesterol esters. The use of avasimibe, an effective inhibitor of ACAT1, remarkably decreased cholesteryl esters stocked in lipid droplets and enhanced free cholesterol amount into the cell in human prostate, pancreatic, lung, glioblastoma, and colon cancer cells leading to inhibition of proliferation, cell cycle arrest, and apoptosis (Yang et al., 2020).

CONCLUSION

As the metabolic needs of cancer cells appear significantly different from non-cancer cells, the metabolic pathways could represent potential innovative targets for a more selective anticancer therapy.

The analysis of literature data confirmed a fundamental role of cholesterol and its metabolism in many and different human pathologies, including atherosclerosis, Alzheimer's disease, and other neurodegenerative disorders, and mounting evidence connected cholesterol homeostasis disorder and cancer. Moreover, dysregulation of the mevalonate pathway has been found to promote cell transformation, thus suggesting the role of oncogene for *HMG-CoA reductase* (Clendening et al., 2010). All of this makes cholesterol a potential attractive pharmacological target also in oncology context. Accordingly, although quite controversial, preclinical and epidemiological data seem to show an antitumor activity of statins, at least against some types of cancer (Solomon and Freeman, 2008).

At present, several drugs targeting cholesterol metabolism besides statins are clinically used to inhibit cholesterol production and increase LDL absorption, to hinder NPC1L1 absorption of dietary cholesterol and LDLR degradation, and to reduce plasma LDLs. For instance, the cholesterol uptake-blocking agent ezetimibe, by decreasing circulating cholesterol levels, has been found to reduce prostate tumor growth (Solomon et al., 2009).

Moreover, an increasing number of preclinical and clinical studies are underway to define as new potential therapeutic target for cancer therapy SREBP and its regulators LXRs, the transporters ABCs, and ACATs.

Increasing evidence showed that also cholesterol-rich raft membrane domains could represent a promising target in cancer therapy (Mollinedo and Gajate, 2006, 2020; Mollinedo et al., 2010a). Indeed, acting as scaffolds for signaling pathways in the cell membrane, lipid rafts promote and facilitate the triggering of both survival and apoptosis by recruiting signaling molecules and death receptors. In this regard, understanding how the different rafts that promote cell survival or death are temporarily formed and disrupted may be an important goal to understand if and how lipid rafts could represent a specific target in cancer therapy.

Finally, it is important to underline the key role played by the intestinal microbiota in the reduction and elimination of

cholesterol, thus contributing significantly to its homeostasis (Zimber et al., 2000; Antharam et al., 2016). An alteration of the bacterial flora could constitute a further risk factor, especially for those tumors particularly dependent on cholesterol homeostasis. Thus, acting on microbiota might also represent a further possibility of therapeutic intervention in the treatment and/or prevention of certain types of cancer.

AUTHOR CONTRIBUTIONS

RV contributed to conceive the idea, drafted the article, and drew figures relative to cholesterol metabolism. EI contributed to design and to draft the article and drew figures relative to lipid rafts. PM conceived the idea, draft and revised the article, coordinated the activity, and concurred to the final version of the review. All authors contributed to the article and approved the submitted version.

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Staphylococcus aureus Infection Influences the Function of Intestinal Cells by Altering the Lipid Raft-Dependent Sorting of Sucrase–Isomaltase

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Staphylococcus aureus is an important nosocomial and community-acquired facultative intracellular pathogen. Many studies have reported that *S. aureus* infections are associated with intestinal symptoms, but little is known about the molecular mechanisms implicated in *S. aureus*-induced alterations of intestinal functions. In this study, we investigated the implication of lipid rafts in the interaction of *S. aureus* with Caco-2 cells. To assess potential alterations in the lipid raft structure and effects on the hydrolytic function, we utilized sucrase–isomaltase (SI) as the major intestinal α-glucosidase that is associated with and sorted to the apical membrane via lipid rafts. Seven days post-confluent, Caco-2 cells were infected with *S. aureus* Newman and further incubated for an additional 2 days. After 48 h, the levels of SI expression as well as the enzymatic function of this protein were assessed in the infected versus non-infected cells. Analysis of the sorting behavior of SI to the apical membrane constituted another crucial aspect in studying the effects of *S. aureus* on Caco-2 cells. For this purpose, the apical membranes or brush border membranes (BBMs; referred to as P2 fraction) were separated in both infected and non-infected cells from the basolateral and intracellular membranes (referred to as P1 fraction) by employing a cationic-based procedure using $CaCl_2$. The data show that there is no significant change in the overall expression levels of SI in the infected versus non-infected cells as assessed by Western blotting analysis using monoclonal anti-SI antibodies. By contrast, a significant decrease in the localization as well as the specific hydrolytic activities of SI toward sucrose and isomaltose (Palatinose) was observed in the BBM (P2 fraction) in Caco-2 cells 48 h post-infection. Concomitantly, the specific SI activities increased in the basolateral membrane/intracellular fraction (P1). Noteworthy, the specific activity of SI in the BBM of infected cells was markedly reduced as compared with that of the non-infected counterparts. The data accumulated from this study strongly suggest that infections with *S. aureus* influence the final step in the lipid raft-associated trafficking of human SI and thereby may trigger secondary functional gastrointestinal disorders.

Keywords: *S. aureus*, human sucrase–isomaltase, brush border membranes, lipid rafts, sucrase–isomaltase

INTRODUCTION

Staphylococcus aureus is a very important nosocomial and community-acquired pathogen. Its infection can involve many organs, and it can cause a broad spectrum of infections, including boils, abscess formation, wound infection, endocarditis, osteomyelitis, and sepsis or septic shock. Furthermore, it is a frequent pathogen in foreign body infections (Lowy, 1998). It is also often responsible for toxin-mediated diseases, such as toxic shock syndrome, scaled skin syndrome, and staphylococcal foodborne disease (Plata et al., 2009).

