STRESS AND IMMUNITY

EDITED BY: Yong-Soo Bae, Eui-Cheol Shin, Yoe-Sik Bae and Willem van Eden
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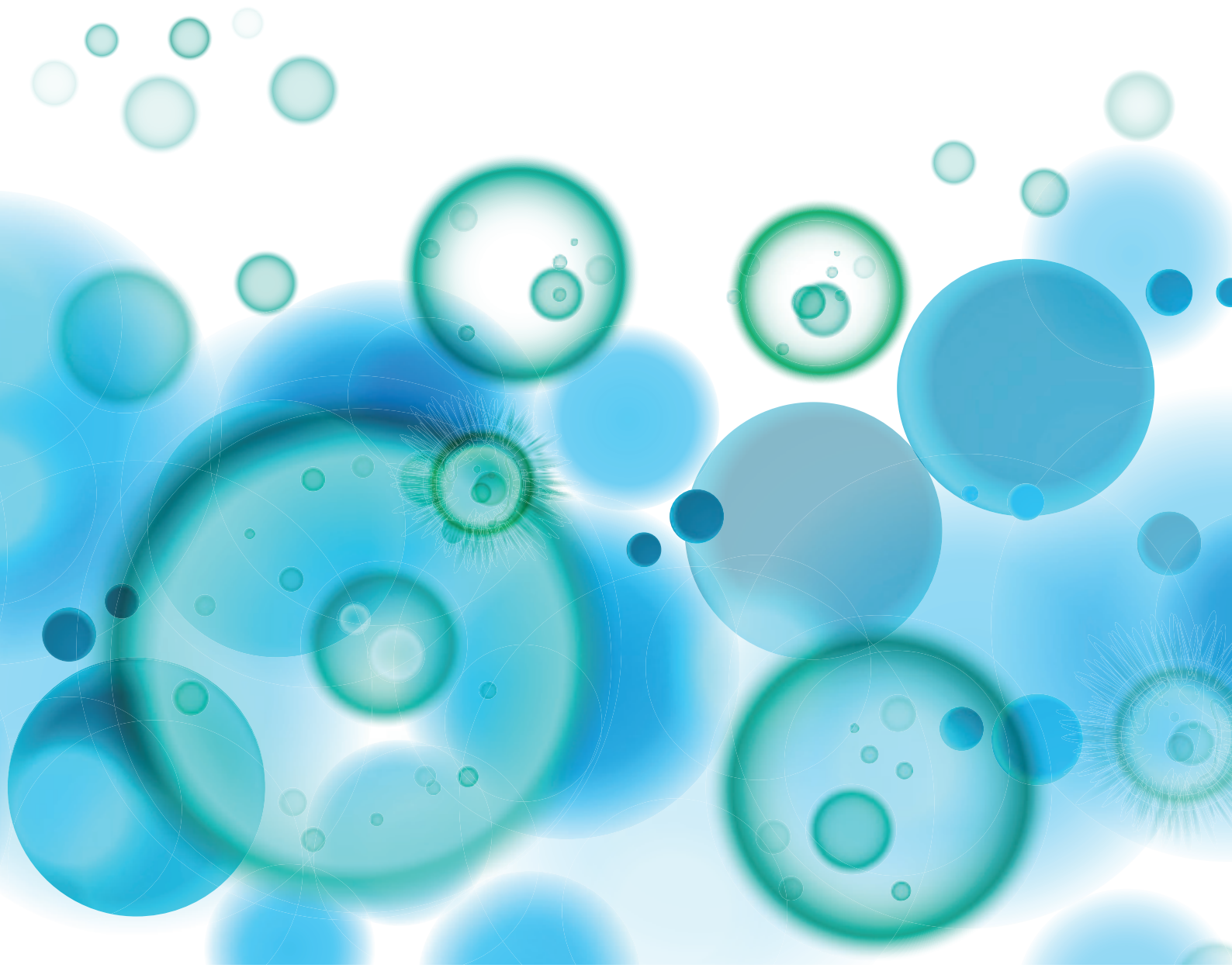

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STRESS AND IMMUNITY

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The "Stress and Immunity" Research Topic includes two distant and seemingly unrelated forms of stress: physicochemical stress and psychological stress. In both forms of stress the body adapts to the changes in the environment. The different chapters of this eBook deal with aspects relevant for the fascinating interplay of various distinct stressors with the immune system.

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EDITORIAL
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Editorial: Stress and Immunity

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Keywords: stress protein, cell stress, psychological stress, heat shock (stress) proteins, unfolded protein response (UPR)

Editorial on the Research Topic

Stress and Immunity

Research regarding stress and its relation to the immune system has always been of interest for the medical and scientific community. In relation to the "Stress and Immunity" Research Topic we have to recognize the existence of two distant and seemingly unrelated forms of stress: physicochemical stress and psychological stress. In both forms of stress we see that the body has a tenacious ability to adapt to the changes in the environment thus adjusting itself accordingly. But the interplay between different stressors and the ever-elusive immune system has made this research increasingly fascinating.

Both forms of stress are worth studying since both have a bearing on the overall health status of an individual. Physicochemical stress results from environmental factors such as food/nutrition, toxins, metabolic disorders, infections, and inflammation. It is known that if the immune system is compromised and cannot properly cope with physicochemical stresses, or if the stress exceeds the regular adaptability of the immune system, this may lead to disease states or fatal conditions. On the other hand, we are all well aware of the effects of psychological stress on the body and the immune system altogether. Long-term and chronic stress leads to persistently high cortisol and corticosteroid levels, which cause resistance to cortisol and impaired anti-inflammatory effects on the immune system. Such effects result in chronic infection, chronic inflammatory autoimmune diseases, or cancers as well as other physiological disorders. Chronic stress also inhibits the cross talk of immune cells and signaling networks.

A relatively large proportion of the contributions in this Research Topic are dealing with the effects of stress in tumor models. It is interesting to note in this era of rising interest in cancer immunotherapies, that both of the aforementioned forms of stress are associated with malignancies and are found to have an impact on tumor immunity and the efficacy of tumor immunotherapies. The role of antidepressants in a murine tumor model was reported by Di Rosso et al. in the pursuit of finding a relationship between psychological-chronic stress and its effects on immune homeostasis and consequently tumor biology. Both of the antidepressants (i.e., fluoxetine and sertraline) were selective serotonin reuptake inhibitors (SSRIs). In this study, authors reported a reciprocal relationship between chronic stress and antitumor immunity resulting in tumor growth and metastasis. Although one may consider there may have been direct effects of the drugs on tumor biology, the inhibition of psychological stress by these drugs appears to restore antitumor immunity.

Another aspect worth exploring is the effect of stress-induced sympathetic adrenergic signaling on the immune system, since adrenergic signaling has been shown to inhibit immune responses in both autoimmune diseases and infection models. Qiao et al. discussed such stress-mediated adrenergic effects on antitumor immunity. They discovered that reduction in adrenergic stress by use of β-blockers reverses immunosuppression and significantly improves responses to checkpoint

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inhibitor immunotherapy. In the case of tumor models, the negative effects of psychological stress on immune performance are seen in a similar manner as consequences of physicochemical stress.

The role of hypoxic stress in the tumor microenvironment is another important facet of untoward anti-tumor immunity. Terry et al. reviewed the many ways in which hypoxic stress can lead to resistance to cell-mediated cytotoxicity and consequently to immune suppression. Talking about hypoxia as a stressor, Ostheimer et al. have introduced osteopontin (OPN) as a new tumor hypoxia-related marker. In patients with non-small-cell lung cancer, serum levels of stress protein HSP70 were found to correlate with OPN and increased levels of these markers were associated with decreased overall survival.

Although the Ostheimer at al. did not discuss the possible immune mechanisms of these correlations, it may be attractive to assume a possible relationship with the issues discussed by Barbera Betancourt et al. In the paper of Barbera Betancourt et al. stress proteins such as HSP70 play a distinctive role in the induction of immune suppressive regulatory T cells. While the evidence for this is obtained from extensive studies in models of chronic autoimmune arthritis, it is likely that similar mechanisms are active in the tumor microenvironment. Alternative and less immunosuppressive effects of stress on cells of the immune system are discussed by Antonangeli et al. Various types of stress are mentioned as triggers for the modulation of NKG2D ligands. Such ligands are recognized by activating NKG2D receptors on, for example, natural killer cells, which through the activation via these receptors can clear stressed mucosal epithelial cells.

In the paper by Yang et al. C/EBP homologous protein (CHOP) is discussed as a main factor in endoplasmatic reticulum stress (ER stress) induced apoptosis and its resulting implication in human diseases, such as cancer, diabetes, neurodegeneration, and fibrosis. CHOP is, besides GRP78 and Atf4, also one of the ER stress proteins studied by Gan et al. The upstream reduction of these proteins was seen in the process of leptin-mediated inhibition of autophagy. Such leptin-mediated autophagy inhibition may offer a potential new means for the therapy of metabolic syndrome in mammals.

The relationship between stress and the immune system is bidirectional and can affect each other in ways that are quite elusive and thus has attracted much attention from the scientific community. The team of Holzer et al. has introduced the reverse path of immune function leading to stress responses, especially from the visceral system to brain function, behavior, and stress coping. Effects of immune activation stress on brain function may well have a bearing on mental health and may lead to novel therapeutic possibilities.

Finally, nutrient deprivation as a stressor for bacterial species and as an adaptation of the fish immune system to limit bacterial invasion was described by Martínez et al. In the notothenoid sub-Antarctic fish model, it was found that bacterial *Piscirickettsia salmonis* infections were reduced by modulation of iron metabolism in the liver and brain.

Altogether, after having seen all individual contributions to this Research Topic, we see that the topic of "Stress and Immunity" is broad and has its roots in an active research field that is fundamental to the biology of health and disease. The far-reaching implications of stress-related immune functioning make this an intriguing subject important for future studies to gain better understanding.

AUTHOR CONTRIBUTIONS

WV and Yong-SooB wrote the paper together. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Adrenergic Signaling: A Targetable Checkpoint Limiting Development of the Antitumor Immune Response

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An immune response must be tightly controlled so that it will be commensurate with the level of response needed to protect the organism without damaging normal tissue. The roles of cytokines and chemokines in orchestrating these processes are well known, but although stress has long been thought to also affect immune responses, the underlying mechanisms were not as well understood. Recently, the role of nerves and, specifically, the sympathetic nervous system, in regulating immune responses is being revealed. Generally, an acute stress response is beneficial but chronic stress is detrimental because it suppresses the activities of effector immune cells while increasing the activities of immunosuppressive cells. In this review, we first discuss the underlying biology of adrenergic signaling in cells of both the innate and adaptive immune system. We then focus on the effects of chronic adrenergic stress in promoting tumor growth, giving examples of effects on tumor cells and immune cells, explaining the methods commonly used to induce stress in preclinical mouse models. We highlight how this relates to our observations that mandated housing conditions impose baseline chronic stress on mouse models, which is sufficient to cause chronic immunosuppression. This problem is not commonly recognized, but it has been shown to impact conclusions of several studies of mouse physiology and mouse models of disease. Moreover, the fact that preclinical mouse models are chronically immunosuppressed has critical ramifications for analysis of any experiments with an immune component. Our group has found that reducing adrenergic stress by housing mice at thermoneutrality or treating mice housed at cooler temperatures with β-blockers reverses immunosuppression and significantly improves responses to checkpoint inhibitor immunotherapy. These observations are clinically relevant because there are numerous retrospective epidemiological studies concluding that cancer patients who were taking β-blockers have better outcomes. Clinical trials testing whether β-blockers can be repurposed to improve the efficacy of traditional and immunotherapies in patients are on the horizon.

Keywords: adrenergic, norepinephrine, antitumor immune response, temperature, stress, β-blocker

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INTRODUCTION

Psychosocial and physical stresses have long been believed to negatively affect health and reduce our resistance to immune-mediated diseases (including cancer), but the mechanisms have been poorly understood (1, 2). A comprehensive review of early work on the effects of stress on tumor growth in animal models highlights the dichotomous results between viral tumors which grow

faster with stress and non-viral/chemically induced tumors which grow more slowly during the stress period (3). In the last decade or so, however, we are beginning to understand the myriad mechanisms by which chronic adrenergic stress molds the immune response and promotes tumor growth (2–9). In this brief review, we will first give examples of how adrenergic signaling impacts immune cells and then how it affects tumor growth. We then summarize how preclinical mouse models are used to study the effects of different stress types on tumor growth, survival, and metastasis, highlighting the idea that tumors attract their own innervation in a process akin to angiogenesis. We emphasize the potential ramifications of the baseline cold stress which is imposed on mice by sub-thermoneutral housing and how this impacts our understanding of the capabilities of the endogenous immune response. In addition, in light of recent excitement about the potential of new immunotherapies to treat cancer, we discuss how adrenergic suppression of the antitumor immune response is a targetable checkpoint which may be blocked to increase the efficacy of immunotherapy. Finally, we summarize retrospective clinical epidemiological studies that support the idea of "repurposing" β-blockers for use in oncology.

NEURAL REGULATION OF THE IMMUNE RESPONSE

Stress can impact the immune response through two major neural pathways, the hypothalamic/pituitary/adrenal (HPA) axis and the sympathetic nervous system (SNS); we have recently reviewed these pathways in depth (10). While stimulation of the HPA axis results in the release of glucocorticoids from the adrenal cortex, the SNS innervates the adrenal medulla and stimulates release of the catecholamine neurotransmitters, epinephrine (Epi) and, to a lesser extent, norepinephrine (NE) (11). Perhaps more importantly for the purpose of this review, NE is also released by postganglionic sympathetic neurons which densely innervate both primary and secondary lymphoid organs (12–15) as well as essentially all other organs. Adrenergic receptors (ARs) for NE and Epi are located on the surface of most cells, including immune cells, and thus in addition to the regulatory influences of chemokines and cytokines, NE released from sympathetic nerve endings also plays a critical role in regulating immune cells. In fact, we are now realizing that neural reflexes may reach immune cells in a fraction of the time (milliseconds) that it takes chemokines and cytokines to circulate and have an effect on these cells (16). While activation of the SNS in response to acute stress such as exercise or injury promotes a rapid immune response (17), chronic ongoing stress often is immunosuppressive (18). As discussed below in the Section "Modeling the Tumor-Promoting Effects of Adrenergic Stress/Signaling in Mice", suppression of the antitumor immune response is one of the consequences of this chronic stress.

Adrenergic receptors are a class of G protein-coupled receptors and are subdivided into two types (α and β) based on their structure, pharmacology, and signaling mechanisms, and these include several subtypes (19). Although there are many studies showing that both α- and β-ARs are expressed by innate immune cells, including neutrophils (20, 21), monocytes (22–24), macrophages (22, 24–26), mature DC (22), NK cells (27–33), and hematopoietic stem cells and progenitors (34), β2-ARs are the most highly expressed subtype on both innate and adaptive immune cells. T and B cells exclusively express β2-AR (35–37); this is also true for hematopoietic stem cells and progenitors (34). Consequently, β_2-ARs are regarded as the main mediators of the immune effects of catecholamines (8). Activation of these receptors activates adenylate cyclase to increase intracellular cAMP, which in turn activates PKA which ultimately activates downstream transcription factors (2, 35, 36, 38–40). The details of this signaling pathway have recently been reviewed for both immune cells (41) and cancer cells (9).

Adrenergic Regulation of Innate Immunity

That activity of innate immune cells is regulated by activation of ARs has been clearly demonstrated. Adrenergic signaling affects macrophage polarization and cytokine production. β-AR signaling promotes macrophage differentiations toward an M2 phenotype which produces anti-inflammatory cytokines (42–45). In one study, gut macrophages in the muscularis mucosa, which are in close proximity to the autonomic myenteric plexus, differentially expressed the β2-AR, which mediated alternative activation of these macrophages, resulting in an M2 anti-inflammatory and tissue-protective profile (46). Both *in vitro* and *in vivo* studies support the conclusion that at least one way in which β-adrenergic signaling can promote breast cancer progression is by polarizing macrophages toward an M2 phenotype (42, 43). Moreover, in response to LPS stimulation, human monocyte-derived macrophages produce reduced amounts of the inflammatory cytokines TNF-α, IL-1β, CCL2, CCL3, and CCL4 (47–49) and decrease IL-27 secretion in response to acute inflammation (50) while, at the same time, increasing production of the anti-inflammatory cytokines IL-4, IL-10, and IL-13 production (44, 50). In contrast to the effects of β-AR signaling in macrophage, α-AR signaling promotes secretion of pro-inflammatory cytokines (24, 51).

Adrenergic signaling has been shown to impact DCs by impairing their maturation, cytokine production, and antigen presentation. Studies have shown that β2-AR activation prevents differentiation of monocytes into DCs (52) and enhances production of the anti-inflammatory cytokines IL-6, IL-10, and IL-33 while decreasing IL-12 and TNF-α production (53–58) by inhibition of NF-κB and AP-1 (54). In addition to altering cytokine production, Epi has also been shown to activate β1-AR/arrestin2–PI3K–MMP9/CCR7 signaling to inhibit migration of human DCs (59) which could impair migration to lymph nodes. Pretreating bone marrow derived DCs (BMDC) with β2-AR agonists before adoptive transfer reduces migration and responses to chemokines. Both *in vitro* and *in vivo* studies show that α2-AR suppresses DC migration by inhibiting type IV collagenase/gelatinase activity (60). The primary function of DCs, antigen presentation, is impaired by treating epidermal Langerhans cells with catecholamines, and this effect is blocked by using a β2-AR antagonist (61). In addition, cross-presentation of proteins by DCs is impaired by activation of β2-AR signaling, which

in turn decreases $CD8^+$ T cell proliferation and IL-2 production (62). Our lab has also found that adrenergic stress impacts the phenotype and function of DCs (63) in ways that could suppress their function.

Several studies have demonstrated that chronic adrenergic signaling can suppress NK cell activity. In a restraint stress mouse model, the number of NK cells is reduced in the intraparenchymal region of the lung and circulation (64). Another study showed that activation of β-ARs reduces the activity (65) of murine NK cells and leads to an increase in tumor metastasis. Interestingly, the two arms of the stress response can cooperate to regulate immune cells. De Lorenzo et al. found that during sleep deprivation, glucocorticoids increase expression of β2-AR in NK cells resulting in reduction of NK cell numbers and cytotoxicity (66). In contrast to the effects observed in these models of chronic stress, some studies have concluded that stress can *increase* NK cell activation and function. In one such study, six episodes of social disruption increased NK cell activity (67). In addition, voluntary exercise reduces the incidence and growth of breast cancer and this effect is mediated by catecholamine induced plasma IL-6, which in this case mobilizes NK cells (17). Enriching the housing environment for mice also increases NK antitumor function (68). Clearly, the effects of stress differ depending on the type and duration.

In addition, there are many reports investigating the regulation of other innate immune cells by adrenergic signaling. For example, a β2-AR agonist was able to prevent eosinophil functions which are induced by exposure to IL-5, LTD4, or IP-10 and which worsen the severity of asthma (69). In another example, the phagocytic efficiency of wound neutrophils was found to be impaired by a pharmacologic dose of NE, in a standard subcutaneous sponge wound model, and this change was found to be mediated by α- and β-ARs and downstream protein kinase A (70).

Adrenergic Regulation of Adaptive Immunity

Norepinephrine also activates ARs on lymphocytes to regulate the differentiation, trafficking/migration, and effector functions of all lymphocyte subpopulations (11). Adrenergic signaling impacts T-cells by directly regulating thymocytes and inhibiting activation, differentiation, and effector function of T-cells directly or indirectly by inhibiting T-cell activating cytokine production by DCs. A study in stressed mice showed that β-AR signaling promotes negative selection in the thymus through the p38 mitogen-activated protein kinase pathway, resulting in decreased numbers of thymocytes (71). In addition, exposure to chronic unpredictable stress reduces the number of double negative thymocytes (72). Adrenergic signaling decreases production of IL-2, IFN-γ, and proliferation of $CD4^+$ T-cell through many mechanisms. β2-AR signaling inhibits cytokines by inhibiting calcineurin in a PKA dependent manner (73). IFN-γ production by $CD4^+$ T-cells can also be affected indirectly by inhibition of cytokine production by DCs. Adrenergic signaling also regulates Th1 and Th2 differentiation (34). Naïve $CD4^+$ T-cells or activated Th1 cells express a detectable level of the β2-AR, while Th2 cells do not, due to differences in histone and DNA modifications within the β2-AR proximal promoter (22, 74). Therefore, Th1 cells can be suppressed by adrenergic signals and β2-AR activation has been found to be involved in directing $CD4^+$ T-cell polarization toward a Th2 phenotype (75). The impact of β2-AR signaling on $CD8^+$ T-cells depends on the stage of differentiation. Memory and effector $CD8^+$ T-cells have significantly higher expression of β2-ARs than naïve T-cells and β2-AR signaling reduces IL-2 and IFN-γ production by memory $CD8^+$ T-cells upon restimulation (76). IL-2 increases β2-AR expression on effector $CD8^+$ T-cells; however, β2-AR signaling suppresses IL-2 production by $CD8^+$ T-cells, which forms a negative-feedback loop in regulating $CD8^+$ T-cell activation and function (77). By contrast, while β2-AR signaling suppresses $CD8^+$ effector T-cell function, β2-AR signaling in regulatory T-cells increases their suppressor activity by increasing expression of the checkpoint molecule CTLA-4 and promotes inducible Tregs by inducing Foxp3 expression in T-cells (55).

Suppression of immune cells by chronic adrenergic stress has long-term negative implications for disease progression and has been well studied in the context of both infectious diseases and autoimmunity. β2-AR signaling impairs $CD8^+$ T-cell-mediated antiviral responses to influenza *in vivo* and blocking the β2-AR with Nadolol enabled development of a robust antiviral response (78). Similarly, β2-AR signaling reduced the $CD8^+$ T-cell response to the vesicular stomatitis virus (79). Dysregulation of the immune response also underlies many autoimmune diseases. Because autoimmune diseases involve overactive immune responses, adrenergic signaling actually helps to suppress autoimmunity, and ablation of sympathetic nerves worsened disease severity in a mouse model of multiple sclerosis (80). An in depth discussion of these studies is beyond the scope of this review but is the subject of a recent review by our group (81).

NEURAL REGULATION OF TUMOR GROWTH

The early observations of Levi Montalcini and collaborators first clearly demonstrated that tumor cells secrete nerve growth factors and can induce neural outgrowth from both sensory and sympathetic ganglia (82). As evidence of the tumor promoting effects of adrenergic signaling accumulated, Entschladen and colleagues hypothesized that in a process analogous to angiogenesis and lymphangiogenesis, tumors might secrete neurotrophic factors to attract their own innervation (83). But it was the elegant study of Magnon et al. (84) that clearly demonstrated both the ability of a developing tumor to attract autonomic innervation and the critical role such innervation plays in development and progression of a tumor. Using an orthotopic xenograft model (PC-3 prostate cells), they showed that the developing tumor attracted new autonomic innervation and that if they severed the hypogastric nerves or chemically sympathectomized the mice, tumor growth was significantly inhibited. Furthermore, they showed that parasympathetic nerves promoted the later stages of invasion and metastasis. Others have shown that NE levels

in tumors are elevated by stress that activates the SNS and that NE is produced locally within tumors (as evidenced by tyrosine hydroxylase positive cells) (81, 85–88).

In the last decade or so there has been a growing effort to understand how adrenergic signaling promotes tumor growth and we now know that β-AR signaling acts through multiple mechanisms (**Figure 1**). ARs are expressed by multiple cell types found in the tumor microenvironment and, therefore, the effects of adrenergic signaling that support tumor survival, growth, and metastasis are complex.

Direct effects include promoting tumorigenesis, tumor cell proliferation, antiapoptotic mechanisms, and promoting metastasis by inducing epithelial to mesenchymal transformation (EMT), motility and invasion. β-Adrenergic signaling activates p53 degradation and DNA damage through the β-AR/ARRB1/PKA pathway (89–91) and activation of oncogenes, such as Src and Her2 (92) and may promote tumorigenesis. In addition, increased psychological stress is associated with decreased telomere length (93), which is associated with increased cancer risk (94). Adrenergic signaling promotes tumor cell proliferation both *in vitro* and *in vivo* (95–101), while blocking adrenergic signaling leads to G1/S phase cell cycle arrest and apoptosis (102, 103). Antiapoptotic mechanisms are upregulated in tumor cells both *in vitro* and in mouse xenografts (84, 85, 104–106). Our lab found that adrenergic signaling increases expression of the antiapoptotic proteins BAD, BCL-2, and MCP-1 in tumors and blocking adrenergic signaling significantly reduces antiapoptotic protein expression and tumor growth (85). In addition, *in vitro*, NE activates the TGF-β pathway in treated cancer cells enhancing migration and invasion (107, 108). Mesenchymal markers such as α-SMA, vimentin, and snail are also increased by β-adrenergic signaling (109, 110). More recently, analysis of exosomes isolated from ovarian patients with high levels of adrenergic pathway activation (associated with low social support) shows upregulation of mesenchymal-characteristic gene transcripts and downregulation of epithelial-characteristic gene transcripts, and this is mediated by the ADRB/cAMP/PKA pathway (111). Adrenergic signaling plays a vital role in promoting of metastasis (112–122) and could directly induce invasive genes such as MMPs by tumor cells (123) and indirectly by causing cancer-associated fibroblasts to produce high levels of collagen and extracellular matrix components, which facilitate tumor invasion and dissemination (124).

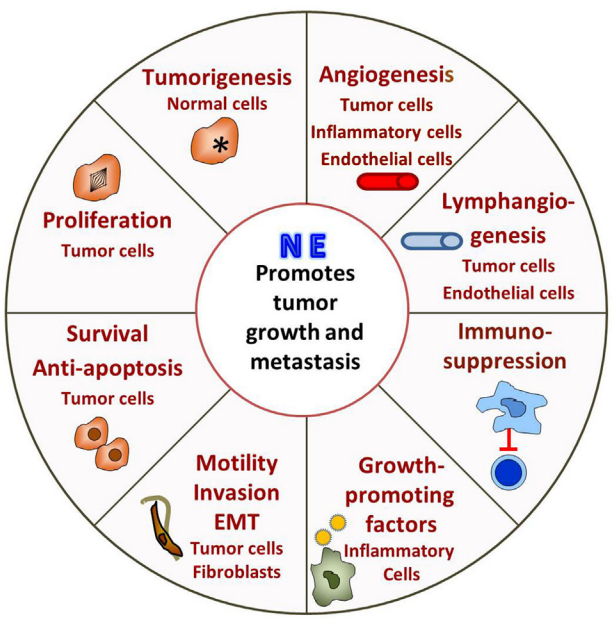

FIGURE 1 | Adrenergic signaling promotes tumor survival, growth, and metastasis. The tumor is innervated by postganglionic nerves of the sympathetic nervous system and, in response to stress, these nerves secrete norepinephrine (NE). Many cells in the tumor microenvironment express adrenergic receptors, and their responses support tumor growth. See text for discussion.

Adrenergic signaling also affects other cells in the tumor microenvironment, which in turn support tumor growth, survival and metastasis. Studies show that adrenergic signaling promotes angiogenesis through increasing VEGF, IL-8, IL-6, PEG2 expression in tumor cells (87, 119, 125–130), and decreasing expression of TSP1, a potent angiogenesis inhibitor (129). A recent study from Zahalka et al. demonstrates that adrenergic signaling also directly alters the metabolism of endothelial cells in the TME, leading to increased angiogenesis (131). In addition, studies demonstrate that adrenergic signaling promotes lymphangiogenesis by inducing VEGFC production by tumor cells and/or macrophages (112, 132). Formation of a pre-metastatic niche in the lung is induced by adrenergic signaling in monocyte/macrophage cells in a breast cancer model (133). In another study, stimulation of bone marrow stromal cells by adrenergic signaling increases breast cancer bone colonization (115). Finally, it is becoming clear that the immunosuppressive effects of adrenergic signaling that have been seen in infection models (78, 79) are also playing a major role in suppressing the antitumor immune response. This inhibits tumor infiltration and function of cytotoxic T-cells (134–136) and promotes infiltration of immunosuppressive cells such as MDSC and Tregs (134, 136–139).

MODELING THE TUMOR-PROMOTING EFFECTS OF ADRENERGIC STRESS/SIGNALING IN MICE

Although the concept of stress is generally understood, it is challenging to define. The original concept of stress, as proposed by Selye, was the response of an organism to a stimulus that challenged its well-being and he called this the General Adaptation Syndrome (140). Briefly, he proposed that an organism had the same response to many different noxious stimuli (swelling of the adrenal cortex, atrophy of the thymus, gastric ulcers) and that people suffering from various diseases had the same symptoms or syndrome. Eventually, he coined the term "stressor" to refer to the stimulus and the "stress response" to refer to the reaction. Stressors can be psychological (such as anxiety, fear, depression), physical (such as injury, surgery), or physiological (environmental factors including temperature) and exposure to stressors

can be either acute (a single short-term exposure—minutes to hours) or chronic (long term, ongoing, or repeated). In general, an acute stress such as an injury leads to a beneficial response including activation of immune cells, whereas chronic exposure is most often deleterious and immunosuppressive (141). Dhabhar (18) has discussed the idea of categorizing stress as "protective, pathological, or regulatory" depending on their effects on overall health.

There is a general view that stress is detrimental to health and in ovarian carcinoma patients with little social support, it has been shown that the stress of social isolation elevates tumor and ascites NE while Epi was undetectable; this elevated NE correlates with more severe disease stages (86). To study how stress impacts tumor growth in preclinical models, protocols for inducing stress in mice have been developed (**Table 1**). It is important to point out that the same method can be used to induce both acute and

TABLE 1 | Effects of different models of stress on tumor growth in mice.

Reference	Tumor model	Effects on tumor growth	Mechanisms
Psychological stress—restraint: confinement, unable to move freely			
Kim-Fuchs et al. (123)	Human pancreatic cancer/nude mice	Increases tumor growth and dissemination of tumor cells to adjacent pancreas and liver	β-Adrenergic receptor (AR) signaling induces expression of tumor invasion genes and matrix metalloproteases, MMP2 and MMP9
Le et al. (132)	Human breast cancer/nude mice Murine breast cancer/BALB/c MMTV-PyMT-C57BL/6	Promotes lymphangiogenesis and tumor cell dissemination and metastasis	β-AR induces tumor-associated macrophages producing inflammatory molecules such as PGE2, which in turn induce tumor cells to produce VEGFC promoting lymphatic remodeling
Hulsurkar et al. (129)	Human prostate cancer/NOD/SCID	Promotes tumor growth	β-AR signaling activates CREB and induces HDAC2 expression by binding to its promoter. HDAC2 repression of TSP1 expression, promotes angiogenesis and prostate cancer progression
Hassan et al. (104)	Prostate cancer/nude mice Hi-Myc mice (and fear)	Antiapoptotic effect on tumor	Increased tumor catecholamine levels, which activates the epinephrine/ADRB2/PKA/BAD antiapoptotic signaling pathway
Nagaraja et al. (119)	Human ovarian cancer injected intraperitoneally or into ovaries (metastasis model)/nude mice	Increases tumor production of inflammatory prostaglandins and tumor metastasis	Increases prostaglandin E2(PGE2) synthesis *via* ADRB2–NF-κB–PTGS2 axis
Nagaraja et al. (124)	Human ovarian cancer/nude mice Murine ovarian cancer/C57BL/6	Increases tumor growth	NE drives cancer-associated fibroblast (CAF) phenotype *via* ADRB2/CREB/INHBA axis. CAFs produce high levels of collagen and extracellular matrix components
Lin et al. (143)	Human colon cancer/nude mice	Increases tumor weight	Increases plasma catecholamine; induces hyper-phosphorylation of ERK1/2, which drives cell proliferation
Psychological stress—social isolation: individual housing			
Thaker et al. (88)	Human ovarian cancer/nude mice (and restraint stress)	Increases tumor burden; more invasive growth of tumor	Increases size of adrenal glands; higher levels of tissue catecholamine; enhances tumor angiogenesis and enhances tumor expression of VEGF, MMP2, and MMP9 by activation of ADRB2/cAMP/PKA pathway
Madden et al. (144)	Human breast cancer/SCID	Increases tumor growth	Increases tumor $F4/80^+$ and $CD11b^+Gr\text{-}1^+$ macrophage populations
Chen et al. (133)	Murine breast cancer i.v./BALB/c; MMTV-PyMT-C57Bl/6 (and chronic unpredictable stressors[a])	Promotes breast cancer metastasis to lung	β-AR signaling induces expression of CCL2 in pulmonary stromal cells and CCR2 in monocytes/macrophages; increases recruitment and infiltration of macrophages into the pre-metastatic lung
Qin et al. (42)	Murine mammary cancer/BALB/c	Increases tumor growth	Increases serum catecholamine levels; increases migration of 4T1 cells *in vitro*; polarizes macrophage to M2 phenotype
Psychological stress: acoustic			
Hou et al. (145)	Murine colon cancer/BALB/c	Promotes tumor progression	Increases serum catecholamine and corticosterone; change Th1 and Th2 cytokines, and shift from Th1 to Th2 response in both circulation and tumor
Partecke et al. (146)	Murine pancreatic cancer/C57BL/6	Increases tumor growth and reduces survival	Increases behave stress; increases serum corticosterone and adrenal tyrosine hydroxylase; reduces Th1 cytokines; increases infiltration of Treg cells in tumor; increases VEGF and TGF-β with greater microvessel densities; increases MMP9 expression

(*Continued*)

TABLE 1 | Continued

Reference	Tumor model	Effects on tumor growth	Mechanisms
Physical/psychological stress: surgery			
Lee et al. (147)	Human ovarian cancer/ nude mice	Increases tumor growth	Increases angiogenesis; increases serum G-CSF, IL-1a, IL-6, and IL-15 concentrations
Physical/physiological stress: housing temperature-induced stress: standard housing at 22°C vs. housing at thermoneutrality 30°C			
Eng et al. (85)	Human pancreatic cancer/SCID Murine pancreatic cancer/C57BL/6	Increases tumor antiapoptosis, resistance to chemotherapy, tumor growth	Increases tumor catecholamines; increases antiapoptotic proteins expression
Bucsek et al. (134)	Murine mammary cancer/BALB/c Murine melanoma/C57BL/6	Increases tumor growth	Increases serum catecholamine; decreases tumor infiltrating $CD8^+$ T cells and $CD4^+$ T cells
Kokolus et al. (136)	Murine mammary cancer, colon cancer/BALB/c Murine melanoma/C57BL/6	Increases tumor growth	Decreases tumor infiltrating $CD8^+$ T cells, $CD4^+$ T cells; increases immunosuppressive cells
Acute stress: restraint			
Dhabhar (18)	Ultraviolet-B (UV) induced squamous cell carcinoma	Decreases tumor incidence and fewer tumors	Increases cutaneous-T-cell-attracting-chemokine (CTACK)/ CCL27, RANTES, IL-12, and IFN-γ gene expression; increases skin infiltrating T cell numbers
Exercise stress: voluntary running			
Pedersen et al. (17)	Melanoma or lung cancer/C57BL/6 DEN-induced liver tumors/NMRI male mice	Reduces tumor incidence and growth	Increases serum catecholamine; increases plasma IL-6: mobilizes NK cells
Dethlefsen et al. (148)	Stage I/II breast cancer patient; human breast cancer/female NMRI-Foxn1nu mice	Reduces tumor growth	Exercise-conditioned human serum decreases breast cancer cell viability and tumorigenic potential; catecholamine induces Hippo tumor suppressor signaling pathway, which inhibits tumor growth
Environmental enrichment[b]			
Song et al. (68)	Murine pancreatic cancer and lung cancer/C57BL/6/Beige mice/$Rag1^{-/-}$ mice	Decreases tumor growth and benefit is lost if mice receive a β-blocker or chemical sympathectomy	β-Adrenergic signaling enhances NK cell-mediated antitumor immune responses; increases expression of CCR5 and NKG2D in NK cells; and increases tumor infiltration of NK cells

[a]*Chronic unpredictable stressors include cage tilt, isolation, crowding, rapid light–dark changes, damp bedding, and overnight illumination.*
[b]*Environment enrichment: mice housed 12/cage in large cages with running wheels, tunnels, wooden toys, small huts, and nesting materials, which were moved 2×/week and changed 1×/week.*

chronic stress and these are distinguished by the duration of exposure. A good example of this is a study that used restraint stress to compare changes in the hippocampal transcriptome following acute vs. chronic stress by looking at samples taken after 1, 8, or 13 days of stress exposure (142).

To model psychological stress, mice have most often been subjected to social isolation or restraint stress. Over 10 years ago, using a mouse xenograft model of ovarian cancer, Thaker's group showed that restraint stress resulted in significant elevation of NE (255–358%) and corticosterone (488–789%) in organs adjacent to the peritoneal cavity in tumor-bearing mice. Tumor growth and metastasis were also significantly promoted in these mice. Mice are social animals and are normally housed at four to five per cage; in fact, special permission is needed if mice are going to be housed singly and in that case, enrichment materials are required. In addition to restraint stress, Thaker's group also subjected mice to social isolation and found enlargement of the adrenal glands as well as confirming the tumor promoting effects of stress. In addition, they showed that these effects could be duplicated by treating mice with the β2-AR *agonist* terbutaline and that the tumor promoting effects of both restraint and social isolation stress could be blocked by the pan β-AR *antagonist* propranolol. Altogether, these results clearly defined a role for adrenergic signaling in tumor growth and this was shown to be mediated by tumor VEGF production and increased angiogenesis (88). Since these seminal studies, the role of adrenergic signaling in tumor progression has been characterized in other models. Other groups have noted increased levels of catecholamines in response to psychosocial stressors. Qin et al. found that Epi was elevated and breast cancer progression was promoted in association with an M2 skewed macrophage population in a social isolation model; however, NE levels were not reported (42). Partecke et al. found that in response to a combination of acoustic and chronic stress, animals' measures of behavioral stress as well as levels of stress hormones (steroids and adrenal tyrosine hydroxylase, the rate limiting enzyme in NE synthesis) were significantly elevated and furthermore, that orthotopic, syngeneic pancreatic tumors grew faster in these mice and there were indications that their immune response was suppressed,

with a trend toward fewer CD4 and increased intratumoral Tregs present (146). Treatment of these mice with propranolol reduced tumor growth and improved overall survival (OS). These studies and several others (see **Table 1**) demonstrate that psychosocial stress promotes tumor growth through various mechanisms and that adrenergic signaling can be blocked by administration of β-AR or specific β2-AR antagonists.

In laboratory studies, the effects of *physical/physiological* environmental stressors on catecholamine levels and tumor growth have not been as well studied as the effects of psychological stresses, but environmental factors have great capacity to induce a stress response and alter internal metabolism and physiology (149). Our lab has recently defined a role for chronic mild cold stress in promoting tumor growth. This environmental stressor is universally imposed on mice as a result of mandated, cool ambient housing temperatures (150), and the impact of this cold stress on the modeling of several non-tumor mouse models of disease has been recently reviewed (151–153). The increasing awareness of this problem is reflected in several additional publications over the last 2–3 years reporting on how ambient housing temperatures affect disease outcomes in atherosclerosis (154–157), Alzheimer's (158), monocyte mobilization into the blood (159), and obesity (160–162). Newer reviews have also drawn attention to this situation (163, 164). We first reported the unexpected discovery that when mice are housed at thermoneutrality (30°C), tumor growth is significantly inhibited in comparison to mice housed at standard temperatures (22°C) even though mice under both conditions maintain a normal core body temperature of 37°C (136). We also demonstrated that this difference in tumor growth was dependent on the adaptive immune response which was significantly suppressed when mice were housed at 22°C. Furthermore, we found that although the effects of cold stress are commonly studied by exposing mice to 4°C, just housing mice at the sub-thermoneutral 22°C was sufficient to cause chronic cold stress and significant elevation of both plasma and tumor NE levels (85, 134). Thus we found that even what was considered mild cold stress at 22°C, is biologically sufficient to induce SNS activation to produce NE and drive non-shivering thermogenesis to maintain a normal body temperature. Furthermore, we have shown that chronic adrenergic signaling in mice housed at 22°C promotes tumor growth in two different ways, both by induction of antiapoptotic signaling molecules and resistance to cytotoxic therapies in tumor cells (85) and by profound immunosuppression of the antitumor immune response which is associated with increases in immunosuppressive cell populations and inhibition of $CD8^+$ T-cell effector phenotypes (134). Therefore, not only are the tumor cells more resistant to being killed, but the ability of the immune cells to kill tumor cells is much less robust when NE levels are increased by baseline housing stress; both of these situations are reversed by administration of the β-blocker propranolol. Interestingly, the recent studies of Wrobel et al. (135) agree with our findings; this group administered propranolol to MT/Ret mice and found inhibition of tumor development, increased $CD8^+$ T-cells in the tumors, and decreased MDSC. However, these authors did not associate this baseline stress with housing temperature and the temperature at which their mice were housed was not reported.

We are particularly concerned about this immunosuppression by baseline adrenergic stress in experimental mice because both the long-term outcome of traditional therapies (165–170) and immunotherapies (171) depend on the development of a robust antitumor immune response. We tested whether the efficacy of immunotherapy, specifically checkpoint inhibitor therapies, was limited by adrenergic stress. In mouse models

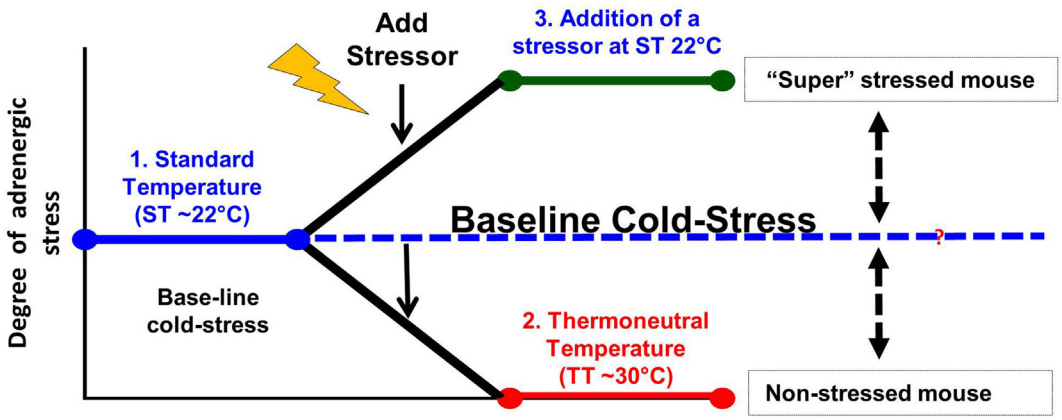

FIGURE 2 | Modeling adrenergic stress in mouse models. 1. Mandated housing of mice at ~22°C imposes chronic cold stress and results in elevated norepinephrine (NE) levels which drive thermogenesis to maintain a normal body temperature of 37°. This "baseline cold stress" is sufficient to inhibit the development of an immune response in several disease models including cancer. 2. Reduction of NE levels and reversal of immunosuppression can be achieved by housing mice at thermoneutrality (~30°C) or administration of β-blockers. Reduction of baseline adrenergic stress significantly improves the antitumor immune response in preclinical models compared with the response in mice housed at 22°C. The improved immune response correlates with a significant improvement in the efficacy of immunotherapy. 3. The majority of studies compare the impaired immune response seen at 22°C with that observed after an additional source of stress is imposed on animals at 22°C. This approach may compromise a full understanding of the capabilities of the endogenous immune response and could also lead to a misunderstanding of the efficacy of therapies depending on an immune response in these models.

of melanoma and breast cancer, we used three separate approaches: housing mice at 30°C, treating mice housed at 22°C with β-blockers, or testing anti-PD-1 in ADRB2$^{-/-}$ mice at 22°C and asked how this impacted in efficacy of anti-PD-1. In each case, it is clear that adrenergic signaling reduces responses to checkpoint inhibitor therapy and the efficacy is significantly improved by reducing this stress.

Taken together, it is clear that the majority of studies on the effects of stress are performed at 22°C when the "control" mice are already "stressed" in comparison to the relatively reduced levels they would be experiencing at 30°C (**Figure 2**). Therefore, restraint stress or other forms of psychosocial stress are imposed on already stressed mice, so that measures of tumor growth, response to therapy or the capabilities of the antitumor immune response in the controls are somewhat compromised. In the future, it will be important to determine if, and/or how, baseline cold stress may be affecting the results of these stress experiments. Although due to the thermal comfort requirements of personnel, it seems unlikely that the ambient temperatures in animal facilities will be changed to 30°C, it may be possible to reduce cold stress in experimental animals by providing nesting materials or constructing special cages in which mice can behaviorally regulate their thermal comfort (172–174).

TABLE 2 | A summary of retrospective studies assessing the beneficial effects of β-blockers, including Propranolol, in patients with different cancers who were also taking β-blockers.

Reference	Cancer type	Patients (numbers)	Survival	OR/HR	95% CI	*P*	Therapeutic effect
β-Blockers (other than propranolol)[a]							
Grytli et al. (188)	Prostate cancer	263		HR: 0.14	0.02–0.85	0.032	Reduced prostate cancer-specific mortality
Grytli et al. (189)	Prostate cancer	3,561		ASR: 0.79	0.68–0.91	0.001	Reduced prostate cancer-specific mortality
Kaapu et al. (190)	Prostate cancer	24,657		OR: 0.73	0.56–0.96	0.038	Decreased risk of advanced prostate cancer
De Giorgi et al. (191)	Thick melanoma	121			0.11–0.54	0.002	Reduce risk of progression of thick malignant melanoma
Lemeshow et al. (192)	Malignant melanoma	4,179		HR: 0.87	0.64–1.20		Increase survival time of patients with melanoma
Diaz et al. (193)	Epithelial ovarian cancer	248	PFS OS	HR: 0.56		0.05 0.02	Reduce chance of death
Wang et al. (194)	Non-small-cell lung cancer	722	DMFS DFS OS	HR: 0.67 HR: 0.74 HR: 0.78		0.01 0.02 0.02	Improved DMFS, DFS, and OS
Botteri et al. (195)	Triple-negative breast cancer	800		HR: 0.42	0.18–0.97		Significantly decreased risk of breast cancer-related recurrence, metastasis, and breast cancer death
Melhem-Bertrandt et al. (196)	Triple-negative breast cancer	1,413	RFS	HR: 0.30	0.1–0.87	0.027	Improve relapse-free survival in all patients with breast cancer and in patients with triple-negative breast cancer
Powe et al. (197)	Breast cancer	466		HR: 0.291	0.119–0.715	0.007	Significantly reduces distant metastases, cancer recurrence, and cancer-specific mortality in breast cancer patients
Jansen et al. (198)	Colorectal cancer	1,975		HR: 0.50	0.33–0.78		Association with longer survival
Monami et al. (199)	Cancer	1,340		HR: 0.33		0.019	Reduce cancer risk
Lin et al. (200)	Cancer	6,771		HR: 0.74	0.63–0.87	<0.001	Reduced upper gastrointestinal tract and lung cancer risk
Propranolol							
Choy et al. (201)	Triple-negative breast cancer	1,029		HR: 0.51	0.23–0.97	0.041	Decreased establishment of brain metastasis
Barron et al. (202)	Breast cancer	5,801		HR: 0.19	0.06–0.60		Reduce breast cancer progression and mortality
Nkontchou et al. (203)	Hepatocellular carcinoma	291		HR: 0.25	0.09–0.65	0.004	Decrease hepatocellular carcinoma occurrence
Chang et al. (204)	Head and neck Esophagus Stomach Colon Prostate	24,238		HR: 0.58 HR: 0.35 HR: 0.54 HR: 0.68 HR: 0.52	0.35–0.95 0.13–0.96 0.30–0.98 0.49–0.93 0.33–0.83		Reduce cancer risk

ASR, adjusted hazard ratio; DFS, disease-free survival; DMFS, distant metastasis-free survival; HR, hazard ratio; OR, odds ratio; OS, overall survival; PFS, progression-free survival; RFS, recurrence-free survival; 95% CI, 95% confidence interval.

[a]*β-Blockers other than propranolol include pindolol, propranolol, timolol, carvedilol, labetalol, nadolol, oxprenolol, alprenolol, sotalol, acebutolol, atenolol, betaxolol, bisoprolol, metoprolol, nebivolol, celiprolol, esmolol, carteolol, penbutolol, and celiprolol.*

CLINICAL RELEVANCE OF PRECLINICAL FINDINGS

How do these findings relate to patients? Studies have demonstrated that several types of patient tumors express ARs including pediatric (175, 176), pancreatic (103), lung (177), melanoma (178), and prostate (179) cancers. More provocatively, as it became apparent that blockade of adrenergic signaling could inhibit tumor growth in preclinical models, researchers looked for evidence of benefit in patients. Several retrospective epidemiological studies have supported the conclusion that patients who are taking β-AR antagonists ("β-blockers") and receiving conventional therapies have better outcomes in terms of both progression-free survival and OS than those who are not (**Table 2**). It should be noted, however, that there are other analyses that failed to identify any benefit of β-blockers (180–186). There are several potential reasons for why some studies show that cancer patients benefit from taking β-blockers while other studies conclude there is no benefit. One important reason is that the type of β-blocker being taken by the patient likely influences the patient's response as well as the overall outcome of the study. There are studies showing that patients taking non-selective β-blockers (blockade of β1 and β2) had improved OS compared with patients taking β1-specific blockers which are the most commonly prescribed β-blockers (180, 183, 184, 187). The heterogeneity of ARs expressed by the tumors themselves may also account for the different responses in cancer patients (180, 184). Besides the type of β-blocker, it is possible that the dosage needed to elicit a favorable antitumor effect is higher than the normal prescription dose for treating cardiovascular diseases (182, 185). In addition, the patient parameters are variable among the studies. Although the most important potential confounders such as sex, stage, treatment were usually adjusted for, there are others, such as socioeconomic status which is proven to be associated with cancer progression, which were not taken into consideration (182, 185). The timing of β-blocker exposure is different between studies. Some studies recruited patients already taking β-blockers before cancer diagnosis, while some studies investigated patients who began taking β-blockers after cancer diagnosis (180, 181, 183, 184). In addition, there are limited records showing whether patients complied with the prescriptions (182, 185). The study design may also influence the result. Some studies used all-cause of mortality as the endpoint, which makes it hard to study the relationship of β-blocker usage and cancer-specific mortality (183). In other studies, patients taking β-blockers who died within 3 months after cancer diagnosis were still included, which is thought to be too short an interval to assess benefit (181–184).