Furthermore, *S. aureus* may cause nosocomial antibiotic-associated diarrhea (Lane et al., 2018). In this regard, decreased stomach acidity can promote the establishment of colonization because of passing the gastric-acid barrier of *S. aureus* (Yoshida, 1999). An overgrowth of bacteria in the intestine can occur, leading to enteritis and/or diarrhea. *S. aureus* is implicated in inflammatory bowel disease (IBD) because gut-derived *S. aureus* antigens could induce inflammatory responses (Lu et al., 2003). Additionally, toxins of *S. aureus* are associated with food poisoning, and the alpha toxin of *S. aureus* can perturb the barrier function of intestinal cells *in vitro* by altering the junctional integrity (Kwak et al., 2012).

The small intestine is the principal organ that is implicated in the digestion of micromolecular nutrients, which is achieved by two families of enzymes, the peptidases and disaccharidases (Hauri et al., 1985; Lin et al., 2012). Of particular importance is the digestion of starch, glycogen, sucrose, maltose, and several other carbohydrates in the intestinal lumen that is achieved by the concerted action of a family of microvillar enzymes, the disaccharidases. The digestion of α-glycosidic linkages of carbohydrates commences by salivary and pancreatic α-amylases and is continued in the small intestine by two major mucosal α-glycosidases, sucrase–isomaltase (SI; EC 3.2.148 and 3.2.1.10) and maltase-glucoamylase (MGAM; EC 3.2.1.20 and 3.2.1.3) (Conklin et al., 1975). The digestive capacities of SI and MGAM cover almost the entire spectrum of carbohydrates that are linked via α-1,2, α-1,4, and α-1,6 linkages and comprise the majority of the typical diet in children and adults. Reduced expression levels or complete absence of intestinal disaccharidases at the cell surface of the enterocytes is associated with carbohydrate maldigestion and malabsorption, most notably described in several cases of genetically determined congenital SI deficiency (CSID) (Moolenaar et al., 1997; Sander et al., 2006; Alfalah et al., 2009; Treem, 2012), which is a primary deficiency of the enzyme SI. SI is the most abundant glycoprotein in the intestinal brush border membrane (BBM) and is exclusively expressed in the enterocytes. Due to its high abundance and its wide substrate specificity, human SI is responsible for about 60–80% of maltase activity in the intestinal lumen (Lin et al., 2012). SI is a heavily N-glycosylated and O-glycosylated protein that is trafficked with high fidelity to the apical membrane (Hauri et al., 1979; Naim et al., 1988a). The sorting of SI is mediated via its association with cholesterol- and sphingolipid-enriched membrane microdomains, known as lipid rafts (LRs) (Alfalah et al., 1999), in the *trans*-Golgi network for which proper O-glycosylation is essentially required (Wetzel et al., 2009).

In addition to primary genetic SI deficiencies, secondary deficiencies can be also induced and occur later in life and are mediated by environmental factors, including bacterial infections that negatively influence the intestinal physiology in general, for example, in IBD. In fact, *S. aureus* infections and toxins produced by *S. aureus* have been shown to be associated with intestinal malfunctioning (Lu et al., 2003; Kwak et al., 2012).

Little is known about the molecular mechanisms implicating *S. aureus* infection of the intestine and how this infection elicits secondary carbohydrate malabsorption. In this article, we have examined the molecular mechanisms that could lead to SI secondary deficiencies and demonstrate that LRs are directly affected by the *S. aureus* infection and that this triggers delayed trafficking and subsequent reduced function of SI at the apical membrane.

MATERIALS AND METHODS

Cell Culture

Human epithelial colorectal adenocarcinoma, Caco-2, cell line (ATCC® HTB-3^{TM}7) was used in the study. Cells were maintained in high glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM; Sigma, Darmstadt, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL, Grand Island, NY, United States), and 50 U/ml of penicillin, and 50 μg/ml of streptomycin (Sigma, Darmstadt, Germany). Caco-2 cells were grown on polystyrene six-well plates (Sarstedt, Nümbrecht, Germany) for 7 days post-confluence to receive optimal SI expression (**Supplementary Figure 1**). Incubations were performed in a tissue culture incubator at 37°C, 5% CO_2 in water-saturated air.

Invasion of *Staphylococcus aureus* Newman Into Caco-2 Cells

The bacterial strain used in this study was the widely used laboratory strain *S. aureus* Newman (GenBank accession number AP009351.1), the respective green fluorescent protein (GFP)-expressing strain (Boero et al., 2021) and the methicillin-resistant *S. aureus* (MRSA) strain USA300 (Berends et al., 2010).

Gentamicin protection assay was used to determine intracellular bacteria after infection (see **Figure 1**). Briefly, bacteria were grown in brain heart infusion (BHI) medium at 37°C with shaking. On the day of infection, fresh overnight cultures were diluted 1:50 in BHI and then grown to mid-exponential growth phase (OD_{600} = 0.7). Ten milliliters of the bacterial culture was added to a 15-ml Falcon tube and was centrifuged at 3,800 × *g* for 10 min (Thermo Scientific Sorvall Legend X1R centrifuge, 4,500 rpm, Falcon rotor 75003658 Thermo Scientific; Thermo Fisher Scientific, Rockford, IL, United States) to discard the bacterial supernatant. The pellet was resuspended in 10 ml of antibiotic-free DMEM supplemented with 10% FCS. The Caco-2 cells were washed twice with antibiotic-free DMEM. Cells were infected with bacteria with multiplicity of infection (MOI) of 10, while negative control cells were left uninfected. After centrifugation of the six-well plates at 142 × *g* (1,000 rpm, plate rotor 75003624

M20; Thermo Fisher Scientific, Rockford, IL, United States) for 5 min, samples were incubated for 90 min at 37°C.

The total growth of bacteria was measured after 90-min incubation to determine the number of cells associated bacteria, using colony-forming unit (CFU) counting. Briefly, the infected Caco-2 cells and non-infected control cells were washed twice with DMEM with 10% FCS to remove non-associated bacteria followed by trypsinization for detachment of cells with 150 μl of trypsin-EDTA per well for 2 min. To each well, 350 μl of 0.1% Triton X-100 was added, and the Caco-2 cells were lysed by pipetting. Cell lysates were diluted and plated in duplicates on blood agar plates followed by an overnight incubation at 37°C. At the next day, colonies were counted, and percentage of associated bacteria at T0 (90 min of infection) was determined.

The cells were washed twice with 1 ml of antibiotic-free DMEM supplemented with 10% FCS to remove non-associated bacteria. For gentamicin treatment, DMEM supplemented with 10% FCS and 100 μg/μl of gentamicin was added to the cells. After further incubation for 1 h at 37°C, 25 μl of the supernatant of each well treated with gentamicin was plated on blood agar to ensure killing of bacteria by gentamicin. Furthermore, bacterial growth inside the cells was determined by counting CFU, as described above, 1, 24, and 48 h after gentamicin treatment. The percentage of invasive bacteria of compared with T0 (90 min) was determined.

Cytotoxicity Assay

After 48 h of gentamicin treatment, lactate dehydrogenase (LDH) release assay was used to measure the viability of *S. aureus* infected and non-infected cells. Control cells were incubated with media only, whereas positive control cells were treated with 1% Triton X-100. Negative control was media without cells. Three microliters of the cell supernatants was added to 47 μl of LDH storage buffer [200 mM of Tris–HCl, pH 7.3, 10% glycerol, 1% bovine serum albumin (BSA)] and were mixed with 50 μl of detection reagent according to the manufacturer (LDH-Glo™, Promega, Madison, WI, United States) and incubated for 60 min at 22°C in a 96-well plate. Luminescence was recorded using plate reader (Tecan, Grödig, Austria) as described in the manufacturer's instructions.

Live/Dead Cell Viability Staining of Caco-2 Cells After Infection With *Staphylococcus aureus*

Caco-2 cells were seeded on glass-bottom 24-well plates and were grown until 7 days post-confluence. The cells were infected as described above with *S. aureus* Newman GFP at the MOI of 10. Forty-eight hours after gentamicin treatment, infected cells and control cells were washed with 1 × PBS; 6.66 μl of ethidium homodimer-1 of the LIVE/DEAD® Viability/Cytotoxicity Assay Kit was added to 3 ml of 1 × PBS. This solution was diluted 1:3 with 1 × PBS and was then added directly to the cells after addition of HOECHST in a dilution of 1:500. After an incubation of 10 min, the samples were analyzed by confocal laser scanning microscopy (CLSM) with the HCX μL APO apochromatic 40 × 1.25 glycerol immersion objective for correlation of infection with bacteria and cell death.

Brush Border Membrane Preparation

Cellular homogenates of infected and non-infected Caco-2 cells were fractionated to apical membrane or BBM (P2 fraction), basolateral and intracellular membranes (P1 fraction), and the soluble and vesicular membranes (S fraction) using the divalent cation precipitation method (Schmitz et al., 1973; Naim et al., 1988b; Hein et al., 2011). The cells were homogenized in the hypertonic homogenization buffer (300 mM of mannitol and 12 mM of Tris–HCl, pH 7.1) supplemented with protease inhibitor mix (antipain 1.48 μM, pepstatin A 1.46 μM, leupeptin 10.51 μM, aprotinin 0.768 μM, soybean trypsin inhibitor 50 μg/μl, and phenylmethylsulfonyl fluoride (PMSF) 1 mM; all were obtained from Sigma, Darmstadt, Germany). The cellular homogenates were depleted from cell debris by centrifugation for 15 min at 5,000 × *g* (the supernatant referred to as H). This supernatant (referred to as H) was treated with 10 mM of $CaCl_2$ at 4°C for 30 min with gentle agitation and centrifuged at 5,000 × *g* for 15 min to yield the pellet (P1). The supernatant was then centrifuged at 25,000 × *g* for 30 min to yield the pellet (P2) and the (S) fraction.

Specific Enzyme Activity

Sucrase activity in H, P1, P2, and S fractions was measured using sucrose as a substrate (150 mM) essentially as described by Wanes et al. (2021); 25 μl of 150 mM of sucrose was added to 25 μl of sample and incubated for 1 h at 37°C followed by incubation for further 20 min at 37°C after adding 200 μl of GOD-POD monoreagent fluid (Axiom Diagnostics, Worms, Germany) to all samples, and the absorbance was measured at 492 nm. Sucrase specific activity was calculated by dividing the measured absorbance over the SI band intensity obtained from Western blotting of a similar sample volume.

Detergent-Resistant Membranes or Lipid Raft Preparation

Lipid rafts or detergent-resistant membranes (DRMs) are known to play an important role in the activity and trafficking of SI. We therefore investigated the status of LRs upon infection of the Caco-2 cells by the *S. aureus* Newman. Forty-eight hours after gentamicin treatment, infected and non-infected control Caco-2 cells were solubilized with 1% (w/v) Triton X-100 in phosphate-buffered saline (pH 7.4) and protease inhibitor mix (mentioned above). The cellular lysates were centrifuged at 5,000 × *g* for 10 min at 4°C to remove cell debris. The supernatant was loaded on a discontinuous sucrose gradient and centrifuged as described by Wanes et al. (2021) to isolate the cholesterol- and sphingolipid-enriched LRs. Ultracentrifugation was performed at 100,000 × *g* for 18 h at 4°C using an SW 40 rotor (Beckman Coulter, Mississauga, ON, Canada). Fractions of 1 ml (10 fractions in total) were collected from the top of the gradient tube. LRs were recovered in the floating fractions of the gradient as assessed by the distribution of flotillin-2 (FLOT2), an LR scaffold protein marker, in Western blotting using anti-FLOT2 [FLOT2 (B-6, 1/6,000) from Santa Cruz Biotechnology, Inc., Dallas, TX, United States].

Lipid Extraction and Lipid Composition Analysis

Total lipids were isolated from sucrose density gradients of Triton X-100 cellular extracts of bacterial infected or non-infected Caco-2 cells, as described previously (Brogden et al., 2014) based on the method of Bligh and Dyer (1959). Cholesterol analysis was performed with a Hitachi Chromaster HPLC, as previously described (Branitzki-Heinemann et al., 2016; Brogden et al., 2017).