To date, there has been a single report of a prospective study treating melanoma patients with propranolol. In this study, De Giorgi and colleagues offered patients the choice of "off-label" treatment with propranolol and found an 80% risk reduction for recurrence for the 19 of 53 patients who chose to receive it (205). Ultimately, the ability of β-blockers to support the development of an antitumor immune response and to improve the efficacy of traditional and immunotherapies will have be determined in blinded, prospective clinical trials, but the results of this study in melanoma lend support for the "repurposing" of β-blockers in oncology.

β-Blockers have been used extensively in the clinic to treat patients with hypertension, angina and anxiety. In general, β-blockers have a good toxicity profile and are well tolerated (206); common side effects include nausea, vomiting, diarrhea, insomnia, weakness and fatigue. However, the safety of using of β-blockers in cancer patients needs to be considered. Although most cancer patients do not have hypertension and/or angina, they do often have increased levels of adrenergic stress and so in that way may be considered to have abnormal adrenergic signaling. In several studies in which cancer patients were taking β-blockers as an off-label treatment, either no drug related adverse effects were recorded (205, 207) or small number of patients had minor side effects which did not require discontinuation (203, 208, 209). However, an understanding of the comparative safety of β-blockers in combination with other cancer therapies remains to be evaluated in the context of clinical trials.

CONCLUSION

Recently developed immunotherapies are showing great promise in cancer treatment, yet many tumor types are not responding and even in sensitive tumors such as melanoma and lung cancer, the majority of patients are not benefiting. Therefore, developing new strategies for improving the therapeutic efficacy of cancer immunotherapies is critical. Immunotherapies and traditional therapies that have an immune-mediated component depend on the development of a robust antitumor immune response, but immunosuppressive factors often limit this. Therefore, a promising strategy to improve response to immunotherapy is to develop additional approaches to reverse immunosuppression that can be used in combination therapies.

A newly identified mechanism of immunosuppression is by stress-induced sympathetic adrenergic signaling, which suppresses many aspects of the immune system including development, differentiation, activation, and function of many types of immune cells. Adrenergic signaling has been shown to inhibit immune responses in both autoimmune diseases and infection models. Sympathetic neurons innervate both primary and secondary lymphoid organs supporting the idea that adrenergic signaling can impact immune cells locally. Recently, it has become clear that adrenergic signaling also affects tumor progression. By using mouse models of both psychosocial and physical stress stress-induced adrenergic signaling was found to be tumor-promoting in many types of cancers. The accumulated evidence demonstrates that adrenergic stress regulates tumor growth directly through multiple mechanisms, including development, proliferation, and protection of tumor cells from treatments. Although less well studied, recent findings show that adrenergic signaling also impacts tumor growth indirectly by regulating antitumor immunity. Our lab recently demonstrated that chronic mild cold stress induces SNS activation which promotes tumor growth; blocking this stress by either housing mice at thermoneutral temperature or adding β-blockers slows

tumor growth. These effects were found to be meditated by $CD8^+$ T-cells. In addition, combining a pan-β-blocker with an immune checkpoint inhibitor (anti-PD-1) significantly increased the therapeutic efficacy of anti-PD-1. Because cancer patients who were already taking β-blockers were found to have better outcomes in several retrospective studies, the preclinical studies provide a rationale for testing this combination in prospective clinical trials. Finally, accumulating evidence shows that external environmental stressors, i.e., housing temperature, creates a level of baseline adrenergic stress that affects experimental outcomes in mouse models. In the future, when designing and analyzing experiments, it will be important to factor in this potentially confounding experimental variable to better understand the biology underlying the results and to improve rigor and reproducibility.

AUTHOR CONTRIBUTIONS

GQ, MC, MB, ER, and BH developed the concepts for this review and were involved in identifying the references and writing and editing the manuscript.

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MINI REVIEW
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Hypoxic Stress-Induced Tumor and Immune Plasticity, Suppression, and Impact on Tumor Heterogeneity

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The microenvironment of a developing tumor is composed of proliferating cancer cells, blood vessels, stromal cells, infiltrating inflammatory cells, and a variety of associated tissue cells. The crosstalk between stromal cells and malignant cells within this environment crucially determines the fate of tumor progression, its hostility, and heterogeneity. It is widely accepted that hypoxic stresses occur in most solid tumors. Moreover, cancer cells found within hypoxic regions are presumed to represent the most aggressive and therapy-resistant fractions of the tumor. Here, we review evidence that hypoxia regulates cell plasticity, resistance to cell-mediated cytotoxicity, and immune suppression. Exposure to hypoxia occurs as a consequence of insufficient blood supply. Hypoxic cells activate a number of adaptive responses coordinated by various cellular pathways. Accumulating data also suggest that hypoxic stress in the tumor microenvironment promotes tumor escape mechanisms through the emergence of immune-resistant tumor variants and immune suppression. Thus, solid tumors seem to build up a hostile hypoxic microenvironment that hampers cell-mediated immunity and dampen the efficacy of the immune response.

Keywords: phenotypic plasticity, tumor microenvironment, hypoxia, antitumor immunity, myeloid-derived suppressor cell, epithelial–mesenchymal transition, NK, T cells

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INTRODUCTION

The tumor microenvironment (TME) is a complex system that contains numerous cell types playing important roles in tumor development and progression. In such system, hypoxia appears as an essential metabolic element that may help to shape cellular plasticity and tumor heterogeneity (1). Hypoxia is characterized by lack of O_2 in a setting where hypoxic tissues are inadequately oxygenated (2). Thus, in cancer, abnormal formation of the vasculature in rapidly growing tumor mass results in heterogeneously distributed areas of low oxygen pressure, generating hypoxic stress. Both non-cancerous and cancer cells adapt to the hypoxic microenvironment by regulating the hypoxia-inducible factor (HIF) family of transcription factors. HIFs are dimeric proteins composed of an O_2-sensitive α subunit (HIF-1α, HIF-2α, or HIF-3α) and a β subunit (HIF-2β). Under hypoxic stress, hypoxia-dependent stabilization of HIF dimers allows for the induction of numerous genes regulating biological processes and functions in cells, including angiogenesis, cell survival, proliferation, pH regulation, and metabolism. Accumulating evidence also points to hypoxia as an important trigger for cancer cell invasion or metastases *via* the activation of hypoxic cascades and HIF-1α. This could at least partly explain associations previously found in human tumors between hypoxic stress and

tumor progression, or other adverse effects (3). Moreover, cancer cell adaptation under hypoxia allows for their survival, maintenance of cancer stem cells (CSCs) (1), and might give rise to heterogeneity and the emergence of therapy-resistant phenotypes (**Figure 1**), with implications for chemo- and radio-resistance, as well as resistance to the immune system. The intent of this minireview is to present recent evidence suggesting that hypoxia influences cancer cell plasticity and cell phenotype that may have consequences on immune resistance and immune suppression in the TME.

HYPOXIA INDUCES RESISTANCE TO CELL-MEDIATED CYTOTOXICITY

Recent advances in immunotherapy approaches have largely improved the survival of many patients with advanced malignancies (**Figure 2**). However, the high prevalence of non-responders also reminds us that we possess only a partial understanding of the mechanisms at play in the immune resistance of tumors. Cancer immunotherapy approaches generally aim to induce a strong cytotoxic T lymphocyte (CTL) response, with the prevailing view that the generation of a sufficiently high frequency of CTL response will result in a clinically significant regression of tumor burden. Yet, even as interest for immunotherapy has grown, it becomes increasingly apparent that tumors can efficiently evade or inactivate even substantial immune responses. This is accomplished through establishing a metabolically hostile microenvironment, by denying T cells access to the tumor, and through selection of immune-resistant cancer cell variants. We previously showed that hypoxia may induce tumor resistance to CTL-induced killing through mechanisms involving increased phospho-STAT3 in target cells (4) or hypoxia-induced autophagy (5). HIF-regulated miR-210 could also be an important mediator of susceptibility to autologous CTL-mediated lysis (6). Interestingly, targeting autophagy in hypoxic melanoma B16-F10-engrafted tumors, improved the efficacy of a TRP-2-peptide cancer vaccine leading tumor regression *in vivo* (4). Previous work also showed that hypoxia could increase ADAM10 expression, with concomitant decrease surface expression of MICA leading to cancer cell resistance to cell-mediated lysis by innate immune effectors such as NK cells (7). Furthermore, activated autophagy under hypoxia has been proposed as a potent mechanism of tumor escape to NK-mediated immune surveillance (8, 9). Indeed, in hypoxic cancer cells, Granzyme B seems to be selectively degraded upon hypoxia-induced autophagy, thereby inhibiting NK-mediated cell lysis (9). The role of autophagy in regulating NK-mediated immune responses was also investigated in a clear cell renal cell carcinoma model harboring mutation in von Hippel–Lindau gene which stabilizes HIF2, and correlating with resistance to NK-mediated killing (10). We observed that inositol 1,4,5-triphosphate receptor type 1 (ITPR1), an endoplasmic reticulum Ca^{2+}-release channel involved in authophagy (11), was overexpressed in mutated cells in a HIF2-dependent manner, while protecting the cells against NK cells attacks by inducing autophagy (10).

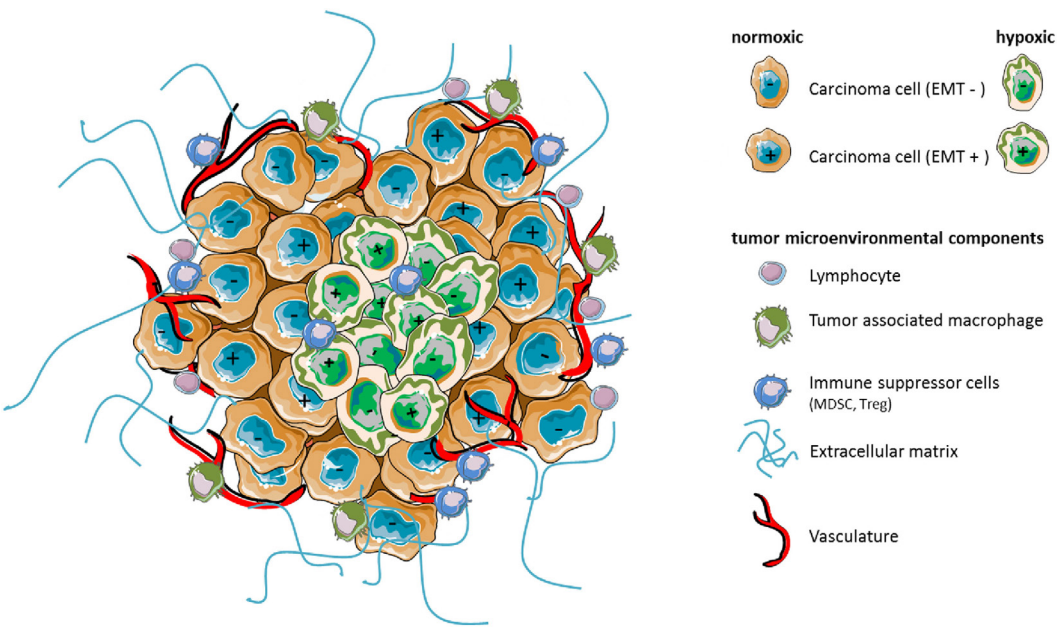

FIGURE 1 | Hypoxia as a major component of the tumor microenvironment shapes stroma reactivity and tumor heterogeneity. Tumors contain distinct cell types that collectively enable tumor growth and progression. Multiple stromal cell types, thus, create microenvironmental conditions that change as tumor is growing. In particular, hypoxia caused by insufficient concentration of oxygen can affect the phenotype (e.g., epithelial EMT− vs mesenchymal EMT+) of certain carcinoma cells leading potentially to increased heterogeneity of the cancer cell population, intrinsic resistance to antitumor immunity, or immunosuppression by affecting the functions, the phenotype, and/or the number of surrounding microenvironmental cell components.

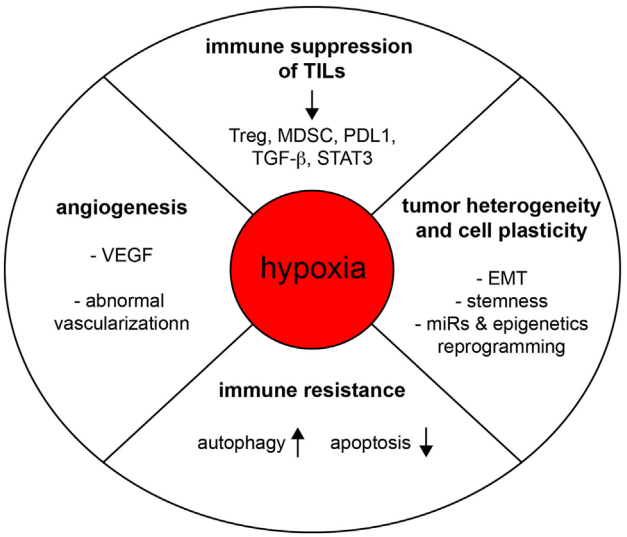

FIGURE 2 | Hypoxia subverts the function of the immune system and promotes tumorigenesis. Hypoxia regulates tumor progression through various mechanisms of action, including the promotion of angiogenesis, tumor heterogeneity, cell plasticity, immune resistance, and intratumoral immune suppression.

HYPOXIA PLAYS A KEY ROLE IN REGULATING IMMUNE SUPPRESSION

Hypoxia has been associated with potentiation of immunosuppression, *via* its role on the activation of an immune suppressive network (**Figure 2**). This has been reviewed in detail elsewhere (12, 13). The effect of hypoxia on intratumoral T CD8 T cells has not yet been fully elucidated (12). Dendritic cells differentiation and maturation seem to be inhibited under hypoxia, distracting them from their T-cell activating functions (14). In a setting where immunosuppression mediated by regulatory T cell (Treg) could represent one relevant mechanism for tumor immune evasion, work by Facciabene and colleagues nicely showed that tumor hypoxia promotes the recruitment of Tregs through inducing chemokine CC-chemokine ligand 28 (CCL28) expression, which, in turn, promotes angiogenesis and tumor tolerance (15). Nonetheless, a direct role of hypoxia in regulating Treg functions deserves further investigations in order to clarify some data discrepancies (16, 17).

Recently, hypoxia-induced Nanog was investigated for a potential role in immune suppression. Nanog is a stemness-associated transcription factor, and is selectively induced in hypoxic areas of B16-F10 tumors (18). Targeting of Nanog in this model system significantly reduced immunosuppressive cells (Treg cells and macrophages) while increasing $CD8^+$ T effector cells in the tumor bed in a manner that seems dependent, at least in part, on TGF-β1 production (18). Thus, Nanog appears to regulate TGF-β1 expression by directly binding the mouse TGF-β1 proximal promoter. In addition to addressing a major role of Nanog in hypoxia-driven immunosuppression, these findings also pointed out a novel mechanistic link between hypoxia-induced Nanog and regulation of TGF-β1.

Tumor-infiltrating myeloid cells, including myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), represent important components of the hypoxic TME likewise known to contribute to tumor-mediated immune escape. In addition to studies reporting a role of HIF-1 and HIF-2 in the promotion of macrophage angiogenic property (19), HIF-1α could also contribute to macrophage-mediated inhibition of T cells (13, 20). Gabrilovich et al. have elegantly shown that hypoxia *via* HIF-1α impedes the function of MDSC in the TME and redirects their differentiation toward TAMs, further providing relevant clues to how different myeloid suppressive cells may cooperate to immune suppression in the TME (21). Recently, we showed that expression of programmed death-ligand 1 (PD-L1) by tumoral MDSCs is upregulated under hypoxia resulting in increased MDSC-mediated T cell tolerance (22). In fact, HIF-1α regulates the expression of PD-L1 by binding directly to a hypoxia response element in the PD-L1 proximal promoter. Moreover, a significantly higher expression of PD-L1 was found on tumor-infiltrating MDSCs than on splenic MDSCs isolated from different tumor-bearing mice. Of importance, PD-L1 can also be induced in cancer cells exposed to hypoxia (22, 23).

HYPOXIA, TUMOR PLASTICITY, AND HETEROGENEITY

The underlying biology of tumor heterogeneity has remained a conundrum for scientists and clinicians alike (**Figure 2**). Yet, there is now a consensus among researchers in support of the idea that some cancer cells have the capacity to transit between epithelial and mesenchymal phenotypes, or states, *via* epithelial–mesenchymal transition (EMT) or the reverse process, mesenchymal–epithelial transition (MET) (24). Since the cells may switch back and forth along the EMT spectrum, these reversible cell state transitions also reflect the so-called plasticity of cancer cells, and as such may represent an important source of phenotypic heterogeneity in the tumor. Cell plasticity seems to be tightly regulated by contextual signals from the TME, vasculature, and anatomic sites. For instance, EMT of carcinoma cells may be induced by the local microenvironment and hypoxia (25). TGF-β, which can be induced and activated under hypoxic conditions might exert important functions in this setting. HIFs and HIF-regulated genes have been associated with marked CSC properties in various cellular contexts including Glioma (26), hematological malignancies (27), breast cancer cells (28), or in circulating tumor cells (CTCs) derived from breast carcinoma MDA-MB-231 engrafted tumors (29). It is noteworthy to add that cancer cells are exposed to chronic or intermittent hypoxic stresses, and depending their location, to various hypoxia levels. This should also contribute to increasing the intratumoral heterogeneity in space and time. In addition, there are also epigenetics determinants involved in cell plasticity (24). Recently, the possibility has emerged that certain cancer cells and cancer clones might use cell plasticity and phenotypic switching to escape immune attacks, and various therapeutic intervention,

including immunotherapies (30–32). Using a model of lung adenocarcinoma cells, we have recently proposed that the transition toward a more mesenchymal phenotype under hypoxia may occur only in a fraction of hypoxia-exposed cancer cells (33), and that carcinoma cells with a more mesenchymal state were functionally more resistant to CTL- and NK-cell-mediated lysis compared to their more epithelial counterparts, which may lead to the emergence of immunoresistant variants (33). In this line, Ricciardi and colleagues observed that exposure to inflammatory cytokines can endow cancer cells undergoing EMT with a number of immunomodulatory effects, including interference with proliferation, differentiation, and apoptosis of NK, T, and B cell populations (34). Hypoxia-induced EMT could also promote an immunosuppressive TME as shown by Yu et al. who reported that hypoxia-induced EMT in hepatocellular carcinoma cells promotes an immunosuppressive TME by increasing expression of CCL20, which acts on monocyte-derived macrophages, eventually promoting metastasis (35). Another intriguing observation is that HIF-1 can stimulate CD47 expression, an important factor for maintaining CSCs, that likewise enables breast cancer cells to avoid phagocytosis by macrophages (36). Future investigations should address molecular paradigms linking hypoxia, cell plasticity, and CD47-mediated resistance to phagocytosis. Moreover, in syngeneic immunocompetent mouse tumor models, CD47 was found to promote evasion from T-cell responses (37).

CONCLUSION

Despite the recent success in the field of immunotherapy, it has become clear that the induction of a good T-cell response is not efficient to control tumor progression and that simply avoiding immune suppression does not necessarily result in the induction of an effective antitumor immune response. Converging evidence suggests that tumor cell plasticity may lead to the emergence of immunoresistant variants. Therefore, we argue that targeting carcinoma cell plasticity should be a new strategy to better control disease progression. Clearly at present, tumor cell plasticity appears to be one of the major obstacles for the cure of malignancies as it makes tumor cells highly adaptable to microenvironmental changes, enables their phenotype switching among different forms, and favors the generation of pro-metastatic cancer cell subsets. All of the events described can be triggered by hypoxic stress. Unlike genetic alterations, which directly modulate tumor cell function, hypoxia influences both tumor and stromal cells, shaping stromal reactivity and tumor vessels (although not discussed in detail here), while interfering with host immunity. More research is now needed to gain knowledge about the crosstalks at play between these components. Clearly, if the immune system plays the music, we believe that the microenvironmental hypoxic stress plays the tune. Therefore, targeting of the microenvironmental components to attenuate its hostility should provide new opportunities to adapt treatments for each individual, develop new combinatorial therapeutic strategies, while improving treatment efficacy. Given the potential of hypoxia to inhibit tumor promoting pathways in both stromal and malignant cells, the development of new agents inhibiting HIF signaling directly or its downstream effectors holds great promise for cancer immunotherapy with more integrative, efficient, and adaptive approaches.

AUTHOR CONTRIBUTIONS

SC directed numbers of the studies that are discussed in this Frontiers Mini Review. SB has significantly contributed to SC's research work. SB and ST prepared Figure 1 and Figure 2, respectively. SC and ST wrote the manuscript and discussed concepts.

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Dynamics of Heat Shock Protein 70 Serum Levels As a Predictor of Clinical Response in Non-Small-Cell Lung Cancer and Correlation with the Hypoxia-Related Marker Osteopontin

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Hypoxia mediates resistance to radio(chemo)therapy (RT) by stimulating the synthesis of hypoxia-related genes, such as osteopontin (OPN) and stress proteins, including the major stress-inducible heat shock protein 70 (Hsp70). Apart from its intracellular localization, Hsp70 is also present on the plasma membrane of viable tumor cells that actively release it in lipid vesicles with biophysical characteristics of exosomes. Exosomal Hsp70 contributes to radioresistance while Hsp70 derived from dying tumor cells can serve as a stimulator of immune cells. Given these opposing traits of extracellular Hsp70 and the unsatisfactory outcome of locally advanced lung tumors, we investigated the role of Hsp70 in the plasma of patients with advanced, non-metastasized non-small-cell lung cancer (NSCLC) before (T1) and 4–6 weeks after RT (T2) in relation to OPN as potential biomarkers for clinical response. Plasma levels of Hsp70 correlate with those of OPN at T1, and high OPN levels are significantly associated with a decreased overall survival (OS). Due to a therapy-induced reduction in viable tumor mass after RT Hsp70 plasma levels dropped significantly at T2 ($p = 0.016$). However, with respect to the immunostimulatory capacity of Hsp70 derived from dying tumor cells, patients with higher post-therapeutic Hsp70 levels showed a significantly better response to RT ($p = 0.034$) than those with lower levels at T2. In summary, high OPN plasma levels at T1 are indicative for poor OS, whereas elevated post-therapeutic Hsp70 plasma levels together with a drop of Hsp70 between T1 and T2, successfully predict favorable responses to RT. Monitoring the dynamics of Hsp70 in NSCLC patients before and after RT can provide additional predictive information for clinical outcome and therefore might allow a more rapid therapy adaptation.

Keywords: heat shock protein 70, osteopontin, radio(chemo)therapy, non-small-cell lung cancer, therapy response, overall survival

INTRODUCTION

Lung cancer is the second most common tumor type in the Western world that accounts for the majority of cancer-related deaths worldwide (1, 2). The lack of specific symptoms limits the possibilities to diagnose lung cancer at an early stage when radical surgery or stereotactic ablative radiotherapy can assure long-term tumor control and cure (3). As a result, most patients are diagnosed in advanced tumor stages where curative-intended treatment options are limited (4, 5). Cure of locally advanced (inoperable) NSCLC after definitive RT (4, 5), as the gold standard, have failed to improve survival significantly (6). Immune-based therapies are in the scientific focus (7) for their remarkable clinical responses that have been reported for some tumor types (8–10). Particularly the treatment with immune checkpoint inhibitors such as nivolumab (11–16) caused a paradigm shift in the therapy of NSCLC. Despite promising remission rates, overall survival (OS) still remains dismal at only 10–20% in almost all patients. This emphasizes the medical need for integration of immune-oncologic approaches into other treatment concepts that are based on an improved patient stratification (17, 18).

Evidence is accumulating that apart from direct cytotoxic effects, RT can elicit systemic antitumor immune responses (19, 20) by modulating the tumor and its microenvironment (21–24). However, the patient's individual immune competence and immune escape mechanisms often hamper radio(chemo) therapy (RT)-induced abscopal effects (25). These findings further accentuate the necessity of a pretreatment patient stratification (26, 27), and a continuous monitoring of immune responses during therapy (28). Presently, circulating proteins, exosomes, microRNAs and immune cell subpopulations are discussed as potential prognostic and predictive markers (29–33). We studied the dynamics of extracellular heat shock protein 70 (Hsp70) levels and correlated it with osteopontin (OPN) plasma levels at diagnosis (T1) as predictors for outcome. Elevated levels of these biomarkers are generally associated with an aggressive tumor phenotype (34, 35). OPN is reported to be associated with reduced intratumoral pO_2 levels, which is prognostic for NSCLC patients (36–38). In a randomized double-blinded trial, elevated OPN plasma levels were able to identify patients with head and neck cancer who showed clinical benefit from a hypoxia sensitizer after RT (39). For NSCLC patients, an additive prognostic value after radical RT could be determined for the co-detection of hypoxia- and angiogenesis-related markers OPN, vascular endothelial growth factor (VEGF), and carbonic anhydrase IX (CAIX) (30). Furthermore, the serial detection of circulating OPN plasma levels provided additional prognostic information for NSCLC patients in stage III with respect to the risk to relapse (31).

Heat shock protein 70 fulfils different tasks, depending on its associated partners and its sub- or extracellular localization. Vaccination with Hsp70-peptide complexes isolated from tumor cells can elicit $CD8^+$ T cell specific antitumor immune responses (40, 41), membrane-bound Hsp70 serves as a tumor-specific target for Hsp70-activated natural killer (NK) cells (42–45), and circulating Hsp70 can act as a biomarker for monitoring outcome in patients with head and neck cancer (46). Extracellular Hsp70 can originate from two major sources, exosomal Hsp70 which is actively released by viable, membrane Hsp70-positive tumor cells and free Hsp70 which most likely originates from dying cells (47, 48). By using lipHsp70 enzyme-linked immunosorbent assay (ELISA), it is possible to detect both forms of Hsp70 quantitatively in serum and plasma (49). Recent data of our group demonstrated a significant correlation of Hsp70 levels and vital tumor mass, but also provided evidence that free extracellular Hsp70 in a pro-inflammatory cytokine milieu can activate innate immunity in NSCLC patients (50).

In this prospective clinical trial, we evaluated the predictive quality of circulating pre- and post-therapeutic Hsp70 and OPN levels at T1 in patients with non-metastasized advanced NSCLC.

PATIENTS AND METHODS

Patients and Treatment

A total of 44 patients with advanced NSCLC (M0) were prospectively recruited into a clinical study at the hospital of Martin Luther University Halle-Wittenberg. The inclusion criteria were (i) age $\geq$ 18 years, (ii) histologically confirmed, nonoperable NSCLC, (iii) no prior treatment, and (iv) indication for RT, as determined by the interdisciplinary tumor board. The Ethics Committee of the Medical Faculty of the Martin Luther University Halle-Wittenberg approved the study protocol. Written informed consent was obtained from all patients before start of the study. All procedures were in accordance with the Helsinki Declaration of 1975 (as revised in 2008). Staging was based on the TNM classification of malignant tumors (7th edition) and treatment was carried out at the Department of Radiation Oncology of the Medical Faculty of the Martin Luther University Halle-Wittenberg. Depending on the World Health Organization performance status and comorbidities, treatment consisted of a three-dimensional conformal, normofractionated (5 Fx/week) definite RT (single dose 2 Gy, total dose 66 Gy, Siemens Primus, Germany) $\pm$ double-agent based chemotherapy (cisplatin 20 mg/m^2 body surface, day 1–5 and 29–33; vinorelbine 25 mg/m^2 body surface, used on day 1 and 29) in treatment week one and five (2 courses). RT was computed tomography-based (GE Healthcare) and all patients received a FDG positron emission tomography-scan prior to RT which was used for target volume delineation (Oncentra Masterplan External Beam software, Nucletron, Elekta, USA). The first follow-up of the patients was performed 4–6 weeks after end of RT to evaluate their post-radiotherapeutic response at the Department of Radiation Oncology, University Hospital Halle-Wittenberg. Thereafter,

Abbreviations: AUC, area under the curve; CAIX, carbonic anhydrase IX; CD, cluster of differentiation; CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; Hsp70, heat shock protein 70; HIF1α, hypoxia-inducible factor 1α; IL-2, interleukin 2; NK cells, natural killer cells; NSCLC, non-small-cell lung cancer; OPN, osteopontin, PAI-1, plasminogen activator inhibitor type 1; PBS, phosphate buffered saline; PET, positron emission tomography; RT, radio(chemo) therapy; ROC curve, receiver operating characteristic; SCC, squamous cell carcinoma; uPAR, urokinase type plasminogen activator receptor; WHO, World Health Organization; VEGF, vascular endothelial growth factor.

patients were followed up regularly every 3 months for a period of 5 years according to RT guidelines. The mean follow-up period in patients alive was 34 (22–48) months. The survival status of patients was continuously monitored in cooperation with local citizen registration offices.

A positive therapy response was defined as complete remission, implying a disappearance of all target lesions, or partial remission which commonly signifies a decrease of at least 30% in the lesion with the largest diameter. A negative therapy response is defined by stable or progressive disease.

Plasma Samples

Blood samples of NSCLC patients were collected by peripheral venous puncture before start of RT (T1) and 4–6 weeks after the end of RT (T2). Briefly, blood was collected in two S-Monovette EDTA KA/9 ml tubes (Sarstedt, Nümbrecht, Germany). Blood was anti-coagulated and centrifuged at 4°C for 10 min with 4,000 rpm. Aliquots of 150–300 µl were prepared and directly stored at −80°C for further analysis.

Detection of Hsp70 and OPN

Heat shock protein 70 concentrations were determined using the lipHsp70 ELISA, which is capable to detect both, lipid-bound and free Hsp70 in serum and plasma. The use of cmHsp70.1 as detection antibody (49, 51) allows quantitative analysis of total content of circulating Hsp70 in the blood. 96-well MaxiSorp Nunc-Immuno plates (Thermo, Rochester, NY, USA) were coated overnight with 2 µg/ml rabbit polyclonal antibody (Davids, Biotechnologie, Regensburg, Germany) directed against human Hsp70 in sodium carbonate buffer (0.1 M sodium carbonate, 0.1 M sodium hydrogen carbonate, pH 9.6). After three washing steps with phosphate buffered saline (PBS, Life Technologies, Carlsbad, CA, USA) with 0.05% Tween 20 (Calbiochem, Merck, Darmstadt, Germany), wells were blocked with 2% milk powder (Carl Roth, Karlsruhe, Germany) in PBS for 1.5 h at 27°C. After another washing step, samples diluted 1:5 in CrossDown Buffer (Applichem, Chicago, IL, USA) were added to the wells for 2 h at 27°C. After another washing, wells were incubated with 4 µg/ml of the biotinylated mouse-anti-human monoclonal antibody cmHsp70.1 (multimmune, Munich, Germany) in 2% milk powder in PBS for 2 h at 27°C. After a last washing step, 0.2 µg/ml horseradish peroxidase-conjugated streptavidin (Pierce, Thermo, Rockford, IL, USA) in 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was added for 1 h at 27°C. Binding was quantified by adding substrate reagent (R&D Systems, Minneapolis, MN, USA) for 30 min at 27°C and absorbance was read at 450 nm, corrected by absorbance at 570 nm, in a Microplate Reader (BioTek, Winooski, VT, USA). Each sample was measured in duplicates in three independent experiments. An eight-point standard curve using recombinant Hsp70 diluted in CrossDown Buffer at concentrations ranging from 0 to 50 ng/ml, as well as reference plasma samples were used as internal controls for each individual assay.

For OPN, the "Human Osteopontin Assay" ELISA (IBL Ltd., Japan) was performed and optical density was measured blinded and in duplicate according to manufacturer's instructions. To determine the OPN concentration, the standard curve supplied by the kit was used and OPN plasma concentration is reported in ng/ml (±1 SD). None of the two markers shows an age- and/or gender-related association.

Statistical Analysis

All statistical analyses were performed using the SPSS PASW software package for windows (SPSS Inc., USA, version 19.0) and statistical significance was accepted with two-sided p-values ($p < 0.05$). Median Hsp70 plasma levels were used as cutoff values.

Non-parametric tests (Mann–Whitney U test, Kruskal–Wallis H test) were used to determine statistically significant differences in patient subgroups with low and high Hsp70 concentration with and without response. Differences in Hsp70 levels in patients with and without therapy response were investigated using Pearson's chi-squared test. Coherences between Hsp70 and OPN as a hypoxia-related marker were evaluated using Pearson's rank correlation coefficient and paired samples test assessed potential differences in plasma Hsp70 levels before and after RT. Survival analysis was performed using the Kaplan–Meier product limit method with the log-rank test. The survival status of the patients was monitored and determined with the help of local citizen registration offices. Overall survival (OS) was calculated from start of radiotherapy until death or last seen in follow-up.

Therapy response was the primary endpoint, classified in responding (complete or partial remission after RT) vs. non-responding patients (progressive or stable disease after RT). For univariate and multivariate analysis, the Cox proportional hazard regression model was used to calculate the relative risk and hazard ratio and its 95% confidence interval (CI). Receiver operating characteristic (ROC) curves illustrate the performance of Hsp70 plasma levels as a binary classifier system in the prediction of therapy response after RT.

RESULTS

Pre-Therapeutic (T1) OPN Levels Correlate with Hsp70 Plasma Levels in Patients with NSCLC

A total of 44 NSCLC patients (6 females, 38 males) with NSCLC (M0) were enrolled into the study for T1. The clinico-pathological characteristics of all patients ($n = 44$) are summarized in **Table 1** and that of non-responding and responding patients is shown in **Table 2**. With respect to tumor volume ($p = 0.086$), age ($p = 0.114$), gender ($p = 0.306$), histology ($p = 0.158$), and UICC

TABLE 1 | Clinico-pathological characteristics of all non-small-cell lung cancer patients ($n = 44$) at M0 (Martin Luther University Hospital, Halle-Wittenberg).

		Counts	
Gender	Female	6	14%
	Male	38	86%
Histological type	Squamous cell carcinoma	23	52%
	Adeno ca	19	43%
	Other	2	5%
UICC stage	I–II	2	5%
	IIIa	16	36%
	IIIb	26	59%

stage ($p = 0.175$), no statistically significant differences have been determined in non-responding and responding patients.

Plasma levels of OPN and Hsp70 were determined pre-therapeutically (T1, before RT) in all patients (**Table 3**). As already shown for a larger patient cohort (30), also in a subgroup of non-metastasized NSCLC patients, high pre-therapeutic OPN plasma levels (above median, $n = 22$) significantly correlated with inferior OS compared to low (below median, $n = 22$) levels (13 [5–66] vs. 23 [5–61]; $p < 0.05$). Both biomarkers, OPN and Hsp70, revealed a positive correlation according to the Pearson's correlation coefficient ($r = 0.422$, $p = 0.005$) for T1. According to the Mann–Whitney *U* test (**Figure 1**), patients whose median Hsp70 values were above 9.30 ng/ml showed significantly higher OPN values, compared to those with median Hsp70 values below 9.30 ng/ml ($n = 43$, $p = 0.021$). A direct comparison of the OPN and Hsp70 values also revealed a correlation ($r = 0.42$), as shown in the Supplementary material (Figure S1 in Supplementary Material).

Hsp70 Plasma Levels Drop after RT in Patients with NSCLC

To investigate the impact of RT on Hsp70 plasma levels, pre- (T1) and post- (T2) therapeutic Hsp70 plasma levels were compared in 26 patients from whom plasma samples were available at both time-points. As summarized in **Table 4**, median Hsp70 levels dropped significantly from 10.35 before RT to 6.05 ng/ml after RT (paired samples test, $p = 0.016$). According to the Pearson's correlation coefficient, a significant positive correlation was determined ($r = 0.659$, $p < 0.0001$).

The drop in circulating Hsp70 after RT was also detected by mean Hsp70 levels (T1 vs. T2: 14.94 vs. 9.02 ng/ml). However, compared to a cohort of 114 healthy donors (7.8 ng/ml) which was published previously (49), mean Hsp70 values in NSCLC patients remained to be significantly upregulated before (T1) and after (T2) RT ($p < 0.05$).

High Post-Therapeutic Hsp70 Plasma Levels Predict Clinical Response to RT

To address the question whether Hsp70 might be predictive for clinical response, pre- (T1) and post- (T2) therapeutic Hsp70 plasma levels were associated with response to RT. As expected, patients who responded to therapy showed a significantly improved OS compared to non-responding patients (23 vs. 9 months, $p = 0.026$,

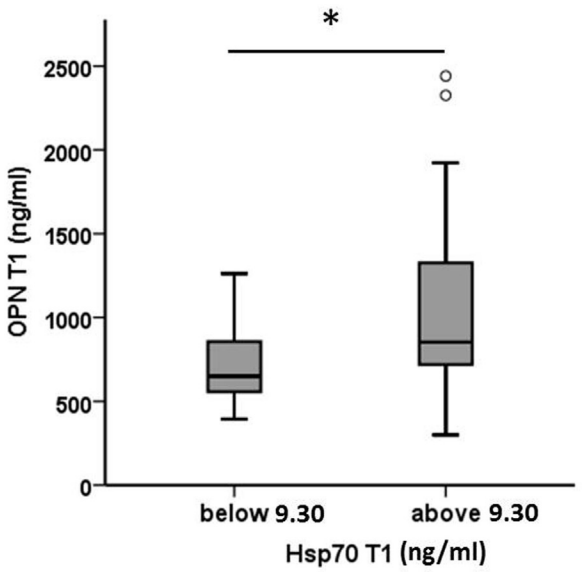

FIGURE 1 | Comparison of pre-therapeutic (T1) osteopontin (OPN) levels in non-small-cell lung cancer patients (M0) with high and low median heat shock protein 70 (Hsp70) plasma levels. According to the median Hsp70 plasma level of 9.30 ng/ml the patient cohort ($n = 43$) was divided into two subgroups with median Hsp70 plasma levels below or above 9.30 ng/ml; Mann–Whitney *U* test, $p = 0.021$.

TABLE 2 | Clinico-pathological characteristics of non-responding and responding non-small-cell lung cancer patients at M0 (Martin Luther University Hospital, Halle-Wittenberg).

		Non-responder		Responder	
		Counts		Counts	
Gender	Female	0		6	17.6%
	Male	10	100%	28	82.4%
Histological type	Squamous cell carcinoma	7	70%	16	47%
	Adeno ca	2	20%	17	50%
	Other	1	10%	1	3%
UICC stage	I–II	1	10%	1	2.9%
	IIIa	4	40%	12	35.3%
	IIIb	5	50%	21	61.8%

TABLE 3 | Comparison of pre-therapeutic (T1) osteopontin (OPN) and heat shock protein 70 (Hsp70) plasma levels in non-small-cell lung cancer patients (M0; $n = 44$) in relation to overall survival.

	OPN T1 (ng/ml)	Hsp70 T1 (ng/ml)
N (missing)	44 (0)	43 (1)
Mean	872.14	12.13
SEM	71.63	2.02
Median	752.45	9.30
SD	475.11	13.26
Maximum	2441.00	67.50
Minimum	299.30	0.20
Paired samples test (overall survival)	$p < 0.05$	

TABLE 4 | Comparison of heat shock protein 70 (Hsp70) plasma levels before (T1) and 4–6 weeks after (T2) radio(chemo)therapy in non-small-cell lung cancer patients (M0).

	Hsp70 T1 (ng/ml)	Hsp70 T2 (ng/ml)
N	26	26
Mean	14.94	9.02
SEM	3.02	1.81
Median	10.35	6.05
SD	15.41	9.24
Maximum	67.50	46.20
Minimum	0.20	0.80
Paired samples test	$p = 0.016$	

log-rank Mantel Cox) who had an increased risk of death ($r = 2.11$, CI [0.94–4.57], $p = 0.58$).

A comparison of Hsp70 plasma levels before (T1) and 4–6 weeks after RT (T2) in non-responding ($n = 7$, 9.76 vs. 4.03 ng/ml) and responding patients ($n = 19$, 16.85 vs. 10.87 ng/ml) demonstrated that in both patient subgroups, mean and median Hsp70 plasma levels declined after RT (**Figure 2**). In general, responding patients had significantly higher (mean/median) Hsp70 plasma levels compared to non-responding patients at T1 and T2 (**Figure 2**).

As depicted in **Figure 3**, all patients who responded to therapy had significantly higher median Hsp70 plasma levels at T2 (median 8.6 ng/ml, range 0.8–46.2) after RT compared to those who showed no response (median 2.8, range 1.5–12.2) 4–6 weeks after therapy (T2) (Mann–Whitney *U* test, $p = 0.013$). The median Hsp70 values, revealed similar results with respect to both time-points in responding (11.10 ng/ml at T1 vs. 8.60 ng/ml at T2) and non-responding (5.30 ng/ml at T1 vs. 2.80 ng/ml at T2) patients (paired samples test $p = 0.034$).

In line with these findings, a subdivision of the patient cohort into subgroups with median Hsp70 plasma levels above and below 5.0 ng/ml at T2 revealed that patients with Hsp70 plasma levels above the threshold had significantly higher response rates than those below the threshold (92.9 vs. 50%, Pearson Chi-Square $p = 0.02$) (**Table 5**). Based on these findings, plasma Hsp70 levels were analyzed for their potential to predict therapy response after RT. The related ROC curve analysis (**Figure 4**) showed a significant predictive function ($p = 0.014$) of plasma Hsp70 levels for therapy response with an area under the curve (AUC) of 0.82. The optimal cutoff value which determines a positive therapy response is a value of ≤4.35 ng/ml with a sensitivity of 0.895 and a false positive rate of 0.143. Plasma Hsp70 levels which were taken before start of therapy (T1) showed a similar trend, but failed statistical significance.

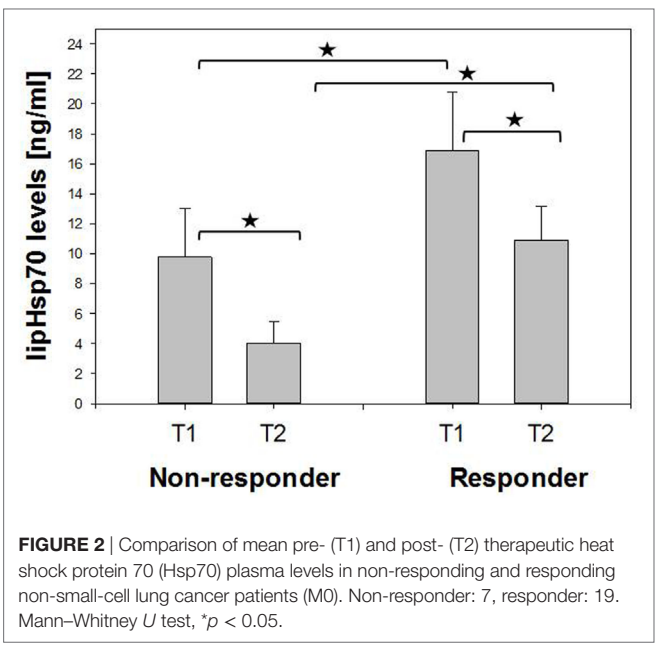

FIGURE 2 | Comparison of mean pre- (T1) and post- (T2) therapeutic heat shock protein 70 (Hsp70) plasma levels in non-responding and responding non-small-cell lung cancer patients (M0). Non-responder: 7, responder: 19. Mann–Whitney *U* test, *$p < 0.05$.

DISCUSSION

Post-Therapeutic Hsp70 Plasma Levels As a Biomarker for Therapy Response

The patient's endogenous immune defense is able to attack tumor cells. However, tumors as well as its microenvironment have developed mechanisms that allow immune escape against tumor cells (52–55). Therefore, novel therapeutic approaches aim to reactivate the patient's immune defense by inhibiting tumor-induced immune checkpoints that impair immune responses against malignantly transformed cells (56, 57). Another strategy to reinforce the patient's immune system is based on the activation of immune effector cells against tumor-specific targets that are overexpressed in tumor cells, presented on the cell surface and released in a tumor-selective manner (58). Tumor cells of different types including NSCLC (34) frequently overexpress Hsp70, present it on their plasma membrane, and actively release

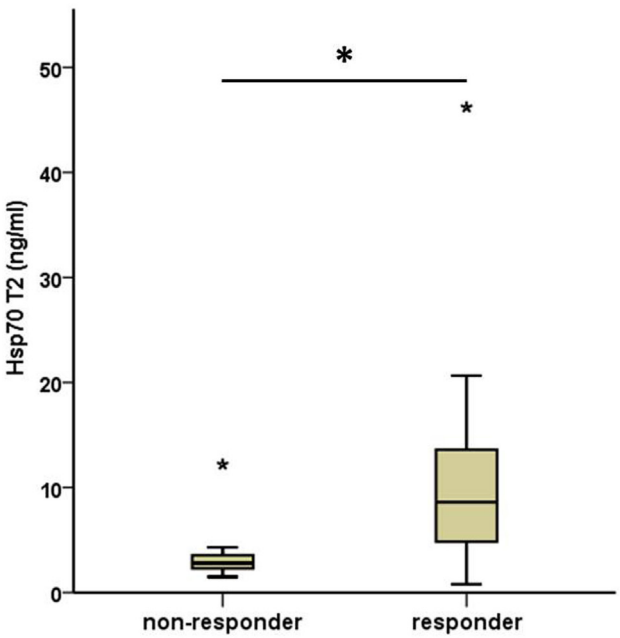

FIGURE 3 | Comparison of median post- (T2) therapeutic heat shock protein 70 (Hsp70) plasma levels in non-responding and responding non-small-cell lung cancer patients (M0). Non-responder: 7, responder: 19. Mann–Whitney *U* test, asterisks above the box plots indicate outliers; *$p = 0.013$.

TABLE 5 | Comparison of post- (T2) therapeutic heat shock protein 70 (Hsp70) plasma levels in non-responding and responding non-small-cell lung cancer patients (M0).

Therapy response (T2)	Median Hsp70 above 5 ng/ml	Median Hsp70 below 5 ng/ml
N	14	12
Non-responder	1 (7%)	6 (50%)
Responder	13 (93%)	6 (50%)
Pearson chi-square	$p = 0.02$	

Patients were divided into two subgroups with high and low median Hsp70 levels.

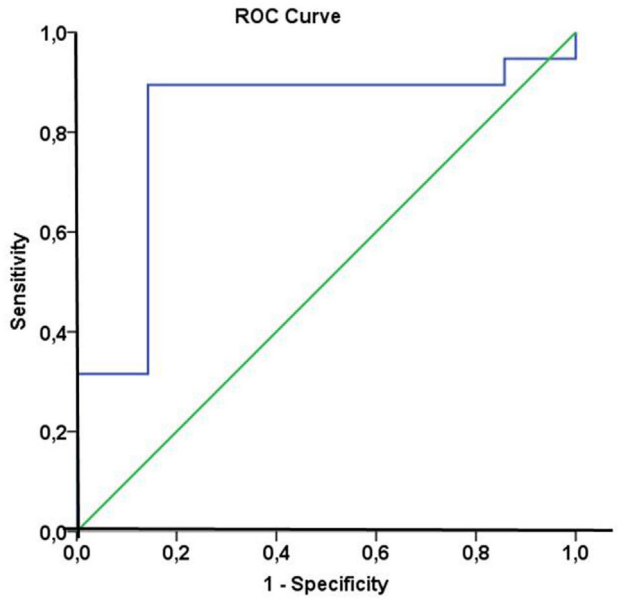

FIGURE 4 | Receiver operating characteristic (ROC) curve analysis of heat shock protein 70 (Hsp70) plasma levels of non-small-cell lung cancer (NSCLC) patients (M0) to predict therapy response. Area under the curve = 0.82, $p = 0.014$. The optimal cutoff value for distinction of responders and non-responders was an Hsp70 plasma level of 4.35 ng/ml with a sensitivity of 0.895 and a false positive rate of 0.143.

it in tumor exosomes (46, 58–60). However, dying tumor cells also release Hsp70, most likely as a free protein (47, 48). High intracellular Hsp70 levels have been found to interfere with apoptotic pathways and thereby protect tumor cells from programmed cell death following stress (61). Hence, we assume that therapy-resistant tumor cells with high cytosolic Hsp70 levels can better compensate RT-induced damage and thus mediate tumor cell survival.

Murakami et al. showed that in addition to cytosolic also membrane-bound Hsp70 supports protection of tumor cells against RT-induced cell death (62), although the expression density of cytosolic and membrane-bound Hsp70 are not associated. Therefore, membrane Hsp70 fulfils dual roles, on the one hand, it acts as a tumor-specific target for Hsp70-activated NK cells (43, 44, 50, 63), on the other hand it mediates therapy resistance. An ongoing phase II clinical trial using *ex vivo* Hsp70-activated NK cells for the treatment of patients with NSCLC after RT is presently testing whether the immunostimulatory capacity of NK cells can overrule therapy resistance of membrane Hsp70-positive NSCLC (44).

In the present trial, we investigated the role of circulating Hsp70 as a prognostic marker to predict outcome of RT in patients with NSCLC (M0) at different time-points. A comparison of pre- and post-therapeutic plasma levels revealed significantly elevated Hsp70 levels in responding compared to non-responding patients.