Western Blotting

Equal volumes of the fractions harvested from the sucrose-density gradients or 20 μg of H, P1, P2, and S of infected and non-infected cells were prepared for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 6 or 8% slab gels transferred onto a polyvinylidene difluoride (PVDF) membrane (Roth, Karlsruhe, Germany) essentially according to Wanes et al. (2021). FLOT2 was detected using purified mouse antibody [FLOT2 (B-6, 1/6,000) from Santa Cruz Biotechnology, Inc., Dallas, TX, United States]; and SI was detected using mAb anti-SI antibody HBB 3/705/60 (Hein et al., 2011). The secondary antibody was anti-mouse peroxidase conjugated secondary antibody (Thermo Fisher Scientific, Rockford, IL, United States). The protein bands were visualized via enhanced chemiluminescent peroxidase substrate and documented with a ChemiDoc MP^{TM} Touch Imaging System (Bio-Rad, Munich, Germany).

Statistical Analysis

Experiments were carried out in duplicates or triplicates and repeated at least three independent times; GraphPad Prism version 8.0.1 (244; GraphPad Inc., San Diego, CA, United States) and Microsoft Excel 2016 (Microsoft, Redmond, WA, United States) software were used. Comparison of data was performed as described in the respective figure legends. p-values of 0.05 or less were considered as significant. Significant values with $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

RESULTS

Non-cytotoxic Survival of *Staphylococcus aureus* Inside the Cells

In the present study, we evaluated the molecular modifications that occur in Caco-2 cells during *S. aureus* Newman infection. We first measured the percentage of invasive bacteria in three different time points (1, 24, and 48 h after gentamicin treatment), and we then tested the potential cytotoxicity due to infection.

Figure 2 shows that *S. aureus* Newman successfully invaded the cells. Based on the lysate CFU count, the invasiveness percentage has been found to be about 1% at 1 h after gentamicin treatment, and increased to 2, and 5.5% in the next 24 and 48 h, respectively. We therefore conclude that the bacteria can invade and multiply within the cell.

To exclude the cytotoxic effect of the bacteria, we performed LDH assay and did not detect differences in LDH production 48 h after gentamicin treatment. Besides LDH, also viability assays using ethidium heterodimer for DNA staining of dead cells were used for microscopic analysis. We used these microscopy techniques since flow cytometry-based quantification by propidium iodide requires a detrimental detachment of cells. Representative images of this assay are shown **Supplementary Figure 2**. The images confirm that low percentage of dead cells occurs in non-infected control cells (A) as well as in infected cells (C), indicating that there is no specific cell death caused by invasive bacteria themselves.

Since the *S. aureus* USA300 strain showed cytotoxic effects after invasion of Caco-2 cells (**Supplementary Figures 3**, **4**), the strain was not used for further experiments. To find effects of host–pathogen interaction on intestinal function independent of cell toxicity, it is the major idea to use a strain that does not result in significant cytotoxic effects. Therefore, all subsequent assays focused on *S. aureus* Newman strain.

Effect of *Staphylococcus aureus* Infection on Sucrase–Isomaltase Sorting to the Apical Membrane

Having excluded a potential cytotoxic effect of *S. aureus* infection on Caco-2 cells, the next question we addressed was whether the infected cells exhibit molecular and functional alterations. In a first set of experiments, we analyzed the function and expression of SI, a differentiation protein marker of intestinal Caco-2 cells, in the BBM. Here, we fractionated the cellular homogenates (H) of infected Caco-2 cells 48 h after treatment with gentamicin using $CaCl_2$ (according to Schmitz et al., 1973, and the modification by Naim et al., 1988b) into BBM that are retained in the P2 fraction, intracellular and basolateral membranes (IM, in P1 fraction), and the cytosolic fraction (S). The expression of SI in these fractions was assessed by Western blotting using similar protein contents. The results shown in **Figure 3** clearly indicate that the expression of SI in the BBM decreased significantly in the infected cells as compared with its counterpart in the non-treated cells. Obviously, this decrease is compatible with a substantial trafficking delay or altered sorting of SI to the basolateral membrane, since the infection itself did not impact the overall expression levels of SI as shown in the cellular homogenates. It rather resulted in an increase of SI in IM (P1) that is enriched in the endoplasmic reticulum (ER), Golgi, and basolateral membranes. In fact, the ratio of SI in BBM (P2) versus IM (P1) is reduced by almost two-fold upon infection, indicative of an intracellular retention or missorting of SI to the basolateral membrane (**Figure 3C**). Another critical parameter we examined is the effect elicited by the infection on the specific enzymatic activity of SI, which was significantly reduced in the P2 fraction (**Figure 4**).

Lipid Raft Alterations Due to *Staphylococcus aureus* Infection

Having demonstrated that the SI levels as well as its specific activity are reduced in BBM, we asked whether these effects are triggered by alterations in cholesterol- and sphingolipid-enriched membrane microdomains or LRs (Shimada et al., 2005; Lindner and Naim, 2009) in infected Caco-2 cells. We have previously shown that LRs are implicated in the apical

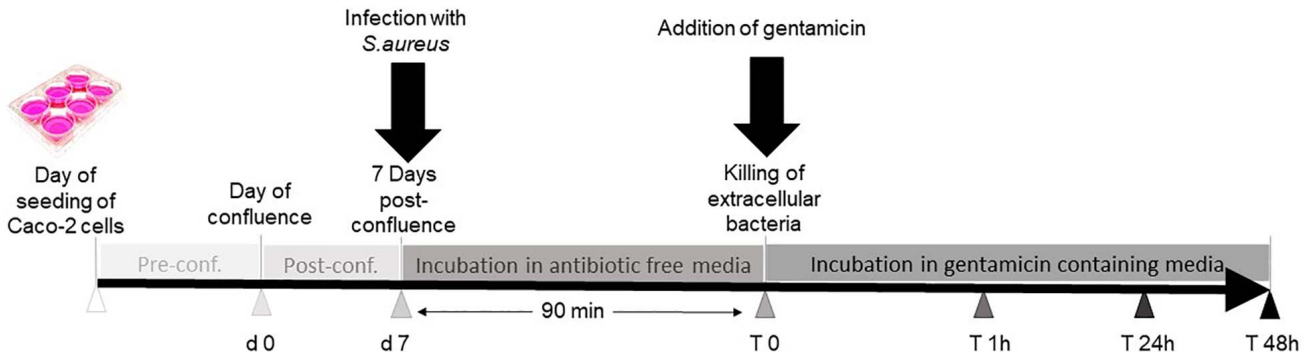

FIGURE 1 | Work follow summary. Caco-2 cells were seeded in six-well plates. Seven days post-confluence, three wells were infected with *Staphylococcus aureus*, and the other three wells were not infected and served as controls. Thereafter, the cells were incubated in antibiotic-free medium for 90 min to allow the bacteria to enter the cells before replacing the medium with gentamicin-containing media to kill extracellular bacteria. The cells were incubated further for 48 h and then harvested for further analysis.

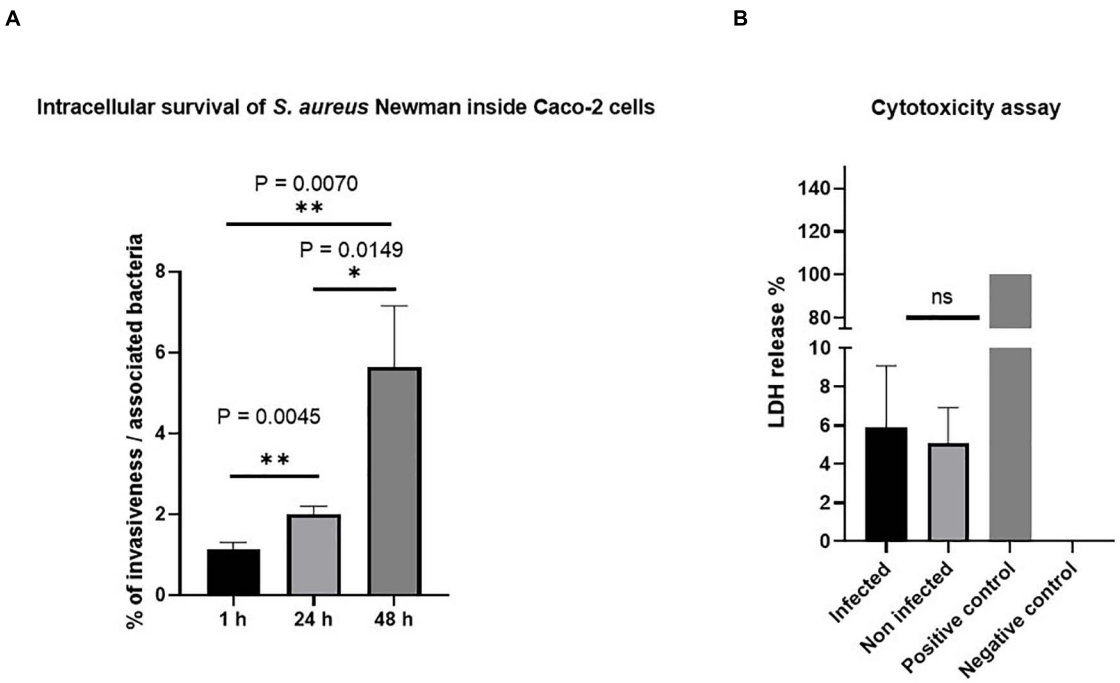

FIGURE 2 | Invasion of *Staphylococcus aureus* Newman in Caco-2 cells. **(A)** *S. aureus* Newman-infected Caco-2 cells survived and multiplied inside the cells without affecting cell viability. The percentage of invasive bacteria showed significant differences between the three different time points after 1, 24, and 48 h of gentamicin treatment. The data were obtained from colony-forming unit (CFU) count of cell lysate infected with a multiplicity of infection (MOI) of 10. **(B)** The viability of Caco-2 cells was not affected upon *S. aureus* infection as estimated by lactate dehydrogenase (LDH) assay. Forty-eight hours post-infection, LDH release was measured in the supernatant of infected or non-infected Caco-2 cells or from cells lysed with 1% Triton X-100 that served as a positive control. ns, non-significant; $^{*}p \leq 0.05$ and $^{**}p \leq 0.005$ by using two-tailed Student's *t*-test and using Tukey's multiple comparison test. Data represent mean ± SEM of three independent experiments.

sorting of SI in intestinal epithelial cells via O-linked glycans that act as a sorting signal (Alfalah et al., 1999). Furthermore, the functional capacity of SI is increased substantially when SI is associated with LRs (Wetzel et al., 2009). We therefore investigated the status of LRs upon *S. aureus* infection by examining the distribution of FLOT2, a LR scaffold protein marker (Langhorst et al., 2005; Browman et al., 2007), in sucrose density gradients of Triton X-100 detergent extracts of infected and non-infected Caco-2 cells. LRs are insoluble in Triton X-100 at 4°C, and by virtue of their buoyant density, they can partition into floating upper fractions of the gradient at low sucrose concentration. Expectedly, the majority of FLOT2 was retained in the top 3 fractions of the gradient, particularly in fraction 2, compatible with its association with LRs (**Figure 5A** and **Supplementary Figure 5**). A smaller proportion of FLOT2 was also found in the soluble non-LR fractions in the bottom of the gradient. Strikingly, upon infection, a substantial proportion of FLOT2 was redistributed to non-LR fractions 8 and 9 that