The lipHsp70 ELISA (52) detects both, lipid-bound and free Hsp70. We hypothesize that high Hsp70 levels at diagnosis predominantly originate from exosomal Hsp70 released by viable tumor cells. This is in line with our finding that Hsp70 plasma levels before start of therapy reflect vital gross tumor volume (50). In contrast, elevated post-therapeutic Hsp70 plasma levels rather originate from dying tumor cells (47, 48) that might be able to stimulate the immune system. Analysis of the concentration of cytosolic proteins in the exosomal versus non-exosomal plasma fraction after ultracentrifugation of responding non-metastasized NSCLC patients in stage IIIa/b ($N = 4$) before (T1) and after RT (T2) showed a significant ($p < 0.05$) protein drop in the exosomal fraction and an increase in the non-exosomal fraction after therapy (data not shown) which reflects the reduction in viable tumor mass after therapy. In summary, pre- and post-treatment Hsp70 levels are indicative for different tumor characteristics such as vital tumor mass, intrinsic tumor aggressiveness, and RT-induced tumor cell death that can cause immunostimulation.

Previous work of our group demonstrated that membrane Hsp70 serves as a target for NK cells that have been pre-stimulated with an Hsp70-peptide plus low-dose interleukin 2 (42). The stimulation of NK cells is associated with an upregulated expression of activatory NK receptors, including the C-type lectin receptor CD94/NKG2C (64) that in turn induces the production of the pro-apoptotic enzyme granzyme B (63). With respect to these findings, we speculate that high post-therapeutic Hsp70 plasma levels derived from dying tumor cells in a pro-inflammotory environment after RT might be able to stimulate Hsp70-reactive NK cells that mediate favorable therapeutic outcome. Elevated pre-therapeutic exosomal Hsp70 plasma levels might be predictive for a superior outcome because they reflect an aggressive, but yet immunogenic tumor type. This is in accordance with the finding that Hsp70-bearing exosomes isolated from membrane Hsp70-positive tumor cells, but not from their Hsp70-negative counterparts, can induce the migratory and cytolytic activity of NK cells (65).

The high predictive value of post-therapeutic Hsp70 plasma levels for the response of an individual patient could be demonstrated by ROC analysis with an AUC of 0.82 ($p = 0.014$). Identical analyses have been performed for Hsp70 plasma levels before start of therapy. Although a similar trend was observed, pre-therapeutic levels failed to show statistical significance as a biomarker for clinical response. This might be explained by the fact that pre-therapeutic Hsp70 plasma levels predominantly originate from viable tumor cells and thus represent vital tumor mass rather than therapy response.

To obtain a better view on the exact dynamics and prognostic relevance of circulating Hsp70 levels, further pre- and post-therapeutic measurements of Hsp70 at different time-points have to be correlated with clinical response in larger patient cohorts.

Role of Hsp70 in the Context of Hypoxia-Related Markers of the Tumor Microenvironment

Another parameter that co-determines OS of patients after RT is the tumor microenvironment. The presence of hypoxic stress impacts prognosis and therapy response to RT adversely. The molecular effects induced by RT involve the production of

DNA radicals which are normally fixed by oxygen. Hence, DNA damage decreased under hypoxic stress and hypoxia-inducible factor 1α (HIF1α) is stabilized which in turn leads to promotion of tumor cell survival. HIF1α also has been shown to impair membrane Hsp70 expression on tumor cells, and therefore might negatively affect NK cell recognition (66).

Tumor hypoxia is also associated with an overexpression of OPN. Physiologically, OPN is involved in the process of bone remodeling (67); however, many tumor cells show an overexpression of this protein (68) as an aggression marker. Plasma levels of OPN correlate with tumor hypoxia in NSCLC (38), and previously we demonstrated that high OPN levels before RT and increasing OPN levels after RT translate into poor OS in NSCLC after radical RT (30, 31). With respect to the prediction of therapy response, OPN as a single marker failed to show significance. Only in combination with VEGF and CAIX, the prognostic impact of OPN could be augmented (30). Hypoxic stress also has been shown to increase the release of exosomes (69) that contain large amounts Hsp70. Therefore, the present study evaluated the prognostic and predictive value of Hsp70 levels in relation to OPN. Due to a positive correlation of Hsp70 and OPN plasma levels at diagnosis, the association of OPN and OS was re-evaluated in the subgroup of non-metastasized NSCLC patients ($n = 44$) that was also analyzed for Hsp70. In line with previous results of a larger, more heterogenous NSCLC patient cohort (31), non-metastastized NSCLC patients also revealed a significant correlation of high OPN values at T1 with a decreased OS.

CONCLUSION

Our findings illustrate the differential prognostic and predictive relevance of pre- and post-treatment Hsp70 levels in NSCLC patients after RT. Being actively released by viable tumor cells in exosomes, high pre-therapeutic Hsp70 levels (T1) are most likely be indicative for viable tumor mass. Therapy response that was associated with a reduction in tumor size results in a significant drop in exosomal Hsp70 plasma levels from 21.7 ± 2.8 to 15.6 ± 1.3 ng/ml ($p < 0.05$, data not shown), as determined by lipHsp70 ELISA in 4 responding patients who were not included into the trial. However, with respect to the immunostimulatory capacity of Hsp70 derived from dying tumor cells, elevated post-therapeutic Hsp70 levels also can predict beneficial outcome to RT. Although elevated OPN levels significantly correlate with decreased OS, reduced lung function, and weight loss (30), OPN as a single parameter is unable to predict therapy response (30, 31). Therefore, the co-detection of both biomarkers before and/or after RT integrates prognostic (OPN) and predictive (Hsp70) information for therapy response that allows a more rapid therapy adaptation to improve clinical outcome of NSCLC patients.

ETHICS STATEMENT

The Ethics Committee of the Medical Faculty of the Martin Luther University Halle-Wittenberg approved the study protocol. Written informed consent was obtained from all patients before start of the study. All procedures were in accordance with the Helsinki Declaration of 1975 (as revised in 2008).

AUTHOR CONTRIBUTIONS

CO and SG equally contributed to the study; CO, SG, and MB conceived and designed the experiments, analyzed data, and wrote the paper; DV gave clinical advice; and GM did proofreading, designed and revised the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01305/full#supplementary-material.

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Beneficial Effect of Fluoxetine and Sertraline on Chronic Stress-Induced Tumor Growth and Cell Dissemination in a Mouse Model of Lymphoma: Crucial Role of Antitumor Immunity

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Clinical data and experimental studies have suggested a relationship between psychosocial factors and cancer prognosis. Both, stress effects on the immune system and on tumor biology were analyzed independently. However, there are few studies regarding the stress influence on the interplay between the immune system and tumor biology. Moreover, antidepressants have been used in patients with cancer to alleviate mood disorders. Nevertheless, there is contradictory evidence about their action on cancer prognosis. In this context, we investigated the effect of chronic stress on tumor progression taking into account both its influence on the immune system and on tumor biology. Furthermore, we analyzed the action of selective serotonin reuptake inhibitors, fluoxetine and sertraline, in these effects. For this purpose, C57BL/6J mice submitted or not to a chronic stress model and treated or not with fluoxetine or sertraline were subcutaneously inoculated with EL4 cells to develop solid tumors. Our results indicated that chronic stress leads to an increase in both tumor growth and tumor cell dissemination. The analysis of cell cycle regulatory proteins showed that stress induced an increase in the mRNA levels of cyclins A2, D1, and D3 and a decrease in mRNA levels of cell cycle inhibitors p15, p16, p21, p27, stimulating cell cycle progression. Moreover, an augment of mRNA levels of metalloproteases (MMP-2 and MMP-9), a decrease of inhibitors of metalloproteases mRNA levels (TIMP 1, 2, and 3), and an increase in migration ability were found in tumors from stressed animals. In addition, a significant decrease of antitumor immune response in animals under stress was found. Adoptive lymphoid cell transfer experiments indicated that the reduced immune response in stressed animals influenced both the tumor growth and the metastatic capacity of tumor cells. Finally, we found an important beneficious effect of fluoxetine or sertraline treatment on cancer progression. Our results emphasize the crucial role of the immune system in tumor progression under stress situations. Although a direct effect of stress and drug treatment on tumor biology could not be ruled out, the beneficial effect of fluoxetine and sertraline appears to be mainly due to a restoration of antitumor immune response.

Keywords: chronic stress, antitumor immunity, fluoxetine, sertraline, tumor invasion, lymphoma

INTRODUCTION

Nowadays, stress affects thousands of people around the world. Stress is defined as a critical, real, or apparent, situation that represents a challenge for homeostasis. To restore this state, a coordinated adaptive response is triggered. Stress mediators involve not only catecholamines and glucocorticoids (the characteristic neuroendocrine hormones of the stress response) but also several other neurotransmitters, cytokines, and growth factors (1). It is important to note that although stress response is an essential survival mechanism, when it is prolonged over time, may affect endocrine, immunological, and behavioral function (1). In particular, epidemiological studies indicate that chronic stress might constitute a risk factor for cancer onset and progression (2, 3).

The role of psychosocial factors in cancer initiation is ambiguous. However, the influence of stress on cancer progression has been demonstrated. Both clinical and experimental studies have shown that the mechanisms involved in stress response are capable of influencing processes related to cancer progression (4–6). Animal models that mimic the pattern of human disease have been used to understand the impact of stress on cancer and other pathologies. These studies put the main focus on the neuroendocrine modulation of the immune response to tumor cells (2, 7–10). Moreover, the direct effect of stress mediators on the proliferation and aggressive behavior of tumor cells, independently of the influence on the immune system, has been analyzed. The group of Sood demonstrated that chronic stress increase catecholamine levels in tumors that in turn promote metastasis of breast (11) and ovarian (12) carcinomas. In addition, in many experimental models, the biological consequences of stress have been shown to be reverted by β-adrenergic blockers (11). In addition, it was proposed that norepinephrine also promotes resistance to anoikis, inhibits apoptosis, and increases chemoresistance (13) of tumor cells. Nevertheless, in general, these studies were developed in athymic nude or SCID mice and analyze the influence of stress on tumor invasion and metastasis independently of the action of immune system (14).

In addition, selective serotonin reuptake inhibitors (SSRIs), as fluoxetine and sertraline, are frequently prescribed for the treatment of stress-associated disorders, such as depression, obsessive–compulsive disorder, panic attack, and bulimia nervosa. The use of antidepressants has been related to immune alterations. Nevertheless, conflictive data have been reported regarding the impact of fluoxetine on the immune system and cancer prognosis (15). In a previous report, we showed that fluoxetine reverts the effect of stress on T helper immunity through compensatory and/or specific mechanisms (16). In addition, fluoxetine was able to enhance the apoptosis/proliferation balance of lymphoma cells and increase T cell immunity in tumor-bearing mice (17).

In this context, the objective of this study was to investigate the effect of chronic stress on tumor progression taking into account both its influence on the immune system and its action on tumor biology. Moreover, we aimed to analyze the influence of two SSRIs, fluoxetine and sertraline, in these effects. For this purpose, we used EL4 T cell lymphoma cells growing as a solid tumor in C57BL/6J mice submitted or not to a variable stress model and treated or not with fluoxetine or sertraline. Our results indicate that tumor growth and metastases are affected by psychological stress. Cellular adoptive transfer approach pointed out that changes in tumor biology were predominantly the result of the influence of stress on the immune function. In addition, treatment with the SSRIs, fluoxetine and sertraline, prevented these effects. These findings strengthen the clinical research about the beneficial effects of the SSRIs prescription in cancer patients.

MATERIALS AND METHODS

Cell Line and Culture Condition

The tumor cell line EL4 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA; Catalog Number TIB-39). Cells were cultured in RPMI 1640 with 10% fetal bovine serum (GIBCO). EL4 cell line was established from a lymphoma induced in a C57BL/6J mouse by 9,10-dimethyl-1,2-benzanthracene (18). Cells were cultured at an optimal concentration ($1–5 \times 10^5$ cells/ml) in RPMI-1640 medium supplemented with 10% v/v fetal bovine serum, 2 mmol/l glutamine, and 100 mg/ml streptomycin (all from Life Technologies), at 37°C in 5% CO_2 atmosphere, as previously described (19).

Animals

Inbred female C57BL/6J (H-2^b) mice, 2–3 months old, were bred and kept at the Instituto de Investigaciones Biomédicas (BIOMED, CONICET-UCA, Buenos Aires, Argentina). Animals were cared for and sacrificed according to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH) (revision 2011) and to the EC Directive 86/609/EEC (revision 2010). The experimental protocol was also approved by the local Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, BIOMED, Argentina).

Chronic Stress Model and SSRIs Administration Protocol

The chronic stress model used consists in the aleatory, intermittent, and unpredictable exposure to different stressors during 5 weeks in C57BL/6J mice. Briefly, animals were randomly and alternately exposed to one of the following stressors for the time indicated for each assay: restraint in well-ventilated tubes for 6 h (20), tail suspension for 5 min (21), forced swimming for 5 min (22), cold temperature exposure (4°C) for 2 h (23, 24), and 2 days of continuous overnight illumination (25).

To analyze the effect of SSRIs, mice were orally given 15 mg/kg/day of fluoxetine (Sigma-Aldrich) (26) or 20 mg/kg/day of sertraline (Sigma-Aldrich) (27), in a fresh solution prepared in the drinking water. The preparation of these solutions was performed taking into account the volume of water drunk daily by each mouse (5 ml) to reach the indicated dose.

Lymphoma Model and Tumor Growth

C57BL/6J syngeneic animals, under different treatments, received subcutaneous injections of 3×10^5 EL4 cells in 200 µl of phosphate-buffered solution (PBS) to generate a solid tumor. Tumor length and width were measured every day using calipers, and tumor volume was calculated as $V = \pi/6 \times \text{length} \times \text{width}$ (7). With the

exception of mice used for the spontaneous metastasis test, mice were euthanized by CO_2 overexposure 14 days post tumor cell injection or when tumor reached the maximum volume allowed by ethical standards (Guidelines for Endpoints in Animal Study Proposals, NIH).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

After 14 days of tumor injection, animals were sacrificed, solid tumors were dissected and instantly homogenized in Tri-Reagent (Genbiotech SRL) to isolate the RNA, following the manufacturer's instructions. The RNA pellets were re-suspended in RNase-free water, and the RNA concentration was quantified by measuring the absorbance at 260 nm in a nanodrop (Nanodrop ND-1000, Thermo Fisher Scientific Inc.). The total RNA was used as a template to generate first-strand cDNA synthesis using the M-MLV Reverse Transcriptase (Invitrogen), random primers (Invitrogen), and dNTPs (Invitrogen). The cDNA amounts present in each sample were determined by a 7500 Real-Time PCR System (Applied Biosystems) using the KAPA SYBR® FAST qPCR Kit Master Mix (2×) Universal (Kapa Biosystems) and following the manufacturer's instructions. Each RT-PCR quantification experiment was performed in duplicate. To verify that the SYBR Green dye detected only one PCR product, all the reactions were subjected to a heat dissociation protocol following the final cycle of PCR. The sequences of mouse-specific primers, the annealing temperature, and the amplicon size are provided in **Table 1**. The primer sequences (Biodynamics SRL), shown in **Table 1**, were designed using the Primer Express Software version 3.0 (Applied Biosystems). To determine the target gene mRNA expression, the comparative cycle threshold (Ct) method was used (28). An average Ct value was calculated from the duplicate reactions and normalized to the expression of β_2-microglobulin, and the $2(-\Delta\Delta Ct)$ value was calculated. It is important to note that similar results were obtained using cyclophilin or glucose-6-phosphate-dehydrogenase (G6PDH) mRNA expression levels as housekeeping (data not shown) (29).

Disaggregation of Solid Tumor

After 14 days of tumor injection, mice were sacrificed, and solid tumors were dissected. To obtain cells from solid tumors, a modification of conventional method of disaggregation by trypsinisation was used (30). Briefly, tumors were fragmented and were

TABLE 1 | Primers sequences for quantitative real-time reverse transcription polymerase chain reaction.

Gene	Accession no.	Sequences	Amplicon size (pb)	Annealing T (°C)
Cyclin A2	NM_009828.2	Fw: 5′-GGCCAGCTGAGCTTAAAGAAAC-3′ Rv: 5′-CGGGTAAAGAGACAGCTGCAT-3′	69	61
Cyclin D1	NM_007631.2	Fw: 5′-CCAAAACCATTCCATTTCAAAG-3′ Rv: 5′-CCAACACACACCAGCAACACT-3′	68	61
Cyclin D3	NM_007632.2	Fw: 5′-TGCGTGCAAAAGGAGATCAA-3′ Rv: 5′-TCACACACCTCCAGCATCCA-3′	68	60
p15/INK4B	NM_007670.4	Fw: 5′-TGGGAAACCTGGAGAGTAGATGA-3′ Rv: 5′-GAATCCCCACACATGACAGTACA-3′	66	58
p16/INK4A	NM_009877.2	Fw: 5′-CTCAACTACGGTGCAGATTCGA-3′ Rv: 5′-CACCGGGCGGGAGAA-3′	57	58
p21/Cip1	NM_007669.5	Fw: 5′-TGTGGCTCCCTCCCTGTCT-3′ Rv: 5′-GCAGGGTGCTGTCCCTTCT-3′	63	58
p27/Kip1	NM_009875.4	Fw: 5′-CCTGGCTCTGCTCCATTTGA-3′ Rv: 5′-ACGGATGGAGCGCAAAAC-3′	71	58
MMP-2	NM_008610.3	Fw: 5′-TCTGGTGCTCCACCACATACAACT-3′ Rv: 5′-CTGCATTGCCACCCATGGTAAACA-3′	90	60
MMP-9	NM_013599.4	Fw: 5′-TGAACAAGGTGGACCATGAGGTGA-3′ Rv: 5′-TAGAGACTTGCACTGCACGGTTGA-3′	121	60
Timp-1	NM_001044384.1	Fw: 5′-GGTGTGCACAGTGTTTCCCTGTTT-3′ Rv: 5′-AAGCAAAGTGACGGCTCTGGTAGT-3′	119	60
Timp-2	NM_011594.3	Fw: 5′-TTTCTAGCCACACCAGGCAGATGA-3′ Rv: 5′-GGTTTGCTGGGAAGGCATTTGAGT-3′	112	60
Timp-3	NM_011595.2	Fw: 5′-ACCACTGCTTTGTCCAGGTGTTTG-3′ Rv: 5′-ATGGAAATGGTTGTGCCTTCTGCC-3′	145	64
β_2-microglobulin	NM_009735.3	Fw: 5′-GCTATCCAGAAAACCCCTCAA-3′ Rv: 5′-CATGTCTCGATCCCAGTAGACGGT-3′	300	58
Cyclophilin B	NM_011149.2	Fw: 5′-CGAGTCGTCTTTGGACTCTTT-3′ Rv: 5′-GCCAAATCCTTTCTCTCCTGTA-3′	87	58
G6PDH	NM_008062.2	Fw: 5′-GAAGCTGCCAATGGATACTTAGA-3′ Rv: 5′-CCACCGTTCATTCTCCACATAG-3′	99	58

incubated at 37°C for 30 min with a solution containing 0.25% trypsin and 0.004% of DNAse in PBS in a relation of 10 ml per 1 ml of tissue. After incubation, the trypsin solution containing dissociated cells was collected into a sterile 50-ml centrifuge tube. Immediately, an equal volume of RPMI medium containing 10% FBS was added to inactivate the trypsin and protect the cells from continued proteolytic digestion. The cell suspension was centrifuged for 5 min at 400 *g* and re-suspended in culture medium. This procedure was repeated two times to obtain the optimal tissue disaggregation. Cell viability was checked by trypan blue exclusion test and settled to the desired concentration.

Evaluation of Metastatic Properties of Tumor Cells

To analyze the metastatic properties of tumor cells, spontaneous and experimental metastasis assays were used (31). One group of solid tumor-bearing mice was used for spontaneous metastasis assessment. These mice were monitored every day and were euthanized when they exhibited characteristic of animals that are about to die such as signs of suffering, hypothermia, and slow locomotion. Animals were sacrificed at day 19 post EL4 cells subcutaneous injection, and the number of metastatic nodules in kidney and liver was determined. For the experimental metastasis tests, mice were inoculated through the tail vein either with 5×10^5 EL4 cells or with solid tumor disaggregated cells from the different experimental groups. After 14 days, mice were killed, organs were removed, and metastatic nodules were counted.

Migration Assay

Tumors from mice of different experimental groups were disaggregated as described in Section "Disaggregation of Solid Tumor" and 5×10^4 cells of each tumor were re-suspended in RPMI culture medium without FBS, seeded into the top well of a transwell chamber with 8.0-μm pores (Jet Biofil), and allowed to migrate toward medium containing 10% of FBS for 24 h. Cells in the upper and in the lower compartment were counted using a Neubauer chamber. Cell migration is presented as percentage of total cell count for each sample (32).

Natural Killer Activity Assay

YAC-1 cells were acquired from ATCC (Catalog number TIB-160). Cells were maintained in supplemented medium as described for EL4 cells. Specific cytotoxic activity against tumor cells was determined according to the just another method (JAM method) as previously reported (7). Briefly, YAC-1 cells were cultured in the presence of 5 mCi [^{3}H]-thymidine for 16 h. Cell suspensions from spleens of mice from different groups were obtained. Briefly, spleens were removed and disrupted through a 1-mm metal mesh, and the cell suspensions were filtered through a 10-lm nylon mesh. The suspensions were depleted of red blood and dead cells using a lysis buffer (NH_4Cl 8.29 g, $KHCO_3$ 1 g, EDTA-2Na 37.2 mg, diluted in distilled water, at pH = 7.4) for 2 min. After three washes in PBS, cells were re-suspended in PBS at final concentration. Cell viability was assessed by trypan blue exclusion assay. A target:effector ratio 1:50 was seeded in 96-well plates at a final volume of 200 μl, and incubated for 3.5 h

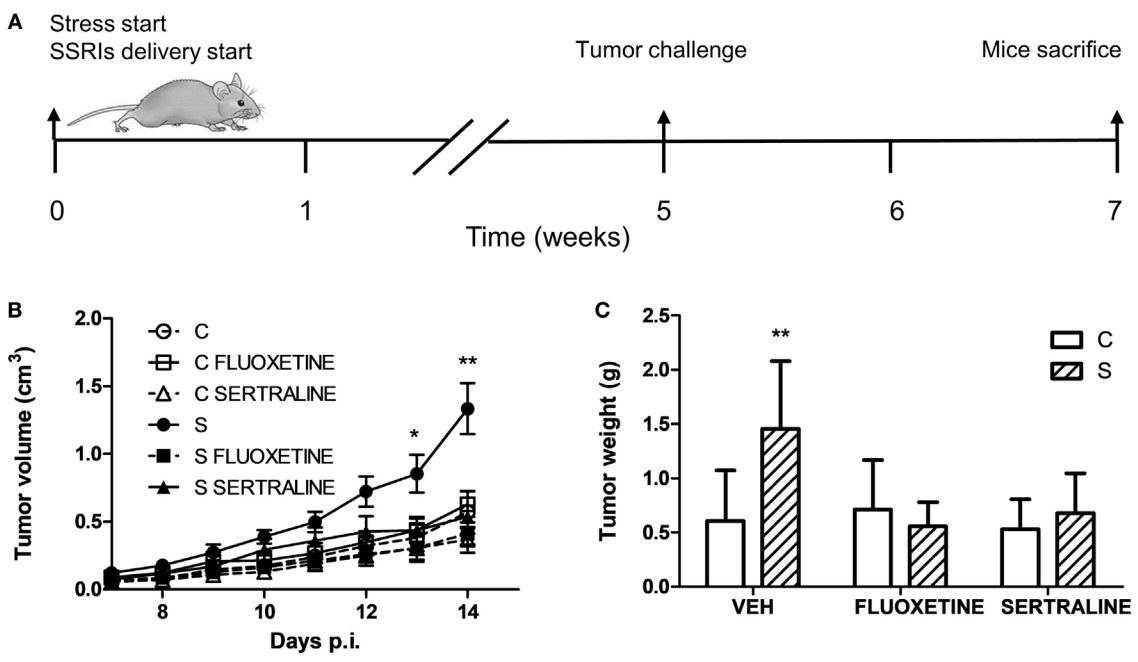

FIGURE 1 | Effect of fluoxetine and sertraline on stress-induced tumor progression. **(A)** Schematic representation of experimental protocol. C57BL/6J mice were treated either with (S) or without (C) the chronic stress protocol, and with or without (VEH) fluoxetine or sertraline. Five weeks later, 3×10^6 EL4 cells were subcutaneously injected to generate solid tumors. Two weeks post injection (p.i.), mice were sacrificed. **(B)** Time course of tumor volume for the different experimental groups. **(C)** Tumor weight at day 14 post EL4 cells injection. Values are expressed as mean ± SEM. $n = 6$ mice per group. $^*p < 0.05$; $^{**}p < 0.01$ respect to control mice.

at 37°C in a 5% CO_2 atmosphere. [^{3}H]-Thymidine incorporation was measured by scintillation counting after retention over GF/C glass-fiber filters (Whatman). NK activity was calculated as 100 × (SR − ER)/SR, where SR is the spontaneous release and ER is the experimental release.

Cytotoxic Activity Assays

Specific cytotoxic activity against tumor cells was evaluated according to the JAM test (7) as previously described. Briefly, EL4 labeled overnight with 5 mCi [^{3}H]-thymidine were co-cultured with spleen cell suspensions from tumor-bearing mice from the different treatments at a target:effector ratio of 1:15 for 3.5 h. The percentages of cytotoxic activity were calculated as the following relation: cytotoxic activity of T lymphocytes = 100 × (SR − ER)/SR, where SR is the spontaneous release and ER is the experimental release.

Total-Body γ-Irradiation and Lymphoid Cell Transplantation

Two-month-old C57BL/6J mice were placed individually into 1-mm thick, rectangular plastic boxes (30 mm × 30 mm × 60 mm) with holes to allow free exchange of air. Mice were exposed to a

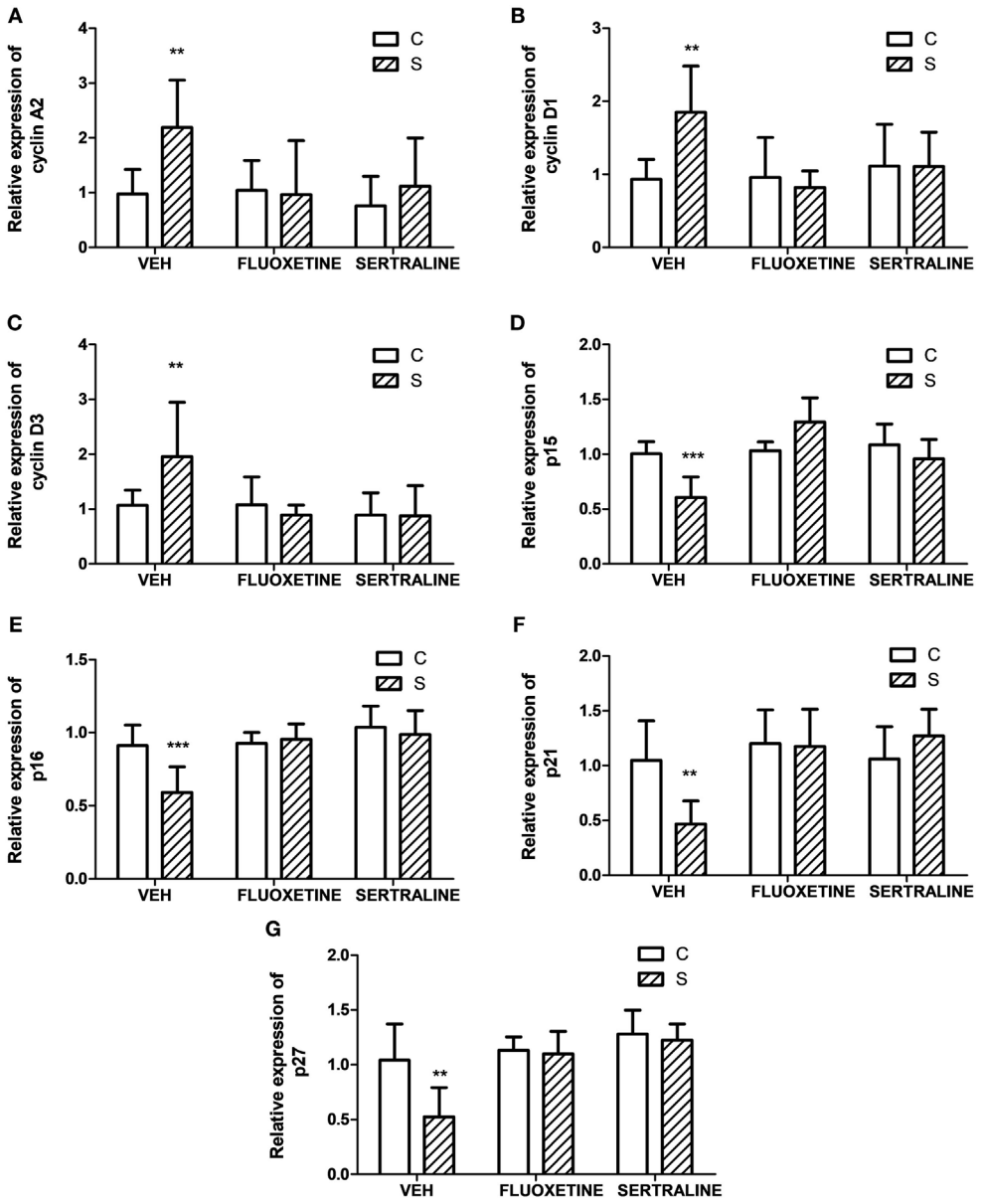

FIGURE 2 | Effect of chronic stress on the expression of proteins associated with cell cycle progression. Action of fluoxetine or sertraline treatment. Quantitative real-time reverse transcription polymerase chain reaction was performed in cDNA obtained from solid tumors excised 14 days post EL4 cells inoculation. **(A)** Cyclin A2, **(B)** D1, **(C)** D3, CDKIs **(D)** p15/Ink4b, **(E)** p16/Ink4a, **(F)** p21/Cip1, and **(G)** p27/Kip1 mRNA relative expression was quantified using β_2-microglobulin as housekeeper. Values are expressed as mean ± SEM. $n = 6$ mice per group. $^{**}p < 0.01$, $^{***}p < 0.001$ respect to control mice.

single dose of 2 Gy applied to the total body at a rate of 0.8 Gy/min. Gamma-irradiation was performed using a vertical beam containing ^{137}Cs source (Cebirsa SA, Buenos Aires, Argentina). This procedure provokes a lymphocyte depression near 80% (33).

On the following day, mice were transplanted with lymphoid cells that were obtained from mice of the different experimental groups. For this purpose, mice were sacrificed and lymph nodes (axillary, inguinal, and mesenteric) were obtained and disaggregated through a 1-mm metal mesh, and the cell suspension was filtered through a 10-μm nylon mesh. After three washes in PBS, cells were re-suspended at final concentration (7). A volume of 0.1 ml of cell suspension containing 8×10^6 lymphoid cells was transplanted into the recipients *via* tail vein injection.

Depletion of Immune Cells in Disaggregated Tumors

After 14 days of tumor injection, mice were sacrificed, and solid tumors were dissected. To purify tumor cells, an immune cell complement depletion protocol was used (34). Noteworthy, EL4 cells are CD4 and CD8 negative, so lymphoma cells are not affected by complement-dependent antibody-mediated lysis. Briefly, tumors were disaggregated as explained in Section "Disaggregation of Solid Tumor." The cell suspension was adjusted to 2×10^7 cells/ml and anti-mouse CD8a, CD4, and F4/80 (BD Biosciences) were added at a proper predetermined dilution. Then, cells were incubated for 30 min at 4°C and centrifuged at 400 *g* for 5 min. The pellet was re-suspended in RPMI containing 10% of low toxicity rabbit complement, incubated for 1 h at 37°C mixing every 15 min, and centrifuged at 400 *g* for 5 min. The cell pellet was then re-suspended either in RPMI-1640 culture for migration assay or in Tri-Reagent (Genbiotech SRL) to isolate the RNA. To confirm the effectiveness of the depletion protocol, a flow cytometric analysis was performed, comparing tumor cell suspensions before and after depletion. Cell suspensions were incubated with CD4, CD8, and F40/80 conjugated antibodies. All three cell populations were reduced after treatment. Macrophages from 15.6 to 1.5%, CD8+ cells from 6.5 to 1.2%, and CD4+ cells 4.7 to 0.6%. Total immune cells from 26.8 to 3.3%.

Statistical Analysis

Data were expressed as the mean ± SEM for each group. All the data were processed using STATISTICA software (StatSoft, Inc., Tulsa, OK, USA). The normality and homogeneity of variance for the dataset were tested using the Shapiro–Wilk test and Levene's test, respectively. Growth tumor data were analyzed with repeated measures two-way ANOVA analysis with condition (control or stressed) and pharmacological treatments (vehicle, fluoxetine, and sertraline) as factors. Other data were analyzed by two-way ANOVA with condition and pharmacological treatment as factors. For experiments using immune cell-depleted suspensions, one-way ANOVA was used. In all cases, if ANOVA showed significant differences between groups, Fisher's *post hoc* test was performed to determine significance level. Student's *t*-test was used for two group comparisons. Non-parametric Mann–Whitney *U* test was carried out to compare the number of metastatic nodules found in control and stressed mice. The binomial distribution test for comparing two proportions was used to analyze the statistical significance of % mice with spontaneous metastasis. $p < 0.05$ was considered to indicate a statistically significant difference.

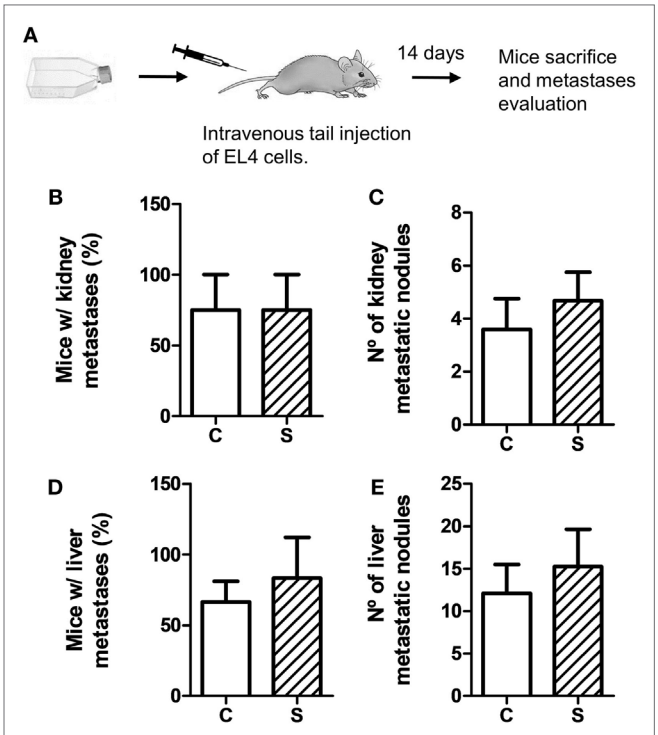

FIGURE 3 | Experimental metastasis assay using EL4 cells. Experimental metastasis were evaluated 14 days after intravenous tail injection of 5×10^5 EL4 cells in mice subjected (S) or not (C) to chronic stress treatment. **(A)** Schematic representation of experimental protocol. Percentage of mice with metastatic nodules in kidney **(B)** or liver **(D)** was calculated. Number of metastatic nodules in kidney **(C)** or liver **(E)** was assessed. Results are the mean ± SEM of three independent experiments ($n = 4$ mice per group).

RESULTS

Fluoxetine and Sertraline Are Able to Prevent the Promotion of EL4 Lymphoma Growth Induced by Chronic Stress

To investigate the effect of chronic stress on tumor growth, 5-week-stressed and control mice were subcutaneously inoculated with EL4 syngeneic lymphoma cells to develop a solid tumor and tumor volume was determined every day. The stress protocol continued until the sacrifice of the mice. To analyze the effect of fluoxetine or sertraline treatment, drugs were dispensed orally simultaneously to stress exposure (see **Figure 1A**). A significant effect depending on time, stress exposure, and SSRIs treatment was found ($F_{14,210} = 5.299$; $p < 0.001$). As it can be seen in **Figure 1B**, data indicated that tumor growth was accelerated in chronically stressed mice respect to control animals after day 13. Interestingly, both fluoxetine and sertraline treatments counteract the stress effects on tumor growth. Accordingly, tumor weight at day 14 post EL4 cells injection was significant depending on

stress exposure and SSRIs treatment ($F_{2,30} = 4.460$; $p = 0.020$) (**Figure 1C**).

To ascertain if proteins involved in the regulation and progression of cell cycle could be altered in parallel with tumor growth, we evaluated the tumor mRNA expression of A2, D1, and D3 cyclins and their inhibitors p15/Ink4b, p16/Ink4a, p21/Cip1, and p27/Kip1. Two-way ANOVA indicated that mRNA expression depended on stress exposure and SSRIs treatment (interaction stress × SSRIs, A2, $p = 0.037$; D1, $p = 0.045$; D3, $p = 0.036$; p15, $p < 0.001$; p16, $p = 0.020$; p21, $p = 0.016$; p27, $p = 0.048$). Results displayed in **Figure 2** indicate that mRNA levels of cyclins A2, D1, and D3 were increased in tumors from animals under stress. In addition, their inhibitors were decreased in tumors from stressed animals. Moreover, both fluoxetine and sertraline treatments restored mRNA expression levels of these regulatory proteins to control values.

Chronic Stress Increases the Tumor Invasion Capacity: Effect of Fluoxetine and Sertraline Administration

To evaluate metastatic dissemination capacity of tumor cells in different experimental groups, we performed experimental metastasis tests according to two experimental designs (see **Figures 3A** and **4A**). In general, intravenous injection into the tail vein results in lung metastasis. However, it has been reported that EL4 cells mainly generate liver and kidney metastasis (35–37). First, we analyzed EL4 cell dissemination in control and stressed mice. For this procedure, EL4 cells cultured in standard conditions were injected in the tail vein (**Figure 3**). As it can be seen in **Figure 3**, the number of mice that presented metastatic nodules in the liver and kidney was not significantly different for control and stressed mice (liver: $t_4 = 0.894$, $p = 0.422$; kidney: $t_4 = 0$, $p = 1$). Moreover, Mann–Whitney U test revealed no significant differences in the number of metastatic nodules in these organs between both groups (liver: $U = 62$, $p = 0.563$; kidney: $U = 59$, $p = 0.453$).

Taking into account that stress was able to modify mRNA expression levels of proteins that regulate tumor growth, we analyzed the possibility that stress exposure could modify the dissemination capacity of tumor cells. For this purpose, solid tumors from different experimental groups were dissected and disaggregated to obtain cell suspensions. These cells were tail vein injected in untreated mice (**Figure 4A**). Results indicate that the incidence in the metastasis development depended on stress exposure and SSRIs treatment of the injected cells, in both kidney and liver (interaction stress × SSRIs: $F_{2,6} = 8.30$, $p = 0.018$;

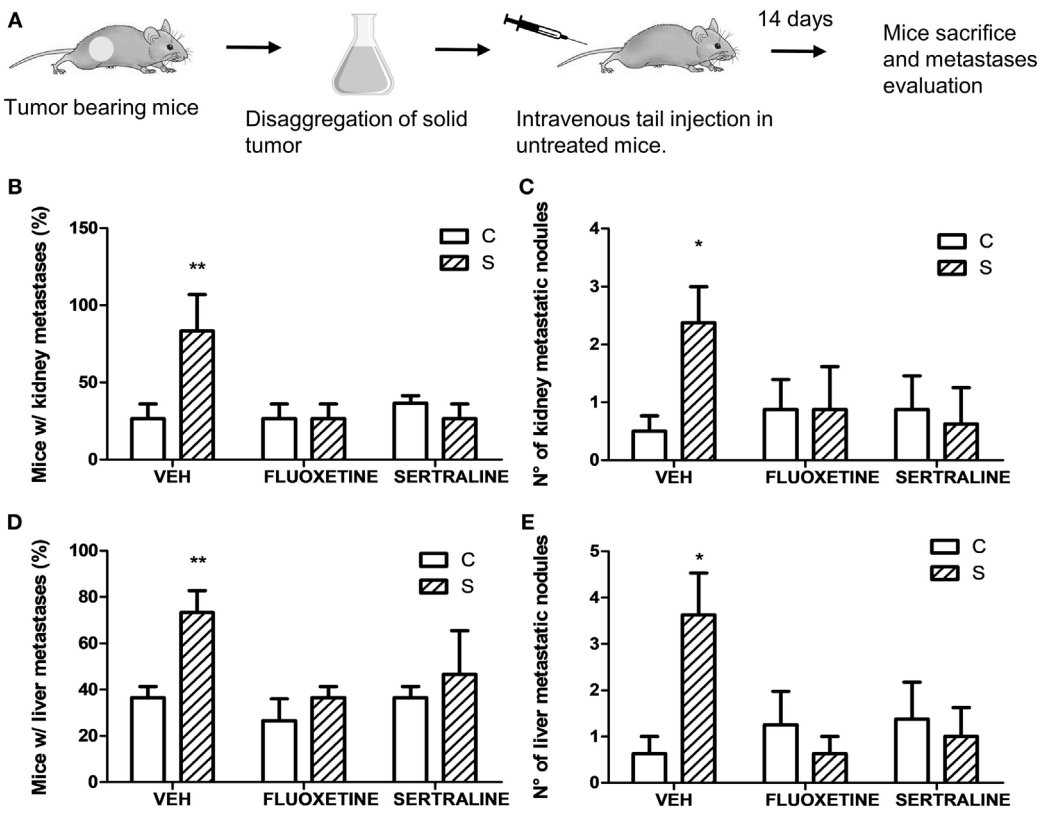

FIGURE 4 | Experimental metastasis assay using cells from solid tumors of different experimental groups. 14 days post subcutaneous injection of EL4 cells in mice from different experimental groups, solid tumors were excised and disaggregated. 5 × 10^5 cells were injected intravenously in untreated mice. Two weeks later, mice were sacrificed. **(A)** Schematic representation of experimental protocol. Percentage of mice with metastatic nodules in kidney **(B)** or liver **(D)** was calculated. Number of metastatic nodules in kidney **(C)** or liver **(E)** was assessed. Results are the mean ± SEM of two independent experiments ($n = 5$ and 4 mice per group). $^*p < 0.05$, $^{**}p < 0.01$ respect to control mice.

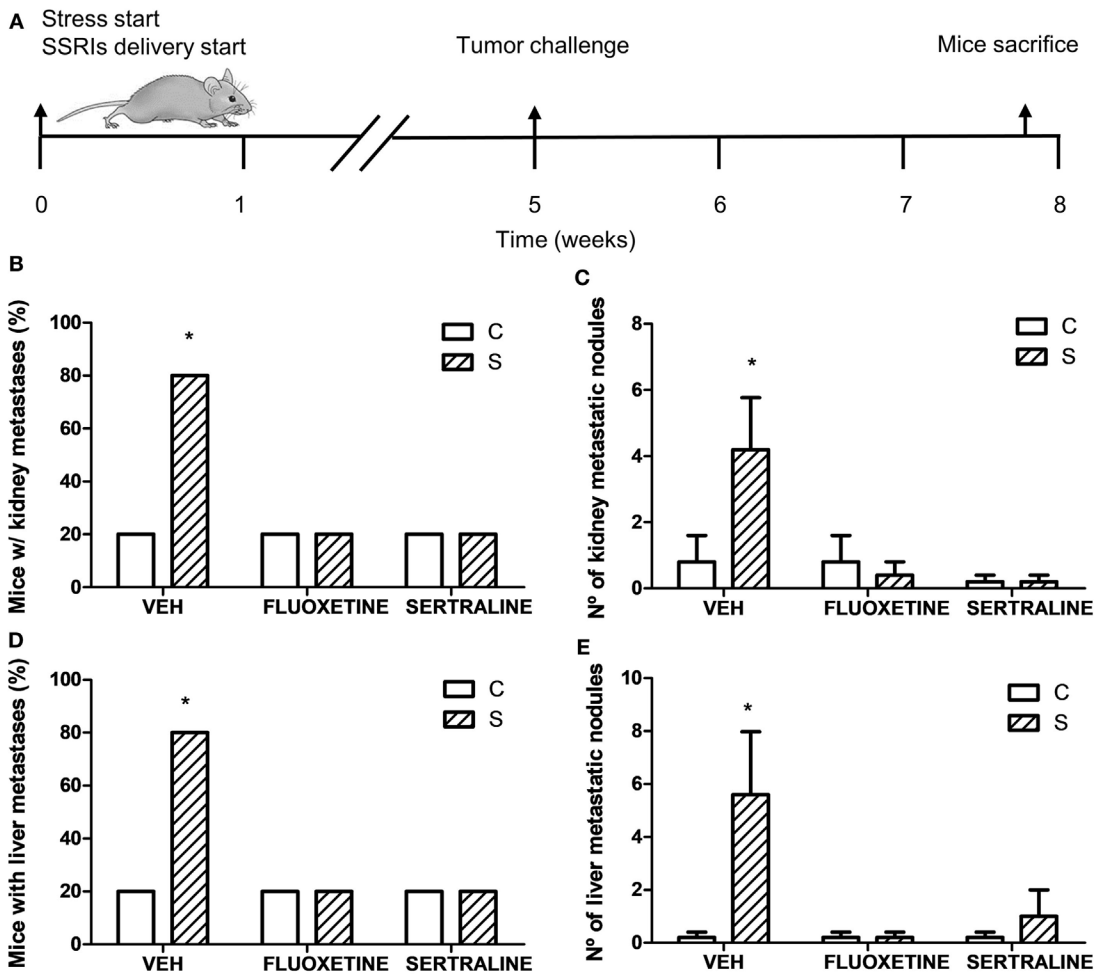

FIGURE 5 | Spontaneous metastasis assay in mice from different experimental groups. **(A)** Schematic representation of experimental protocol. 19 days post subcutaneous injection of EL4 cells in mice from different experimental groups, mice were sacrificed and percentage of mice with metastatic nodules in kidney **(B)** or liver **(D)** was calculated. Also, number of metastatic nodules in kidney **(C)** or liver **(E)** was assessed. Values are expressed as mean ± SEM. $n = 5$ mice per group. $^*p < 0.05$ respect to control mice.

$F_{2,6} = 8.03$, $p = 0.02$, respectively). *Post hoc* analyses indicated that the percentage of mice with both kidney and liver metastasis was significantly higher in mice injected with cells from tumor of stressed mice (**Figures 4B,D**). Non-parametric analyses of the number of metastatic nodules found in liver and kidney, revealed a greater number of metastatic nodules in mice injected with cells from tumor of stressed mice respect to control mice (liver, $U = 8.5$, $p = 0.013$; kidney, $U = 10.5$, $p = 0.024$) (**Figures 4C,E**).

Considering these results, we performed one experiment to determine spontaneous metastasis to evaluate the ability of cells to spread from a tumor implanted subcutaneously. For this purpose, mice were sacrificed 19 days after EL4 cells subcutaneous injection (**Figure 5A**). The binomial distribution test for comparing two proportions showed that the percentage of mice with metastasis was significantly higher in the stressed mice compared to control mice in both liver and kidney ($p = 0.047$) (**Figures 5B,D**). Mann–Whitney test showed a greater number of metastatic nodules in stressed mice respect to control group (liver, $U = 3.5$, $p = 0.044$; kidney, $U = 4$, $p = 0.050$) (**Figures 5C,E**). Interestingly, fluoxetine or sertraline treatment prevented these effects.

In accordance with these results, two-way ANOVA of transwell migration assay data showed that the percentage of migration depended on stress exposure and SSRIs treatment (interaction stress × SSRIs, $F_{2,23} = 7.143$, $p = 0.004$). *Post hoc* analysis indicated that cells from tumors of stressed animals have a major migration capacity in a transwell chamber using FBS as attractant. As expected, fluoxetine or sertraline treatment eliminated this effect (**Figure 6**).

Altogether, these findings indicate that stress-induced alterations in the biological behavior of tumors, and fluoxetine and sertraline were able to prevent these changes. In this context, invasion-related genes such as metalloprotease 2 (MMP2) and MMP9, and their inhibitors (TIMP 1, 2, and 3) were determined. As it can be seen in **Figure 7**, chronic stress significantly upregulated

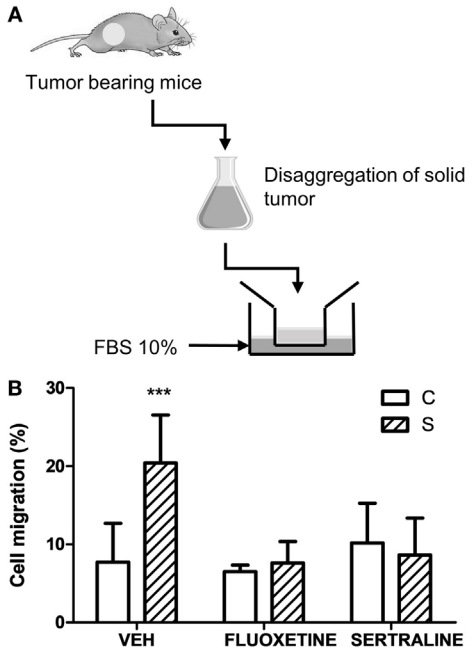

FIGURE 6 | Effect of chronic stress and antidepressant treatment on tumor cells migration. **(A)** Schematic representation of the experimental protocol. 14 days post subcutaneous injection of EL4 cells in mice from different experimental groups, solid tumors were excised and disaggregated. 5×10^4 cells were seeded in the upper well of transwell migration chamber and RPMI with 10% FBS as attractant. After 24 h, cells in the upper and in the lower chamber were counted. **(B)** Cell migration is presented as percentage of total cell count for each sample. Values are expressed as mean ± SEM. $n = 5$ mice per group. $^{***}p < 0.001$ respect to control mice.

the expression of MMP2 and 9 in tumors and downregulated the expression of TIMPs. Also, fluoxetine and sertraline impeded these effects (interaction stress × SSRIs: MMP-2, $p = 8.079$; MMP-9, $p < 0.001$; TIMP-1, $p < 0.001$; TIMP-2, $p = 0.005$; TIMP-3, $p = 0.046$).