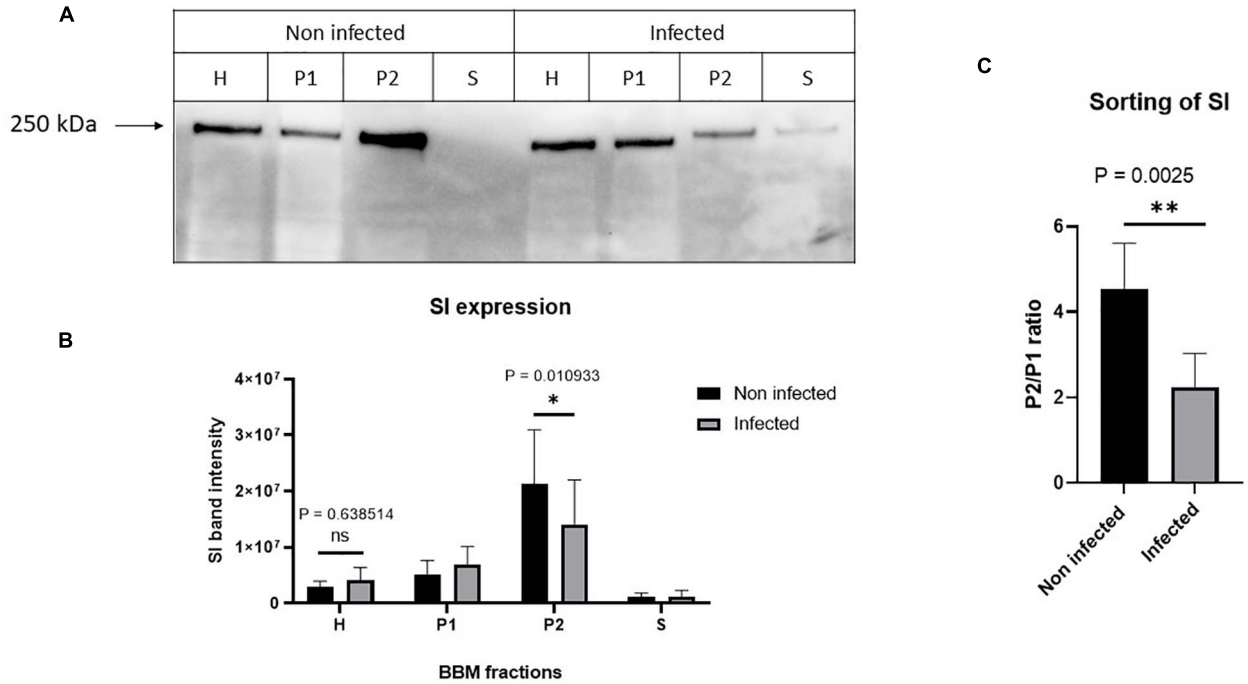

FIGURE 3 | *Staphylococcus aureus* Newman infection of Caco-2 cells induced impaired trafficking of sucrase–isomaltase (SI) to the brush border membrane (BBM). Caco-2 cells were infected or not infected with *S. aureus* Newman. Forty-eight hours post-infection, Caco-2 cellular homogenates (H) were partitioned into intracellular and basolateral membranes (P1), brush border membranes (BBM) (P2), and vesicular and soluble fraction (S). Equal protein amount from the different fraction was analyzed by Western blotting using monoclonal anti-SI antibody **(A)**. While the infection did not significantly affect the overall expression of SI, the trafficking to the BBM (P2) was significantly reduced **(B)**. The sorting efficiency of SI to the BBM after infection with *S. aureus* was analyzed via comparing the expression of SI in the P2 versus P1. The ratio P2/P1 showed that the sorting of SI to the apical membrane is significantly reduced after *S. aureus* infection **(C)**. ns, non-significant; and $^{*}p \leq 0.05$ or $^{**}p \leq 0.01$ using two-tailed Student's *t*-test. Data represent mean ± SEM of three independent experiments with samples in duplicates or triplicates.

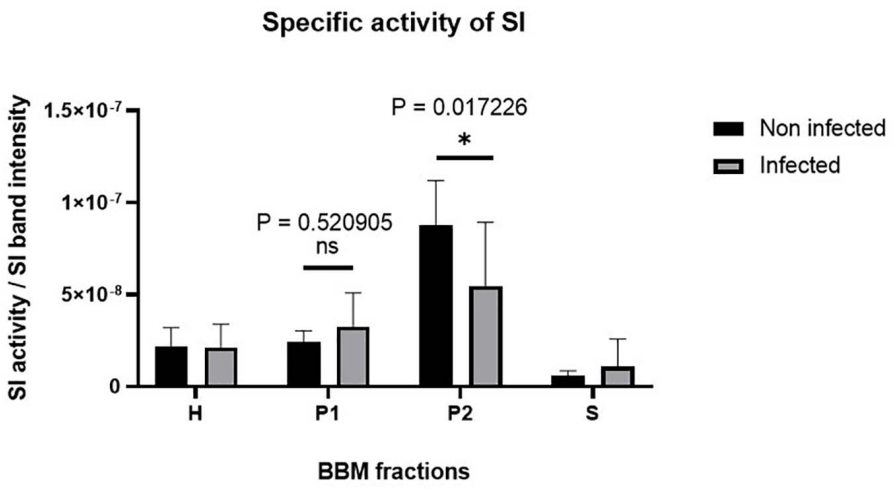

FIGURE 4 | The specific activity of sucrase–isomaltase (SI) is substantially reduced in Caco-2 cells upon *Staphylococcus aureus* Newman infection. Caco-2 cells were infected or not infected with *S. aureus* Newman. Forty-eight hours post-infection, Caco-2 cellular homogenates (H) were partitioned into intracellular and basolateral membranes (P1), brush border membranes (P2), and vesicular and soluble fraction (S). Specific activity of SI in the different membrane fractions was analyzed and found to be significantly reduced in the P2 fraction in infected Caco-2 cells as compared with the non-infected. ns, non-significant; and $^{*}p \leq 0.05$ using one-tailed Student's *t*-test. Data represent mean ± SEM of three independent experiments with samples in duplicates or triplicates.

resulted in a dramatic increase in the proportion of non-LRs versus LRs (**Figure 5A** and **Supplementary Figure 5**). An association of SI with LRs was also detected peaking in fraction 3 of the upper three fractions (**Figure 5B** and **Supplementary Figure 6**). Unlike FLOT2, the proportion of SI in the non-LRs was substantially higher than in the LRs. This observation reflects the biosynthesis and trafficking pathways of SI and its distribution in cellular membranes that are not enriched in LRs, such as the ER and several Golgi cisternae. Nevertheless, and similar to FLOT2, the proportion of SI in the non-LRs increased markedly upon infection (**Figure 5**). Together, these findings point to an alteration in the membrane lipid composition of LRs in infected cells.

We therefore assessed the variations in cholesterol, one of the major two lipids in LRs, by comparing its content in the LRs versus non-LRs in non-infected and infected cells (see **Figures 6A,B**). **Figure 6C** shows that the ratio of non-LRs in the three bottom fractions of the gradient to the LRs in the upper three fractions is increased by almost two-fold in infected cells, compatible with a substantial reduction of cholesterol that normally assembles with sphingolipids to form LRs in cellular membranes. Since intact LR-enriched vesicles constitute an absolute requirement for efficient sorting of SI to the apical membrane (Alfalah et al., 1999), our data indicate that the reduced expression levels of SI in BBM are linked to delayed trafficking and missorting of SI to BBM due to distorted LRs (represented by P2 in **Figure 3**).

DISCUSSION

Staphylococcus aureus infections are among the most common human bacterial infections worldwide that affect several tissues, including the gastrointestinal tract. The current study sheds light on intestinal cell infection by *S. aureus* and the potential mechanism associated with the symptoms elicited by this infection. *S. aureus* Newman was chosen for experiments due to its origin from a human infection as well as its importance and frequency in laboratory use worldwide. This strain is methicillin-sensitive and displays robust virulence properties in animal models of disease and has already been extensively analyzed for its molecular traits of staphylococcal pathogenesis (Baba et al., 2008). Caco-2 cells were used as a suitable *in vitro* experimental model to study the inflammatory conditions caused by *S. aureus*. Previously, invasion and intracellular survival for 120 h of *S. aureus* in Caco-2 cells was observed for several *S. aureus* strains, including *S. aureus* RN6390 and *S. aureus* 502A. In these studies, the intracellular survival of *S. aureus* was associated with apoptosis (Hess et al., 2003) in contrast to our study, which demonstrated via CFU, LDH, and live/dead staining that *S. aureus* Newman strain can invade the intestinal cells and multiply inside the cells without causing significant cytotoxic effects at 48 h after infection. To find effects of host–pathogen interaction on intestinal function independent of cell toxicity, it was the major idea to use a time point and strain that does not result in significant cytotoxic effects. Accordingly, we hypothesized that this microorganism might induce alterations at the membrane and cellular levels that ultimately culminate in affecting the enzymatic function of intestinal cells leading to symptoms like those observed in carbohydrate malabsorption, such as diarrhea. The most prominent intestinal enzyme that is implicated in processing carbohydrates in the gut is SI. The digestive capacities of SI cover almost the entire spectrum of carbohydrates that are linked via α-1,2, α-1,4, and α-1,6 linkages and comprise most of the typical diet in children and adults. SI is synthesized and processed exclusively in intestinal cells. It is a heavily N- and O-glycosylated protein that is sorted almost exclusively to the apical membrane, where it exerts its digestive function. We show here that the high sorting fidelity of SI to the apical membrane is distorted in Caco-2 cells that have been infected with *S. aureus* as assessed by the substantial reduction of SI in the BBM fraction concomitant with an increase in the proportion of SI in the intracellular membrane and basolateral membranes. The switch of SI from the apical membrane to the intracellular and basolateral membranes suggests that the sorting machinery has been affected in the infected cells. The sorting mechanism of SI to the apical membrane occurs in the *trans*-Golgi network and utilizes cholesterol- and sphingolipid-enriched LRs (Alfalah et al., 1999). SI is packaged into specific LR-enriched sorting carriers that segregate from another vesicle type and trafficked along microtubules and the actin cytoskeleton to the apical membrane (Jacob and Naim, 2001; Jacob et al., 2003). As shown here, the infection with *S. aureus* has affected the LRs and has thus distorted the integrity of these vesicles, at least in part, impairing thus an efficient trafficking of SI to the apical membrane. The question that arises is whether the LR distortion and subsequently missorting of SI is a direct effect of *S. aureus* infection or is secondary to another mechanism that is affected by the infection. One potential mechanism could implicate actin depolymerization and redistribution as well as re-guidance of vesicle traffic as has been described for bacterial protein toxins (Schwan and Aktories, 2016). We have previously demonstrated that gliadin toxic peptides, which are implicated in the pathogenesis of celiac disease, interact directly with the actin cytoskeleton and elicit its depolymerization via competing in the binding of actin to Arp2/3 (Reinke, 2009; Reinke et al., 2011). The consequences are impaired trafficking of intestinal proteins that depend on an intact actin network. Structural alterations in the actin cytoskeleton that are associated with impaired trafficking to and reduced expression of SI at the cell surface have been also described in rotavirus-infected intestinal cells (Jourdan et al., 1998). Taking all this together, it can be postulated that an impaired trafficking of LR-enriched vesicles harboring SI—and other membrane proteins—and their intracellular retention leads to an intracellular accumulation of the two main lipid components comprising LRs, cholesterol, and sphingolipids. This in turn could elicit redistribution and alterations in the membrane lipid composition as has been demonstrated in several lysosomal storage diseases, such as Niemann-Pick Type C and Fabry diseases (Brogden et al., 2017, 2020; Shammas et al., 2019). In addition to altered membrane and protein trafficking in infected Caco-2 cells, *S. aureus* infection resulted in a reduction of the overall enzymatic activity of SI, most likely due to altered LRs. It has been shown before that