Fluoxetine or Sertraline Administration Prevents the Decrease of Antitumor Immune Response Induced by Chronic Stress

To investigate if stress exposure and drug treatment affect antitumor immune responses, we evaluated the NK activity in mice that were not exposed to the tumor challenge (**Figure 8A**) and the specific cytotoxicity against EL4 cells in tumor-bearing mice (**Figure 8C**). Two-way ANOVA of NK activity data revealed that the % of lysis of YAC-1 cells depended on stress exposure and SSRIs treatment (interaction stress × SSRIs, $F_{2,16} = 4.008$, $p = 0.039$). As it is shown in **Figure 8B**, splenocytes from stressed mice showed a decreased cytotoxic activity mediated by NK cells. This impaired NK activity was prevented by fluoxetine and sertraline administration. Two-way ANOVA of the specific cytotoxicity assay showed that immune cells from spleens of tumor-bearing animals of the different experimental groups were able to lyse the EL4 cells depending on stress exposure and SSRIs treatment (interaction stress × SSRIs, $F_{2,36} = 6.354$; $p = 0.004$). As it can be seen in **Figure 8D**, the percentage of EL4 cell lysis was significantly lower when EL4 cells were incubated with splenocytes from stressed mice compared to control mice. Once again, fluoxetine and sertraline treatments counteracted this effect.

Finally, to determine if alteration of antitumor immune responses could be involved in the promotion of tumor growth and tumor invasion capacity induced by stress exposure, adoptive transfer experiments were performed.

For this purpose, irradiated mice were tail vein injected with lymphoid cells from control and stressed animals treated or not with fluoxetine or sertraline. After this procedure, mice were inoculated with tumor cells and the tumor growth and spontaneous metastasis were determined (see scheme in **Figure 9A**). A significant effect depending on time, stress exposure and SSRIs treatment of the injected cells was found ($F_{22,352} = 5.207$; $p < 0.001$). As it can be seen in **Figure 9B**, data indicated that tumor growth was increased after day 16 in mice injected with lymphocytes from chronically stressed mice when compared with those transferred with immune cells from control animals. Furthermore, mice injected with immune cells from stressed animals treated with fluoxetine and sertraline did not show this effect. Accordingly, tumor weight at day 18 post EL4 cells injection was significant depending on stress exposure and SSRIs treatment of the transferred cells ($F_{2,32} = 3.586$; $p = 0.039$) (**Figure 9C**). In addition, the assessment of spontaneous metastasis indicated that animals transferred with lymphoid cells from stressed animals had a higher incidence of liver metastasis ($p = 0.023$) (**Figure 9D**) and a major number of liver metastatic nodules (**Figure 9E**) respect to animals transferred with cells from control animals ($U = 3.5$, $p = 0.027$). These differences were not significant in kidney metastasis incidence ($p = 0.740$) (**Figure 9F**) or number of metastatic nodules ($U = 7.5$, $p = 0.089$) (**Figure 9G**). In addition, tumors from animals that were transferred with cells from stressed animals that had received fluoxetine or sertraline administration showed a similar biological behavior than those transferred with cells from control animals.

Experiments Using Immune Cell-Depleted Tumor Cell Suspensions Demonstrate That Molecular Alterations Observed in the Total Tumor Mass Are Mainly due to Changes in the Cancer Cells

To further ascertain whether the alterations in cell cycle gene expression, cell migration, and MMPs expression described above were originated by the direct effect of treatment on cancer cells, or if the immune cells present in the tumor microenvironment were responsible for these changes, molecular alterations were assessed in immune cell-depleted tumor cell suspensions.

One-way ANOVA showed significant differences between groups for cyclin A2 ($F_{2,14} = 7.483$; $p = 0.008$), D1 ($F_{2,14} = 6.519$; $p = 0.012$), and p16 ($F_{2,14} = 4.437$; $p = 0.036$) expression levels. Results displayed in **Table 2** indicate that mRNA levels of cyclins A2, D1 were significantly increased and p16 decreased in immune cell-depleted suspensions from animals under stress. Treatment with fluoxetine prevented these changes.

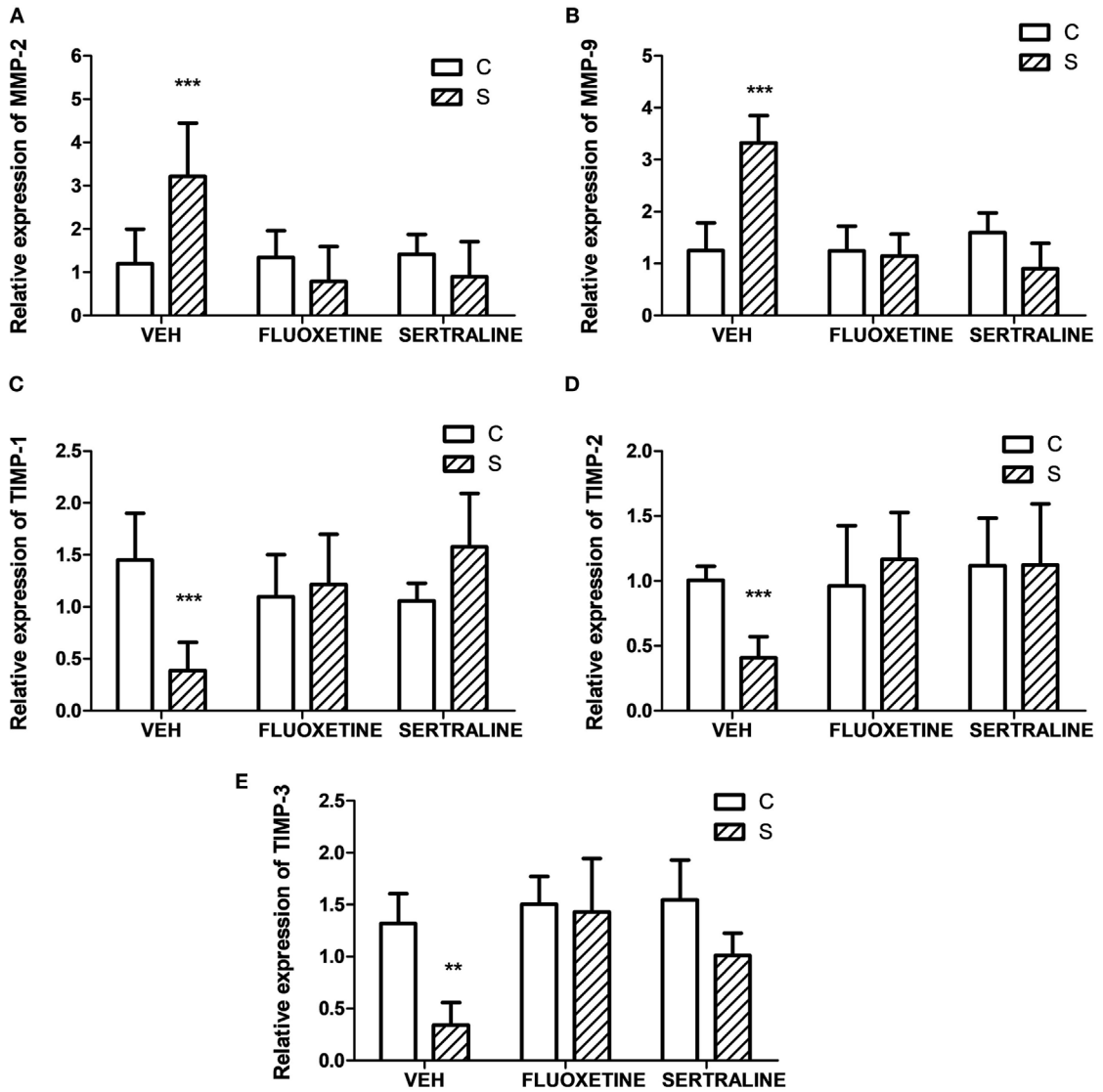

FIGURE 7 | Effect of chronic stress on the expression of proteins associated with cell invasion. Action of fluoxetine and sertraline. Quantitative real-time reverse transcription polymerase chain reaction was performed in cDNA obtained from solid tumors excised 14 days post EL4 cells inoculation. **(A)** MMP-2, **(B)** MMP-9 and their inhibitors **(C)** TIMP-1, **(D)** TIMP-2, and **(E)** TIMP-3 mRNA relative expression was quantified using β_2-microglobulin as housekeeper. Values are expressed as mean ± SEM. $n = 6$ mice per group. $^{**}p < 0.01$; $^{***}p < 0.001$ respect to control mice.

Similarly, significant differences between groups were found for MMP-2 ($F_{2,14} = 5.151$; $p = 0.024$), MMP-9 ($F_{2,14} = 5.826$; $p = 0.017$), and TIMP-1 ($F_{2,14} = 7.627$; $p = 0.007$) mRNA expression. *Post hoc* analysis indicated a significant increase in MMPs and a decrease in TIMP-1 levels induced by stress that were not observed in stressed animals treated with fluoxetine (**Table 2**).

In accordance with these results, one-way ANOVA of transwell migration assay data showed that the percentage of migration was significantly different depending on the treatment ($F_{2,14} = 5.199$; $p = 0.024$). *Post hoc* analysis indicated that immune cell-depleted suspensions from stressed animals have a greater migration capacity in a transwell chamber using FBS as attractant. Again, fluoxetine treatment impeded this effect (**Table 2**).

DISCUSSION

Epidemiologic and experimental animal research have indicated that stress may influence tumor progression (2–4, 7). However, the biological interactions between mediators of stress response, immune system, and tumor biology are not well understood. In particular, the role of the immune system in controlling solid tumor growth and dissemination has been considered unclear. In this context, our results showed a relevant role of antitumor immunity in solid tumor growth and in the invasion and dissemination of tumor cells. Our results indicated that chronic stress induces an alteration of immune homeostasis that in turn leads to an increase in both tumor growth and tumor cell dissemination.

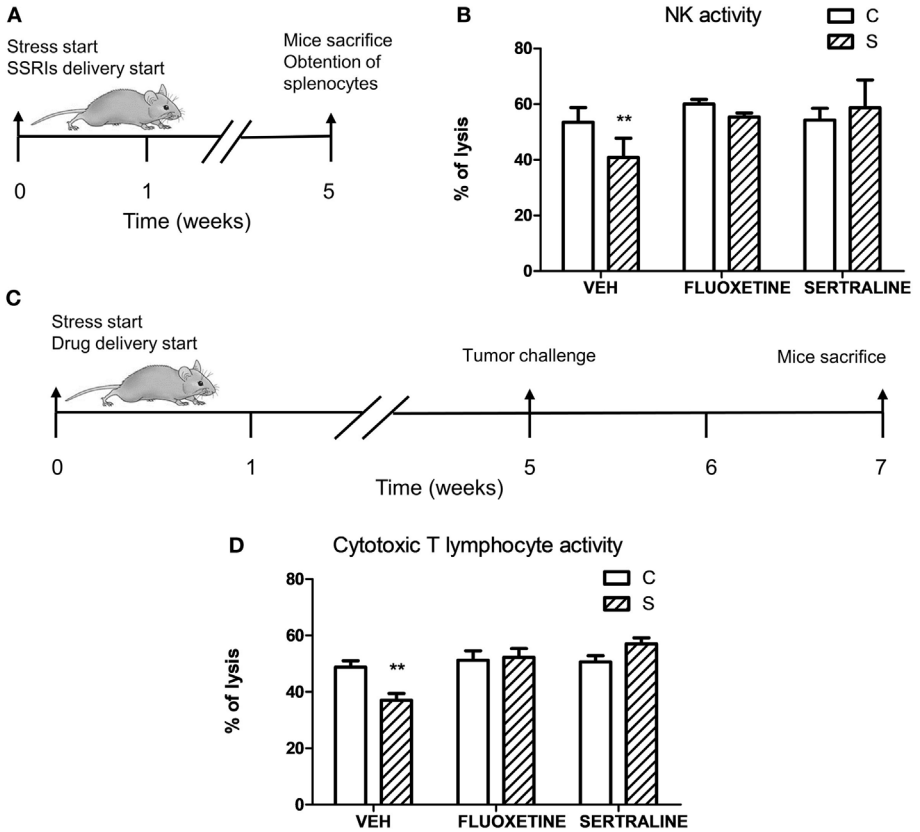

FIGURE 8 | Influence of chronic stress and antidepressant treatment on antitumor immunity. **(A)** Schematic representation of experimental protocol. Mice were treated either with (S) or without (C) the chronic stress protocol, and with or without (VEH) fluoxetine or sertraline. Five weeks later, mice were sacrificed. **(B)** Splenocytes of mice from different experimental groups were co-incubated with YAC-1 cells labeled with [3 H]-thymidine at a target:effector ratio 1:50, cultured for 3.5 h and harvested. $n = 4$ mice per group. **(C)** Schematic representation of experimental protocol. C57BL/6J mice were treated either with (S) or without (C) the chronic stress protocol, and with or without (VEH) fluoxetine or sertraline. Five weeks later, 3×10^6 EL4 cells were subcutaneously injected to generate solid tumors. Two weeks post injection (p.i.), mice were sacrificed and spleen cell suspensions were obtained. **(D)** Specific cytotoxic activity against tumor cells was evaluated co-culturing spleen cells suspensions from tumor-bearing mice and labeled EL4 cells with [3H]-thymidine at a target:effector ratio 1:15 for 3.5 h and harvesting. Percentages of NK or cytotoxic activity were calculated as 100 × (SR − ER)/SR, where SR is the spontaneous release and ER is the experimental release. $n = 7$ mice per group. Values are expressed as mean ± SEM. $^{**}p < 0.01$ respect to control mice.

In addition, an important beneficious effect of fluoxetine or sertraline treatment was found.

Chronic stress exposure resulted in an increase of tumor growth. This effect was related to an enhancement of cell cycle progression through the modulation of cell cycle regulatory proteins. We observed an increase in the mRNA levels of cyclins A2, D1, and D3 in the tumors from stressed animals. A decrease in mRNA levels of cell cycle inhibitors p15/Ink4b, p16/Ink4a, p21/Cip1, and p27/Kip1 was also found. Much evidence have pointed the involvement of cyclins D1 and D3 in T-cell lymphomagenesis, and they have been highlighted as relevant molecular markers of oncogenic power in T cell lymphomas (38, 39). In addition, an increase of cyclin D3 has been linked to a high proliferation rate and with reduced levels of p27/Kip1 (40). Cyclins D1 and D3 upregulation has been related to a poor outcome in lymphoma bearing patients (41–43).

A robust correlation between a big tumor load, higher tumor growth, and increased chance of metastasis has been demonstrated in many human cancers (44, 45). In accordance with this association, our results showed an important increase of the incidence and number of spontaneous metastasis in stressed animals. In addition, we analyzed experimental metastases after intravenous injection into the lateral tail vein. Our results indicated that no differences were found in the incidence and number of nodules in the kidney and liver when control and stressed animals were injected with EL4 cells from culture. However, when untreated animals were injected with cells obtained from tumors that had been grown in stressed animals, the incidence and number of metastatic nodules were significantly higher than those obtained when injecting cells from tumors that had been dissected from control animals. These results indicate that stress modifies the capacity of cells to give metastatic colonization in distant tissues.

To metastasize, cancer cells have to migrate, overpass the extracellular matrix (ECM), invade blood vessels, adhere to a remote place, and extravasate to originate a distant foci. MMPs are a zinc-dependent endopeptidases family that are able of disrupting the main components of the ECM and that have a relevant role in pathological situations that course with a significant degradation of ECM, such as tumor invasion, and tumor metastasis (46). In

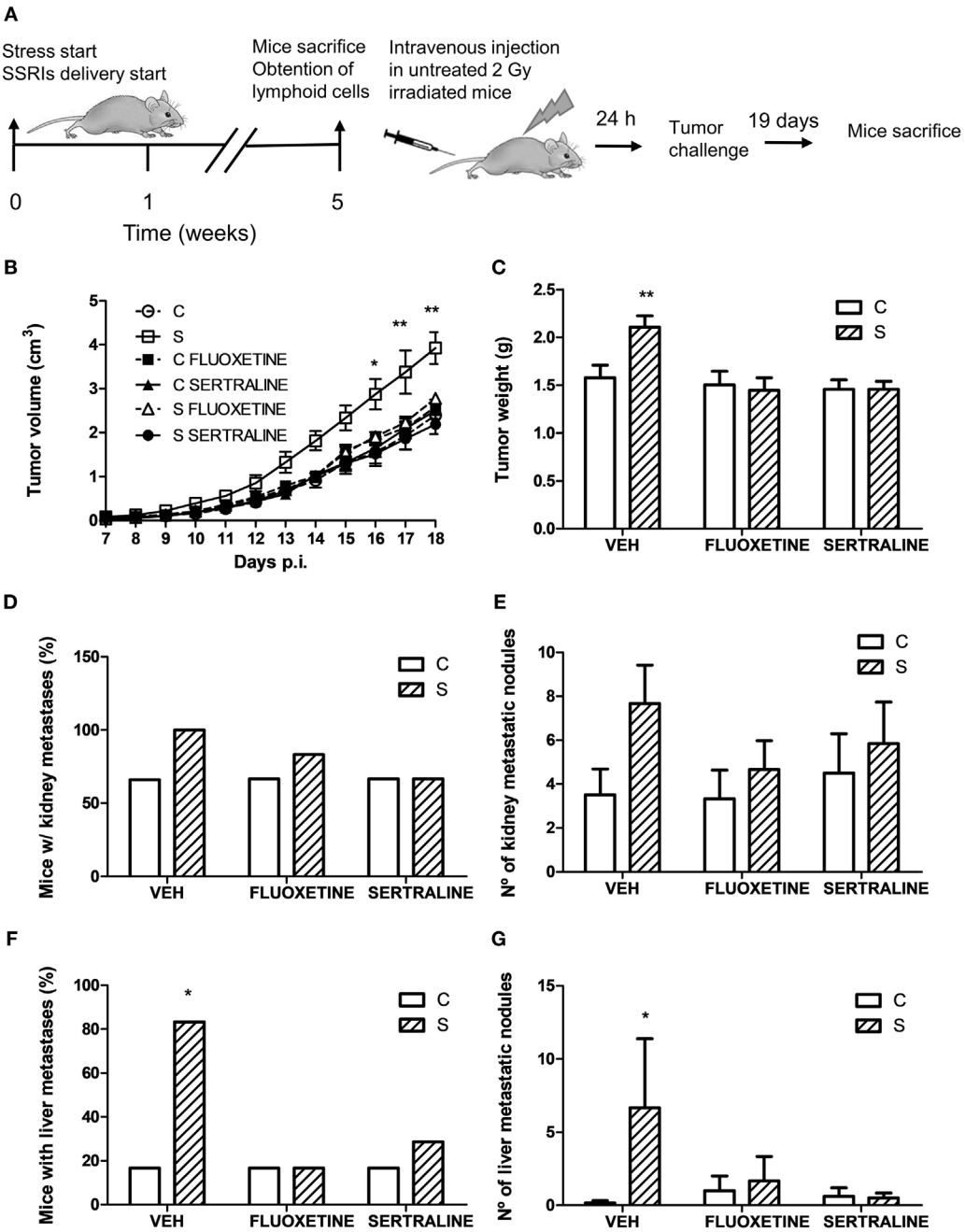

FIGURE 9 | Evaluation of antitumor immunity role in tumor biological behavior using adoptive cell transfer strategy. **(A)** Schematic representation of experimental protocol. Mice were treated either with (S) or without (C) the chronic stress protocol, and with or without (VEH) fluoxetine or sertraline. Five weeks later, mice were sacrificed and lymphoid cells from different experimental groups were tail vein injected in untreated irradiated mice. After 24 h, EL4 cells were subcutaneously injected to generate solid tumors. 19 days post subcutaneous injection, mice were sacrificed, and spontaneous metastasis was evaluated. **(B)** Time course of tumor volume for the different experimental groups. **(C)** Tumor weight at day 19 post EL4 cells injection. **(D)** Percentage of mice with metastatic nodules in kidney or liver **(F)** was calculated. Also, number of metastatic nodules in kidney **(E)** or liver **(G)** was assessed. Values are expressed as mean ± SEM. $n = 6$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ respect to control mice.

addition, despite MMPs are expressed by multiple cell types in tumors, it has been probed that they exert broad pro-tumoral functions and their increase in tumors indicate a high-metastatic capacity (47). The action of MMPs is partially regulated by TIMPs, and the MMPs/TIMP activities balance is relevant for ECM turnover (48). Our results showed greater MMP-2 and MMP-9 and lower TIMP 1, 2, and 3 mRNA levels in tumors from stressed animals compared with control. Moreover, cells from tumors of stressed animals have a major ability to directionally respond to chemoattractants in the transwell migration assays.

TABLE 2 | Effect of chronic stress and fluoxetine treatment on molecular changes and migratory ability in immune cell-depleted tumor cells.

		C	S	S Fluoxetine
mRNA expression of cell cycle proteins	A2	0.999 ± 0.096	2.075 ± 0.376**	1.170 ± 0.117
	D1	0.996 ± 0,084	1.688 ± 0.177*	1.076 ± 0.166
	p16	1.080 ± 0.181	0.530 ± 0.114*	1.087 ± 0.151
mRNA expression of MMPs and TIMPs	MMP-2	1.124 ± 0.079	1.927 ± 0.147*	1.290 ± 0.277
	MMP-9	1.024 ± 0.204	2.414 ± 0.558*	1.032 ± 0.082
	TIMP-1	1.153 ± 0.247	0.341 ± 0.061**	1.110 ± 0.131
Cell migration (%)		5.649 ± 0.905	9.752 ± 1.331*	5.745 ± 0.756

*Quantitative real-time reverse transcription polymerase chain reaction was performed in cDNA obtained from purified tumor cells suspensions from control (C), stressed (S), and fluoxetine-treated stressed (S Fluoxetine) mice. mRNA relative expression was quantified using β_2-microglobulin as housekeeper. Moreover, isolated cells from solid tumors were seeded in the upper well of transwell migration chamber and RPMI with 10% FBS as attractant. After 24 h, cells in the upper and in the lower chamber were counted. Cell migration is presented as percentage of total cell count for each sample. Values are expressed as mean ± SEM. n = 5 mice per group. *p < 0.05, **p < 0.01 respect to control mice.*

It is important to consider that the determinations of cell cycle, MMP, and TIMP gene expression levels were performed using total tumor RNA. However, it is known that in the tumor microenvironment there is a complex variety of cells that express these genes. In particular, recent reports demonstrated that antigen-specific CD8+ tumor-infiltrating lymphocytes are actively proliferating, but also have a high apoptosis rate (49). Moreover, among the innate and adaptive immune cells recruited to the tumor site, macrophages are particularly abundant and are present at all stages of tumor progression (50). In this context, the stress-induced alterations observed in the whole tumor could be, at least in part, due to the immune cells from the tumor microenvironment. To ascertain if the molecular changes took place in cancer cells or in the infiltrating immune cells, we performed experiments using tumor cell suspensions depleted of the main infiltrating immune cell subsets, namely CD4+ and CD8+ T lymphocytes and macrophages (19, 50, 51). Our results showed that cell cycle gene expression changes induced by stress were similar in both, immune-depleted and not depleted tumor cell suspensions. However, a higher increase in MMP-2 expression levels was found in total tumor cells (% of increase, stressed vs control: 169) respect to immune cell-depleted suspensions (72%). Non-significant differences were observed in MMP-9 and TIMP-1 expression levels. Transwell assay results also indicated that the increased migration, induced by stress, of total tumor cells was higher than the observed for immune cell-depleted cell suspensions (160 vs 72%). Taken together, these results indicate that stress-induced tumor growth could be mainly related to molecular changes in cancer cells and that the greater invasive capacity of tumors from stressed animals is related to molecular changes in both cancer and tumor-infiltrating immune cells. Noteworthy, fluoxetine treatment reverted the effect of stress in both total tumor cell suspensions and immune cell-depleted suspensions.

Many findings have suggested a dynamic bidirectional dialogue between tumors and the immune system that modulates tumor growth and metastasis (52). The concept of cancer immunosurveillance (53) argues that cells of the innate and adaptive immune systems eliminate tumor cells thus protecting the host against tumor development. However, as cancer progress, tumor variants that are able to evade immune-mediated elimination appear and generate clinically apparent neoplasms. This evidence lead to a new assumption, the cancer immunoediting hypothesis, which emphasizes the dual role of immune system: host protective and tumor modeling on developing tumors (54).

Our results indicate that antitumor immunity was decreased in mice submitted to chronic stress. It could be possible to postulate that stress decreases immune response, thus favoring tumor growth. However, due to the unclear performance of the immune system in managing solid tumor progression under stress situations, the possibility that stress mediators, in particular the activation of the sympathetic nervous system, may straightly regulate the tumor behavior has been investigated (14, 55). Experimental analyses in animal models have found that behavioral stress induced an accelerated progression of pancreatic (56), prostate (57), breast (58), and ovarian (12) carcinomas and malignant melanomas (59). In addition, it was demonstrated that the biological action of stress could be effectively inhibited by β-adrenergic antagonists and simulated by β-agonists (11, 60, 61).

To elucidate if the effects of stress were due to a direct action of hormonal stress mediators on tumor cells or an indirect action through the alteration of the immune response, we performed an adoptive immune cell transfer experiment. Our results indicated that when irradiated animals were transferred with immune cells from stressed animals, a higher tumor growth and an increased number of spontaneous metastasis were observed compared with animals transferred with immune cells from control animals. It is important to note that irradiated animals were not submitted to stress in the whole experiment. These results indicated that, in our experimental model, the effect of stress on tumor progression was mediated mainly by immune cells.

Finally, our results indicate that fluoxetine or sertraline treatment were able to inhibit the effect of stress on tumor progression. Antidepressants are frequently used in cancer patients to treat their emotional disorders, such as depression and dysthymia. Nevertheless, clinical studies have not revealed clear effects of treatment with antidepressant in patients with cancer (62).

Nowadays, there are evidences that suggest that SSRIs could be useful in either treating cancer administered alone or in combination with standard chemotherapies (63). In addition, antidepressants, and more specifically SSRIs have been shown to reduce the risk of certain cancers (64–67). Moreover, these antidepressants have been shown to be oncolytic *in vitro* and *in vivo*, through a mechanism that involves an increase of the intracellular influx of Ca^{2+} (68–70) and/or a disruption of the mitochondrial membrane potential as well as the generation of reactive oxygen species (68, 71, 72). In general, these studies have been focalized on the direct action of SSRIs on tumor biology without taking into account the effect on antitumor immunity.

Our results emphasize the crucial role of the immune system in tumor progression under stress situations. Although a direct effect of stress and SSRIs treatment on tumor biology could not be

ruled out, the beneficial effects of fluoxetine and sertraline appear to be mainly due to the restoration of the antitumor immune response. It is important to carry out investigations that allow to identify SSRIs targets outside their primary mechanism of action and thus encourage the performance of clinical studies leading to find significant benefits of the SSRIs prescription in cancer patients.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Guide for the Care and Use of Laboratory Animals" (NIH) (revision 2011) and of the EC Directive 86/609/EEC (revision 2010). The protocol was approved by the local Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, Instituto de Investigaciones Biomédicas-UCA-CONICET, Argentina).

AUTHOR CONTRIBUTIONS

GC and AG contributed to the conception of the work. MD and AG designed the study. MD performed the *in vivo* experiments. MD and HS carried out the *in vitro* experiments. MD and AG analyzed and interpreted the data and drafted the manuscript. HS and GC critically revised the manuscript. All the authors read and approved the final manuscript.

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T Cell-Mediated Chronic Inflammatory Diseases Are Candidates for Therapeutic Tolerance Induction with Heat Shock Proteins

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Failing immunological tolerance for critical self-antigens is the problem underlying most chronic inflammatory diseases of humans. Despite the success of novel immunosuppressive biological drugs, the so-called biologics, in the treatment of diseases such rheumatoid arthritis (RA) and type 1 diabetes, none of these approaches does lead to a permanent state of medicine free disease remission. Therefore, there is a need for therapies that restore physiological mechanisms of self-tolerance. Heat shock proteins (HSPs) have shown disease suppressive activities in many models of experimental autoimmune diseases through the induction of regulatory T cells (Tregs). Also in first clinical trials with HSP-based peptides in RA and diabetes, the induction of Tregs was noted. Due to their exceptionally high degree of evolutionary conservation, HSP protein sequences (peptides) are shared between the microbiota-associated bacterial species and the self-HSP in the tissues. Therefore, Treg mechanisms, such as those induced and maintained by gut mucosal tolerance for the microbiota, can play a role by targeting the more conserved HSP peptide sequences in the inflamed tissues. In addition, the stress upregulated presence of HSP in these tissues may well assist the targeting of the HSP induced Treg specifically to the sites of inflammation.

Keywords: heat shock proteins, tolerance, T regulatory cells, rheumatoid arthritis, inflammatory eye diseases, diabetes mellitus, type 1

In many cases, chronic inflammatory diseases are autoimmune diseases that are caused by a loss of tolerance to self-antigens due to inappropriate activation of the immune system. Collectively, autoimmune diseases affect 4–5% of the population, being females affected with a higher incidence than males (3:1 ratio) (1).

Genome-wide association studies have underscored the genetic association of the major histocompatibility complex (MHC) region with autoimmune diseases, in which case various predisposing alleles have been found (2, 3). The main function of MHC molecules is to present processed peptides for the recognition of antigen-specific T cells. And such T cells have the capacity to damage healthy tissues when they are not tightly controlled. The exact mechanisms triggering autoimmune diseases are unknown, but the presence of pro-inflammatory T cells in target organs as well as the strong link with MHC loci highlights the important role for adaptive immune responses in their development. The most accepted hypothesis proposes that for the initiation of an autoimmune disease,

an immune response with pro-inflammatory characteristics needs to be directed against specific tissue antigens in genetically susceptible individuals. Regulatory mechanisms exist in the periphery to control such effector responses to avoid excessive tissue damage (4). Mechanisms include the following: regulatory T cells (Tregs), direct inactivation of effector T (Teff) cells by induction of anergy or apoptosis and activities mediated by tolerogenic antigen-presenting cells (APCs). However, there is an increasing understanding that pro-inflammatory responses directed to self-antigens become chronic in autoimmune diseases because regulatory mechanisms fail to control them.

PERIPHERAL TOLERANCE MECHANISMS

$CD4^+CD25^{high}Foxp3^+$

$CD4^+CD25^{high}FoxP3^+$ Tregs can prevent autoimmune diseases by maintaining the tolerance to self-antigens. FoxP3 constitutes the most specific marker for these cells and is to some extent indispensable to develop a Treg phenotype and for their suppressive function (5). The development of autoimmune diseases when $CD4^+CD25^+$ cells are depleted in normal rodents or when rodents and humans have mutated FoxP3 genes highlights the role of Tregs in the prevention of such diseases (6, 7). As illustrated in **Figure 1**, when activated by their cognate antigen, Treg cells display a broad range of suppressive mechanisms, which endow them with the ability to control immune responses. The potential of controlling T- and B-cell responses with different specificities as well as the modulation of the maturation status of APCs by Tregs makes them attractive targets for the development of therapeutic strategies. Apart from $CD4^+CD25^{high}Foxp3^+$ Tregs, there are several subsets of $CD8^+$ T cells that are able to downregulate $CD4^+$ T-cell effector responses by different mechanisms including the induction of anergy in APCs and T cells as well as the secretion of anti-inflammatory cytokines (8). $CD8^+CD28^-Foxp3^+$ Treg cells are probably the subset best characterized (9). The activation of these cells is antigen specific [major histocompatibility complex (MHC)-I class-restricted], and their suppressor mechanism

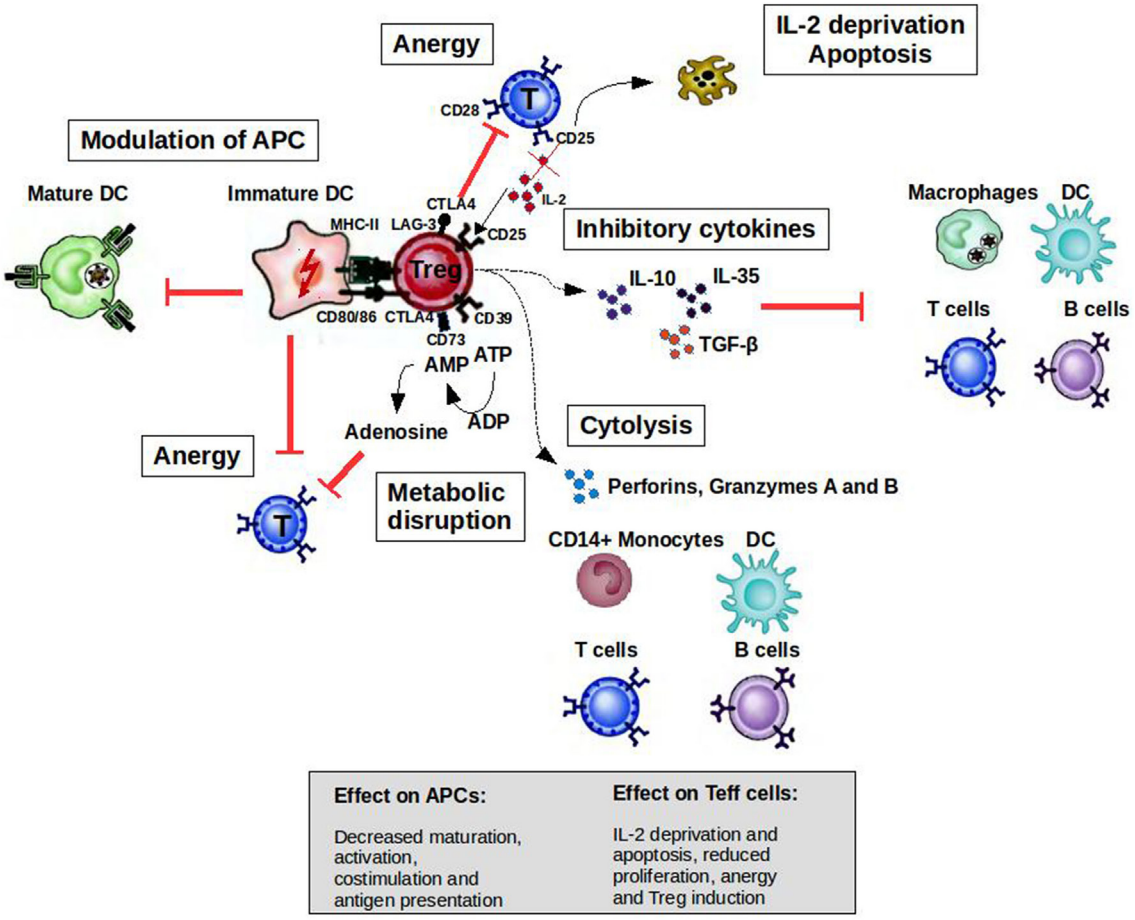

FIGURE 1 | Mechanisms of suppression by Treg cells to control immune responses. A broad range of molecular mechanisms contribute to the suppressive function of Tregs. Mechanisms include the following: apoptosis/cytolysis (IL-2 deprivation, granzyme A/B, perforins); antigen-presenting cell (APC) modulation (CTLA4, LAG-3); inhibitory cytokines (IL-10, IL-35, and TGF-β); and metabolic disruption (CD73/39 and ATP/adenosine mechanism). Abbreviations: CTLA4, cytotoxic T lymphocyte-associated antigen 4; DC, dendritic cell; CD, cluster of differentiation; IL, interleukin; Treg cell, regulatory T cell; LAG-3, lymphocyte activation gene 3; TGF, transforming growth factor; MHC, major histocompatibility complex.

involves the induction of a tolerogenic phenotype in APCs by the increased expression of immunoglobulin-like transcript 3 (ILT3) and ILT4. ILT3 and ILT4 suppress the activation of nuclear factor-κB mediated by CD40, which in turn reduces the transcription of co-stimulatory molecules such as CD80 and CD86 (9–11). These tolerogenic APCs in turn promote an anergic phenotype on naive $CD4^+$ and $CD8^+$ T cells, which could acquire similar regulatory functions spreading the induction of tolerance (10).

However, some studies have shown that patients with autoimmune diseases have less effective or fewer $CD4^+CD25^{high}Foxp3^+$ Treg cells compared with healthy individuals [reviewed in Ref. (12)]. Numbers and/or function of $CD8^+$ Tregs have been also found to be defective in animal models of autoimmunity and in patients (13). Defects in the capacity of Teff cells to be controlled by Tregs have also been found in the context of autoimmune diseases (12). Collectively, these findings suggest that Treg malfunction might be a factor promoting the development or chronicity of autoimmune diseases. Therefore, approaches to expand regulatory populations in autoimmune diseases have therapeutic potential (14, 15).

Anergy

T cells are activated when their T-cell receptors (TCRs) recognize antigenic peptides presented by MHC molecules expressed on the surface of APCs. Secondary signals like the one provided by CD28 expressed by T cells and B7.1 (CD80) or B7.2 (CD86) expressed by APCs are essential to initiate IL-2 production and T-cell proliferation. However, the activation of T cells without second signals induces a state of anergy where these clones are not able to respond to antigenic stimulus because they cannot produce IL-2. Cytotoxic T lymphocyte-associated antigen-4 (CTLA4) is a cell surface molecule related to CD28 that has the ability to block CD28-dependent T cell activation (16). The critical role of this molecule in controlling T-cell activation and maintaining peripheral tolerance was supported by the development of a massive lymphoproliferative disorder and autoimmune disease being fatal by 3–4 weeks of age in CTLA-4-deficient mice (17). Activated T cells transiently increase the expression of CTLA4, which is important to limit the expansion of activated T cells during an immune response. This cell surface molecule is expressed constitutively by Tregs endowing them with the potential to control T-cell activation through CD28 blockade (**Figure 1**). The inhibition of the CD28-dependent T cell activation has been used as a therapeutic tool for several autoimmune diseases. The blockade of the CD28 pathway with CTLA-Ig in animal models of autoimmune diseases prevented the progression of the disease [reviewed in Ref. (18)]. Abatacept (CTLA4-Ig) has been approved by the FDA for use in rheumatoid arthritis (RA) patients with an inadequate response to one or more of the disease-modifying antirheumatic drugs.

Apoptosis

Apoptotic cell death is another important regulatory mechanism operating in the thymus and periphery to delete self-reactive T cells or activated pathogenic T-cell clones, respectively. During the development of T cells in the thymus, clones bearing autoreactive TCRs are eliminated by apoptosis in a process known as negative selection. However, T cells with potential autoreactive receptors escape to the periphery where these clones should be kept in check by regulatory mechanisms such as Tregs, anergy, or deletion. In the periphery, activated T cells express death receptors belonging to the tumor necrosis factor (TNF) family (e.g., Fas/Fas-ligand) making them susceptible to activation-induced cell death (AICD) (19, 20). Memory T cells, Tregs, and Th2 cells are less susceptible than Th1 cells to AICD (21, 22), allowing the polarization of the immune response to protective responses (Th2/Treg) in the periphery. On the other hand, by inducing IL-2 deprivation and secreting perforins and granzymes, Tregs at the site of inflammation increase the susceptibility of Teff cells and other cells such as B cells and monocytes to cell death (23, 24).

Tolerogenic APC

Tolerogenic APCs present antigens to T cells but since they display low numbers of co-stimulatory molecules such as CD80, CD86, and CD40, antigen presentation leads to T-cell anergy (25). Tolerogenic APCs can be induced and enhanced using different compounds such as rapamycin, corticosteroids, interleukin-10 (IL-10), and transforming growth factor beta 1 (26). Several studies have shown the therapeutic effect of tolerogenic APCs induced *ex vivo* in experimental animal models [reviewed in Ref. (27, 28)]. Treg cells can also modulate the maturation status of APCs. For example, these cells can decrease the expression of co-stimulatory molecules on APC affecting their capacity to activate T cells (29). In addition, ligation of CTLA4 to CD80 and CD86 induces APC to express an immunosuppressive molecule (indoleamine 2,3-dioxygenase), which is able to abolish T-cell activation (30, 31). Lymphocyte activation gene 3 (LAG-3) is another molecule expressed by Tregs that could affect APC function. This is a CD4 homolog with a high affinity for MHC class II molecules. The binding of LAG-3 to MHC class II induces an inhibitory signaling pathway, which leads to the inhibition of APC maturation (**Figure 1**) (32).

MHC-ASSOCIATED DISEASES ARE T CELL-MEDIATED AND POSSIBLE TARGETS FOR INDUCTION OF HEAT SHOCK PROTEIN (HSP)-DRIVEN THERAPEUTIC TOLERANCE

The strong link of autoimmune diseases with MHC loci and the presence of pro-inflammatory T cells in target organs highlight the important role for adaptive immune responses in their development. In such cases, therapeutic tolerance may become established through the induction of Tregs with bystander regulatory activities leading to the inhibition or modulatory skewing of these pro-inflammatory self-antigen-specific T cells. Examples of MHC-associated, primarily T-cell driven autoimmune diseases are RA, type 1 diabetes (T1D), and several eye diseases.

Rheumatoid Arthritis

Rheumatoid arthritis is a chronic inflammatory disease characterized by joint inflammation and synovial hyperplasia, which leads to cartilage and bone destruction (33).

The HLA-DRB1 gene has been associated with the susceptibility of this disease, especially with the shared epitope (SE) coding alleles (HLA-DRB1*0401*, *0404*, *0405*, *0408*, *0101*, *0102*, *1402*, and *1001*). The SE is a five amino-acid sequence motif found in residues 70–74 of the HLA-DRβ chain that encodes a conserved positively charged residue at position 71 (34). The latter seems to guide the nature of the amino acid that can be accommodated in the P4 pocket of these HLA-DR molecules. Although the susceptibility of this disease appears to be determined genetically, the onset might depend on other factors such as environmental, epigenetic or posttranslational events factors (35).

As expected by the strong association of HLA-DRB1 and RA, $CD4^+$ T cells are enriched in synovia of these patients and seem to play a critical role in the perpetuation of inflammation [reviewed in Ref. (36)]. Susceptibility to RA has also been linked to other pathways implicated in the activation of T cells, such as PTPN22, PTPN2, CTLA4, IL2RA, IL-2RB, among others [reviewed in Ref. (29)]. Specifically, a $CD4^+$ T cell subset that produces IL-17, 21, 22, and TNF-α has been in the center of the attention in recent years. Emerging data have suggested that active RA might result from an imbalance between defective Tregs and pro-inflammatory Th17 cells (37–39). Nevertheless, the mechanisms governing such imbalance that could contribute to RA chronicity have remained unclear.

Tumor necrosis factor-α has been shown to be the master element of inflammation in RA (40). Consequently, the blockade of this cytokine has emerged as the main tool for its treatment. Although the exact mechanism underlying clinical effects of anti-TNF-α therapy in patients is not completely understood it is apparent that it can have an effect on other pathways associated with tolerance (41). For instance, it has been reported that the treatment with infliximab increases the percentage of $CD4^+CD25^+$ Tregs in RA patients who responded to therapy (42). Further studies showed that infliximab induced a distinct Treg population *in vitro* that could compensate the compromised Tregs detected in RA (43). Despite excellent results in patients responding to anti-TNF-α therapy, there is an increased susceptibility to serious adverse effects including: infectious diseases, malignancies and demyelination (44). In addition, only partial responses are achieved with this treatment and a continuous treatment is required.

Diabetes Mellitus Type 1

Pancreatic β cells producing insulin are the targets for antigen-specific T cells in T1D. Epidemiologic studies suggest that the incidence of this disease is rising (45). The updated estimates of the incidence (20.04 per 100,000 per year) and prevalent cases (129,350) of T1D in children 0–14 years old in Europe for 2013 (46) reflect an increasing trend of 3–4% per annum during the past 20 years (47).

HLA-DRB1*0401-DQB1*0302 and HLA-DRB1*0301-DQB1*0201 have been associated with T1D susceptibility whereas the haplotypes HLA-DRB1*1501 and HLA-DQA1*0102-DQB1*0602 confer resistance (48). However, most people bearing the haplotypes associated with the greatest susceptibility do not develop the disease. In addition, despite the finding of islet-specific T cells in the blood of healthy individuals, one study showed that these cells secrete IL-10 instead of interferon gamma (IFN-γ) (49), indicating that regulatory mechanisms should fail to develop T1D. Indeed, there is evidence supporting that regulation is impaired in this disease, where patients seem to have a decreased Treg suppressive functionality compared with non-diabetic controls (50, 51).

The exact mechanism by which β cells are destroyed in the pancreas is not fully understood, but genetic and environmental factors appear to predispose individuals with defective regulatory mechanisms to develop the disease. Similar to other chronic inflammatory diseases, T1D onset requires $CD4^+$ and $CD8^+$ T cells [reviewed in Ref. (52)]. The latter has been demonstrated in experiments in which the precipitation or prevention of diabetes was achieved in the non-obese mice model by transfer or elimination of $CD4^+$ or $CD8^+$ T cells, respectively. Both cell types are able to infiltrate the pancreatic islets in mice and humans and are considered to be the final executors of the destruction of insulin-producing β cells (52). $CD4^+$ and $CD8^+$ T cells can induce the death of pancreatic β cells. However, as β cells only express HLA-class I, direct cytotoxicity can be only mediated by $CD8^+$ T cells able to recognize appropriated peptides displayed on β-cell class I molecules. $CD8^+$ T cells are able to kill β cells through different mechanisms including granzyme B and perforins, pro-inflammatory cytokines, and/or Fas/FasL interactions (52).

No drugs have been approved to halt the autoimmune process that causes the destruction of β cells in T1D (53). The main goals are the induction of a residual β-cell function. Different approaches to treat this disease have been used so far [reviewed in Ref. (54)]. One of the therapeutic approaches showing promise in T1D is the use of anti-CD3 monoclonal antibodies that have been shown to interfere antigen-specific T cell activation. However, after promising clinical trials (phase 1 and 2) in T1D patients with a recent onset, phase 3 trials fail to meet primary endpoints (55, 56).

MHC-Associated Inflammatory Eye Diseases

Various studies have confirmed that eye diseases, such as idiopathic uveitis (57), birdshot retinochoroidopathy (BSR) (58), and sympathetic ophthalmia (59), have an association with MHC. Uveitis is the most common form of inflammatory eye disease and one of the leading causes of visual impairment and blindness. The association of the MHC class I molecule HLA-B27 with uveitis was first noted in 1973 (60). The precise molecular and pathogenic mechanisms behind the association between uveitis and HLA-B27 have remained unclear. HLA-B27 encompasses around 105 known subtypes (HLA-B*27:01 to HLA-B*27:106 thus far identified) that are encoded by 132 alleles (61). HLA-B27 subtypes have a varied prevalence in different races and regions of the world. HLA-B*2705 and B*2702 are the main HLA-B27 subtypes in northern Europe, whereas HLA-B*2704 and B*2706 are the most widespread subtype among Asian populations (62). A study from China found that among northern Chinese people, ankylosing spondylitis (AS) patients with B*2704 have a stronger risk of developing uveitis than those with B*2705 in Ref. (63). Conversely, a Japanese study showed that HLA-B27 anterior

uveitis (AAU) patients with the B*2704 subtype seemed to be less susceptible than patients with B*2705 (64). This suggests that HLA-B*2704 and HLA-B*2705 may be the most prevalent HLA-B27 subtypes, with observed conflicting results on the role of this molecule in AAU caused by different races and regions, genetic background, or environmental factors. Remarkably, the majority of individuals who carry susceptibility conferring HLA subtypes never develop uveitis or other systemic autoimmune disease, implying that HLA-B27 is a genetically predisposing factor for uveitis but that other genetic or environmental factors contribute to the development of uveitis. HLA-B27-associated uveitis is also closely related to other systemic autoimmune syndromes, such as AS and systemic sarcoidosis. Several studies have shown that HLA-B27-positive AS patients are more susceptible to uveitis than HLA-B27-negative patients (65, 66). 20–30% of patients with sarcoidosis were affected by uveitis (67).

Except for uveitis, also specific other ocular inflammatory diseases show a strong association with HLA. BSR and idiopathic retinal vasculitis are associated with HLA-A29 (68), with HLA-A*29.01 and HLA-A*29.02 representing the most common A29 subtypes found in BSR patients (69). The HLA-A*29.01 subtype is more frequent among Asians, whereas HLA-A*29.02 is more common among Caucasians (70). In addition, Behcet's disease (BD) is an inflammatory disease affecting multiple organs that also include a relapsing and remitting pan-uveitis, which is strongly associated with HLA-B*51 (71). HLA-B5101 is the predominant subtype associated with BD in Japanese and Iranian patients. The association of HLA-B*5108 and BD was also found in Greek and Spanish patients. A study of Israeli showed that HLA-B*52 may also be associated with BD (72).

In humans, more and more evidence reveals that cytokines produced by autoreactive Teff cell play a pivotal role in the pathogenesis of autoimmune uveitis. Early studies suggested that the imbalance of anti- and pro-inflammatory Th2 and Th1 subsets is responsible for the pathology of uveitis. However, in recent studies, emphasis was laid on Th17 and $CD4^+CD25^+FoxP3^+$ T regulatory cells, which produce IL-17 and IL-10, respectively. The ratio of Th17/Treg was distinctly increased at the progression of uveitis in patients and in experimental autoimmune uveitis (EAU) disease models (73, 74), and imbalance of Treg cells over Th17 cells was observed at the recovery phase of EAU (73). Th1 cells play central roles in early phase of uveitis, whereas Th17 cells act in the late phase of uveitis (75), Treg and inducible Treg cells suppress both Th1 and Th17 cell responses by counterbalancing pro-inflammatory activities of these T cells. This implies that increasing the number of Treg cells may be a promising and safe way to control MHC-associated eye diseases.