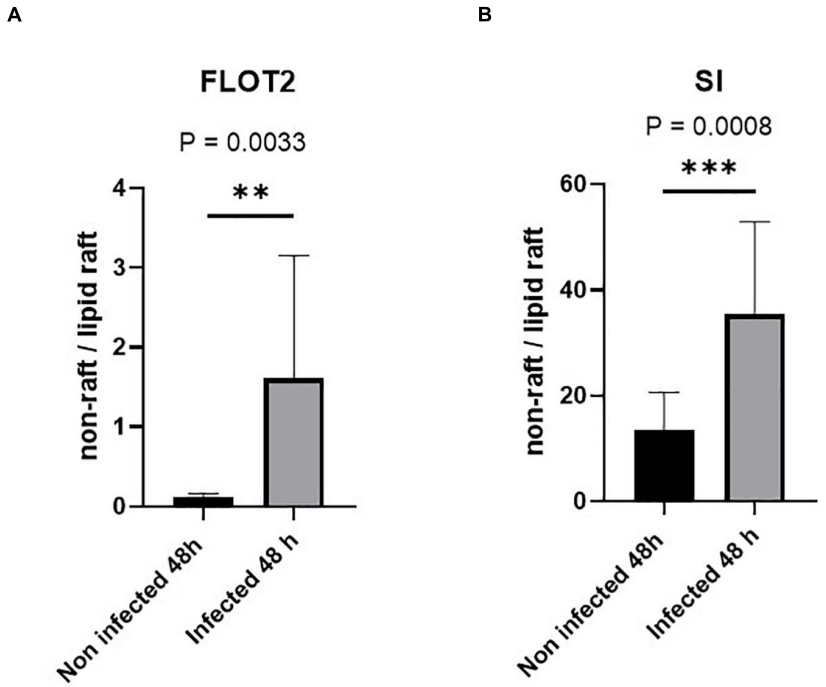

FIGURE 5 | Lipid rafts (LRs) are altered in Caco-2 cells infected with *Staphylococcus aureus* Newman. Control non-infected or *S. aureus* infected Caco-2 cells were solubilized with 1% (w/v) Triton X-100 and separated on sucrose density gradients. Ten fractions were collected and used for further analysis in which the LRs are found in the top fractions (fractions 1–3) due to their low buoyant density. The distribution of the LR markers flotillin-2 (FLOT-2) **(A)** and sucrase–isomaltase (SI) **(B)** in the bottom three fractions (non-rafts) and in the top three fractions (LRs) in the control non-infected cells was compared with that of respective values from the same fractions in cells infected with *S. aureus* using by Western blotting. Raw data are shown in **Supplementary Figures 5**, **6**. The redistribution suggests an altered LR composition in *S. aureus*-infected Caco-2 cells. Statistical significant differences are shown as ** $p < 0.01$ or ***$p < 0.001$.

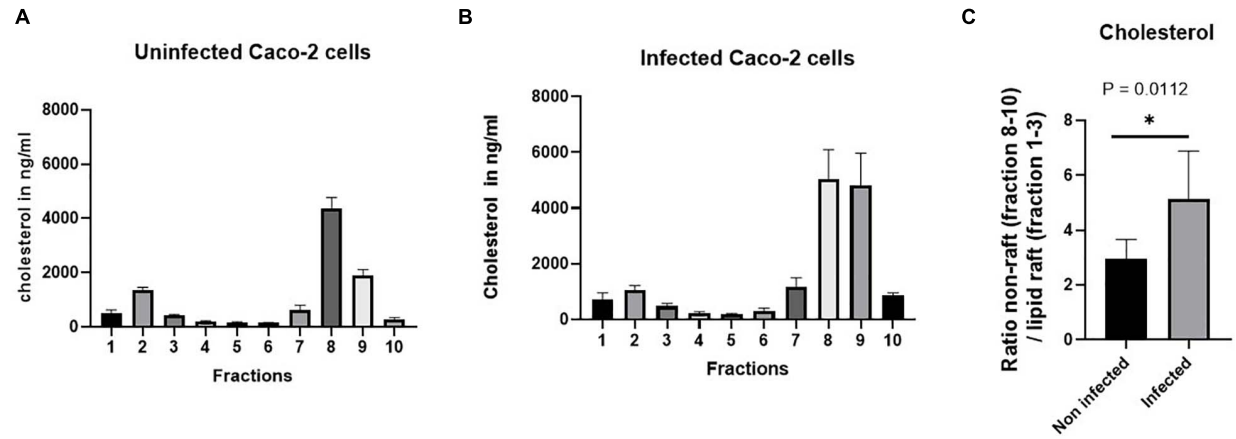

FIGURE 6 | The proportion of cholesterol. For the separation of the lipid rafts (LRs) from the non-LR fractions, sucrose gradient fractionations were used, in which the LRs are found in the top fractions (fractions 1–3) due to their low buoyant density. The level of cholesterol in the bottom three fractions (non-rafts) and in the top three fractions (LRs) in the control non-infected cells was compared with that of cholesterol from the same fractions in cells infected with *S. aureus*. **(A,B)** The raw data of the proportion of cholesterol in the control non-infected cells compared with cells infected with *S. aureus*. Cholesterol analysis was performed by high-performance liquid chromatography (HPLC). **(C)** A significant reduction (almost 50%) in the cholesterol levels was observed in the LRs of Caco-2 cells infected with *S. aureus*. The data represent mean ± SEM of two independent experiments with samples in duplicates or triplicates ($n = 5$). Statistical significant differences are shown as *$p < 0.05$.

association of SI into LRs increases activities of both sucrase and isomaltase, by almost three-fold, since SI clusters into LRs, exhibits a cooperative hydrolytic activity, and increases substrate accessibility (Wetzel et al., 2009). Reduced enzymatic activities of SI and also another LR-associated disaccharidase, maltase-glucoamylase, have been reported in intestinal biopsy specimens

of patient with Niemann-Pick Type C, in which the trafficking or SI and LRs is markedly affected. The substantial reductions in the catalytic activity and cell surface expression of SI lead collectively to a substantial loss of function of SI at the cell surface. *In vivo*, these functional deficits can trigger symptoms, such as diarrhea, flatulence, and abdominal cramps, which are associated with functional gastrointestinal disorders (FGIDs), such CSID and irritable bowel syndrome. Unlike the secondary effects on SI function in *S. aureus* infection, FGIDs are genetically determined disorders in which SI is directly affected and constitutes therefore the primary cause of these diseases. The common features of these primary and secondary disease phenotypes are the impaired trafficking of SI and also altered association with LRs, which altogether lead to enzyme malfunction.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AM, DW, NS, and KB-H performed the experiments and interpreted the results. AM analyzed the data statistically and drafted the first version of the manuscript. HN and MK-B developed the concept of the study and analyzed the results. HN, MK-B, and DW wrote the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.699970/full#supplementary-material

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