HSP AND THE INDUCTION OF THERAPEUTIC TOLERANCE

HSP Proteins or Peptides As Inducers of Tregs

Initial studies reported that several HSP families were able to induce both pro-inflammatory and anti-inflammatory effects. Pro-inflammatory cytokine production mediated by HSP70 appears to be linked to the activation of toll-like receptor 2 (TLR2) and TLR4 signaling pathways on innate immune cells (76, 77). Pathogen-associated molecular patterns such as lipopolysaccharide or other proteins present in recombinant HSP produced in bacteria have been suggested to be responsible for the observed pro-inflammatory effects [reviewed in Ref. (78)]. In line with this idea, HSPs often fail to induce an inflammatory effector response in highly purified preparations (79, 80). On the contrary, other studies using non-recombinant Hsp70, boiling treatments (which cause the degradation of HSP) or antibiotics have led to the conclusion that HSPs are responsible for the activation of innate immune cells as well as T cells through TLR signaling pathways (81, 82). It seems that whether these proteins have an activating or immunosuppressive role depends on several factors including their local concentration, the nature of the HSP itself (self or microbial), among others [reviewed in Ref. (83)]. In the context of autoimmune diseases, HSP proteins have been considered as target molecules involved in their pathogenesis in part because they become highly available at sites of inflammation (83). The other main reason is the high homology between species whereby microbial HSPs can active immune responses that can be cross-reactive with self-HSPs, which in theory could provoke autoimmunity. However, autoreactivity to self-HSPs has been also found in healthy individuals (84, 85), which means that these proteins are under a tight regulation network. The latter also means that autoreactivity to self-HSPs is not a synonym of autoimmunity. In fact, self-HSP reactivity appears to be a physiological mechanism for controlling the inflammatory process (86). In this regard, several studies in mice and humans support the fact that HSP, and specially conserved epitopes have the potential for attenuating rather than triggering inflammatory responses (87, 88).

The initial indication of a possible role of HSP in the induction of therapeutic tolerance was obtained in the model of adjuvant arthritis in rats. T cells collected from diseased animals were found to respond to mycobacterial HSP60 (89). When the recombinant mycobacterial HSP60 protein was used for immunizations, no arthritis was seen to develop. Interestingly, induction of adjuvant arthritis in these immunized animals appeared not to be possible anymore. Subsequent experiments revealed that the same protection against adjuvant arthritis induction was obtained by immunizing the animals with only a conserved sequence (peptide) of mycobacterial HSP60 (90). On the basis of these latter experiments, it was concluded that the conserved peptide induced T cells that were cross-reactive with self (mammalian) HSP upregulated at the site of inflammation. In various additional studies, the regulatory nature of these cross-reactive T cells was recognized, since they were producing regulatory cytokines such as IL-10.

More recent studies have shown the HSP mediated induction of T cells with regulatory potential, which showed the actual phenotypic characteristics of the currently known Tregs (91). This was among others the case for an HSP70 derived conserved mycobacterial peptide called B29. When BALB/c mice were immunized with B29, responding T cells were collected on the basis of CD25 expression and transferred into naïve syngeneic recipient mice. Subsequently, these T cells were found to inhibit

disease activity after induction of arthritis and to persist in various organs, including the joints, for more than 50 days. When during this time period, the presence of these cells was interrupted by infusion of a Treg depleting antibody, disease returned, which showed the actual disease suppressive activity of these HSP70-specific Tregs. When B29-specific T cells were selected on the basis of LAG-3 expression, it sufficed to transfer only 4,000 of these T cells to fully inhibit arthritis. Several explanations are possible for the capacity of conserved microbial HSP peptides to induce Tregs. An obvious explanation can be found in the contact of the immune system with microbiota in the gut. It is known that APCs lining the gut mucosa ingest bacteria from the microbiota. This causes transport to mesenteric lymph nodes, where the derivative microbial antigens are presented to T cells, a phenomenon that must contribute to mucosal tolerance. Since ingestion of bacteria will lead to a stress response, both in bacteria and in the APC of the host, MHC molecules will be loaded with HSP fragments in this process. By these mechanisms, both microbial and the self-cross-reactive T cells will be activated. And since such events happen in the environment of the tolerance promoting mucosa, induction of peripheral Tregs seems a direct and physiological consequence. Given the evolutionary conservation of the HSP molecules present in the complete kingdom of prokaryotes, it seems unavoidable that through the repeated contacts with bacteria, the immune system develops a focus on the conserved parts of the molecules. And by this same focus on the shared sequences between bacterial and mammalian HSPs, Tregs induced by bacterial HSP may easily cross-respond to self-HSP (over-)expressed in the inflamed tissues. Herewith the regulation, which is of a bystander nature, will be targeted toward the sites of inflammation.

Endogenous HSP-Loaded MHC Molecules

Apart from the possibility that mucosal tolerance creates a repertoire of HSP-specific Treg, there is also reason to think that HSPs are a default antigen for Tregs in the context of healthy tolerogenic APCs in the absence of co-stimulation activating microorganisms. In various studies, HSP70 has been found to belong to the most frequent cytosolic/nuclear MHC class II natural ligand sources (92). In other words, MHC elution studies have revealed that sequences of HSP70 family members are relatively often present in the proteome obtained from the antigen-binding clefts of human and mouse MHC-II molecules. And especially in the case of cell stress, such as the stress caused by inflammatory mediators, HSP70 fragments have been seen to become preferentially uploaded into MHC-II molecules [reviewed in Ref. (87)]. Dengjel et al. (93) have analyzed the sequences eluted from human B cell-derived HLA-DR4 molecules under amino-acid deprivation as the cell-stress factor. It was shown that under such conditions chaperone-mediated autophagy became operative, which led to involvement of HSP70, which is one of the molecular participants in the process of chaperone mediated autophagy. In general terms, it was seen that under stress, the presentation of peptides from intracellular and lysosomal source proteins was strongly increased on MHC-II in contrast to peptides from membrane and secreted proteins. For these reasons, it was concluded that their study illustrated a profound influence of autophagy on the class II peptide repertoire and suggested that this finding had implications for the regulation of CD4(+) T cell-mediated processes. Interestingly, also the mammalian homologs of our earlier defined HSP70-derived B29 peptide were eluted from this HLA-DR4 molecule (HLA DRB1*0401). Knowing this, it seems reasonable to think that HSPs and HSP70 family members in particular are frequently seen by Tregs in the context of MHC-II molecules. In this manner, they could well serve as a default antigen for Tregs, especially when presented by tolerogenic APC.

Given the stress-inducible nature of various HSP70 family members, we have attempted to raise the abundance of HSP70 fragments in MHC-II molecules of APCs by administering a so-called HSP co-inducing compound (94, 95). And indeed, in the experimental model of proteoglycan-induced arthritis (PGIA) in mice, we have seen such an intervention with an HSP co-inducer to lead to a T cell-mediated resistance against arthritis. The experiments were carried out with carvacrol, an essential oil obtained from Oregano plants. Initial studies *in vitro* had indicated that incubation of cells before further exposure to classical stressors, such as raised temperature or arsenite, caused a raised expression of endogenous HSP70. When given orally, carvacrol was found to lead to a raised expression of HSP70 family members in the Peyer's patches, the lymphoid organs of the gut. In addition, when analyzing the T cell responses to HSP70, it was found that oral carvacrol had raised the frequency of HSP70-specific T cells and that such cells showed to an enhanced degree the $CD4^+CD25^+Foxp3^+$ phenotype of Tregs. Transfer of these cells into naïve recipients inhibited subsequently induced PGIA. Thus, the experiments with carvacrol have indicated that for the purpose of generating therapeutic tolerance, the co-induction of HSPs, even by dietary measures, may be a possible and attractive strategy.

How to Induce HSP-Based Therapeutic Tolerance in T Cell-Mediated Autoimmune Diseases

Effective interventions in animal models are usually based on disease inhibition by administering therapeutics before disease induction. In other words, effective interventions are mostly preventive and not therapeutic. Successful therapeutic interventions, when overt disease has been established, are notoriously difficult to reach. The reason for this is that established inflammation is known to cause a relative resistance to therapy, among others by a supposed resistance of effector cells to the regulation by Tregs (96). For these reasons, it seems essential for therapeutic tolerance to become an effective intervention, to treat chronic inflammatory diseases in an early phase of the disease.

Initial clinical studies in patients with autoimmune diseases with a recent diagnosis using peptides derived from HSP have shown that the treatment is safe and also the possibility to skew the pro-inflammatory profile of pathogenic T-cell clones has been noted [reviewed in Ref. (97–99)]. For example, dnaJP1 is a 15 mer peptide, derived from bacterial HSP40, that shares homology with the "shared epitope" sequence present in some HLA-class II molecules associated with RA, which confers susceptibility to develop the disease. The peptide was identified

as a pro-inflammatory T cell epitope in patients with active RA (100, 101), and authors hypothesized that it could be involved in the amplification of the inflammation due to the loss of regulatory mechanisms. In the double-blind, placebo-controlled phase II trial, dnaJP1 was administrated to active RA patients (<5 years of diagnosis) with the aim of inducing mucosal tolerance to this pro-inflammatory epitope. The treatment consisted in the administration of 25 mg of the peptide by oral route daily for 6 months. A decreased percentage of $CD3^+$ T cells producing TNF-α in response to restimulation *in vitro* with dnaJP1 was observed in patients treated with the peptide but not with placebo. A trend toward increased levels of IL-10 was seen only in clinical responders. The expression of certain molecules associated with the downregulation of the immune response before the therapy was found necessary for successfully tolerization using this peptide. Finally, authors reported significant differences between treatment groups on day 140 for both American College of Rheumatology (ACR)20 and ACR50 responses (102).

More recently, a randomized placebo-controlled double-blind phase I/IIA trial was performed in patients with unresponsive active RA using binding immunoglobulin protein (BiP). BiP is an endoplasmic reticulum resident chaperone and stress protein with strong tolerogenic effects in the collagen-induced arthritis model (103). A single i.v. infusion of BiP (1, 5, or 15 mg) was well tolerated. The efficacy in this study was confounded by a high clinical response in the placebo group. However, at the end of the follow up period (12 weeks), remission was only achieved by some patients receiving 5 and 15 mg of BiP. Decreased C-reactive protein levels, VEGF, and IL-8 were decreased in patients receiving BiP compared with placebo at that time point (104). Finally, it was concluded that a large study is required to find the optimum dose and frequency of BiP administration.

Antigen-specific tolerance using HSP-derived peptides has also been explored in T1D. DiaPep277 is a 24-amino-acid peptide derived from the 437–460 sequence of HSP60. The treatment of newly diagnosed diabetic patients with DiaPep277 was well tolerated. In some patients, the treatment may delay the loss of the C-peptide production thereby decreasing the demand for exogenous insulin when compared with placebo groups in phase I and II clinical trials (105). The study of the T-cell populations of patients treated with DiaPep277 but not with placebo showed a shift toward a Th2 phenotype characterized by reduced levels of IFN-γ and increased expression of IL-4, IL-10, and IL-13 (106).

In general, immunological effects often correlate with a trend to clinical efficacy compared with placebo groups. However, the clinical efficacy has been less than expected. Therefore, it may turn out necessary to combine various strategies. For example, anti-TNF-α drugs may be combined with HSP peptide-based vaccination to have a synergic effect of inhibition of inflammation in combination with a Treg inducing strategy. A recently probed intervention was utilizing autologous tolerogenic dendritic cells (DCs) loaded with (autologous) synovial fluids in patients with progressive forms of RA (107). This first phase clinical trial showed the safety and the attainability of the approach. Although in some patients also a beneficial clinical effect was noted, it now seems needed to repeat such an intervention in patients with less advanced forms of the disease. Since it will be less practical to obtain synovial fluids from such patients, an attractive alternative possibility will be the use of HSP70 peptide B29. Besides the fact that B29 has shown a capacity to induce HSP70-specific Tregs, an additional advantage of using a well-defined antigen, such as B29, is that this will provide an opportunity to monitor the effect of the intervention precisely at the level of peptide-specific T cells. A clinical trial exploring the effect of B29 in combination with tolerogenic DCs in patients with RA is under development.

Although clearly in its infancy, therapeutic tolerance is expected to become a reality. In the case of RA, therapeutic progress has been significant until now. From the first pain killers, such as aspirin that was already available in the end of the nineteenth century, gold preparations since the 30s of the previous century, prednisone since World War II and biologics more recently, a very significant progress was made. The typical anatomical joint aberrations as they were seen frequently in RA patients are fully avoidable these days. Nonetheless, none of these interventions leads to cure. When therapy is halted, disease returns. Knowing this, the real challenge for the coming years will be the development of interventions that lead to a permanent remission based on regained self-tolerance. Given their supposed physiological role as targets for T cell regulation, HSPs may provide us possibly with the means to achieve true therapeutic tolerance.

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AB, QL, and WE did the writing. FB, AS, and VR were involved with the design of the paper.

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MINI REVIEW
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How Mucosal Epithelia Deal with Stress: Role of NKG2D/NKG2D Ligands during Inflammation

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Mucosal epithelia encounter both physicochemical and biological stress during their life and have evolved several mechanisms to deal with them, including regulation of immune cell functions. Stressed and damaged cells need to be cleared to control local inflammation and trigger tissue healing. Engagement of the activating NKG2D receptor is one of the most direct mechanisms involved in the recognition of stressed cells by the immune system. Indeed, injured cells promptly express NKG2D ligands that in turn mediate the activation of lymphocytes of both innate and adaptive arms of the immune system. This review focuses on different conditions that are able to modulate NKG2D ligand expression on the epithelia. Special attention is given to the mechanisms of immunosurveillance mediated by natural killer cells, which are finely tuned by NKG2D. Different types of stress, including viral and bacterial infections, chronic inflammation, and cigarette smoke exposure, are discussed as paradigmatic conditions for NKG2D ligand modulation, and the implications for tissue homeostasis are discussed.

Keywords: NKG2D, natural killer cells, toll-like receptor, inflammation, stress, gut, epithelia, innate lymphocytes

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INTRODUCTION

Mucosal epithelia represent the frontline of multicellular organisms, and they are continuously exposed to several types of stress. Pathogens and environmental stress (thermal, oxidative, and chemical) can result in cell damage and loss of tissue function. To control inflammation and promote tissue repair, different mechanisms for the detection and elimination of stressed cells have evolved, including activation of the immune system.

NKG2D is a C-type lectin-like activating/co-stimulatory receptor expressed by innate and adaptive lymphocytes, such as natural killer (NK) cells, $CD8^+$ αβ T cells, γδ T cells, and iNKT cells. Engagement of NKG2D triggers the cytolytic function of effector $CD8^+$ T cells independently of TCR recognition in some circumstances (1–4) while directly activates the effector functions of NK cells, namely, cytolytic granule release and IFNγ secretion. Thus, expression of NKG2D ligands is strictly linked to the immunosurveillance of stressed cells by innate lymphoid cells, especially NK cells (5–7). In humans, NKG2D ligands are MICA and MICB (MHC class I chain-related proteins A and B), encoded by genes in the MHC region, and ULBP1-6 (UL16-binding proteins), with the encoding genes located on chromosome 6. Murine NKG2D ligands include five different isoforms of RAE-1 (retinoic acid early inducible-1), MULT-1 (murine ULBP-like transcript-1), and three different isoforms of H60 (histocompatibility 60) (8). These molecules are present at low or undetectable levels on normal cells (9, 10) but are rapidly induced upon cellular stress

and are frequently upregulated in virus-infected and neoplastic cells (11–13). Even if it is known that the promoter region of the *MICA* gene contains a heat-shock element able to respond to cellular stress (14), only recently the molecular mechanisms driving NKG2D ligand expression during cellular stress have more deeply been investigated (15).

Expression of NKG2D ligands has been shown in tissues from patients with chronic inflammatory diseases, including rheumatoid arthritis (16, 17), type 1 diabetes (18), and atherosclerosis (19); much less is known regarding NKG2D ligand expression and relevance in mucosal epithelia during both normal and stress conditions.

This review focuses on different conditions that are able to modulate NKG2D ligands on epithelial cells, and, in particular, on the role of NKG2D/NKG2D ligands in controlling the homeostasis of the gut and lung epithelia during inflammation. Recent findings link toll-like receptor (TLR) signaling to NKG2D ligand expression, and we can now think of epithelial and immune cells as an integrated system able to deal promptly with environmental stress.

SENSING THE STRESS: INTERPLAY BETWEEN TLRs AND NKG2D LIGANDS

Among the pattern recognition receptors, TLRs play a key role in innate immunity, serving as first line sensors of structurally conserved bacterial and viral components, the so-called pathogen-associated molecular patterns. It is clear that TLRs can be triggered also by endogenous ligands, such as nucleic acids released by necrotic cells and matrix components generated during tissue injury, collectively called damage-associated molecular patterns. As TLR engagement leads to the production of inflammatory cytokines and chemokines, which contribute to local inflammation and leukocyte recruitment, the recent findings showing that TLR signaling is able to modulate the NKG2D axis and thus lymphocyte activation are of great interest (**Table 1**).

Rotaviruses are causative of intestinal alterations leading to diarrhea, and it has been reported that in mice viral dsRNA from rotavirus genome induces severe intestinal injury *via* TLR3 activation (20). Indeed, upon stimulation, TLR3 forces intestinal epithelial cells (IECs) to express both IL-15 and RAE-1, which promote mucosal damage by activating intraepithelial lymphocytes (IELs), in particular CD8αα$^+$ T cells, engaging their NKG2D receptor (21). In this scenario, Tada and colleagues have shown that probiotics belonging to *Lactobacillus* strains are able to reduce the levels of IL-15 and RAE-1, and at the same time to increase the level of IL-10 in the intestine, thus performing immunomodulatory activities (22) (**Figure 1**). Interestingly, the NKG2D axis is modulated in an opposite way by TLR9 during *Salmonella typhimurium* infection. *S. typhimurium* is an important food-derived pathogen and unmethylated CpG-containing DNA from *S. typhimurium* is recognized by TLR9 (23). TLR9 triggering promotes the accumulation of IkBα, resulting in strong inhibition of the NF-kB pathway and thus controlling intestinal tissue inflammation. On the contrary, in TLR9-deficient mice, TLR9 signal deficiency releases its inhibition on NF-kB and leads to pro-IL-1β expression in IECs. In addition, lack of TLR9 signal causes activation of NLRP3 inflammasomes, resulting in pro-IL-1β processing and IL-1β secretion. Secreted IL-1β acts in an autocrine way stimulating IECs to expose NKG2D ligands (namely, RAE-1, MULT-1, and H60) and with a paracrine mechanism induces the expression of NKG2D on IELs (24). This positive loop augments the susceptibility of IECs to the cytotoxicity of IELs leading to the breaking of the epithelial barrier and to the spread of *S. typhimurium* infection (**Figure 1**).

TLR-dependent NKG2D ligand expression has also been observed in mouse kidney during renal ischemia–reperfusion injury (25). HMGB1 is a DNA-binding protein showing inflammatory function after ischemia–reperfusion injury by binding TLR4 (26–28). Chen and colleagues have reported that HMGB1 can induce RAE-1 and MULT-1 upregulation on injured kidney cells through a TLR4/MyD88-dependent signaling (25), suggesting a contribution of the NKG2D axis during tissue damage after ischemia. Accordingly, NK cell depletion has been demonstrated to ameliorate kidney damage following ischemia, with NK cells having a direct role in killing tubular epithelial cells *via* perforin secretion (29).

TABLE 1 | Modulation of NKG2D ligand expression following different types of stress and pattern recognition receptor (PRR) involvement.

Type of stress	Tissue	PRR (if any)	NKG2D ligand modulation	Reference
Rotavirus	Gut	TLR3	↑ RAE-1	(21)
Lactobacillus	Gut	–	↓ RAE-1	(22)
Salmonella typhimurium	Gut	TLR9	↓ RAE-1 ↓ MULT-1 ↓ H60	(24)
Ischemia	Kidney	TLR4	↑ RAE-1 ↑ MULT-1	(25)
Crohn's disease (chronic inflammation)	Gut	–	↑ MICA	(31)
Escherichia coli (pathogenic)	Gut	–	↑ MICA	(32)
Celiac disease (chronic inflammation)	Gut	–	↑ MICA/B	(39)
ER stress	Gut	–	↑ MULT-1	(40)
Akkermansia muciniphila	Gut	–	↓ MICA/B	(56)
Oxidative	Lung	–	↑ MICA/B ↑ ULBP2	(59)
Pseudomonas aeruginosa	Lung	–	↑ RAE-1	(60)
Cigarette smoke	Lung	TLR3/7/9	↑ RAE-1 ↑ MICA	(62–65)

↑, *stands for upregulation;* ↓, *stands for downregulation on the indicated tissue; ER, endoplasmic reticulum.*

MODULATION OF NKG2D LIGAND EXPRESSION DURING CHRONIC INFLAMMATION: THE GUT MODEL

The gut system represents a peculiar challenge for the immune system, as it is continuously exposed to commensal bacteria and

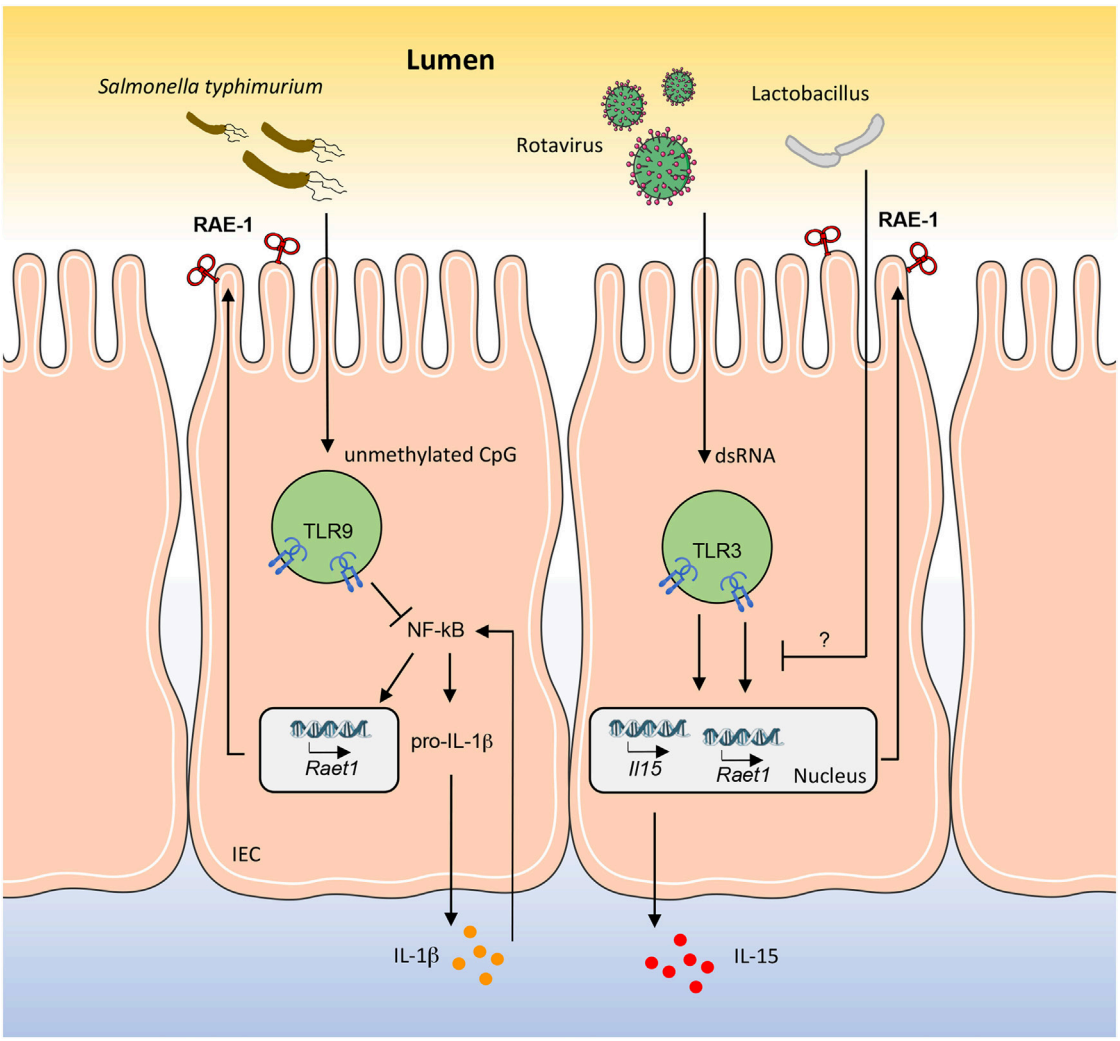

FIGURE 1 | RAE-1 modulation in mouse intestinal epithelial cell (IEC) by gut pathogens and commensal bacteria. How RAE-1 (encoded by *Raet1*) is regulated after toll-like receptor (TLR) engagement is schematically depicted. The apical localization of RAE-1 is inferred based on its similarity to human ULBP1–3 structure. The mechanisms through which *Lactobacillus* strains are able to reduce the levels of RAE-1 and IL-15 are only partially known.

food antigens. IECs constitutively express MICA and MICB, even if at low levels (14, 30), while much less is known regarding ULBP proteins. In physiological conditions, this expression does not lead to an immune response, but during chronic inflammatory diseases, MICA/B-expressing cells become target of IELs, namely, intraepithelial NK cells, γδ T cells, and $CD8^+$ αβ T cells. Although with different etiology, both Crohn's disease and celiac disease are characterized by chronic inflammation. This type of stress strongly relies on the relationship between the environment and the immune system.

Crohn's Disease

MICA expression has been found significantly increased on IECs isolated from patients with Crohn's disease, with higher levels in the macroscopically affected areas (31). Whether this upregulation is causative of the inflammation or a consequence of tissue damage is still unclear. Nevertheless, the upregulation of MICA has been associated with the expansion of a mucosal $NKG2D^+CD4^+$ T cell population able to promote a Th1 response, thus contributing to tissue inflammation and damage (31). There is evidence of a direct link between persistent MICA expression, innate lymphocyte activation and Th1 cytokine production in a model of pathogenic *Escherichia coli* infection (32), suggesting that this host–bacteria interaction may be relevant to the pathogenesis of Crohn's disease, as adherent *E. coli* strains have been isolated from inflammatory bowel disease (IBD) patients (33, 34). The presence of adherent pathogenic *E. coli* triggers a rapid increase of MICA expression on the surface of intestinal

cells after the interaction of the microbial adhesin AfaE with the cellular protein CD55, also known as the decay-accelerating factor (32). This event leads to NK cell activation with production of high levels of IFN-γ. As other human enteric pathogens, such as enteroviruses, use CD55 for cell entry (35, 36), it is possible to speculate that the *in loco* MICA overexpression occurring during chronic inflammation in unresolved infections contributes to lymphocyte activation associated with Crohn's disease. Based on these findings, targeting the interaction between NKG2D and MICA may be seen as a promising strategy to reduce inflammation. Results of a randomized controlled trial for the use of an anti-NKG2D monoclonal antibody (NNC0142-0002) in active Crohn's disease have reported no major improvement, but further investigations regarding dose ranging and dose regimen are needed (37). Furthermore, NKG2D ligands may have a wider role than stress sensors, contributing to the homeostatic control of the immune system, since a study by La Scaleia and colleagues has shown that NKG2D ligands, namely MICA/B and ULBP1–2, are upregulated not only on the epithelium of gut but also on the immune infiltrate in IBD lesions (38).

Celiac Disease

Celiac disease is an immune-mediated disease characterized by damage to the small intestine in response to gluten exposure. Interestingly, there is evidence for a direct link between the expression of NKG2D ligands in the inflamed mucosa and cellular stress. Indeed, a similar MICA/B pattern of expression in the gut of celiac patients and in different *in vitro* models of cellular stress has been observed. In both cases, MICA/B were located in stress granules commonly observed after oxidative and endoplasmic reticulum (ER) stress (39). A recent study by Hosomi and colleagues has disclosed a molecular mechanism through which ER stress is associated with MULT-1 upregulation in IECs. In a mouse model of ER stress, the ER stress-related transcription factor C/EBP homology protein, a major component of the unfolded protein response, induces the transcription of the *Ulbp1* gene leading to MULT-1 cellular surface expression. MULT-1 upregulation has been linked to the activation of intraepithelial group 1 innate lymphoid cells (NK cells and ILC1) and innate-like T cells (such as CD8αα$^+$ T cells), which contribute to mucosal inflammation in an NKG2D-mediated manner (40). Expression of NKG2D ligands in celiac disease can also be induced by cytokines, among which IL-15 seems to play a major role (41–45). Indeed, IL-15 has been shown to rapidly induce the expression of MICA and to relocate it from the cytosol to the surface membrane of enterocytes, where can be engaged by NKG2D-expressing IELs (3, 46, 47). On the other hand, MICA/B expression has been found also in the cytoplasm of intraepithelial and *lamina propria* lymphocytes (39). This intracellular localization of MICA/B in T cells during active celiac disease has been postulated to avoid overactivated T cell homeostatic regulation, thus contributing to tissue inflammation and damage.

An interesting issue to be considered for both Crohn's disease and celiac disease regards NKG2D ligand polymorphism. MICA gene is highly polymorphic with more than one hundred alleles, which affect both RNA and protein expression levels (48). Some studies have assessed the prevalence of specific MICA alleles in IBD patients, with contrasting or not conclusive results (49). MICA allele*007 was associated with ulcerative colitis but not with Crohn's disease in a British population (50), but this finding was not confirmed in a German cohort (51). MICA*008, instead, has been found overrepresented in celiac disease patients, but this could be ascribed to the linkage disequilibrium between HLA-B*08 and MICA*008 (52). Notably, MICA isoforms containing a methionine at position 129 bind NKG2D with high affinity, whereas those with a valine bind NKG2D with low affinity (53). Whether this diversity influences IBD is still unclear. A higher frequency of MICA-129met/met and a lower frequency of MICA-129val/met genotypes was observed in Spanish IBD patients compared with healthy controls (54). On the other hand, a study conducted in Chinese patients showed a higher frequency of the MICA-129val/val genotype in patients with ulcerative colitis (55).

The broad expression of NKG2D ligands on both epithelial cells and lymphocytes of the intestinal tract not only in pathological but also physiological conditions is intriguing but still unexplained. It may represent a state of alert ready to become active in response to stress, or, in more general terms, be part of tissue homeostasis regulation. On the other hand, it may be accountable for the state of permanent mild inflammation that characterizes the gut due to microbiota and food antigens. In support of the idea that the microbiota can be a main force driving the expression of NKG2D ligands on IECs is the observation that antibiotic administration and feeding are able to strongly modify NKG2D ligand expression (56). Germ-reducing conditions, such as ampicillin treatment, induce higher levels of NKG2D ligands, while food intake (i.e., xylooligosaccharides) or drug treatment supporting gut colonization by *Akkermansia muciniphila* decrease NKG2D ligand expression. An interesting association between the above conditions and the intestinal levels of IL-15 has been postulated, once again linking the inflammatory *milieu* to NKG2D ligands (56). Dietary contribution in the protection of gut inflammation has been investigated also in mice fed with a gluten-free diet. This food regimen leads to a reduced expression of NKG2D on DX5$^+$ NK cells as well as to an increased number of CD8$^+$ γδ T cells expressing TGF-β and the inhibitory receptor NKG2A, thus performing immunomodulatory functions (57, 58). Altogether, these findings reveal the importance of the NKG2D system in the homeostatic regulation of the intestinal mucosa.

ROLE OF NKG2D IN THE AIRWAY EPITHELIUM FOLLOWING STRESS

The respiratory tract is often under attack of environmental pathogens and is equipped with different populations of NKG2D-expressing lymphocytes. Bronchial airway epithelial cells constitutively express MICA, MICB, and ULBP1–4 transcripts, while

cell surface expression is largely absent in normal conditions. However, upon oxidative stress, NKG2D ligands become visible at the protein level on the cellular membrane, with a molecular mechanism based on the ERK pathway (59). The capability of stressed airway epithelial cells to rapidly express NKG2D ligands suggests that they are able to directly engage and activate the immune system. This finding has been confirmed in mice with lung *Pseudomonas aeruginosa* infection. Acute infection stimulates the alveolar epithelium to express RAE-1, and epithelial cell death (likely due to the activation of cytotoxic NKG2D-bearing lymphocytes) is associated with increased bacterial clearance (60). Pulmonary *P. aeruginosa* infection is also associated with increased levels of IL-1β, TNF-α, and IFN-γ, which may be another effect of NKG2D engagement. Thus, NKG2D ligand expression leads to a competent host response that can be blocked with NKG2D-specific antibody (60).

The NKG2D system plays an important role in lung homeostasis also in the event of physicochemical stress, as demonstrated in chronic obstructive pulmonary disease (COPD). COPD is a severe form of airway epithelium inflammation largely due to cigarette smoke exposure. Cigarette smoke is known to induce necrotic and apoptotic cell death with concomitant nucleic acid release (61). This leads to TLR3/7/9 activation and, with an unknown mechanism, to RAE-1 expression on mucosal airway epithelial cells. The consequent inflammation and NKG2D-driven cytotoxicity is responsible for further tissue damage, worsening the initial cigarette smoke exposure. NK cells become hyperresponsive breaking the balance between injury and repair (62–64). Notably, NKG2D ligand overexpression in transgenic mice is sufficient to induce pulmonary emphysema, strongly suggesting that NKG2D/NKG2D ligand axis *via* cytotoxic lymphocyte activation plays a major role in alveolar epithelial injury (65). The strong relationship between NKG2D ligand expression and COPD has been further confirmed by the finding of enhanced MICA expression in the airway epithelium specimens from smokers (65).

CONCLUDING REMARKS

Expression of NKG2D ligands can be achieved in several circumstances, ranging from viral and bacterial infections to physiochemical stress (**Table 1**). This versatility is needed to face a plethora of stressful stimuli with common pathways leading to the involvement of the immune system. As a result, intracellular insults are associated with intercellular responses, with great advantage for the whole organism. However, only recently the molecular mechanisms underlining these processes have been investigated, and many questions remain unanswered:

I. Which ligand is displayed? Is this choice stress type dependent?
II. Do different ligands engage the receptor in different ways?
III. What is the contribution of NKG2D ligands to the pathogenesis of gut and lung inflammatory diseases? Does NKG2D ligand polymorphism play a role in IBD?

Indeed, both NKG2D and NKG2D ligand genes are highly polymorphic. This polymorphism has been associated with host–pathogen coevolution, with great relevance for viral infections, but functional consequences have also been described during hematopoietic transplantation and cancer immunosurveillance (48, 66, 67). The impact of this variability in the context of stress response and inflammation still needs to be fully elucidated.

Another important point to be considered is the strict polarity of epithelial tissues. In the gut, the apical side of the epithelial layer displays different functions from the basolateral side, where IELs are located. Human ULBP1–3 and murine RAE-1 are anchored to the cell membrane *via* a glycosylphosphatidylinositol molecule and thus are supposed to be trapped in the lipid raft-enriched apical side of epithelial cells (**Figure 1**), while MICA presents a basolateral-targeting motif in his structure (68). Hence, MICA expression is prone to a rapid recognition by NKG2D-bearing IELs, while ULBP1–3 and murine RAE-1 become available only in the event of epithelial polarity breaking, as result of infection or autoimmunity (69). Remarkably, MICA allele*008, due to its truncated cytoplasmic tail, lacks the basolateral-targeting motif and changes its localization, leading to hypothesize a different NKG2D engagement (68, 69). Furthermore, it is now clear that NKG2D ligand transcript and even protein expression does not always mean cell surface exposure, with obvious functional implications (70, 71).

Great interest is growing regarding the role of the microbiome in human pathophysiology, as commensal bacteria can establish an immune regulatory milieu in the intestine (72, 73). The articles here reported suggest that, beside the production of TGF-β and IL-10, modulation of the NKG2D axis is a key function in this context. Indeed, it is now emerging that the NKG2D receptor and NKG2D ligands not only function as stress sensor molecules but also play a pivotal role in shaping innate and adaptive lymphocyte populations, thus contributing to the homeostatic regulation of the mucosal immune system.

AUTHOR CONTRIBUTIONS

FA searched for literature articles; conceived and wrote the manuscript. ANS conceived and wrote the manuscript. ALS, CC, and GS revised and critically contributed to the manuscript drafting. FA, ALS, CC, GS, and ANS approved the final version of the manuscript.

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REVIEW
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Transcription Factor C/EBP Homologous Protein in Health and Diseases

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C/EBP homologous protein (CHOP), known also as DNA damage-inducible transcript 3 and as growth arrest and DNA damage-inducible protein 153 (GADD153), is induced in response to certain stressors. CHOP is universally acknowledged as a main conduit to endoplasmic reticulum stress-induced apoptosis. Ongoing research established the existence of CHOP-mediated apoptosis signaling networks, for which novel downstream targets are still being determined. However, there are studies that contradict this notion and assert that apoptosis is not the only mechanism by which CHOP plays in the development of pathologies. In this review, insights into the roles of CHOP in pathophysiology are summarized at the molecular and cellular levels. We further focus on the newest advances that implicate CHOP in human diseases including cancer, diabetes, neurodegenerative disorders, and notably, fibrosis.

Keywords: C/EBP homologous protein, apoptosis, endoplasmic reticulum stress, fibrosis, cancer, neurodegenerative disorders, diabetes

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INTRODUCTION

C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible protein 153 (GADD153), belongs to the CCAAT/enhancer-binding protein (C/EBP) family. Much of our understanding of CHOP originates from the roles it plays during endoplasmic reticulum (ER) stress (1) and amino acid limitation (2). It was gradually discovered as a stress-responsive transcription factor during growth arrest, DNA damage, nutrient deprivation, hypoxia, genotoxic agents, etc. CHOP expression is induced by unfolded protein response (UPR) and integrated stress response (ISR) (3, 4), primarily through the PRKR-like ER kinase (PERK) pathway. As a nuclear transcription regulator, CHOP also controls numerous genes involved in multifaceted cellular processes including inflammation, differentiation, autophagy, and apoptosis. A considerable aspect of CHOP's involvement in disease is evident in the fact that sustained CHOP activation has long been accepted as a pivotal trigger for ER stress-related apoptosis.

In eukaryotic cells, the ER is a specialized organelle with the capacity for synthesis and storage of calcium as well as the folding and transport of secretory proteins to maintain cellular proteostasis. However, intrinsic and extrinsic insults, such as perturbations in calcium homeostasis and redox status, disturb ER proteostasis and cause accumulation of unfolded or misfolded proteins, collectively termed ER stress. In response, cells activate a series of adaptive pathways, namely the UPR, to restore homeostasis. Another innate protective pathway to proteostatic regulation is the ISR (5).

Literature on the role of ER stress (or protein misfolding) and UPR in numerous disease states, such as cancer, neurodegenerative disease, metabolic disease, and genetic disorders, has been well reviewed (6–8). To penetrate into the significance of CHOP in pathological processes, it is important to have a full overview of several aspects of CHOP. In this review, we delineate its structure and characteristics. The regulating mechanisms of CHOP at the transcriptional level and its functions–primarily apoptosis are summarized in detail. Finally, the latest studies targeting CHOP will be highlighted in four classifications of human disease, with special attention to fibrosis, for which the targeting of CHOP as a therapeutic approach has not yet been reviewed.

CHARACTERIZATION AND MOLECULAR FUNCTION OF CHOP

C/EBP homologous protein, encoded by the DNA damage-inducible transcript 3 (*Ddit3*) gene, is one of the six identified members of C/EBP trans-acting factors that bind to the CCAAT box motif present in several promoters. CHOP is characterized by transcriptional activation/repression domains at its N-terminus and a C-terminus basic-leucine zipper (bZIP) domain which contains a basic region mediating sequence-specific DNA binding along with a leucine zipper motif for dimerization. The N-terminal region is necessary for proteasomal degradation of CHOP. A serine/threonine-rich motif (97–100) in its transactivation domain can be recognized by speckle-type POZ protein (SPOP), which triggers CHOP degradation *via* the ubiquitin–proteasome pathway (9). Similarly, macrophage AMP-activated protein kinase α1 mediates CHOP ubiquitination and proteasomal degradation *via* phosphorylation at the serine residue (30) (10). Two serine residues (79, 82) are responsible for CHOP phosphorylation by p38 mitogen-activation protein kinase (p38 MAPK) (11). This phosphorylation event enhances its transactivation activity and is required for CHOP-induced apoptosis in macrophages (12) (**Figure 1**). It is well known that the conservation of CHOP's bZIP domain provides a platform for the formation of heterodimers. Furthermore, the basic region of CHOP holds proline and glycine residues that interrupt DNA-binding activity of the protein, causing increased heterodimerization with other C/EBPs (13). The homotypic heterodimers uniquely bind to the sequence (A/G) TGCAAT(A/C)CCC in response to stress (14). CHOP can also dimerize with members of another bZIP subgroup, the CREB/activating transcription factor (ATF) family. Consistently, a C/EBP-ATF-binding site is present in the amino acid response elements (AARE) of *CHOP* promoter (15). It has also been reported that the bZIP domain is required for CHOP-induced apoptotic processes (16, 17). Tribbles-related protein 3 (TRB3) recognizes the region between amino acid (aa) 10 and 18 to interact with CHOP.

C/EBP homologous protein serves as a double-edged transcription factor. It was originally proposed to be a dominant-negative regulator for other C/EBP-induced transcription by forming dimers and impairing their DNA-binding activity (18). However, CHOP also negatively regulates ATF4-dependent induction of the *ASNS* gene during ER stress or amino acid deprivation (19). Indeed, microarray analysis shows CHOP overexpression inactivates the expression of most of the target genes, serving as a dominant-negative factor by sequestration of dimer forming transcription factor partners (20). Nevertheless, subsequent studies have shed light on the positive role of CHOP–C/EBP interaction in transcriptional activation (21, 22) and have also revealed that CHOP–ATF4 heterodimers induce the expression of numerous stress-responsive genes (23).

REGULATION OF CHOP

C/EBP homologous protein is a cellular stress sensor that can be induced in response to a series of physiological or stress conditions such as ER stress, nutrient deprivation, DNA damage, cellular growth arrest, and hypoxia (1, 2, 24). It expresses at a very low level in normal physiology, but cellular stress leads to high-level expression. CHOP is acknowledged as a specific and convergent transcription factor of ER stress and its expression is generally modulated at the transcriptional level. CHOP transcription can be regulated *via* ER stress response elements (ERSE) and the C/EBP-ATF response element (CARE) of its promoter in response to cellular stress (15, 25), and *via* amino acid response elements (AARE) under amino acid starvation conditions (26) (**Figure 2**).

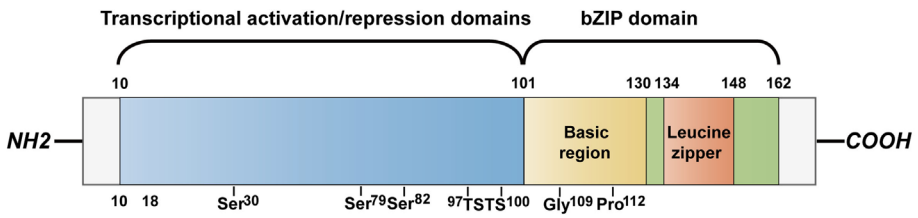

FIGURE 1 | CHOP structure. CHOP is a protein containing 169 amino acids that divide into N-terminus transcriptional activation/repression domains and a C-terminus bZIP domain including a basic region for DNA binding and a leucine zipper region for dimerization. The motif between aa 10 and 18 is for interaction with TRB3. The transactivation domain contains a serine residue (30) that is phosphorylated by AMPKα1 to trigger the proteasomal degradation of CHOP in macrophages. It is also degraded by SPOP that recognizes the serine/threonine-rich motif between aa 97 and 100. Phosphorylation at two serine residues (79, 82) by p38 MAPK enhances the transcriptional activation by CHOP. The basic region holds glycine (109) and proline (112) substitutions interrupting the DNA-binding activity. CHOP, C/EBP homologous protein; bZIP, basic-leucine zipper; SPOP, speckle-type POZ protein; p38 MAPK, p38 mitogen-activation protein kinase; aa, amino acid.

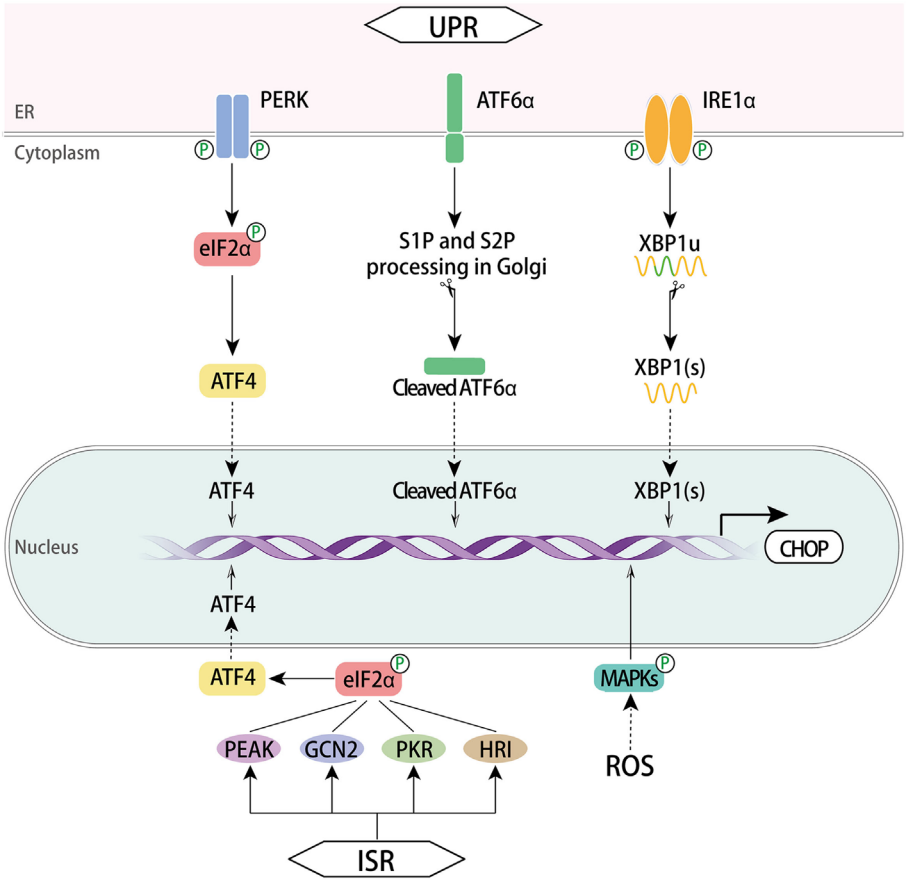

FIGURE 2 | Regulation of CHOP. The three signaling branches of UPR lead to CHOP transcription respectively. Once activated *via* dimerization and trans-autophosphorylation, PERK phosphorylates eIF2α, which enables ATF4 translation. Subsequently, CHOP is activated by ATF4 trafficking to the nucleus. In the presence of misfolded proteins, ATF6α translocates to the Golgi apparatus where it was processed by the protease SP1 and SP2, thus producing a cytosolic fragment ATF6f to regulate CHOP activation in the nucleus. Activation of IRE1α RNase domain processes unspliced XBP1 mRNA to create activated XBP1(s), which enters the nucleus and controls the expression of CHOP. Another pathway involves ISR. This response is initiated with GCN2, PKR, HRI, and PEAK that converge on the phospho-eIF2α/ATF4 pathway and CHOP induction ensues. A ROS-dependent mechanism also activates CHOP *via* MAPKs. CHOP, C/EBP homologous protein; UPR, unfolded protein response; ISR, integrated stress response; ATF, activating transcription factor; ATF6α, activating transcription factor 6α; PERK, PRKR-like ER kinase; XBP1, X box-binding protein 1; GCN2, general control nonderepressible 2; PKR, RNA-dependent protein kinase; HRI, heme regulated inhibitor; ROS, reactive oxygen species; MAPKs, mitogen-activated protein kinases; eIF2α, eukaryotic translation initiator factor 2α.

Unfolded Protein Response

Endoplasmic reticulum stress induces UPR, an adaptive mechanism that controls cell fate between survival and death in an intensity time-dependent manner. It involves three signal transduction pathways initiated by three ER transmembrane proteins: PERK, inositol requiring protein 1α (IRE1α, also known as ERN1), and activating transcription factor 6α (ATF6α) (27, 28). Each of the three maintains an inactive state in combination with the ER chaperone BiP (also named GRP78) in resting cells. Under chronic or overwhelming ER stress, all three mammalian UPR pathways uniquely lead to the initiation of CHOP transcription through binding sites within *CHOP's* promoter. The cis-acting AARE1 and AARE2 as well as the composite CARE (29) are bound by ATF4, while ATF6α and X box-binding protein 1 (XBP1) bind to ERSE1 and ERSE2.

The PERK pathway is predominant in CHOP activation. Upon the luminal binding of misfolded proteins, PERK is activated through dimerization and trans-autophosphorylation (30). It phosphorylates eukaryotic translation initiator factor 2α (eIF2α), which then attenuates global protein synthesis. Upon activation, ATF4 translocates into the nucleus and transcriptionally upregulates *CHOP* as well as many UPR genes that are vital for amino acid metabolism and redox processes (31).

The activation of IRE1α is similar to PERK in that its luminal domains are first dimerized and then are trans-autophosphorylated. Activated IRE1α creates spliced XBP1(s) by cleaving a 26-nucleotide intron from the mRNA of unspliced XBP1 using a cytoplasmic RNase domain. IRE1α also mediates regulated IRE1-dependent decay of selective mRNAs (32). XBP1(s) enters the nucleus and induces the transcription of genes correlated with protein-folding capacity and ER-associated degradation. Hence, CHOP expression is upregulated by XBP1(s) (33, 34).

In the ATF6α branch, the type II ER located protein ATF6α is transported to the Golgi apparatus where it is processed by

Site-1 and Site-2 proteases (SP1 and SP2, respectively). As a consequence of this processing, a cytosolic fragment of ATF6α is produced and enters the nucleus to regulate the expression of target genes, including *BiP* and *CHOP* (35, 36). Along with XBP1(s), ATF6f contributes to the augmentation of ER size and ER protein-folding capacity through target genes.

Integrated Stress Response

Integrated stress response serves as another cytoprotective mechanism against various stressors, such as ER stress, nutrition stress, oxidative stress, proteasome inhibition, hyperoxia, or viral infection (5, 37, 38). This common adaptive response initiates with four kinases consisting of general control nonderepressible 2, RNA-dependent protein kinase (39), heme regulated inhibitor, and PEAK, all four of which then converge on a core event, the phospho-eIF2α/ATF4 pathway, which in turn increases the transcription of CHOP (4, 40).

Reactive Oxygen Species (ROS)–MAPKs

Reactive oxygen species disturb redox status and ER homeostasis, thus inducing ER stress responses. ROS have been reported to activate CHOP through the AP-1 element in the CHOP promoter (41). The MAPKs consisting of JNK, p38 MAPK, and ERK are canonical downstream mediators of ROS (42). There are a number of studies establishing the signaling axis of ROS-induced CHOP upregulation *via* MAPKs signaling pathways in different cells. The ROS–MAPKs–CHOP pathway has been reported to suppress migration of hepatocellular carcinoma (HCC) cells (43) and mediate the downstream death receptor pathway in a number of cancer cells (44–47). Moreover, a scavenger of ROS diminished the PERK/eIF2α/CHOP pathway (48). IRE1 can recruit TRAF2 to activate ASK1, which can, through different pathways, induce expression of JNK and p38 MAPK (49). Phosphorylation of CHOP by p38 MAPK is required for its activation. Thus, the way by which ROS activates MAPKs and CHOP may be the IRE1α or PERK pathway of UPR (50). The ATF4/ATF3 axis was also reported to induce CHOP expression under ROS-dependent ER stress (51, 52).

Others

Several members of the CREB/ATF transcription factor family are capable of regulating CHOP expression. ATF3 can interact with the CARE elements within the *CHOP* promoter (29), while ATF5 activates the AARE1 site (53) under arsenite exposure. ATF2 binds the AARE sequence to regulate *CHOP* transcription in response to amino acid starvation (54). Conversely, some factors can inhibit the expression of CHOP, thereby reducing its detrimental effects. At early stage of ER stress, miR-211 expression, induced by PERK activation, can suppress *CHOP* transcription through histone methylation at its promoter (55). CHOP expression can also be repressed through toll-like receptor (TLR)–TRIF-dependent pathway which increases the activity of eIF2B to counteract the effect of p-eIF2α under the treatment of LPS, a TLR4 ligand *in vitro* and *in vivo*. Furthermore, the activation of eIF2B by TLR–TRIF signaling is attributed to serine dephosphorylation of eIF2Bε by protein phosphatase 2A. When TRIF was deficient in mice, CHOP induction, apoptosis, and organ dysfunction ensued (56, 57).

CELLULAR FUNCTION OF CHOP

In addition to its pro-apoptotic role, the function of CHOP in regulating other cellular processes has recently come to light. CHOP serves as a multifunctional transcription factor that contributes to cellular functions including apoptosis, autophagy, inflammation, cell differentiation, and proliferation. Under non-stressed conditions, the subcellular location of CHOP is mainly in the cytoplasm where it negatively affects cell migration-associated genes, while stress conditions lead to its nucleus translocation, partly *via* LIP, a C/EBPβ isoform (58), and its DNA-binding capacity therefore allows it to regulate gene expression. Nuclear CHOP can induce a transient cell cycle arrest in G_1 phase (20). During ER stress, CHOP also inhibits the growth arrest-specific p20K genes, which are a group of genes that activate reversible G_0 arrest to regulate cell proliferation (59). Generally, it is known as an important node in the transcription factor network that dominates stress-inducible regulation of specific target genes. *CHOP* deficiency does not produce a substantial phenotype without a stress signal.

CHOP in Apoptosis Modulation and Signaling

During a stress situation, UPR attempts to increase protein-folding capacity and remove misfolded and unfolded proteins. If the remedy is inadequate to restore homeostasis under chronic ER stress, terminal UPR will trigger apoptosis through abundant signaling mechanisms, mainly mediated by CHOP, JNK, and caspase-12, with CHOP as the most widely studied (60). It is notable that CHOP expression itself is not sufficient to induce apoptosis unless exposed to a stress signal. Studies in both cellular and animal models with *CHOP* gene deficiency have shed light on the pro-apoptotic role of CHOP during cellular stress (61, 62). Synoptically, CHOP-dependent apoptosis is mainly mediated by altering the expression of pro-apoptotic or anti-apoptotic genes, either directly or indirectly (1, 63). Both the intrinsic, mitochondrial pathway and extrinsic, death receptor pathway of classic apoptosis can be activated by CHOP and proceed with a set of initiator caspases and common executioner caspases (64). Overall, the apoptotic pathways mediated by downstream targets of CHOP form networks (**Figure 3**), wherein the molecular interaction mechanisms remain to be further understood.

Bcl-2 Family

The B-cell lymphoma 2 (BCL2) family of proteins includes anti-apoptotic members, such as BCL2-like, and pro-apoptotic members, such as BH3-only and BAX-like. As a widely cited mechanism for the mitochondrial apoptotic pathway, CHOP induces the upregulation of certain BH3-only proteins, such as BIM (65), PUMA (66), while inhibiting the expression of BCL2 to release its sequestration of BH3-only proteins (67). Thus, they regulate BAX–BAK homo-dimerization and consequent mitochondrial outer membrane permeabilization, causing release of cytochrome *c* and stimulation of an apoptotic signaling cascade (68). A recent study pointed to a role for BOK, another BAX-like protein, in regulating ER stress-induced apoptosis through CHOP

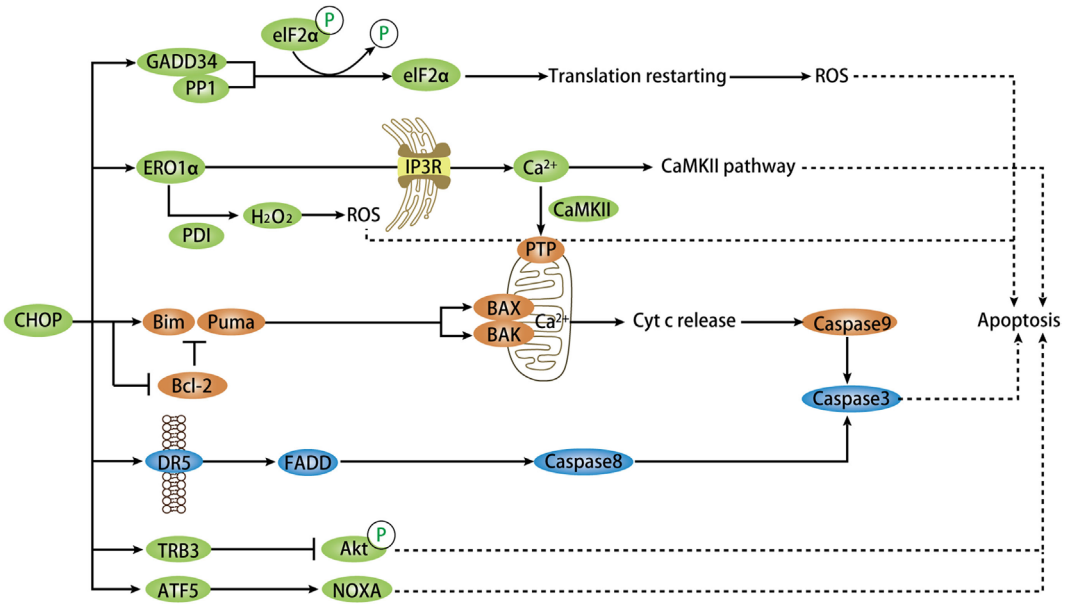

FIGURE 3 | Model depicting targets of CHOP-dependent apoptosis. During chronic ER stress, CHOP activation mediates pro-apoptosis signaling *via* numerous targets and pathways directly or indirectly. CHOP triggers the intrinsic apoptotic pathway through inhibition of BCL-2 and upregulation of BIM and PUMA, which regulate BAX–BAK-mediated mitochondrial outer membrane permeabilization. This leads to cytochrome *c* release and caspase cascade. CHOP also directly induces the expression of DR5-mediating extrinsic apoptotic pathway *via* FADD and caspase8-mediated cascade. In normal conditions, CHOP-dependent ERO1α induction oxidizes PDI to produce ROS that plays a critical role in apoptosis. The ERO1α–IP3R–Ca^{2+}–CaMKII pathway, in addition to ROS, can trigger several apoptotic pathways, primarily the Ca^{2+}-dependent mitochondrial apoptosis *via* PTP. GADD34 is a key target of CHOP and ATF4 and combines with PP1 to promote dephosphorylation of phospho-eIF2α. This event renews protein translation that promotes apoptosis in certain stress settings. Another target is TRB3 that prevent Akt phosphorylation in this apoptotic pathway. ATF5, downstream of CHOP, facilitates apoptosis through activation of some pro-apoptotic genes, such as NOXA. CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; ATF, activating transcription factor; ROS, reactive oxygen species; DR5, death receptor 5; FADD, Fas-associated death domain; GADD34, growth arrest and DNA-damage-inducible protein 34; PP1, protein phosphatase 1; PDI, protein disulfide isomerase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; TRB3, tribbles-related protein 3; ERO1α, ER oxidase 1α.

as evidenced by diminished activation of CHOP, diminished activation of BIM and apoptosis in $Bok^{-/-}$ mice (69).

Death Receptor 5 (DR5)

C/EBP homologous protein has been proven to directly control the transcription of the TNF family member cell-surface DR5, which activates the adaptor Fas-associated death domain to trigger caspase8-induced apoptosis (70). The CHOP–DR5 model sensitizes several chemically challenged cancer cells to extrinsic apoptosis mediated by ROS *in vitro* (44, 71), and ATF4 both *in vivo* and *in vitro* (72). During this process, the upstream signaling of CHOP includes ATF4, ROS–MAPKs, or the ATF4–ATF3 axis (46, 52).

Growth Arrest and DNA-Damage-Inducible Protein 34 (GADD34)

During prolonged ER stress, PERK-induced CHOP expression directly upregulates the transcription of GADD34, which forms a complex with its cofactor protein phosphatase 1 to facilitate dephosphorylation of phospho-eIF2α and protein translation (63, 73). However, if the feedback protein synthesis increase does not revert proteostasis, GADD34 upregulation can cause further misfolded proteins aggregation and ROS production to promote apoptosis.

ER Oxidase 1α (ERO1α)

Reactive oxygen species-dependent oxidative stress in ER stress is induced by the CHOP target ERO1α (74). Normally, ERO1α is responsible for disulfide bond formation by oxidizing protein disulfide isomerase. This process is coupled with the production of hydrogen peroxide (H_2O_2), which raises ROS generation and causes cell death if excessively produced (75). This is in line with another report on CHOP knockdown that showed decreased H_2O_2 formation and ROS-induced apoptosis (76). Faced with stress, CHOP activates calcium-mediated apoptosis through ERO1α, which activates inositol-1,4,5-trisphosphate receptor (IP_3R), the ER calcium channel that mediates Ca^{2+} efflux (77). As a result, the cytosolic Ca^{2+} activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) that triggers multiple apoptotic pathways including JNK signaling. The ROS signal generated from one of these pathways can conversely amplify CHOP activation *via* a positive feedback loop (31). Another pathway is that of CaMKII, which promotes the uptake of Ca^{2+} by mitochondria through mitochondrial permeability transition pores to activate a mitochondrial apoptotic pathway (78). Collectively, ERO1α induces oxidative stress and Ca^{2+}-mediated mitochondrial impairment in ER-stressed cells, which lead to CHOP-dependent apoptosis.

Tribbles-Related Protein 3 (TRB3)

Past research has demonstrated that CHOP–ATF4 cooperates to transactivate the transcription activity of TRB3 that contributes to CHOP-induced apoptosis in various cells types, such as cardiomyocytes (79). TRB3 binding to prevent Akt phosphorylation is probably the underlying mechanism of its pro-apoptotic function (80). The binding site of CHOP overlaps the amino-acid response elements in TRB3 promoter and respective specific regions in CHOP and TRB3 protein are responsible for their interaction (81).

Han and his colleagues have proposed a novel mode of CHOP-induced apoptosis where CHOP–ATF4 heterodimer binds to promoters of genes involved in protein synthesis, such as Gadd34, Trb3, Atf3, and Wars. Forced expression of CHOP and ATF4 evokes increased protein synthesis, consequent ATP depletion, and oxidative stress, thereby leading to cell death (23). This is in line with previous research on CHOP knockouts that showed less protein aggregation, ROS, and apoptosis (63, 82). Advances made in understanding the mechanism of ER stress-induced apoptosis have recently gained new impetus for the analysis of microRNAs in cells challenged with the ER stressor tunicamycin (TUN). For example, the miR-216b is proved to be a direct target of CHOP and thereby executes its pro-apoptotic activity by suppression of c-Jun expression (83). Full induction of ATF5 expression requires the upstream regulation of CHOP and the CARE element of its promoter can be bound by ATF4 and CHOP. As such, ATF5 potentiates CHOP-dependent apoptosis through activation of pro-apoptotic genes, including NOXA, during proteostasis imbalance (84). Another novel target of CHOP-mediated apoptosis is p21, a prominent cell cycle regulator with strong anti-apoptotic activity. The direct suppression of p21 transcription by CHOP is important for the pro-apoptotic pathway *in vivo* and *in vitro* (85, 86). CHOP also binds to the promoter of lipocalin 2, which mediates apoptosis in lung cancer cells in response to ER stress (87).

Recent research has also shown that CHOP does not elicit apoptotic processes under stress conditions in some specific cell types, such as myeloid-derived suppressor cells (MDSCs) (88) and myelinating glial cells (89). In light of the data, a distinct model of apoptosis proposed with an UPR cycle places CHOP in an obligatory step upstream of GADD34 that dephosphorylates p-eIF2α and resumes global protein synthesis, which is the decisive matter of cell fate (89). In this context, cells attempt to restore homeostasis while events including calcium loss, ATP depletion, and oxidative stress, eventually lead to cell death. If cells survive these events, protein aggregation due to subsequent stress drives another UPR cycle. This is consistent with a previous study in mouse embryonic fibroblasts (MEFs) overexpressing CHOP that implies forced expression of CHOP alone is not sufficient to induce apoptosis (23).

Autophagy

To cope with an inadequate protein-folding environment in ER, cells activate autophagy, an early stress-adaptive self-eating process, responsible for lysosome-dependent degradation of protein aggregates and other cellular material through UPR signaling (90). Many cellular stresses can trigger autophagy or apoptosis depending on specific circumstances and autophagy usually precedes apoptosis. CHOP has been implicated in autophagy induced by amino acid starvation, ER stress, virus infection, and hypoxia.

During amino acid starvation and ER stress, CHOP binds to the promoters of a set of autophagy genes (91). The time course analysis provides a further understanding that its upregulation of autophagy genes is within a short period of leucine starvation in cells. However, as time goes on, CHOP turns to inhibit the activation of these genes. It also inhibits autophagic flux and the conversion of microtubule-associated protein 1A/1B-light chain 3B (referred to as LC3), which is a key step for autophagy formation. The UPR-controlled balance of cell fate is therefore inclined toward cell death (92). Moreover, CHOP modulates the induction of autophagosomes during ER stress, as evidenced by the inhibition of LC3-II expression and GFP-LC3B dots (93). The results also showed that CHOP upregulated IRE1α, which contributed to autophagy induction, but their specific role and relation in this autophagy pathway remain unknown. Similarly, CHOP-mediated Licochalcone A-induced autophagy in non-small cell lung cancer cells and HeLa cells, while knockdown of CHOP reversed autophagy by reducing LC3-II and GFP-LC3 expression (94). Another study implied that UPR-activated CHOP elicited complete autolysosome maturation in hepatitis C virus-induced autophagy *via* LC3B-II-dependent mechanism (95). In the context of hypoxia, CHOP induces the expression of the autophagy gene *Atg5* by directly binding to its promoters (96).

Cell Differentiation

C/EBP homologous protein is involved in the block of differentiation in mesenchymal lineages. It is a fundamental regulator of adipogenesis, a role that has been supported by numerous experiments. The terminal differentiation of adipocytes is necessary for efficient lipid storage. CHOP was initially found to negatively regulate adipocyte differentiation *in vitro*, in response to metabolic stress (97), hypoxia (98), and when phosphorylated by stress-induced p38 MAPK (11). In subsequent studies, both *in vivo* and *in vitro* adipocyte differentiation is inhibited by CHOP expression after PERK–eIF2α activation during ER stress (99). As for the specific mechanisms, under polyamine depletion, CHOP exerts the inhibitory effect through interaction with C/EBPβ, thus impairing its role in the execution of mitotic clonal expansion process and in the transcriptional activation of peroxisome proliferator-activated receptor γ (PPAR-γ) and C/EBPα; the predominant regulators of adipogenesis (100). This is consistent with the molecular mechanism whereby CHOP impairs the differentiation of preadipocyte in response to aging (101). Higher levels of CHOP have led to hyperplasia of adipose tissue with less differentiated adipocytes in mouse models and downregulation of CHOP mRNA is required for complete adipocyte differentiation of MEFs (102).

Besides adipocytes, CHOP was also identified as a negative modulator for osteoblast differentiation. In terms of the mechanism, CHOP inhibits the binding activity of C/EBPβ against Runx2, thus suppressing their induction of the osteocalcin (*Ocn*) gene and phosphatase activity (103). This negative modulation is also supported by recent experiments. Osteoblast proliferation

and differentiation can be regulated by ADP-ribosylation-like factor 6 interacting protein 5 (Arl6ip5), whereas CHOP is required for reduction of the above two events and induces apoptosis in Arl6ip5-knockdown osteoblasts (104). The promotion of osteoblast differentiation by the transcription factor EB is coupled with reduced expression of ATF4 and CHOP. However, their expression was upregulated by TFEB overexpression in the stimulation of bone morphogenic protein 2 (BMP2), a potent inducer of osteoblast differentiation (105). It was previously proposed that CHOP may indirectly promote BMP2-induced osteogenesis (103) and enhance osteoblast differentiation of mesenchymal progenitor cells especially in the presence of BMP2 (106). Consistent with the finding, the PERK–eIF2α–ATF4 pathway promotes BMP2-induced osteoblast differentiation (107). Taken together, expression of CHOP exerts a dual role in osteoblast differentiation. It may be that the inhibitory action of CHOP is not sufficient to affect BMP2-induced differentiation processes. The specific association between CHOP and BMP2 in osteoblasts remains a topic of further exploration. Moreover, myoblast differentiation is inhibited by CHOP, which suppresses the transcription of myogenic regulatory factor in myoblasts by binding to its transcription regulatory sequences and affecting histone acetylation (108). Recent evidence has also unveiled that CHOP blocks the progression of myeloid lineage in granulomonocytic progenitors (109).

CHOP IN DISEASES

C/EBP homologous protein has widely documented roles in metabolism, neurodegeneration, and therioma. Recent findings have added the condition of fibrosis to this list of diseases mediated by CHOP. CHOP-mediated cellular apoptosis leads to organ dysfunction and may thus be involved in a wide range of diseases. We herein highlight recent advances that implicate CHOP in the occurrence, development, and outcome of diseases; in addition to outlining potential treatment strategies that target CHOP.

Fibrosis

Fibrosis progresses as an eventual pathological outcome of three stages of general wound-healing responses; injury, inflammation, and repair to persistent organ injury. A pathological hallmark of fibrosis is the excessive deposition of extracellular matrix (ECM) in the tissues. This increased protein synthesis may disturb ER homeostasis and induce the expression of CHOP (110). An emerging role of CHOP in promoting fibrotic response of internal organs is supported by alleviation of fibrosis in *CHOP*-knockout mice (111). Given that apoptosis is a common cellular event that leads to organ remodeling and fibrosis after insult, CHOP-initiated pro-apoptotic activity may be partly the underlying mechanism. Moreover, alternatively activated phenotype (M2) macrophages are considered to participate in promoting collagen deposition and fibrogenesis. In recent years, research advances have indicated that CHOP likely regulates the activation of M2 macrophages to trigger tissue fibrosis (112). These M2 macrophages would release various cytokines to create a microenvironment that favors fibrogenesis, including high levels of TGF-β1, which is a key factor that promotes the activation of ECM-producing myofibroblasts (113). Modulation of CHOP expression may be a potential treatment for organ fibrosis. Here, we show the vital role of CHOP in the formation of specific fibrotic disorders.

Lung Fibrosis

The crucial events in the pathogenesis of lung fibrosis include TGF-β activation and alveolar epithelial cells (AECs) apoptosis, which then trigger resident fibroblast proliferation and differentiation of ECM-producing myofibroblasts (114). Research in the past decade has established a relation between ER stress and lung fibrosis (115). CHOP, the key player in ER stress, was found to mediate methamphetamine (MA) and thrombin-induced apoptosis of AECs in chronic pulmonary injury or fibrotic lung tissue (116, 117). Likewise, analysis of lung tissue from mouse fibrosis models induced by bleomycin (BLM) and from patients with IPF has manifested altered CHOP expression along with ER stress. Indeed, the loss of CHOP expression protected mice from BLM-induced pulmonary injury and fibrosis (112, 118). Mechanistic investigation indicates that CHOP regulates the production of M2 macrophages and subsequent TGF-β1 signaling involved in lung fibrosis. Further studies dissected the mechanism through which *CHOP* deficiency reduced M2 macrophage infiltration, for it upregulated the STAT6 inhibitors SOCS1 and SOCS3, thus repressing STAT6/PPAR-γ signaling (112). Meanwhile, a different viewpoint has been posited that CHOP-mediated macrophage apoptosis provides protection for *Grp78*$^{+/-}$ mice against BLM-induced fibrosis (119). Nevertheless, both of these competitive findings support the role of CHOP in regulating macrophage to participate in the progress of lung fibrosis.

Kidney Fibrosis

Renal fibrosis, which includes glomerulosclerosis and/or tubulointerstitial fibrosis, is a common pathogenic consequence of chronic progressive renal diseases. Overwhelming expression of ER stress markers including CHOP are associated with fibrosis in rat kidneys subjected to unilateral ureteral obstruction (UUO) (120). Zhang and colleagues first noted that *CHOP*$^{-/-}$ mice were protected from UUO-induced renal fibrosis, wherein loss of CHOP decreased UUO-induced apoptosis of tubular cells and the Hmgb1/TLR4/NFκB/IL-1β signaling. Thus, the IL-1β downstream TGF-β1/Smad2/3 signaling was also inhibited, eventually ameliorating renal fibrosis (121). Consistent results were also obtained that indicate that *CHOP* deletion attenuated renal tubulointerstital fibrosis in the mouse UUO model. In addition, there are novel findings about these mechanisms that *CHOP* deficiency not only lessens tubular cell apoptosis but also abates profibrotic factors, oxidative stress, and recruitment of inflammatory cells including macrophages (111). Recently, both in patients with renal fibrosis resulting from chronic kidney disease (CKD) and a mouse fibrosis model of hypertensive CKD, an increase in *CHOP* gene was demonstrated. Furthermore, inhibition of CHOP by an ER stress inhibitor, 4-phenylbutyric acid, attenuated renal interstitial fibrosis, as well as macrophage infiltration and TGFβ1 expression. *CHOP*-knockout mice developed less renal fibrosis accompanied by lower macrophage infiltration (122). Moreover,

Pan et al. found that M2 macrophages specifically enhance epithelial-to-mesenchymal transition and subsequent renal fibrosis by high production of TGFβ1 in a mouse UUO model (113). In addition, a function of CHOP in inducing fibronectin production in tubule cells was identified, indicating its role in promoting tubulointerstitial fibrosis during diabetic nephropathy (DN) (123).

Liver Fibrosis

Hepatic stellate cells (HSCs) are the principal cell-type responsible for ECM production and collagen deposition during liver fibrogenesis in which it is activated into myofibroblast in a TGF-β1-dependent manner. An early study on hepatic fibrosis in cholestatic liver injury caused by bile duct ligation showed that *CHOP* deletion alleviated hepatocyte death and hepatic fibrosis, with inhibitory effect on TGF-β1 induction and HSCs activation (124). *CHOP* deficiency also attenuated liver fibrosis in HCC induced by diethylnitrosamine (DEN) (125), and a fat-loading, methionine-choline-deficient diet (126). As illustrated by the aforementioned studies, hepatocyte apoptosis is a cellular mechanism underlying the promotion of CHOP for hepatic fibrotic response (127). Moreover, CHOP was significantly upregulated in the liver from animals with CCl_4-induced fibrosis (128). Another study showed that pronounced CHOP expression was stimulated by hepatitis B virus (HBV) surface proteins and correlated with increased liver injury and fibrosis in HBV transgenic mice on BALB/c background, as compared to C57BL/6 (129). Ablation of *CHOP* attenuated hepcidin suppression and ensuing iron overload in a mouse liver fibrosis model induced by thioacetamide (130). In contrast, liver fibrosis induced by dietary steatohepatitis was greater in *CHOP*$^{-/-}$ mice, due to lessened CHOP-induced apoptosis of activated macrophages (131).

Taken together, in both mouse renal fibrosis and pulmonary fibrosis models, the deletion of the *CHOP* gene resulted in a marked decrease in inflammatory infiltration of macrophages, embodying a reduced differentiation of M2 macrophages, which are considered as a new cell-type involved in fibrogenesis. To the best of our knowledge, no studies have yet found that CHOP can regulate M2 macrophages in hepatic fibrosis, while it has been verified that M2 macrophages play an important role in schistosomiasis-induced liver fibrogenesis *via* IL-13/STAT6 signaling pathways (132). Hepatic fibrosis during schistosomiasis may represent a class of special cases where macrophage is actively involved in host immune responses against schistosome infection. Macrophages have long been accepted as profibrotic in schistosome infection. However, there are reports showing that restorative macrophages, characterized by an anti-inflammatory anti-fibrogenic expression profile, are in fact key to the remodeling and resolution of liver fibrosis (133, 134). Therefore, further studies investigating the role of CHOP in different macrophage subsets during liver fibrosis may advance our understanding of the involvement of CHOP in liver fibrosis.

Cardiac Fibrosis

It has also been suggested that CHOP may be involved in cardiac myocyte apoptosis, cardiac hypertrophy, and heart failure (135, 136). A high-fat diet fed to metabolically healthy, obese minipigs activates oxidative stress and ER stress with increasing expression of CHOP in myocardial fibrosis within the minipigs (137). In mice subjected to a transverse aortic constriction operation, ablation of CHOP can attenuate cardiac hypertrophy, cardiac dysfunction, and fibrosis with less apoptotic cell death (138) and alleviate myocardial reperfusion injury *via* attenuated myocardial apoptosis and inflammation (139).

Cancer

In states of uncontrolled proliferation and insufficient vascularization (e.g., in cancer), conditions of low nutrient supply, such as hypoxia and oxidative stress, may trigger ER stress and subsequent UPR activation that have been documented in various human cancers (140). Studies have shown evidence of CHOP activation in various types of cancer cells (125, 141). Thus, CHOP-induced apoptosis in ER stress has significant implications for cancers. Furthermore, *CHOP* mutations are found in some human tumors, although whether or not they contribute to tumorigenesis remains unknown (142). Accumulating data suggest that CHOP impinges upon several aspects of cancer including tumor formation as well as progression of tumors once formed. Nonetheless, how CHOP activation exerts tumor-supporting or tumor-suppressive roles remains to be elucidated.

The Role of CHOP in Carcinogenesis

Most of the evidence supports an anti-oncogenic function of CHOP-induced apoptosis in a stressful environment. Indeed, apoptosis is a critical mechanism for maintaining tissue homeostasis through selective elimination of cells once they are damaged, mutated, or pose a threat to the organism, such as precancerous cells. During stress conditions, the human hematopoietic stem cell (HSC) pool maintains integrity by elimination of individual HSCs through PERK–eIF2α–ATF4–CHOP–GADD34 signaling induced apoptosis, hence preventing persistent cloning of oncogenic mutations and decreasing the risk of leukemogenesis (73). CHOP induction triggers apoptosis of premalignant cells to prevent malignant progression in a mouse lung cancer model (143). Hepatocyte-specific *CHOP* ablation increased tumorigenesis in high fat diet-induced steatohepatitis and HCC. This effect indicates a tumor-suppressive role of CHOP, perhaps *via* apoptosis of initiated hepatocytes in preneoplastic lesions (144). However, CHOP tends to promote specific oncogenic processes, at least in one case; when fused with FUS/TLS or EWS protein by genomic rearrangement (145, 146). The FUS–CHOP oncoprotein has been newly proved to induce metastasis *via* transcriptional induction of tumor-associated proteases, both in liposarcoma and fibrosarcoma cell lines, as well as an *in vivo* model (147). Specifically, DeZwaan-McCabe et al. (125) proposed that ISR-induced CHOP provokes inflammation and fibrosis followed by compensatory proliferation to promote chemical hepatocarcinogenesis (148). CHOP was upregulated both in genetic and DEN-induced mouse models of HCC, as well as human HCC. *CHOP*$^{-/-}$ mice were protected from DEN-induced oncogenesis in liver, which was also proven by Scaiewicz et al. (149). The latter further found a marked reduction of IFNγ levels and macrophages in CHOP-knockout tumors and ATF6 activation upstream of CHOP, implying that CHOP induction regulates inflammation and

macrophage infiltration to promote hepatocarcinogenesis after DEN treatment. In actuality, the carcinogen DEN evokes DNA damage and the apoptosis induced by this damage partly contributes to chronic inflammation and release of tumor-promoting cytokines. Collectively, CHOP may play an anti-oncogenic role in the precancerous cells and an oncogenic role when expressed in macrophages.

CHOP in Cancer Development and Progression

During the progression of tumors, CHOP triggers the death of a number of tumor cells, which has been reviewed (3). In the case of hepatoma cells, CHOP mediates the autophagic apoptosis induced by apoptosis-stimulating protein of p53-2 (150). However, the tumor-supporting functions of CHOP have been newly indicated in certain cells. Thevenot et al. found that the MDSC with immunosuppressive activity within tumors aberrantly expressed CHOP without completely undergoing apoptosis. *CHOP* deficiency induced antitumor effects in a MDSC-dependent manner, suggesting an important role of CHOP in tumor tolerance and potential benefits of its inhibition for tumor immunotherapy (88, 151). Moreover, SPOP mutations fail to mediate CHOP degradation and suppress CHOP-induced apoptosis, which indicates CHOP involvement in the progression of prostate cancer is associated with SPOP mutations (9). A common hallmark of a tumor microenvironment is hypoxia, during which cancer cells can activate pathways to develop and progress, such as immune responses. CHOP expression, in the context of ER stress and TLR agonists, increases dendritic cell expression of IL-23 (152), which supports T helper 17 cell propagation and its function to promote immune response and tumor growth (153). Induction of autophagy by ATF4 and CHOP helps several human cancer cell lines adapt to hypoxia (96), whereas a previous study suggests that cyclophilin B mediates the adaptation of tumor cells to hypoxia through ubiquitin-dependent degradation of CHOP (154). Overall, further understanding of the pro- and anti-oncogenic roles of CHOP and information on the cell types where CHOP is activated or suppressed in different stages of cancer may provide insight into different carcinogenesis modalities and promote its implication for cancer therapy.

CHOP in Cancer Therapy

Here, we also highlight recent advances in therapeutic strategies for cancer treatment that involve CHOP. CHOP-induced cell death has been widely suggested as one of the strategies to ameliorate cancer (**Table 1**). First, experiments with cancer cells have demonstrated that CHOP-mediated DR5 expression is responsible for the caspase8-mediated apoptotic pathway (70). Therefore, various natural and synthetic products that enhance CHOP–DR5 signaling have been presented for treatment of a series of cancers (45, 71). Second, a selective CHOP inducer, sulfonamidebenzamide, was identified with pro-apoptotic and antiproliferative effects in multiple cancer cell lines (155). Asparagine was found as a CHOP inhibitor with anti-apoptotic function, and suppression of asparagine synthetase may restore CHOP-induced cell death and exert therapeutic benefit in solid tumors (156). Finally, CHOP forms a complex with C/EBPβ and decreases C/EBPβ-dependent ALDH1A3 expression in chemoresistant cell subpopulations. This mechanism may be responsible for butein-induced enhancement of chemoresistant cell apoptosis (157) and contributes to the treatment of non-small cell lung cancer with garcinol (158). The suppression of STAT3–NFκB activity by butein is a prerequisite for high levels of CHOP expression (157). Moreover, CHOP downregulated the anti-apoptotic p21 in cancer cells treated with TUN, thus enhancing chemotherapeutic drug efficacy (86). During radiotherapy with high-LET carbon ions, chloroquine co-treatment enhances apoptosis *via* IRE1–CHOP signaling *in vitro* and *in vivo* (159). Besides apoptosis, CHOP regulated androgen receptor degradation in prostate cancer cells treated with rosemary extract (160). All types of oncogenic FUS–CHOP fusion proteins can be inactivated by trabectedin through blockage of their binding to target promoters, both in a mice xenograft model and human cell lines, thereby exerting a selective antitumor activity (161).

Diabetes

In diabetes mellitus, the glucostatic cycle to maintain normoglycemia is dysregulated due to an insufficient mass of functioning pancreatic β-cells to synthesize the needed amounts of insulin for metabolism (162). For type 2 diabetes (T2D), insulin resistance under stress of excess nutrients, including hyperglycemia and hyperlipemia, causes progressive β-cell failure (163). Dissimilarly, in type 1 diabetes (T1D), β-cells are attacked by autoimmune activity and the workload of the remaining β-cells increases. Collectively, high demand of insulin synthesis and secretion overwhelms the capacity of β-cell ER and thereby activates UPR to compensate. As the process continues, terminal UPR leads to apoptosis of β-cells and the onset of diabetes (164).

Apoptosis has been the main focus of studies on β-cell dysfunction during diabetes, among which CHOP-induced apoptosis is the most studied, as it is a key event in the pathogenesis of diabetes (165). Indeed, numerous studies have found that islet cells from mice and patients with T1D or T2D manifests elevated levels of CHOP (7, 166). In 2002, studies conducted with Akita mice proved that genetic removal of CHOP alleviated β-cell loss and hereditary diabetes, which vividly links CHOP to β-cell apoptosis for the first time (167). *CHOP* deficiency also prevents oxidative damage with reduced ROS and thereby improves ER function in β-cells (168), while oxidative stress is proven as an important factor that gives rise to β-cell dysfunction in diabetes (169). Thus, past studies have identified drugs, such as vildagliptin (170), that promote β-cell survival by decreasing CHOP expression in diabetic mouse models, along with downregulation of ATF4 and TRIB3 in T2D db/db mice (171).

For T2D, it has been evidenced that CHOP is responsible for β-cell apoptosis and dysfunction both in genetic and diet-induced mouse models of T2D, as well as *in vitro* (168, 172). The PERK/eIF2α/CHOP signaling mediated β-cell sensitization to lipotoxicity and apoptosis under the challenge of palmitate (173, 174) and guanabenz (175). Ubiquitination and degradation of CHOP by cellular inhibition of apoptosis protein-1 prevented palmitate-induced lipotoxicity (173). Consistent with this notion, the inhibition effect of CHOP on adipocyte differentiation interferes with effective fatty acid storage, which may cause lipotoxicity. Furthermore, the human islet amyloid polypeptide (h-IAPP),

TABLE 1 | Strategies to target C/EBP homologous protein (CHOP)-mediated cell death for cancer treatment in preclinical models.

Cancer type	Treatment agents	Involved mechanisms and phenotype	Research models	Reference
Pancreatic cancer	CGK733	Induces calcium sequestration in reversible vesicles through PRKR-like ER kinase (PERK)-CHOP signaling and subsequent non-apoptotic/necrotic cell death	Cells	(207)
Hepatocellular carcinomas	IMB-6G	Induces mitochondrial-dependent apoptosis *via* PERK–CHOP signaling Induces IRE1α–ASK1–JNK mediated apoptosis	Cells	(208)
	Piperlongumine	Increases reactive oxygen species (ROS) and activates endoplasmic reticulum (ER)–mitogen-activated protein kinases (MAPKs)–CHOP signaling *in vivo* and *in vitro* to trigger cell death Suppresses migration/invasion	Cells, mice (xenografts)	(43)
Triple-negative breast cancer	YM155 (surviving suppressant)	Upregulates p38 mitogen-activation protein kinase (p38 MAPK)- and CHOP-mediated DR5 expression to induce apoptotic response Impairs cell growth and increases cytotoxic effect	Cells, mice	(209)
Non-small cell lung cancer	Obovatol	Activates CHOP-induced apoptosis	Cells	(210)
	Licochalcone A	Induces CHOP-dependent apoptosis and autophagy	Cells	(94)
Ovarian carcinoma	Tanshinone IIA	Activates extrinsic apoptosis by JNK–CHOP–DR5 signaling	Cells	(44)
Colon carcinoma	Apigenin	Activates CHOP-mediated intrinsic and extrinsic apoptotic pathways with ROS generation and Ca^{2+} release Exerts anti-proliferation and cell cycle arrest role	Cells	(211)
Colorectal cancer	Rapalogs and ATP-competitive mTOR inhibitors	Activates CHOP–DR5 axis-dependent extrinsic apoptosis pathway	Cells	(212)
Multiple myeloma	Histone deacetylase 4 inhibitor	Activates activating transcription factor (ATF)4–CHOP-induced apoptosis Enhances the cytotoxicity of ER stressor	Cells, mice	(213)
T-cell lymphoblastic lymphoma and T-cell acute lymphoblastic leukemia	LAT1 selective inhibitor	Induces ATF6, ATF4, eIF2α, growth arrest and DNA-damage-inducible protein 34, p38 MAPK expression and triggers CHOP-dependent apoptosis Decreases activation of Akt and mTORC1 Decreases cell viability and proliferation	Cells, mice (xenografts)	(214)
T-cell acute lymphoblastic leukemia	Inhibitor of CK2α	Activates apoptosis induced by IRE1α and CHOP Downregulates PI3K/Akt/mTOR signaling and the levels of GRP78 Exerts cytotoxic and cytostatic effects	Cells	(215)
Oral squamous cell carcinoma	Celastrol	Induces cell death through PERK–eIF2–ATF4–CHOP signaling	Cells, murine embryonic fibroblasts	(216)
Human esophageal cancer	Neddylation inhibitors (MLN4924)	Induces ATF4–CHOP–DR5-mediated extrinsic apoptosis Triggers ATF4–Noxa axis-mediated intrinsic apoptosis	Cells, murine	(72)
Glioblastoma multiform	Isochaihulactone	Induces CHOP–NAG1-mediated apoptosis independent of PERK	Cells, mice (xenografts)	(217)

also characteristic of T2D, induced dysfunction of autophagy and apoptosis through CHOP, but inhibition of CHOP alone may not be a durable therapeutic strategy for the β-cell toxicity of h-IAPP, considering multiple stress pathways are activated during this process (176). To unveil the specific mechanisms underlying β-cell apoptosis in T2D, researchers found that CHOP regulation of puma is essential for the apoptotic pathway during glucotoxicity T2D (177). Another critical event is that CHOP downregulates p21 to trigger β-cell apoptosis due to glucotoxicity, thus promoting the onset of T2D (85, 178). As such, the chemicals that inhibit CHOP expression protect β cells from apoptosis and dysfunction, such as 1,2,3-triazole derivatives (179).

For insulinopenic T1D, autoimmunity triggers an inflammatory response along with cytokine release which induces ER stress in β-cells. CHOP contributes to cytokine-induced apoptosis of β-cells *via* mitochondrial apoptotic pathways and indirect pro-inflammatory responses, indicating the role of CHOP in T1D. Mechanistic studies have shown that CHOP knockdown in insulinoma cell lines protected against the downregulation of anti-apoptotic BCL-2-like proteins, Bcl-2 and Mcl-1, while decreasing NF-κB activity and expression of its target genes, including inducible NO synthase (iNOS) and TNF receptor superfamily member 6 (FAS) (180). CHOP blocking by siRNA partially protected human beta cells against cytokine-induced apoptosis independent of NO, whereas CHOP induction was NO dependent and could be inhibited by iNOS blocker in rat insulin-producing cells (181). Moreover, CHOP acted as a mediator of β-cell apoptosis in islets deficient for Gata4, which belongs to a group of β-cell survival factors that contribute to T1D risk when they undergo mutations (182).

Overall, cumulating evidence suggests that CHOP is involved in the pathogenesis of diabetes, predominantly T2D, in response

to glucotoxicity, lipotoxicity, as well as oxidative stress and islet amyloid derived from IAPP. With regard to diabetic complications, there is also some relevance to CHOP. For example, during murine DN, ATF6-dependent CHOP activation was induced by defective insulin signaling due to impaired nuclear translocation of sXBP1 in podocytes (34), whereas *CHOP*-null mice gained protection from DN (183). Tubules of diabetic mice and patients showed increased levels of CHOP protein, and other than apoptosis, CHOP-induced expression of fibronectin in tubule cells (123). As for diabetic cardiovascular complications, including diabetic cardiomyopathy, IL-1β-induced myocyte apoptosis was mediated by the IRAK-2/CHOP pathway (184). CHOP-induced apoptosis was partly targeted by Ginsenoside Rg1 to ameliorate diabetic myocardial damage (185). Correspondingly, myocyte apoptosis and cardiac dysfunction induced by methylglyoxal were attenuated in *CHOP*-null mice (62). Furthermore, streptozotocin-induced diabetic $CHOP^{-/-}$ mice manifested not only reduced hyperglycemia but also lessened severity of oxidative-nitrative stress in their sciatic nerve and in their eventual diabetic peripheral neuropathy (186).

Neurodegeneration

Neurodegenerative diseases are hallmarked by progressive loss of neuronal function. Many risk factors including aging, oxidative stress, and gene mutations of neurodegenerative process can cause toxic accumulation of misfolded proteins, which ultimately leads to neuronal cells undergoing ER stress-induced apoptosis. As a key player in ER stress and oxidative stress, CHOP expression is found to be elevated in many disorders related to neurodegeneration, such as the Parkinson disease (PD) (187), and CHOP induces neuronal apoptosis, which has been proposed as a target of treatments for some neurodegenerative diseases (188). In some cases, disruption of CHOP exerts a neuroprotective role through yet unknown mechanisms (189). We list here important studies on CHOP involvement in neurodegenerative diseases in recent years.

A prominent clinical hallmark of Alzheimer's disease (AD) is progressive cognitive impairment. AD is attributed to pathological deposits of neurofibrillary tangles formed by hyperphosphorylated tau aggregates and abnormal aggregation of amyloid-β (Aβ) plaques. In the AD brain, ATF4 synthesis in axons locally exposed to $A\beta_{1-42}$ triggered retrograde cell loss through CHOP, and conversely, *CHOP* deletion hindered $A\beta_{1-42}$-mediated neurodegeneration (190). In the mouse model of AD and neuroblastoma cells, researchers analyzed the effect of palmitate and noticed that CHOP indispensably mediated increased β-site APP-cleaving enzyme 1 (BACE1) activity and ensuing Aβ production, but only partially (191). In agreement with this, silencing *CHOP* expression attenuated NF-κB activation and its binding to the BACE1 promoter, thus reducing Aβ production induced by 27-hydroxycholesterol (192). CHOP knockdown also alleviated the negative regulation of C/EBPα binding to the leptin promoter and subsequent leptin expression, which is able to decrease Aβ genesis and tau phosphorylation (193). As for PD, CHOP and ATF4 play a key role in regulating Trib3 and apoptosis in cellular models of PD, as evidenced by the protective role of CHOP and ATF4 knockdown in 6-OHDA and MPP(+) models (194). Inhibition of CHOP-mediated crocin-induced neuroprotection in the PD model through Wnt pathway *in vitro* (195).

In S63-deletion mice of Charcot–Marie–Tooth (CMT) disease type 1B, *CHOP* deletion decreased demyelination and rescued their motor deficit (196). Mechanistic studies subsequently revealed that CHOP targeted GADD34 to reactivate translation in the nerves of this model (197). Surprisingly, CHOP ablation did not rescue the abnormalities of Schwann cell development in R98C mouse model of type 1B CMT (198). Prion-related diseases are another type of neurodegenerative disorder, and accumulation of prion protein (PrP) defines the pathobiology. Upregulation of CHOP through the PERK pathway is a pathogenic factor of neurodegeneration induced by the membrane-tethered flexible tail of PrP (199). A role of CHOP in mild spinal cord injury is indicated by enhanced neuronal functional recovery in CHOP-deficient mice, partly due to decreased oligodendrocyte apoptosis (200). Moreover, CHOP and caspase12 induced neuron apoptosis at later stages of chemical hypoxia (201). In the context of neurodegeneration in retinas, a sustained upregulation of CHOP can result from optic nerve injury and *CHOP* deficiency increased the survival of retinal ganglion cells (202). Apoptotic cell death of photoreceptors was also mediated by CHOP in retinas deficient in autophagosomes (203). Likewise, in brain astrocytes, MA mediated CHOP upregulation downstream of the activation of all three ER stress pathways, which together lead to apoptosis *via* intrinsic caspase cascade (204). This arises as the mechanism of MA-mediated neurodegenerative effects. The PERK–eIF2α–ATF4–CHOP pathway mediated sevoflurane-induced neuroapoptosis in neonatal brains (205). Moreover, CHOP expression in the brain plays a pivotal role in the negative regulation of two neurotrophic cytokines, leptin and insulin-like growth factor-1 by palmitate (206).

SUMMARY AND PERSPECTIVES

In summary, induction of CHOP is converged from the regulation of UPR, ISR, and MAPKs signaling in response to various cellular stress conditions, including ER stress and ROS. CHOP can be protective for cell survival *via* regulating autophagy in early stages (before irreversible ER stress). This stress-responsive transcription factor has been extensively recognized as the link between prolonged protein-folding stress, namely ER stress and apoptosis. To the best of our knowledge, no studies have yet shown that CHOP directly leads to apoptosis. CHOP indirectly regulates apoptosis by controlling the expression of pro-apoptotic or anti-apoptotic genes. Therefore, we have constructed a signal network depicting canonical and emerging targets of the CHOP-dependent apoptotic pathway, including the BCL-2-mediated intrinsic and DR5-mediated extrinsic apoptotic pathway. Collectively, they can result in protein aggregation, disturbance of redox status, and mitochondrial function to culminate in apoptosis. Inhibition of CHOP is an approach to improve the survival and function of cells. However, in certain conditions, CHOP expression does not induce cell apoptosis. Given the dual role of CHOP, whether it is

more a pro-apoptotic or a protective factor remains to be defined in specific cell types. Cells behave differently to CHOP induction owing to the intensity and duration of stress, as well as distinct cell and disease context.

C/EBP homologous protein impinges upon different process such as autophagy, apoptosis, and cell differentiation. From an overview of its impact on different diseases so far, apoptosis is a major cellular function of CHOP that involves it in pathological processes for a wide range of diseases. CHOP-dependent apoptosis may exert amelioration or aggravation effects on different diseases. It has been increasingly implicated as a treatment strategy in the context of cancer and more *in vivo* research is needed to evaluate the efficacy. In addition, the emerging roles of CHOP in the progress of fibrosis and regulation of macrophage polarization open up new avenues for future research. The broader functions and molecular mechanisms of CHOP in physiopathology will continue to be unveiled to target it in potential therapeutic strategies.

AUTHOR CONTRIBUTIONS

Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content.

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Reduced Endoplasmic Reticulum Stress-Mediated Autophagy Is Required for Leptin Alleviating Inflammation in Adipose Tissue

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Leptin is an adipocyte-derived hormone and maintains adipose function under challenged conditions. Autophagy is also essential to maintain cellular homeostasis and regulate characteristics of adipose tissue. However, the effects of leptin on autophagy of adipocyte remain elusive. Here, we demonstrated endoplasmic reticulum (ER) stress and leptin were correlated with autophagy and inflammation by transcriptome sequencing of adipose tissue. Leptin-mediated inhibition of autophagy was involved in upstream reduction of ER stress proteins such as Chop, GRP78, and Atf4, since blockage of autophagy using pharmacological approach had no effect on tunicamycin-induced ER stress. Moreover, we determined *KLF4*, the potential transcriptional factor of *Atf4*, was required for the leptin-mediated autophagy in the regulation of adipocyte inflammation. Importantly, ATF4 physically interacted with ATG5 and subsequently formed a complex to promote adipocyte autophagy. Further analysis revealed that Atg5, a core component of autophagosome, was the target for leptin-mediate autophagy. In addition, leptin alleviated ER stress-induced inflammation by reducing autophagy-mediated degradation of IκB in adipocytes. Exogenous leptin treatment also ameliorated autophagy and inflammation of white adipose tissue in *ob/ob* mice. Taken together, our results indicated that leptin inhibited ER stress-mediated autophagy and inflammation through the negatively regulation of Atf4/Atg5 complex in adipocytes. These findings identify a new potential means for intervention of autophagy to prevent or treat obese caused metabolic syndrome of mammals.

Keywords: leptin, endoplasmic reticulum stress, autophagy, Atf4/Atg5, inflammation, adipocyte

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INTRODUCTION

Metabolic disorders, especially obesity and diabetes, become the most important public health concern in worldwide. The pathophysiology seems to be largely attributable to endoplasmic reticulum (ER) stress, inflammation and cytokines resistance (e.g., leptin resistance and insulin resistance) in adipose tissue. Therefore, dynamic improvement of these disorders in adipose tissue provides an effective therapeutic strategy to alleviate severe systemic metabolic disorders.

Leptin is a hormone produced by peripheral adipose tissue. The ectopic serum leptin level is usually considered as a symbol of metabolic disorders (1–3). In particular, leptin signal is essential to mediate protective function in adipocytes under challenged conditions (1, 4, 5). Meanwhile,

obesity is usually associated with mild chronic inflammation in adipose tissue. Notably, inflammatory stimulation by tumor necrosis factor-alpha (Tnfα) and other cytokines initiate adipose inflammation through elevating unfolded protein response in the ER (6, 7). ER plays a critical role in protein synthesis, folding and transportation. Certain conditions, such as accumulation of misfolded proteins and uncontrolled energy homeostasis perturb ER homeostasis and lead to a condition defined as ER stress (8–10). Recent studies show that ER stress serves as a key etiologic component causing chronic inflammation in adipose tissue of obese human and mice (11, 12). Current evidence suggests that ER stress promotes inflammation through positive transcription regulation of inflammatory genes and coordinating the activities of critical inflammatory kinases such as nuclear factor-κB (NF-κB), c-Jun N-terminal kinase, and protein kinase R (13–15). Our previous study has shown that tunicamycin (TM) induces ER stress and reduces adiponectin by increasing the *activating transcription factor 2* (*Atf2*) transcription in mice adipose tissue (12). Since leptin is an adipocyte-derived hormone involved in obesity and inflammation (5, 16), the specific role of leptin in the progress of ER stress-induced adipose inflammation remains to be determined.

Autophagy is a highly regulated process that functioned in the maintenance of cellular homeostasis (17, 18). Recently, studies indicate activation of ER stress could initiate autophagy and inflammation in astrocytes (19, 20). Many studies also determine that ER stress triggers autophagy and subsequent lysosomal lipolysis in hepatocytes (21, 22). Furthermore, autophagy can directly degrade lipid droplets through hydrolysis of triglycerides into free fatty acids in adipose tissue (23, 24). But the relationship between ER stress and autophagy in adipocytes remains elusive. Generally, the autophagy-related genes (Atg) recruit damaged organelles to form autophagosomes and recycle the cargo by lysosomal degradation (23, 25, 26). Deletion of autophagy-related gene 7 (Atg7) blocks autophagy and promotes hepatic lipid accumulation (27). Adipocyte-specific deletion of Atg genes exhibit markedly decreased plasma concentration of leptin (28, 29). These findings suggest leptin may be correlated with autophagy by regulating ER stress in adipocytes.

In this study, we investigated the effects of leptin on autophagy and inflammation of adipose tissue. We show that leptin alleviates ER stress-induced inflammation through the activating transcription factor 4 (Atf4)/Atg5-mediated autophagy in mice adipocytes. Thus, our study has revealed the mechanistic link between leptin, autophagy and ER stress, providing novel insights into the pharmacologically therapeutic target for obesity and inflammation.

MATERIALS AND METHODS

Animal Experiment

Eight-week-old C57BL/6J male mice and *ob/ob* mice were purchased from the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, China). Mice handling protocols were conducted following the guidelines and regulations approved by the Animal Ethics Committee of Northwest A&F University (Yangling, China). Mice were provided *ad libitum* water and a standard laboratory chow diet purchased from Animal Center of the Fourth Military Medical University. Body weight was recorded weekly. The animal room was maintained at 25 ± 1°C, humidity at 55 ± 5%, and 12 h light/dark cycles.

Mice ($n = 24$) were randomly divided into four groups ($n = 6$ each). Half of the mice were intraperitoneally injected with saline (control), and the other half injected with TM (1 mg/kg, Sigma-Aldrich, MO, USA, T7765) or thapsigargin (Tg, 0.5 mg/kg, Sigma-Aldrich, MO, USA, T9033) for 3 and 2 days separately before the dark onset (12, 30–32). The TM injection was used to create ER stress in the mice adipose tissue. To address the effect of leptin on ER stress of adipose tissue, half of the mice that received saline or TM or Tg injection were further injected with 1 mg/kg recombinant murine leptin (Peprotech, NJ, USA, 450-31) into the tail vein two times a day for 7 days (15, 33, 34), and the other half still received saline injection. For the experiment of 4-phenylbutyric acid (4-PBA, 0.5 g/kg, Sigma-Aldrich, MO, USA, P21005) injection (35), mice ($n = 24$) were all injected with TM (1 mg/kg) and randomly divided into four groups ($n = 6$ each). Half of these mice received saline injection (control), and the other half received 4-PBA injection (0.5 g/kg) for 7 days. And then half of the mice that received 4-PBA or saline injection were injected with recombinant murine leptin (1 mg/kg) two times a day for 7 days. For the rapamycin (5 mg/kg, Sigma-Aldrich, MO, USA, V900930) injection experiment (36), we performed the same procedures as in the 4-PBA injection experiment. 4-PBA injection was used to block ER stress and rapamycin injection was used to activate autophagy. In the *ob/ob* mice experiment ($n = 18$), mice were randomly divided into three groups ($n = 6$ each group), one group of mice was the C57BL/6J mice [wild type (WT)], the other two groups were *ob/ob* mice. Half of the *ob/ob* mice were injected with saline (control) and the other half injected with 1 mg/kg recombinant murine leptin (1 mg/kg) two times a day for 7 days. Mice were then euthanized by ethyl ether. The epididymal white adipose tissue (eWAT) was dissected and was used for the following studies. Serum leptin level was measured by the commercial enzyme-linked immunosorbent assay (ELISA) kit from Sigma (Sigma-Aldrich, MO, USA, RAB0334,), and the measurement kits of interleukin (IL)-18 and IL-1β were from Abcam (UK, ab216165, ab100704) following the manufacturer's instructions and our previously study (37).

Primary Adipocyte Culture and Virus Vectors Infection

The connective fiber and blood vessels of eWAT tissues were removed, and washed three times with PBS buffer containing penicillin and streptomycin (Sigma-Aldrich, MO, USA, P3032, S6501). The adipocyte culture was carried out according to our previous publication (38). Briefly, adipocytes were seeded onto 35-mm culture dishes at 30% (v/v) confluence, and incubated at 37°C under a humidified atmosphere of 5% CO_2 and 95% air for subsequent experiments. After reaching 95% confluence, preadipocytes were induced to differentiate using DMEM/F12 (Gibco, CA, USA, 12500062) with 15% FBS and 100 nM insulin for 5–6 days until exhibiting a massive accumulation of fat droplets. Leptin (Peprotech, NJ, USA, 450-31) was added

into culture medium at a final concentration of 100 ng/mL and incubated for up to 24 h. Adipocytes were preincubated with 3-methyladenine (3-MA, 5 mM for 2 h, Selleck, China, S2767) or 4-phenylbutyrate (4-PBA; 50 mM for 1 h, Sigma-Aldrich, MO, USA, SM0309). Then followed treated with leptin for 12 h or 16 h separately, then TM (2 μg/mL, Sigma-Aldrich, MO, USA) or rapamycin (100 nM, Selleck, China, S1039) were further added into the culture medium for 12 or 8 h, respectively. For autophgic flux detection, adipocytes were exposed to leptin or TM followed by treatment of cells with an inhibitor of autophagosome-lysosome fusion, bafilomycin A1 (BafA1) (400 nM in the last 4 h of the 24 h treatment period), and assessed for accumulation of LC3II. For vectors infection study, adipocytes were infected with overexpression adenovirus or interference lentiviral recombinant vectors of *leptin* (pAd-*Leptin* or si-*Leptin*), *Atg5* (pAd-*Atg5* or si-*Atg5*), or Krüppel-like factor 4 (*KLF4*, pAd-*KLF4* or si-*KLF4*) for 48 h at the titer of 1×10^9 IFU/mL, and then treated with leptin. The control vectors were pAd-GFP or pGLVU6-GFP. All the vectors were constructed by Gene Pharma (China).

Detection of Autophagy Incidence by Flow Cytometry (FACS)

After treatment with different conditions, the adipocytes were incubated with 0.05 mM monodansylcadaverine (MDC, Sigma-Aldrich, MO, USA, 30432) at 37°C for 30 min and then washed three times with PBS. Intracellular MDC was measured by flow cytometry (FACS) within 30 min after incubation. Fluorescence intensity of cells was determined by BD FACScan (BD Biosciences, NJ, USA) and data were analyzed using Cell Quest software (BD Biosciences).

GFP-LC3 Analysis and Subcellular Localization

Cells were transfected with GFP-LC3 plasmid by using X-treme GENE HP Reagent (Roche, Switzerland) according to the manufacturer's instructions. After 48 h transfection, cells were washed with OptiMEM I (Invitrogen, CA, USA, 51985042) and subjected to staining. The cells were stained with LysoTracker® Green DND probe (Thermo Scientific, CA, USA, L7526) as recommended by the manufacturer. The formation of GFP-LC3 punctate and Tracker fluorescence were visualized and analyzed using Cytation3 Cell Imaging Multi-Mode Reader (BioTek, VT, USA).

Transmission Electron Microscopy (TEM)

At room temperature eWAT or adipocytes were fixed in 2.5% glutaraldehyde in PBS (pH = 7.2) for 24 h, postfixed in 1% osmium tetroxide in water for 2 h. After dehydrated in an ascending series of ethanol (30, 50, 70, 80, 90, and 100%) for 10 min each, the samples were then embedded in Durcopan ACM (Fluka Chemie AG, Switzerland, 44611). Sections were cut with a diamond knife at a thickness of 50–60 nm. These sections were stained with uranyl acetate and lead citrate, and examined with a TEM (HT7700, 80 kV, Hitachi, Japan). Images were recorded on film at 30,000× magnification. The percentage of mitochondrial integrity was determined by dividing the number of normal mitochondria by the total number of mitochondria per image.

RNA-Seq Analysis

Total RNA from the eWAT was prepared with RNAiso Reagent (Takara, China, D312) and the RNA-seq analysis was performed as previous described (39). Briefly, quantification and quality control of the sample libraries were assessed by Agilent 2100 Bioanalyzer and ABI StepOnePlus real-time PCR system. RNA sequencing was performed using Hiseq 4000 instrument (Illumina). Real-time analysis was used for base calling. Fastq files were mapped to the mouse genome (NCBI37/mm9) using TopHat (version 2.0.4). Mapped reads were then assembled *via* Cufflinks (version 2.0.2) with the default settings. Assembled transcripts were then merged using the Cuffmerge program with the reference genome. Analysis of mRNA levels was carried out using the Cuffdiff program, with samples being grouped by treatment condition, three replicates per group. Volcano plots comparing log10 (statistical relevance) to log2 (fold change) were generated using R (version 3.1.1), using the base plotting system and calibrate library. Gene Ontology (GO) and pathway enrichment analysis were performed to categorize the considerably enriched functional classification or metabolic pathways in which DEGs operated.

Plasmids Construction and Dual-Luciferase Reporter Assay

A 1,267 bp mouse *Atf4* promoter was cloned by PCR amplification of C57BL/6J mouse genomic DNA and inserted in the pGL-3 basic vector. The resulting reporter was named $Atf4_{1267}$-Luc. Further deletion of the $Atf4_{1267}$-Luc generated $Atf4_{720}$-Luc, $Atf4_{560}$-Luc and $Atf4_{130}$-Luc reporters contained of 720, 560, and 130 bp of *Atf4* promoter, respectively. HEK293 cells were cotransfected with luciferase reporter plasmid, pRL-TK reporter plasmid (control reporter), and KLF4 plasmid (pc-*KLF4*) using X-tremeGENE™ transfection reagent (Roche, Switzerland, 06366236001). After transfection for 24 h, cells were harvested and measured using the dual-luciferase reporter assay system (Promega, WI, USA, E1910), and luciferase activity was divided by the Renilla luciferase activity to normalize for transfection efficiency.

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation assay was performed as previous described using a ChIP assay kit (Abcam, UK, ab500) according to the manufacturer's protocol (12). Primary antibodies of Atf4 and IgG (Abcam, UK, ab172730) were used. DNA–protein crosslinking complexes were collected, and purified DNA was subjected to qPCR with SYBR green fluorescent dye (Invitrogen, CA, USA).

Coimmunoprecipitation (Co-IP) Analysis

HEK293 cells were transfected with plasmids using X-tremeGEN™ transfection reagent (Roche, Switzerland, 06366236001) as previous described (32). After 24 h transfection, cells were then

snap-frozen in lipid nitrogen. Whole cell lysate was harvested in lysis buffer with a protease inhibitor. Cells were then sonicated for 10 s and the whole cell lysate was precleared with Protein A for 2 h and incubated with 2 μg primary antibody overnight at 4°C. Immune complexes were pulled down with Protein A agarose for 2 h at 4°C with shaking. Beads were washed once with lysis buffer and three times with wash buffer, and then eluted by boiling in SDS sample buffer followed by detection of Western blot.

Nuclear Protein Extraction

Nuclear and cytoplasmic fractions were prepared using the protocols from Liu et al (40). In brief, cells were lysed with 400 μL of cytoplasmic lysis buffer. The lysates were incubated for 5 min on ice and vortexed 2 times for 10 s. The lysates were centrifuged for 30 s at 16,000*g*, and supernatants were collected as cytoplasmic fractions. The pellets were re-suspended in 50 μL of nuclear extraction buffer and sonicated 3 times on ice. The nuclear fractions were centrifuged for 5 min at 16,000*g*, and the supernatant was collected to obtain nuclear proteins. The proteins were denatured by boiling at 100°C and kept for further studies.

Real-time Quantitative PCR Analysis

Total RNA of eWAT or adipocytes were extracted with TRIpure Reagent kit (Takara, China) according to previous study (41). 500 ng of total RNA was reverse transcribed using M-MLV reverse transcriptase kit (Invitrogen, USA, 28025013). Primers were synthesized by Invitrogen (China). Quantitative PCR was performed in 25 μL reaction system containing specific primers and AceQ qPCR SYBR Green Master Mix (Vazyme, China, Q111-02). Amplification was performed in the ABI StepOne Plus™ RT-PCR System (CA, USA). The levels of mRNA were normalized in relevance to GAPDH. The expression of genes was analyzed by method of $2^{-\Delta\Delta Ct}$.

Protein Extraction and Western Blot Analysis

Western blot was performed as previously described (31). Adipocytes were solubilized in lysing buffer. Protein samples (30 μg) were separated by electrophoresis on 12 and 5% SDS-PAGE gels using slab gel apparatus, and transferred to PVDF nitrocellulose membranes (Millipore, USA). Antibodies including LC3II (ab48394), Atg5 (ab108327), Beclin1 (ab62557), SQSTM1 (ab51416), signal transducer and activator of transcription 3 (STAT3, ab68153), p-STAT3 (ab76315), JAK2 (ab108596), p-JAK2 (ab32101), Atf4 (ab184909), glucose-regulated protein 78 (GRP78, ab21685), C/EBP homologous protein (Chop, ab11419), p65 (ab16502), IκB (ab32518), p-IκB (ab92700), NLRP3 (ab214185), IL-18 (ab71495), PCNA (ab29), GAPDH (ab8245), anti-HA tag (ab18181), and anti-His tag (ab18184) were purchased form Abcam (UK) and IL-1β (12426) from cell signaling technology (USA), the appropriate HRP-conjugated secondary antibody (Boaoshen, China) were used. Proteins were visualized using chemiluminescent peroxidase substrate (Millipore, USA), and then the blots were quantified using ChemiDoc XRS system (Bio-Rad, USA).

Statistical Analysis

Statistical analyses were conducted using SAS v8.0 (SAS Institute, NC, USA). Data were analyzed using one-way or two-way ANOVA. Comparisons among individual means were made by Fisher's least significant difference. In consideration of multiple testing, the significance level was corrected using the Bonferroni method. Data were presented as mean ± SEM. $p < 0.05$ was considered to be significant.

RESULTS

Leptin Produces a Transcriptional Signature Distinct from TM and Reduces Genes Associated With Autophagy and ER Stress in Mice Adipose

We used RNA-seq and functional enrichment analysis to compared white adipose transcriptomes from leptin and TM-injected mice. Notably, a total of 12,732 genes were found to be significantly altered, and 68% (8,650 out of 12,732) of the genes were increased in leptin-injected mice, whereas 32% (4,081 out of 12,732) were decreased (Figure S1A in Supplementary Material). Similarly, TM injection altered 12,588 genes expression, and 62% (7,767 out of 12,588) of the genes were increased, whereas 38% (4,821 out of 12,588) were decreased (Figure S1A in Supplementary Material). GO analysis showed 22% of these genes were mainly functioned in signal transduction, 18% functioned in protein digestion, and 14% functioned in inflammation response (Figure S1B in Supplementary Material). Subsequent analysis showed leptin downregulation of 4,081 genes and TM upregulation of 7,767 genes contemporarily, and the genes were enriched in those encoding factors involved in autophagy (Figure S1C in Supplementary Material), suggesting leptin and TM treatment resulted in a regulation of autophagy. Further pathway analysis of the TM and leptin coregulated genes revealed that autophagy, ER stress and inflammation pathways were all highly enriched (**Figure 1A**), demonstrating that genes regulated by TM and leptin were correlated with autophagy and inflammation processes in adipose tissue. The data also showed the transcription factor cluster of multiply genes were altered, suggesting the existence of transcription regulation between these genes (**Figures 1B,C**). mRNA expression measurement established that autophagy marker genes *autophagy-related gene 5* (*Atg5*), *autophagy-related gene 12* (*Atg12*), and *Beclin1* were decreased in leptin group but increased in TM group; while the cotreatment of leptin and TM reduced these genes expression compared with that in TM alone group (**Figure 1D**). Inflammation indicators *IL-18*, *IL-1β*, and *NLR family, pyrin domain containing 3* (*NLRP3)* were also decreased after leptin treatment, but increased with TM injection; cotreatment of TM and leptin further decreased the levels of these genes (**Figure 1D**). These results were also confirmed with another ER stress inducer, the Tg treatment (Figures S2B,C in Supplementary Material). These findings indicated ER stress accelerated autophagy, but leptin played the opposite role in the regulation of both ER stress and autophagy in adipose tissue.

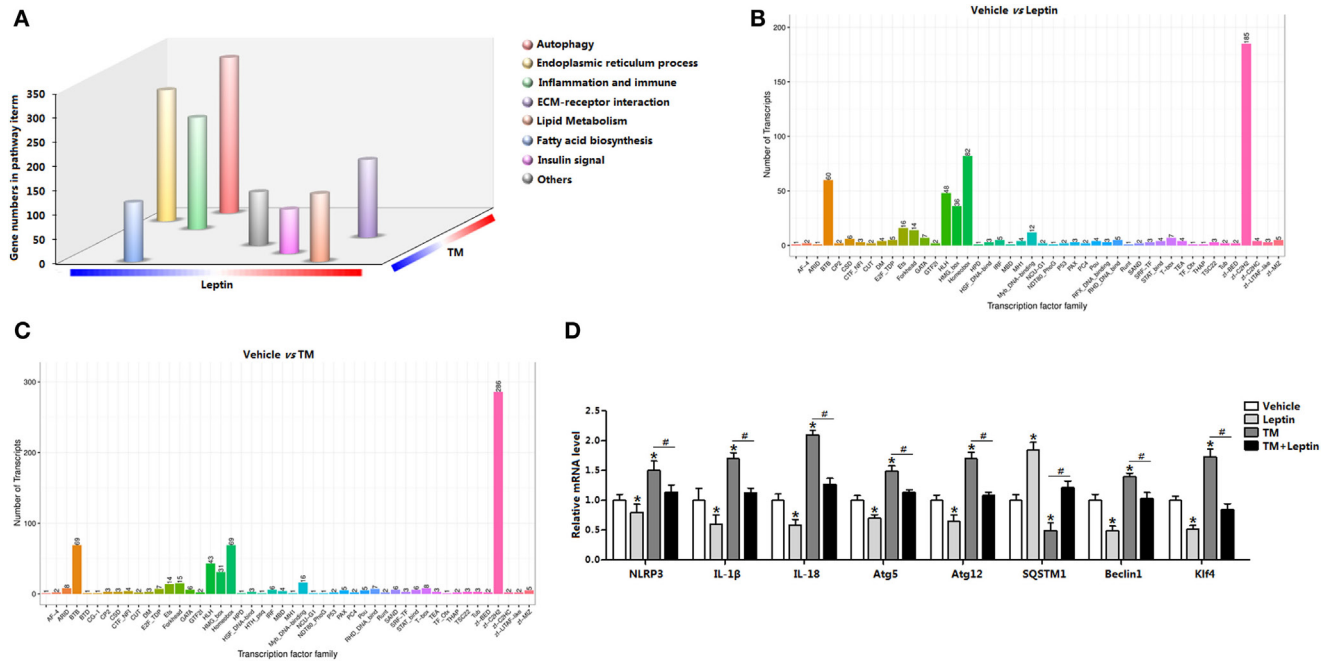

FIGURE 1 | Leptin treatment produces a transcriptional signature distinct from tunicamycin treatment and is defined by the induction genes associated with autophagy and endoplasmic reticulum (ER) stress in mice adipose. **(A)** Gene Ontology (GO) analysis of the genes set from RNA-seq data ($n = 3$). **(B)** Transcription factor family in genome after leptin treatment ($n = 3$). **(C)** Transcription factor family in genome after TM treatment ($n = 3$). **(D)** Changes in the expression level of genes associated with autophagy and inflammation, which were significantly altered from RNA-seq analysis ($n = 3$). Values are means ± SEM. $^{*}p < 0.05$ compared with the vehicle group, $^{\#}p < 0.05$ compared with the tunicamycin (TM) group, $^{\&}p < 0.05$ compared with the leptin group.

Leptin Reduces ER Stress-Induced Autophagy and Alleviates Inflammation Cytokines Secretion in Mice Adipose Tissue

The distinct responses of leptin and ER stress to autophagy were further demonstrated with the cotreatment of leptin and TM *in vivo*. Leptin markedly enhanced the phosphorylation levels of STAT3 and kinase 2 (JAK2) which were blunted by TM injection, indicating leptin signal was sensitive to leptin and TM treatments (**Figure 2A**). As expected, TM treatment triggered adipose ER stress and the addition of leptin attenuated the elevation of *Chop*, *GRP78*, *Atf4*, and *inositol-requiring enzyme 1* (*IRE1*) (**Figure 2B**). And Tg treatment also showed the consistent results (Figure S2A in Supplementary Material). As shown in **Figure 2C**, protein levels of classic autophagic markers LC3II, Beclin1, and Atg5 were significant induced by TM incubation whereas the level of SQSTM1 protein, a marker for autophagic flux, showed a decrease with TM treatment, suggesting the induction of autophagy by TM-induced ER stress. However, the addition of leptin showed the opposite results, indicating the reduction of autophagy (**Figure 2D**). Analyzing of autophagosomal structures of adipose tissue by TEM demonstrated autophagosome formation was increased in TM injected mice and decreased with leptin treatment, and changed in the cotreatment group, implying leptin reduced the autophagosome formation (**Figure 2C**). We also examine inflammation status with the cotreatment of leptin and TM. TM-induced ER stress promoted serum secretion of IL-18 and IL-1β, while the addition of leptin had the opposite effect (**Figures 2E,F**). Taken together, these observations indicated leptin treatment reduced ER stress-induced autophagy and alleviated inflammatory cytokines secretion of mice adipose tissue.

Leptin Decreases ER Stress-Induced Autophagy Flux by Reducing LC3II Turnover and SQSTM1 Degradation in Mice Adipocytes

Having shown leptin was capable of reducing ER stress-induced autophagy in mice adipose tissue, we next sought to confirm this by measuring the autophagosome formation in adipocytes *in vitro*. We first transfected adipocytes with overexpression GFP-LC3 plasmid followed incubated cells with TM or leptin, and subsequently detected the green fluorescent LC3II puncta. TM exposure prominently triggered the formation of characteristic punctate GFP-LC3, suggesting the recruitment of GFP-LC3 during autophagosome formation (**Figure 3A**). Cotreatment of TM and leptin resulted in the decrease in the percentage of cells with punctate GFP-LC3 compared with the cells treated with TM alone (**Figure 3A**), confirmed the reduction of autophagosome formation by leptin. We then analyzed the TEM pictures of adipocytes pretreated with TM or leptin. Notably, autophagosome formed in the TM and leptin cotreated cells were smaller than that in the TM exposure cells, and much more than that in leptin group (**Figure 3B**). These findings were

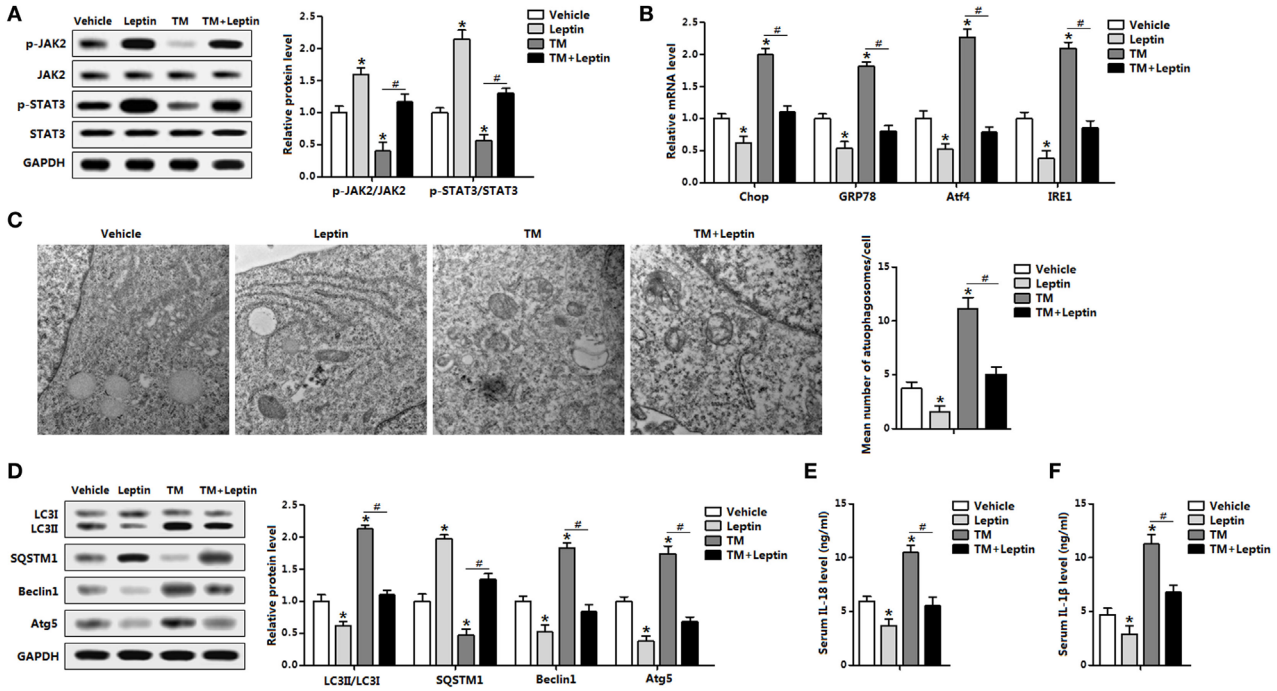

FIGURE 2 | Leptin treatment reduces endoplasmic reticulum (ER) stress-induced autophagy and alleviates inflammation cytokines secretion in mice adipose tissue. Mice were injected with tunicamycin (TM) or leptin, the white epididymal adipose tissue (eWAT) or serum was used for this study ($n = 6$). **(A)** Leptin sensitivity markers [phosphorylation of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3)] were examined by Western blot analysis. **(B)** Gene expression levels of ER stress markers: C/EBP homologous protein (*Chop*), glucose-regulated protein 78 (*GRP78*), activating transcription factor 4 (*Atf4*), and inositol-requiring enzyme 1 (*IRE1*). **(C)** Representative electron micrographs (30,000×) of eWAT treated with TM or leptin. The graph showed on the right was the quantification of mean number of autophagosomes in the EM images. **(D)** Autophagy was evaluated by the conversion of LC3I to LC3II, the protein levels of Beclin1, Atg5, and SQSTM1 were detected by Western blot analysis. **(E)** Serum interleukin (IL)-18 level measured by enzyme-linked immunosorbent assay (ELISA) test. **(F)** Serum IL-1β level measured by ELISA test. Full scans of uncropped blots are included in Figure S3. Values are means ± SEM. $^{*}p < 0.05$, $^{\#}p < 0.05$ compared with the TM group.

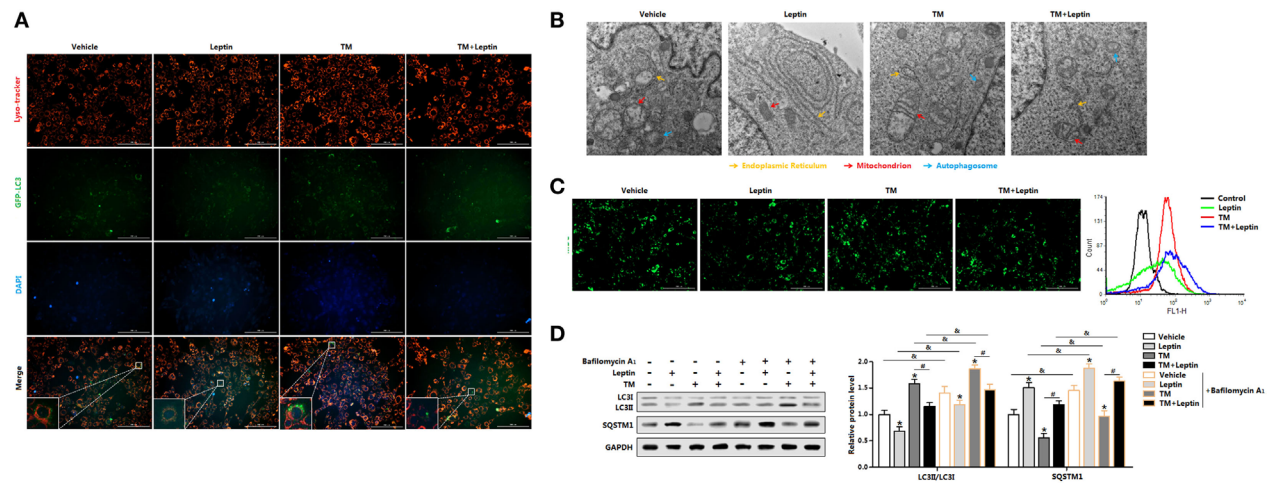

FIGURE 3 | Leptin decreases endoplasmic reticulum (ER) stress-induced autophagy flux by reducing LC3II turnover and SQSTM1 degradation in mice adipocytes. **(A)** Representative fluorescent photomicrographs showing the GFP-LC3 puncta formation in adipocytes transfected with GFP-LC3 plasmid and stained with Lyso-tracker Red. Cells were treated with tunicamycin (TM) or leptin. The nuclei were stained with DAPI shown in blue ($n = 3$). **(B)** Representative electron micrographs (30,000×) of adipocytes. Yellow arrowhead: endoplasmic reticulum, red arrowhead: mitochondria, blue arrowhead: autophagosome ($n = 3$). **(C)** Representative pictures of autophagosome formation monitored by MDC staining. Cell autophagy was analyzed by flow cytometry ($n = 3$). **(D)** Representative Western blots showing the protein levels of LC3II and SQSTM1 in adipocytes treated with leptin or TM followed by treatment with 400 nM bafilomycin A1 (BafA1), which was added in the last 4 h of the treatment period ($n = 3$). Full scans of uncropped blots are included in Figure S3. Values are means ± SEM. $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the TM group, $^{\&}p < 0.05$ compared with the BafA1 group.

further validated by the MDC staining and FACS measurement that leptin reduced the autophagosome formation of adipocytes (**Figure 3C**).

Since leptin decreased the number of autophagosome in adipocytes, it is needed to distinguish whether the reduction of autophagosome is due to the de-activation of autophagy or rather due to an enhanced of autophagosome-lysosomal fusion at later stages (21). To determine this, we exposed the cells to the lysosomal V-ATPase inhibitor BafA1, which blocks LC3II/autophagosome degradation and reveals changes in autophagosome synthesis (21). Autophagic flux was measured through LC3II turnover assay, by measuring LC3II degradation in adipocytes treated with TM or leptin, and followed exposed with or without BafA1. As shown in **Figure 3D**, exposure of adipocytes with TM followed by treatment with BafA1 resulted in significant increase of LC3II levels compared with BafA1 alone group; but the addition of leptin showed the opposite result. The decreased accumulation of LC3II protein indicated the reduced autophagic flux in cells exposed to leptin. We next assessed the degradation of SQSTM1 by autophagy to monitor the autophagic flux. It was evident that SQSTM1 level was decreased in TM-treated adipocytes, but increased in the TM and leptin cotreatment cells (**Figure 3D**). Further measurements demonstrated SQSTM1 protein level was significantly increased in TM and leptin cotreated adipocytes followed treated by BafA1, compared with cells without BafA1 (**Figure 3D**). These findings underpin the potential of leptin to downregulated autophgic flux. Therefore, reduced autophagic flux is responsible for SQSTM1 degradation in leptin-treated adipocytes.

Leptin-Mediated Autophagy and Inflammation Involved Upstream Activation of ER Stress in Mice Adipocytes

We next determined the effect of ER stress inhibitor, 4-PBA, on leptin-mediated autophagy and adipocyte inflammation. As shown in **Figure 4A**, TM-induced ER stress was inhibited in cells with 4-PBA preincubation through the downregulation of *Chop*, *GRP78*, and *Atf4*. Compared with TM group, cotreatment of leptin and TM further strengthen the inhibition of ER stress by 4-PBA (**Figure 4A**) The protein levels of autophagy markers, LC3II and Atg5 were significantly decreased in TM and leptin cotreatment group compared with those in the control group without 4-PBA, but the level of SQSTM1 was increased (**Figure 4B**). Moreover, 4-PBA exposure led to the alleviation of adipocytes inflammation. As shown in **Figure 4C**, the mRNA levels of *IL-18*, *Tnfα*, and *IL-1β* were reduced significantly in leptin and TM cotreated cells which were pretreated with 4-PBA than that of alone cotreated cells.

Next, we explore the effect of 3-MA, a pharmacological autophagy inhibitor, on leptin-mediated autophagy and adipocytes inflammation. As presented in **Figure 4D**, protein levels of LC3II and Atg5 were inhibited in adipocytes incubated with 3-MA. Leptin-mediated the blockage of autophagy was further strengthened with the 3-MA treatment (**Figure 4D**). However,

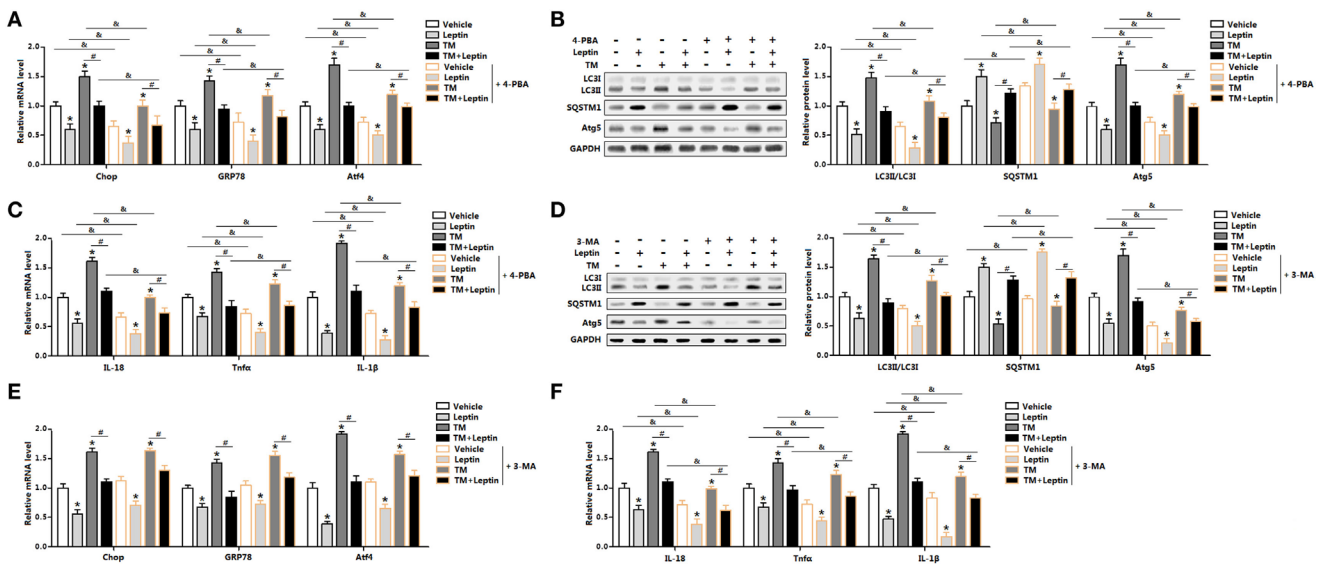

FIGURE 4 | Leptin-mediated autophagy and inflammation involved upstream activation of endoplasmic reticulum (ER) stress in mice adipocytes. Adipocytes were pretreated with 4-phenylbutyric acid (4-PBA, 5 mM for 2 h) or 3-methyladenine (3-MA, 50 mM for 1 h), followed incubated with recombinant leptin for 24 h or tunicamycin (TM) for 12 h ($n = 3$). **(A)** Gene expression profile of C/EBP homologous protein (*Chop*), glucose-regulated protein 78 (*GRP78*), and activating transcription factor 4 (*Atf4*) in adipocytes treated with or without 4-PBA. **(B)** Representative Western blots showing the protein levels of LC3II, SQSTM1, and Atg5 in adipocytes treated with or without 4-PBA. **(C)** Gene expression profile of proinflammatory cytokines, such as *IL-18*, *Tnfα*, and *IL-1β* in adipocytes treated with or without 4-PBA. **(D)** Representative Western blots showing the protein levels of LC3II, SQSTM1, and Atg5 in adipocytes treated with or without 3-MA. **(E)** Gene expression profile of *Chop*, *GRP78*, and *Atf4* in adipocytes treated with or without 3-MA. **(F)** Gene expression profile of *IL-18*, *Tnfα*, and *IL-1β* in adipocytes treated with or without 3-MA. Full scans of uncropped blots are included in Figure S3. Values are means ± SEM. $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the TM group, $^{\&}p < 0.05$ compared with the 4-PBA group or 3-MA group.

the mRNA expression profiles of ER stress markers, such as *Chop*, *GRP78*, and *Atf4* did not change significantly in leptin and TM cotreated cells preincubated with 3-MA than that of no 3-MA-treated cells (**Figure 4E**). Additionally, inflammatory markers were decreased in cells preincubated with 3-MA and followed treated with leptin and TM (**Figure 4F**). These findings confirmed that ER stress is the upstream for leptin-mediated autophagy and inflammation in mice adipocytes.

Leptin Inhibited ER Stress-Induced Autophagy and Inflammation in Mice Adipocytes

We had demonstrated that signature genes regulated by leptin were associated with autophagy and inflammation from the volcano plot (**Figure 5A**). To confirm that leptin inhibited ER stress-induced autophagy and alleviated adipose inflammation, we forced expression or silenced of *leptin* (**Figure 5B**). Leptin signal sensitivity was significantly altered after leptin vectors infection as represented by the change of phosphorylation of JAK2 and STAT3 (**Figure 5C**). Consistently with the exogenous leptin treatment data, overexpression of *leptin* decreased the protein levels of Chop, GRP78, and Atf4 (**Figure 5C**). By contrast, interference of *leptin* observed the opposite results (**Figure 5C**). Next, we monitor autophagosome formation in adipocytes infected with pAd-*leptin* or si-*leptin*, the GFP-LC3 fusion protein was utilized. As expected, overexpression of *leptin* reduced the formation of exogenous GFP-LC3 puncta, and we found the consistent results in BafA1-treated group, indicating there was a decrease accumulation of autophagosome (**Figure 5D**). We next measured the autophgic flux *via* LC3II turnover assay and detected the degradation of SQSTM1. Cells infected with pAd-*leptin* followed by BafA1 resulted in a significant decrease of LC3II level. In addition, the evident upregulation of SQSTM1 in the present of pAd-*leptin* and BafA1 confirmed the decreased autophgic flux by leptin treatment in adipocytes (**Figure 5E**).

We next determined the effect of 3-MA on leptin mediated autophagy. Monodansylcadervarine (MDC) staining indicated overexpression of *leptin* significantly reduced autophagy incidence in the cells preincubated with 3-MA (**Figure 6A**). The autophagy indicators LC3II and Atg5 were decreased with overexpression of *leptin* followed by 3-MA, while protein of SQSTM1 showed the opposite result (**Figure 6B**). Furthermore, *leptin* attenuated proinflammatory cytokines expression in cells pretreatment with 3-MA, similarly to the results showed in control group (**Figure 6C**). Overall, these findings implicated that leptin blocked ER stress-induced autophagy and alleviated inflammation in mice adipocytes.

Leptin Reduces ER Stress *via* the Blockade of *Atf4* Transcription in Mice Adipocytes

Having confirmed that leptin inhibited ER stress-induced autophagy in adipocytes, we next sought to explore the regulation of leptin on ER stress. As shown in **Figures 6D,E**, overexpression of *leptin* downregulated autophagy and inflammation both in the control group and in the 4-PBA group. And as indicated cluster of transcription factors of multiply genes were altered with leptin treatment (**Figure 1B**). In order to analyze the underlying mechanisms of leptin on ER stress, we firstly considered transcriptional-level control. Our results showed leptin increased

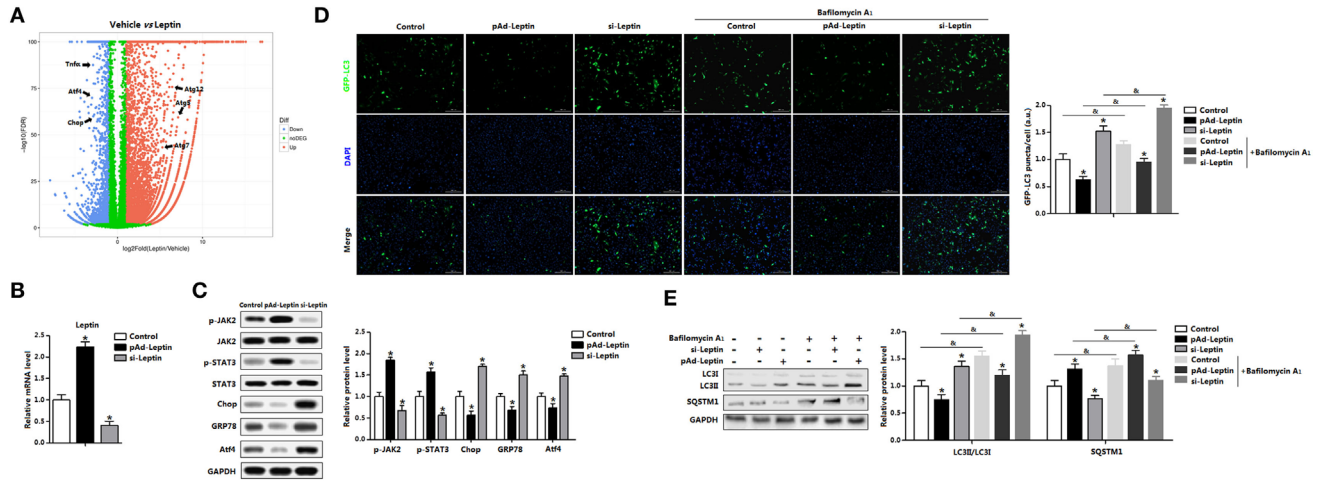

FIGURE 5 | Overexpression of leptin inhibited autophagy in mice adipocytes. **(A)** Volcano plot of transcriptome in the white epididymal adipose tissue (eWAT) of mice injected with or without leptin ($n = 3$ each). Individual endoplasmic reticulum (ER) stress, autophagy, and inflammation signature genes were highlighted. Red: upregulated, blue: downregulated. **(B)** Gene expression level of *leptin* in adipocytes infected with pAd-*Leptin* or si-*Leptin* ($n = 3$). **(C)** Protein levels of phosphorylation of Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), C/EBP homologous protein (Chop), glucose-regulated protein 78 (GRP78), and activating transcription factor 4 (Atf4) in adipocytes infected with pAd-*Leptin* or si-*Leptin* ($n = 3$). **(D)** Representative pictures of GFP-LC3 punctate structures in adipocytes expressing GFP-LC3, in the presence of pAd-*Leptin* or si-*Leptin* and pretreated with TM then incubated with or without bafilomycin A1 (BafA1). The nuclei were stained with DAPI shown in blue in adipocytes ($n = 3$). **(E)** Representative Western blots showing the protein levels of LC3II and SQSTM1 in adipocytes infected with pAd-*Leptin* or si-*Leptin* that pretreated with TM and followed treated with BafA1 or not ($n = 3$). Full scans of uncropped blots are included in Figure S3. Values are means ± SEM. $^{*}p < 0.05$ compared with the control group, $^{\&}p < 0.05$ compared with the BafA1 group.

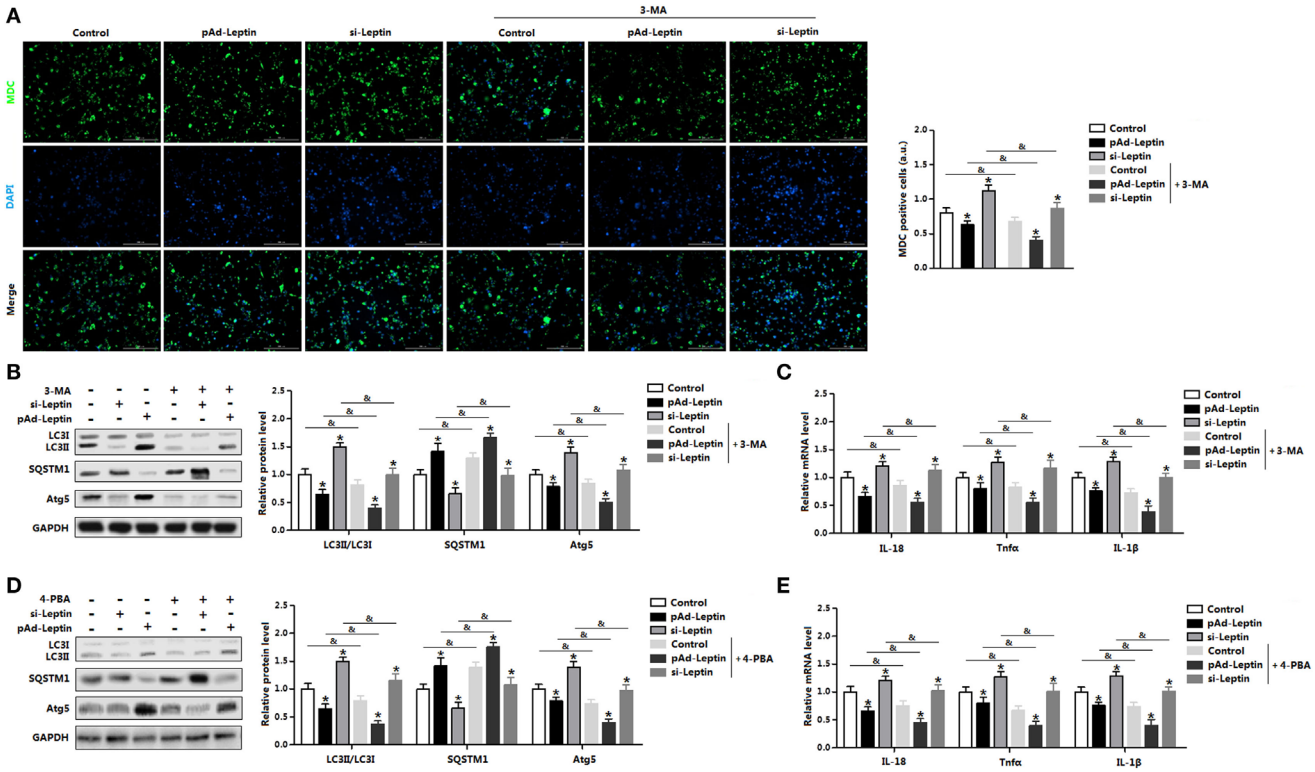

FIGURE 6 | Leptin signal is essential for endoplasmic reticulum (ER) stress-induced autophagy and inflammation in mice adipocytes. Adipocytes were preinfected with pAd-*leptin* or si-*leptin* and incubated with tunicamycin (TM), then followed treated with 4-phenylbutyric acid (4-PBA, 5 mM for 2 h) or 3-methyladenine (3-MA, 50 mM for 1 h) ($n = 3$). **(A)** Representative pictures of autophagosome formation monitored by monodansylcadervarine (MDC) staining in adipocytes with or without 3-MA treatment. The nuclei were stained with DAPI shown in blue. **(B)** Representative Western blots showing the protein levels of LC3II, SQSTM1, and Atg5 in adipocytes treated with or without 3-MA. **(C)** Gene expressions of inflammation markers in adipocytes treated with or without 3-MA. **(D)** Representative Western blots showing the protein levels of LC3II, SQSTM1 and Atg5 in adipocytes treated with or without 4-PBA. **(E)** Gene expressions of inflammation markers in adipocytes treated with or without 4-PBA. Full scans of uncropped blots are included in Figure S3. Values are means ± SEM. $^{*}p < 0.05$ compared with the control group, $^{\&}p < 0.05$ compared with the 3-MA group or 4-PBA group.

the expression of *KLF4*, but TM treatment had the opposite effect (**Figure 7A**). Further analysis demonstrated the *Atf4* promoter region contained three potential binding domains of *KLF4* (**Figure 7B**). And measurements revealed that the binding site, 560–130 bp upstream of the initiation site of *Atf4* functioned (**Figures 7B,C**). We next treated adipocytes with the overexpression recombinant adenovirus vector of *KLF4* (pAd-*KLF4*) or interference recombinant lentiviral vector of *KLF4* (si-*KLF4*), in the presence of leptin or not. **Figure 7D** showed cotreatment of leptin and pAd-*KLF4* reduced the mRNA levels of *Atf4* and *Chop* significantly (**Figure 7D**). The data also indicated autophagy indicators *Beclin1* and *Atg5* were decreased with the overexpression of *KLF4*, and leptin addition further strengthened this trend (**Figure 7D**). Thus, these findings suggested *KLF4* inhibited the transcription of *Atf4*, and leptin enhanced the inhibition effect of *KLF4* and reduced ER stress of adipocytes.

Atf4-Atg5 Complex Regulates Leptin-Mediated Autophagy in Mice Adipocytes

The transcriptional regulation of *Atf4* by *KLF4* led us to hypothesize whether ER stress affected adipocytes autophagy through a transcription regulation either. However, we failed to show this regulation network (data were not shown). The increased protein levels of autophagy-related proteins caused by TM treatment prompted us to further hypothesize that ER stress regulated autophagy by direct modification, through a physical interaction. Firstly based on the bioinformatics analysis and previous data sheet, we showed Atf4 interacted with Atg5 (**Figure 7E**). Then by protein–protein measurement, Atf4 protein interacted strongly with transfected Atg5 in both HEK293 cells and in adipocytes (**Figures 7F,G**). Thus, these data suggest that Atf4 and Atg5 directly bind, and then regulated autophagy progress in adipocytes.

To determine whether Atg5 is responsible for leptin-mediated autophagy, we next employed a genetic approach, overexpression recombinant adenovirus vector of *Atg5* (pAd-*Atg5*) or interference recombinant lentiviral vector of *Atg5* (si-*Atg5*), to explore the underline mechanisms. As expected, interference of *Atg5* inhibited autophagy of adipocytes and the addition of leptin further reduced the punctate GFP-LC3 formation (**Figure 8A**). Consistently, LC3II protein level was downregulated in adipocytes cotreated with leptin and si-*Atg5* compared with that in the si-*Atg5* alone group (**Figure 8B**). Similar to

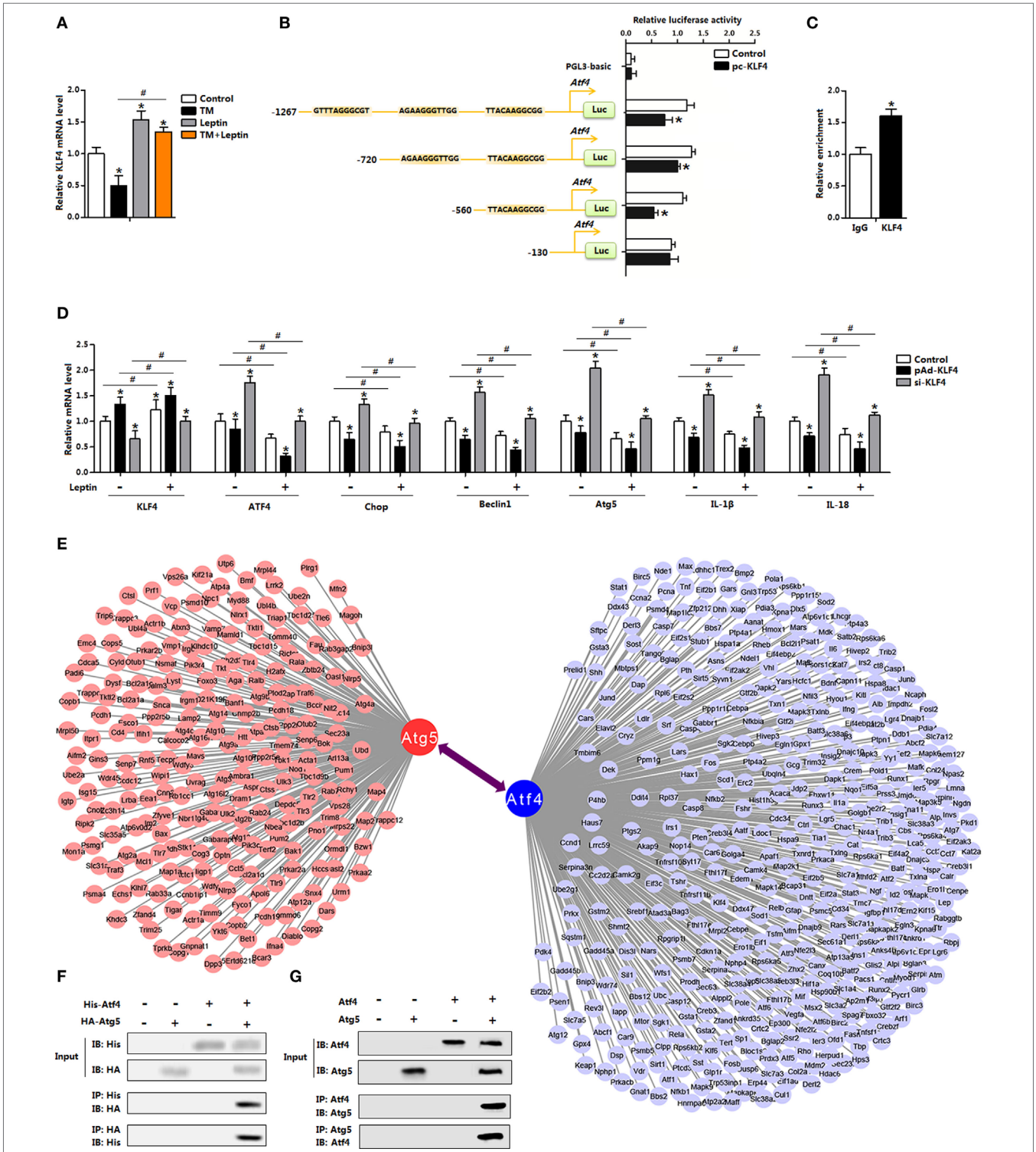

FIGURE 7 | Leptin reduces endoplasmic reticulum (ER) stress *via* the blockade of activating transcription factor 4 (*Atf4*) transcription in mice adipocytes. **(A)** Gene expression of Krüppel-like factor 4 (*KLF4*) in adipocytes incubated with tunicamycin (TM) or leptin ($n = 3$). **(B)** Dual luciferase reporter assay of *KLF4* and *Atf4*. Cells were transfected with PGL3-basic or PGL3-*Atf4* plasmids, and pc-*KLF4* plasmid ($n = 3$). **(C)** Chromatin immunoprecipitation (ChIP) analysis between Atf4 and KLF4 ($n = 3$). **(D)** Gene expression levels of *KLF4*, *Atf4*, *Chop*, *Beclin1*, *Atg5*, *IL-18*, and *IL-1β* in adipocytes infected with pAd-*KLF4* or si-*KLF4* and incubated with leptin or not ($n = 3$). **(E)** Graphic representation of a network of the target genes. Bioinformatics analysis of the protein–protein interaction. **(F)** Atg5 interacted with Atf4. Coimmunoprecipitation (Co-IP) analysis was done in HA-Atg5 and His-Atf4 transfected HEK293 cells. **(G)** Co-IP analysis was done in pAd-*Atg5* and pAd-*Atf4* transfected adipocytes with no infection were used as control. Values are means ± SEM. $^{*}p < 0.05$ compared with the TM group, $^{\#}p < 0.05$ compared with the leptin treatment group.

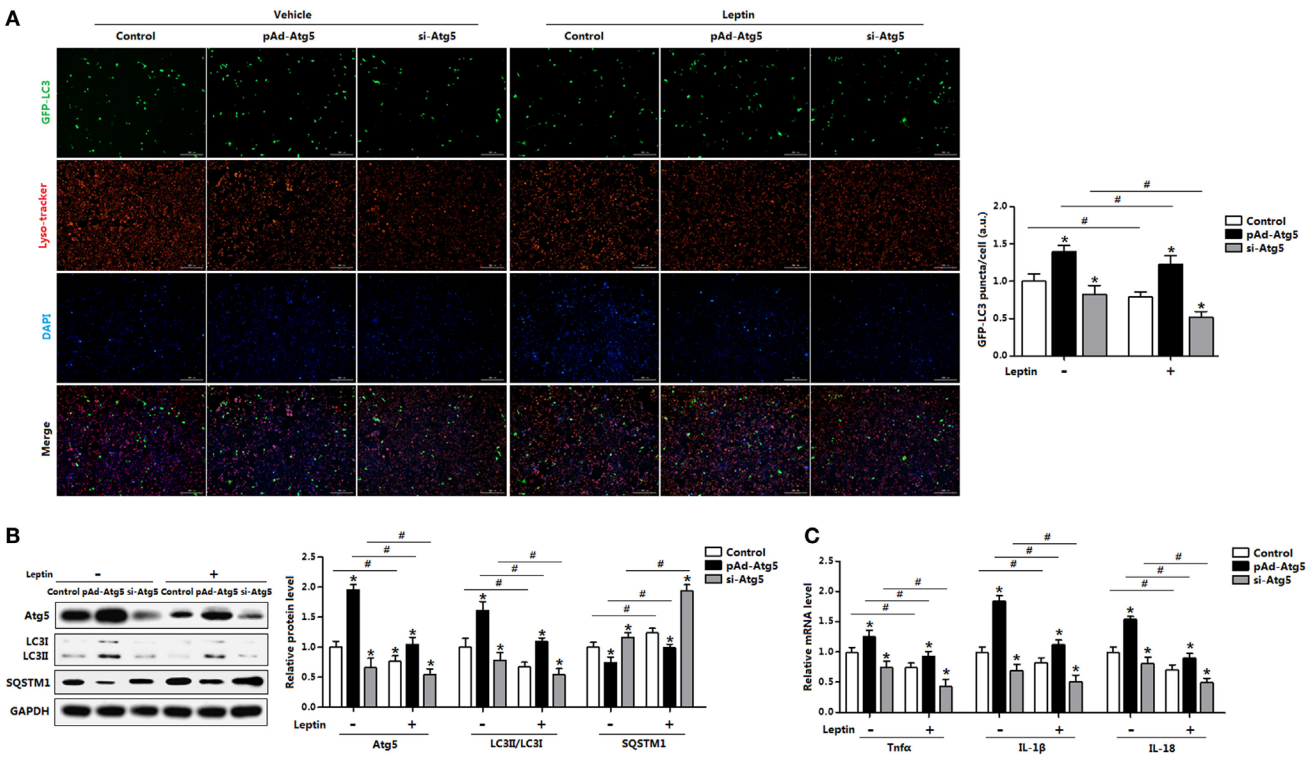

FIGURE 8 | Activating transcription factor 4 (Atf4) forms a complex with Atg5 and drives leptin-mediates autophagy in mice adipocytes. Adipocytes were preinfected with pAd-*Atg5* or si-*Atg5* and incubated with tunicamycin (TM), followed treated with or without leptin ($n = 3$). **(A)** Representative fluorescent photomicrographs showing the GFP-LC3 puncta formation in adipocytes transfected with GFP-LC3 plasmid and stained with Lyso-tracker Red. The nuclei were stained with DAPI shown in blue. **(B)** Representative Western blots showing the protein levels of Atg5, LC3II, and SQSTM1. **(C)** Gene expression of *Tnfα*, *IL-1β*, and *IL-18* in adipocytes. Values are means ± SEM. $^{*}p < 0.05$ compared with the TM group, $^{\#}p < 0.05$ compared with the leptin group.

the 3-MA blockage, gene-silencing of *Atg5* combine with leptin treatment also significantly inhibited the increasing of proinflammatory cytokines in adipocytes (**Figure 8C**). Overall, these findings implicated Atf4-Atg5 complex played key role in the process of leptin-mediated autophagy and inflammation in mice adipocytes.

Atg5 Activates IκB Signaling Pathway in Autophagy-Mediated Adipocyte Inflammation

Having confirmed that Atg5 functioned in leptin-mediated autophagy and inflammation, we then sought to examine the underlying regulation mechanism of Atg5 in adipocytes inflammation. Overexpression of *Atg5* significantly increased autophagy of adipocytes that preincubated with 3-MA (**Figure 9A**); it also enhanced inflammation by elevating the expression of *Tnfα*, *IL-1β*, and *IL-18* which were inhibited with 3-MA incubation (**Figure 9B**). We then measured the protein levels of phosphorylation IκB, NLR family, pyrin domain containing 3 (NLRP3), IL-18, and IL-1β. Results showed the inflammatory indicators such as p-IκB, NLRP3, and IL-1β were all increased with pAd-*Atg5* alone treatment, and still increased when cells were cotreated with *Atg5* and 3-MA (**Figure 9C**). We then investigated whether the effect of Atg5 on inflammation was correlated with the degradation of IκB protein. Forced expression of *Atg5* greatly increased the protein level of IκB in the cytoplasm and the protein level of p65 in the nuclear extracts (**Figure 9D**). And this also happened when cells were pretreated with 3-MA (**Figure 9D**). These findings thus initiated underscore that Atg5-mediated autophagy attenuated inflammation *via* IκB signaling pathway.

Leptin Administration Reduces Autophagy and Inflammation of Adipose Tissue *In Vivo*

To further explore the *in vivo* relevance of our findings, we next determined the induction of ER stress and autophagy in mice treated with leptin. As shown in **Figure 10A**, leptin injection significantly reduced body weight in leptin genetic deficiency mice (*ob/ob* mice). The replenish of leptin induced a markedly decrease in the levels of ER stress markers, while increased the level of *KLF4* (**Figure 10B**). Consistently, autophagic marker proteins LC3II, Beclin1, and Atg5 were all reduced with leptin injection compared with those in the *ob/ob* mice (**Figure 10C**). As expected, the gene expressions of pro-inflammatory cytokines were also reduced with leptin injection (**Figure 10D**).

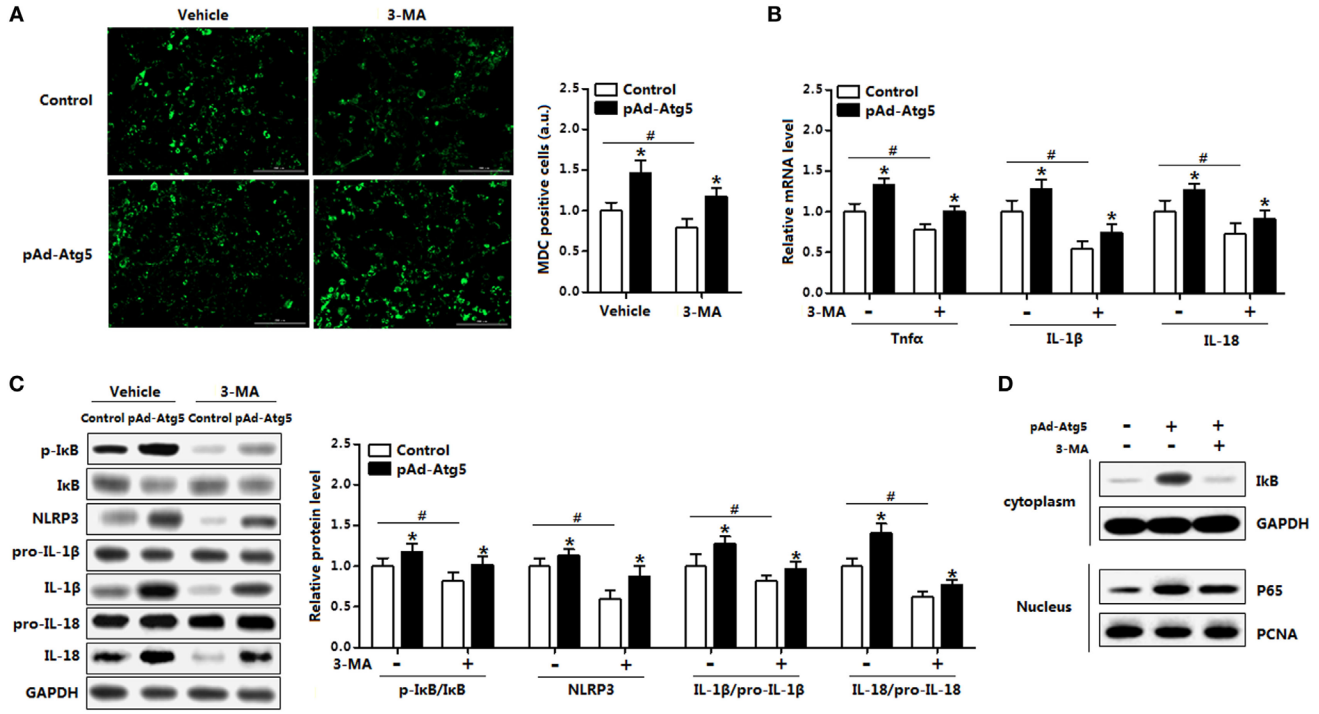

FIGURE 9 | Autophagy-related gene 5 (Atg5) activates IκB signaling pathway in autophagy-mediated adipocyte inflammation. Adipocytes were infected with pAd-*Atg5* and incubated with tunicamycin (TM), followed with or without 3-methyladenine (3-MA) treatment ($n = 3$). **(A)** Representative pictures of autophagosome formation monitored by MDC staining. **(B)** Gene expression of *Tnfα*, *IL-18*, and *IL-1β*. **(C)** Protein level of phosphorylation IκB, NLRP3, IL-1β, and IL-18. **(D)** IκB and GAPDH protein levels in cytoplasm and P65 and PCNA protein levels in nuclear extracts of the adipocytes. Full scans of uncropped blots are included in Figure S3. Values are means ± SEM. $^{*}p < 0.05$ compared with the TM group, $^{\#}p < 0.05$ compared with the 3-MA treatment group.

Next, we used leptin administration in the WT mice which had been trigged ER stress by TM injection. Leptin treatment further reduced ER stress in 4-PBA pretreated mice (**Figure 10E**). In addition, 4-PBA administration decreased the protein levels of LC3II and Atg5, but increased SQSTM1 level. The addition of leptin further strengthened the autophagy blockage and similarly reduced adipose inflammation (**Figures 10F,G**). As shown in **Figures 10H,I**, leptin treatment significantly reduced rapamycin-induced autophagy, along with the downregulation of ER stress. Additionally, the addition of leptin inhibited adipose inflammation in rapamycin injected mice (**Figure 10J**). Thus, these data verify the role of leptin in the administration of ER stress and autophagy *in vivo*.

DISCUSSION

Autophagy is an essential lysosome-mediated bulk degradation pathway for cellular survival, development and homeostasis (17, 42). Particularly, autophagy is essential for by endolysosomal degradation and elimination of misfolded proteins and damaged organelles during ER stress (43, 44). Although recent investigations have revealed that ER stress can either stimulate or inhibit autophagy in different cell types, it still does not determine crosstalk between ER stress and autophagy in adipocytes (43, 45–48). Meanwhile, the ER stress-associated molecular cues that control the switch of autophagy are also obscure. In this study, we demonstrated that exogenous TM and Tg stimulated ER stress and autophagy in adipose tissue. These findings are consistent with our previous and other studies that ER stress is sufficient to trigger autophagy and reduce adiponectin expression in adipocytes (12, 49, 50). Moreover, ER stress also initiated inflammation but decreased leptin level in adipocytes. Interestingly, autophagy-deficient adipose tissue has drastically reduced leptin secretion (51). We then investigated the expression profile of core autophagy and inflammation genes in leptin-treated or TM-treated adipose tissue. Our data showed TM elevated but leptin reduced the autophagy-related genes such as *Beclin1*, *Atg5*, and *Atg12*. Consistently, kinds of biological process and pathways correlated with cellular inflammation response were enriched in the GO analysis. Furthermore, we uncovered an unexpected result that leptin inhibited ER stress-induced autophagy and inflammation of adipocyte, though the status of inflammation and autophagy were both elevated in TM-induced ER stress. Previous studies show that ER stress induces adipocyte inflammation and insulin resistance by activating NF-κB pathways (52, 53). Although several previous studies investigate the link between ER stress and autophagy, it remains unclear how leptin affects the crosstalk between ER stress and autophagy (54, 55). It is interesting to note that leptin might be an upstream regulator and be essential for inhibiting ER stress-induced autophagy and alleviating adipocyte inflammation in downstream.

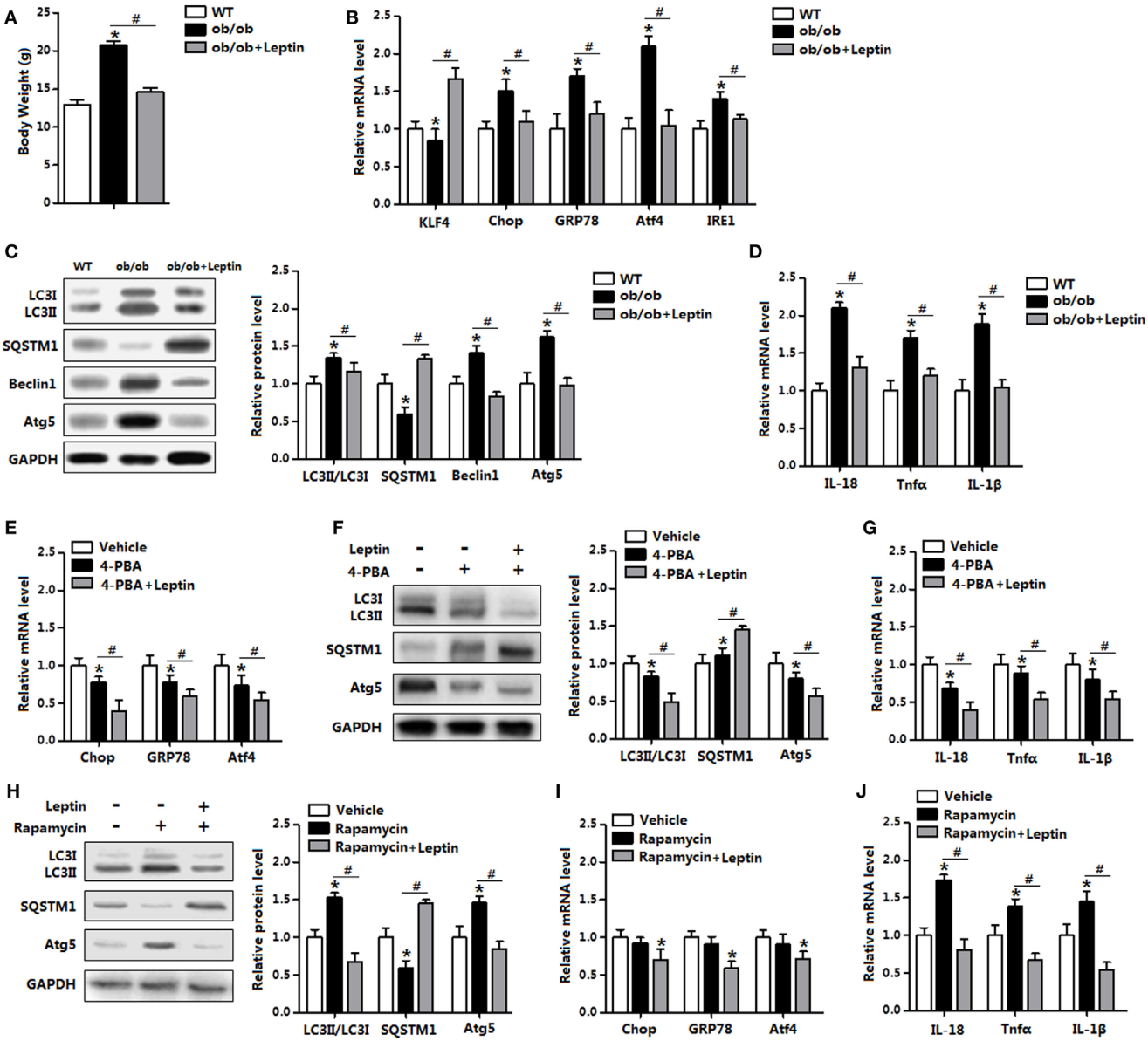

FIGURE 10 | Leptin administration reduced autophagy and inflammation of adipose tissue *in vivo*. From **(A–D)** wild-type (WT) mice and *ob/ob* mice were injected with tunicamycin ™, and followed treated with recombinant leptin protein or not ($n = 6$). **(A)** Body weight of the mice. **(B)** Gene expression level of Krüppel-like factor 4 (*KLF4*), C/EBP homologous protein (*Chop*), glucose-regulated protein 78 (*GRP78*), activating transcription factor 4 (*Atf4*), and inositol-requiring enzyme 1 (*IRE1*) in the mice white epididymal adipose tissue (eWAT). **(C)** The conversion of LC3I to LC3II and protein levels of Atg5, Beclin1, and SQSTM1 were examined by Western blot in the eWAT of mice. **(D)** Gene expression level of *IL-18*, *Tnfα*, and *IL-1β* in mice eWAT. From **(E–G)** WT mice were pretreated with TM and 4-phenylbutyric acid (4-PBA), and followed treated with recombinant leptin protein or not ($n = 6$). **(E)** Gene expression of ER stress markers in the eWAT of mice. **(F)** Representative Western blots showing the protein levels of Atg5, LC3II, and SQSTM1 in the eWAT of mice. **(G)** Gene expression of *Tnfα*, *IL-18*, and *IL-1β* in the eWAT of mice. From **(H–J)** WT mice were pretreated with TM and rapamyclin, and followed treated with recombinant leptin protein or not ($n = 6$). **(H)** Representative Western blots showing the protein levels of Atg5, LC3II, and SQSTM1 in the eWAT of mice. **(I)** Gene expression of ER stress markers in the eWAT of mice. **(J)** Gene expression of *Tnfα*, *IL-18*, and *IL-1β* in the eWAT of mice. Full scans of uncropped blots are included in Figure S3. Values are means ± SEM. $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the leptin group.

Based on these findings, we first explored the regulatory role of leptin on adipocyte autophagy. Leptin is a well-known adipocyte-derived hormone involved in food intake and energy metabolism (1, 56). Several reports have implicated increased accumulation of autophagosomes possibly functions as a pathogenic signal contributing to induction of ER stress-mediated inflammation (57, 58). In this study, we preliminarily determined that leptin inhibited ER stress-induced autophagy in adipocytes and adipose tissue with downregulating the autophagic flux by LC3II turnover, SQSTM1 degradation and

reduced autophagosome clearance. These findings are consistent with other studies that leptin inhibits canonical autophagy in peripheral tissues including skeletal muscle, heart and liver by reducing LC3II, Beclin1, and Atg5 (59, 60). Accordingly, we also employed the approaches of pharmacological inhibition which impeded either early autophagosome formation (by 3-MA) or late autolysosome formation (by BafA1) phases of autophagy signaling, or the ER stress inhibitor (4-PBA) to specifically determine the roles of ER stress and autophagy in mediating the effects of leptin on adipocyte inflammation. Our findings demonstrated that autophagy inhibitor, 3-MA further accelerated leptin-reduced autophagy in adipocytes, while still maintaining leptin-mediated downregulation of ER stress. These findings thus suggested that leptin-mediated inhibition of ER stress was the upstream of autophagy response, which ultimately resulted in the reduction of inflammation as evidenced by decreasing the proinflammatory cytokines expression. Moreover, Saroj et al. find that leptin induces autophagy and promotes apoptosis in cancer cells (61). Indeed, we speculate that cellular events, i.e., autophagy, ER stress and apoptosis, respond to leptin very differently in normal and cancer cells (62, 63). Similar to these findings, it is thought that ER stress initiates autophagy only when aggregated proteins become excessive enough to overwhelm the canonical ubiquitin-proteasome-dependent ER-associated degradation (64, 65). Moreover, studies indicate the transcriptional upregulation of LC3 and Atg5 depends on Atf4 and Chop induction during ER stress-induced autophagy (66, 67). Intriguingly, our present study found that Atf4 directly interacted with Atg5 and the complex was formed to mediate autophagy and inflammatory response in adipocytes. Furthermore, we showed that *KLF4* bound to the *Atf4* promoter region then inhibited the transcription of *Atf4*, suggesting that leptin inhibited ER stress of adipocytes through activating JAK2/STAT3 pathway and promoting *KLF4* transcriptional inhibition of *Atf4*. Recent studies also confirm that KLF4 mediates leptin's effects in hypothalamic arcuate nucleus (68, 69).

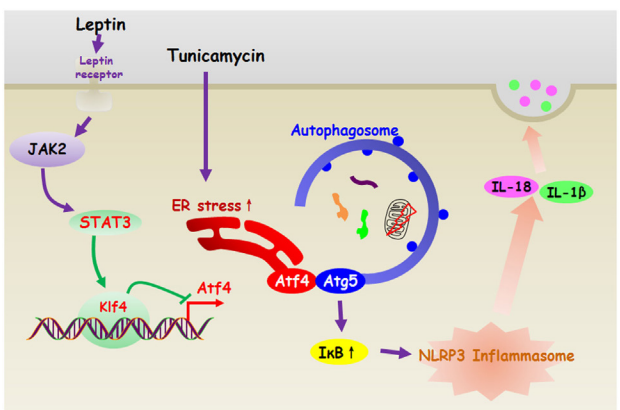

FIGURE 11 | Leptin inhibited endoplasmic reticulum (ER) stress-mediated autophagy and inflammation through the negatively regulation of activating transcription factor 4 (Atf4)/Atg5 complex in adipocytes. And leptin is essential for transcriptional regulation of Atf4/Atg5 signal during the NLRP3 inflammasome degradation in adipocytes.

Another intriguing observation from the present study was that Atf4 had a protein–protein interaction with Atg5 and subsequently initiated adipocyte autophagy. The main events of autophagy, such as phagophore formation and maturation, are substantially maintained by the ATG12-ATG5 conjugate (70–72). This observation was further confirmed by the results that inhibition of Atg5 abolished leptin signaling-mediated autophagy induction in adipocytes. Collectively, our findings provide the first evidence that leptin signal regulates autophagy of adipocytes through the Atf4-Atg5 signal, although how leptin regulate protein activity of Atf4-Atg5 complex still requires further investigation.

One question remains to be answered is that how leptin inhibited ER stress-induced inflammation by activating autophagy. Increasing evidence demonstrates the importance of NLRP3 inflammasome in the regulation of adipocyte inflammation (73, 74). IκB kinase is one of the most important kinases that mediates the effects of general inflammatory stimuli inside adipocyte and is a major upstream regulator of NLRP3 inflammasome (8, 75, 76). In the current study, we demonstrated that leptin reduced NLRP3 protein level and inflammatory factors expression by inhibiting Atg5-mediated autophagy. These findings are consistent with the studies proposed the essential relationship between autophagosomes formation and NLRP3 inflammasome activation (77, 78). Unexpectedly, our result also showed that reduction of autophagy by 3-MA ameliorated inflammation as leptin functioned in adipocyte. Though TM-induced ER stress can directly activate NLRP3 inflammasome and mediate p65 translocation in nucleus which has been confirmed by our data and other studies, further investigations are needed to resolve the detail molecular mechanisms (79, 80).

In summary, our present study demonstrates that leptin inhibits ER stress-induced inflammation through reducing Atf4-Atg5-mediated autophagy in adipocytes. Moreover, leptin is essential for transcriptional regulation of Atf4/Atg5 signal during the NLRP3 inflammasome degradation in adipocytes (**Figure 11**). Thus, our results provide a novel therapeutic option for exploring the proteasome or autophagy activator to reverse obesity-related metabolic disorders.

DATA ACCESSION

The raw data have been deposited to NCBI Sequence Read Archive (SRA). The NCBI SRA accession: SRP119756.

ETHICS STATEMENT

Mice handling protocols were conducted following the guidelines and regulations approved by the Animal Ethics Committee of Northwest A&F University.

AUTHOR CONTRIBUTIONS

LG and CS designed research. All authors performed research. ZL and DL analyzed data. QR, HW, and CL contributed reagents/analytic tools. LG and ZL wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01507/full#supplementary-material.

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REVIEW
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Visceral Inflammation and Immune Activation Stress the Brain

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Stress refers to a dynamic process in which the homeostasis of an organism is challenged, the outcome depending on the type, severity, and duration of stressors involved, the stress responses triggered, and the stress resilience of the organism. Importantly, the relationship between stress and the immune system is bidirectional, as not only stressors have an impact on immune function, but alterations in immune function themselves can elicit stress responses. Such bidirectional interactions have been prominently identified to occur in the gastrointestinal tract in which there is a close cross-talk between the gut microbiota and the local immune system, governed by the permeability of the intestinal mucosa. External stressors disturb the homeostasis between microbiota and gut, these disturbances being signaled to the brain *via* multiple communication pathways constituting the gut–brain axis, ultimately eliciting stress responses and perturbations of brain function. In view of these relationships, the present article sets out to highlight some of the interactions between peripheral immune activation, especially in the visceral system, and brain function, behavior, and stress coping. These issues are exemplified by the way through which the intestinal microbiota as well as microbe-associated molecular patterns including lipopolysaccharide communicate with the immune system and brain, and the mechanisms whereby overt inflammation in the GI tract impacts on emotional-affective behavior, pain sensitivity, and stress coping. The interactions between the peripheral immune system and the brain take place along the gut–brain axis, the major communication pathways of which comprise microbial metabolites, gut hormones, immune mediators, and sensory neurons. Through these signaling systems, several transmitter and neuropeptide systems within the brain are altered under conditions of peripheral immune stress, enabling adaptive processes related to stress coping and resilience to take place. These aspects of the impact of immune stress on molecular and behavioral processes in the brain have a bearing on several disturbances of mental health and highlight novel opportunities of therapeutic intervention.

Keywords: gut–brain axis, gut microbiota, immune–brain axis, immune stress, intestinal inflammation, lipopolysaccharide, mental health, neuropeptide Y

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INTRODUCTION

In a general context, stress is considered to be a dynamic process in which the physical and/or mental homeostasis of an organism is challenged, the outcome depending on the type, severity, and duration of stimuli (stressors) involved, the stress responses triggered and the stress susceptibility/resilience of the organism. Homeostatic disturbances can be triggered by both exogenous and endogenous

stressors. There is abundant evidence that the immune system is involved in stress responses, given that both physical and psychosocial stressors have an impact on immune function. It needs to be emphasized, however, that the interaction between stress and immune system is a bidirectional process, implying that alterations in immune function themselves can elicit stress responses. Such bidirectional interactions have been identified to occur in the gastrointestinal (GI) tract in which there is a close cross-talk between the gut microbiota and the local immune system (1–4), governed by the permeability of the GI mucosa. On the one hand, external stressors impact on the gut microbiota and its relationship with the GI mucosal, immune, endocrine, and nervous system. On the other hand, this disturbance of gut homeostasis is signaled to the central nervous system (CNS) *via* multiple communication pathways constituting the gut–brain axis, ultimately eliciting stress responses and perturbations of brain function (5).

It has been known for some time that infection-related as well as infection-independent immunological stimuli can evoke stress responses as reflected by an increased activity of the hypothalamic–pituitary–adrenal (HPA) axis, resulting in enhanced plasma concentrations of adrenocorticotropic hormone (ACTH) and cortisol/corticosterone (6, 7). Pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS) have been extensively studied in their ability to stimulate the innate immune system *via* binding to toll-like receptor-4 (TLR4), cause the formation of proinflammatory cytokines, activate the HPA system (8–10), and alter brain function and behavior. Cytokines generated in response to, e.g., LPS trigger a complex behavioral response, encompassed in the terms "sickness behavior" or "illness response," which comprise fever, anorexia, somnolence, decrease in locomotion, exploration and social interaction, hyperalgesia, and delayed depression-like behavior (11–15). These cerebral effects are brought about by multiple signaling mechanisms: direct access of cytokines to the brain, activation of vagal afferent neurons, and neuroinflammatory processes in the brain (11, 12, 14, 16). Once acute sickness subsides, depression-like behavior may ensue, in which cytokine-induced HPA axis hyperactivity plays a particular role (17).

Given the abundance of the gut microbiota (18), it is commonly assumed that a large part of the circulating levels of LPS and related PAMPs derive from bacteria in the GI tract (19) and that the effects of intraperitoneally (IP) administered LPS replicate primarily the reactions to increased translocation of LPS from the gut lumen under conditions of enhanced mucosal permeability. The intestinal mucosal barrier is subject to many influences that regulate its cellular and paracellular permeability, among which stress is an important factor. de Punder and Pruimboom (19) hypothesize that the stress-induced increase in mucosal permeability serves to meet the enhanced metabolic demand under conditions of stress. At the same time, a persistent increase in the translocation of LPS to the circulation is associated with pathologies such as chronic GI inflammation (20) and non-alcoholic fatty liver disease (21) but also with chronic fatigue syndrome (22), depression (23), and autism spectrum disorder (24). A minor part of circulating PAMPs may also derive from other microbe-colonized organs, such as oral cavity, respiratory system, and genitourinary tract as well as from food (19). It has, in addition, been argued that there are dormant bacterial reservoirs in the blood and certain tissues, including the brain, and that PAMP production in these reservoirs may contribute to chronic inflammatory disease (25).

In view of these facts and conditions, the present article sets out to highlight some of the interactions between peripheral immune activation, especially in the visceral system, and brain function, behavior, and stress coping. These issues are exemplified by the way the intestinal microbiota and its metabolites communicate with the immune system and CNS, on the one hand, and the mechanisms whereby overt inflammation in the GI tract impacts on brain function, pain sensitivity, and stress coping, on the other hand. As the interactions between the peripheral immune system and the brain take place along the gut–brain axis, the major pathways of this communication system are also briefly dealt with. Furthermore, novel insights into the molecular signaling processes in the brain that occur under conditions of peripheral immune stress are discussed, and adaptive processes related to stress coping and resilience are considered. In concluding, these novel aspects of immune–brain interaction are put into perspective with disturbances of mental health that become manifest under conditions of stress and with emerging opportunities of therapeutic intervention.

MULTIPLE COMMUNICATION PATHWAYS ALONG THE GUT–BRAIN AXIS

The communication network between GI microbiota, mucosa, endocrine system, immune system, and enteric nervous system, on the one hand, and the brain, on the other hand, uses at least five information carriers (**Figure 1**): gut microbiota-derived molecules, immune mediators, gut hormones, vagal afferent neurons, and spinal afferent neurons (5). As the interaction between gut and the brain is bidirectional, there are also at least four information carriers that signal from the CNS to the GI tract: parasympathetic efferent neurons, sympathetic efferent neurons, neuroendocrine factors involving the adrenal medulla, and neuroendocrine factors involving the adrenal cortex (5). Additional relays include the blood–brain barrier (BBB) and distinct brain circuits that process the information the CNS receives from the periphery.

It is important to note that these circulation-based (endocrine) and neuronal communication routes do not operate in isolation but are closely interrelated with each other. Microbial metabolites, microbe-associated molecular patterns (MAMPs), and PAMPs can act both on GI endocrine and/or immune cells and sensory neurons. This is exemplified by the short-chain fatty acids (SCFAs) comprising acetic, *n*-butyric, and propionic acid, which are generated from otherwise indigestible carbohydrate fibers through microbial fermentation. SCFAs are multi-target messengers that act on GI endocrine, mucosal, and immune cells as well as on brain microglia (26, 27). SCFAs are important energy sources for the microbiota and mucosa, exert antiinflammatory effects through their action on macrophages, neutrophils,

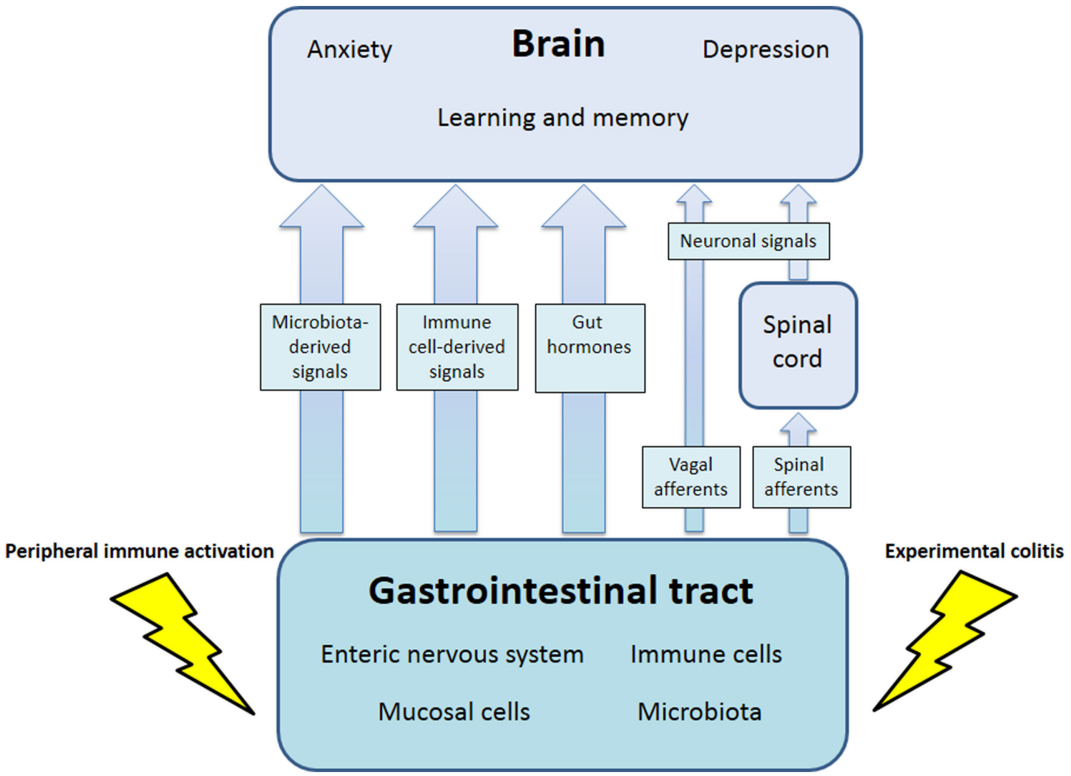

FIGURE 1 | Pathways involved in the behavioral disturbances associated with visceral immune activation and inflammation. There are multiple communication pathways between gut and brain: microbiota-derived signals, immune cell-derived signals, gut hormones, and vagal and spinal afferents. In the course of experimental colitis or microbe-evoked peripheral immune activation, signaling along these pathways is altered, ultimately influencing brain functions, such as anxiety, depression-like behavior, learning, and memory.

dendritic, and regulatory T cells, and have a fortifying influence on the GI epithelial barrier (27) as well as BBB (28).

Most of the cellular effects of SCFAs are mediated by G protein-coupled receptors (GPRs), such as GPR41 (also known as FFAR3), GPR43 (also known as FFAR2), and GPR109A (also known as HCAR2) (26, 27). This is also true for the impact SCFAs have on enteroendocrine cells in the GI mucosa. By stimulating GPR41 and GPR43 on L cells in the distal ileum and colon, SCFAs release the gut hormones peptide YY (PYY), glucagon-like peptide-1 (GLP-1), and GLP-2 (29–31). Through this route, enteroendocrine cells convey messages of the gut microbiota within the digestive system as well as to distant organs, including the brain. Following their release from L cells, PYY and GLP-1 inhibit gastric motility, improve glucose homeostasis, induce satiety (29, 32), and alter behavior (33, 34). It is likely that other appetite-regulating hormones, such as ghrelin, cholecystokinin, and leptin, are also under the influence of the gut microbiota (35–37). Gut hormone activity may be coupled with intestinal immune processes, as proinflammatory prostaglandins (PGs) acting *via* EP_4 receptors enhance the release of GLP-1, GLP-2, and PYY (38), and enteroendocrine cell activity is increased in Crohn's disease affecting the small bowel (39). In addition, enteroendocrine cells may be involved in hormone-independent ways of gut–brain communication, given that misfolded α-synuclein could be transferred to the brain through direct connections between enteroendocrine cells and neural circuits, thus contributing to the pathogenesis of Parkinson's disease (40).

A number of gut hormones including PYY, GLP-1, and ghrelin signal to the brain to affect appetite and energy homeostasis but also impact on mood and emotional-affective behavior (29, 32–34). This messaging is not only accomplished *via* a circulatory route but also through activation of vagal afferent neurons (5, 41). The vagus nerve appears in fact to play a particular role in the signaling of microbial, endocrine, and immune signals to the brain, which is consistent with its predominant sensory nature, given that the vast majority (80–90%) of the axons in the vagus nerve are afferent nerve fibers (42–44). Vagal afferents are thought to tonically deliver information from visceral organs to the brain, this massive sensory input being relevant not only to the autonomic regulation of digestion and energy balance but also to interoception (45, 46). Also termed the "sixth sense" (45), interoception refers to the integrated sense of the physiological condition of the body (46) and the representation of the internal state in the brain (47). GI interoception includes a wide range of conscious sensations, such as pain, nausea, GI discomfort, GI tension, hunger, and thirst, as well as signaling processes that go virtually unnoticed although they impact on emotional-affective and cognitive processes (48–50).

IMMUNE STRESS SIGNALING FROM THE GUT TO THE BRAIN

Immune Signaling *via* Microbial Factors

The gut microbiota is a rich source of potential messenger molecules: primary metabolites generated by microbial cells, MAMPs, and PAMPs shed from microbial cells, and secondary metabolites generated by microbial fermentation of food components or transformation of host molecules such as bile acids (27, 51–55). Apart from the MAMPs and PAMPs, many of the other microbial metabolites, such as SCFAs, trimethylamine-*N*-oxide, p-cresol, aryl hydrocarbon receptor ligands, formyl peptides, flagellin, polyamines such as spermidine, 4-ethyl phenol sulfate (4-EPS), and polysaccharide A produced by *Bacteroides fragilis* (27), have an effect on the immune system, may influence sensory nerve activity or travel by the blood stream to distant organs including the brain (**Table 1**). While we know many factors that govern the composition, diversity, and function of the gut microbiota, we still lack a full comprehension of the signaling systems that govern the homeostatic interaction between the gut microbiota and the local immune system as well as the resilience of this homeostatic cross-talk. In a wider perspective, a dysbalance in the micobiota–immune relationship represents itself a stress scenario which, if this "immune stress" is transmitted to the brain, will elicit a systemic stress response. As alluded to before, the intestinal mucosal barrier (27) plays an important role in the interaction between the gut microbiota and the intestinal immune system. Spadoni and colleagues (56) have recently characterized some of the structural and functional characteristics of the gut–vascular barrier in mice and humans that controls the translocation of microbial macromolecules into the bloodstream and denies entry of microbial cells. They identified Wnt/β-catenin signaling in gut endothelial cells as an important control mechanism which, when downregulated, may enable certain pathogenic bacteria such as *Salmonella typhimurium* to enter the bloodstream (56).

Although the list of identified chemical messengers derived from the gut microbiota is steadily increasing, only a limited number of these molecules have been investigated in their effects on gut–brain and immune–brain signaling: PAMPs such as LPS, lipoteichoic acid (LTA), and peptidoglycan components, SCFAs and 4-EPS (**Table 1**). The latter metabolite is markedly increased in a mouse model of autism spectrum disorder which is caused by maternal immune activation and characterized by enhanced gut permeability, altered microbial composition, altered serum metabolomic profile, and defects in communicative, stereotypic, anxiety-like, and sensorimotor behaviors (57). Some of these behavioral abnormalities are reproduced by 4-EPS, while treatment with the human commensal *Bacteroides fragilis* has a beneficial effect (57).

Immune Signaling Across the Blood-Brain Barrier (BBB)

The BBB is an important checkpoint for the entry of molecules and cells into the brain and in this capacity shares many similarities with the gut–vascular barrier (56, 58). Both boundaries are

TABLE 1 | Effects of PAMPs and other microbial metabolites on emotional-affective and cognitive behavior.

PAMP/metabolite	Main receptor	Dose	Species (sex)	Behavioral effects	Additional effects	Reference
MALP-2 FSL-1	TLR2/6	100 µg/kg IP	Wistar rats (male)	Sickness behavior: anorexia, adipsia, hypoactivity	Hypo- and hyperthermia, upregulated levels of proinflammatory cytokines in plasma	(137)
Pam3CSK4	TLR2/1	200 ng/2 µl in mice, 1 µg/3 µl in rats, ICV	Mice and rats (male)	Sickness behavior: anorexia, hypoactivity	Hypothalamic inflammation and microglia activation, increased POMC neuron activity, hyperthermia	(138)
LTA	TLR2	20 mg/kg IP	C57BL/6N mice (male)	No effect 3 h after LTA injection	Upregulated levels of proinflammatory cytokines in plasma (proteins) and brain (mRNA), decreased expression of tight junction-associated proteins in the brain, increased circulating corticosterone levels	(97)
LPS	TLR4	0.83 mg/kg IP	C57BL/6 mice (male)	Acute sickness (6 h) and delayed depression (24 h)	Expression of acute (c-Fos) and chronic (ΔFosB) cellular reactivity markers	(13)
			Crl:CD1 mice (male)	Depression-like behavior prevented by minocycline or IDO antagonist 1-MT	Enhanced kynurenine/tryptophan ratio in plasma and brain normalized by minocycline or 1-MT	(191)
		0.8 ng/kg IV	Healthy human volunteers (male)	Anxiety, depressed mood, and decreased memory performance	Increased circulating levels of IL-6, TNF-α, soluble TNF receptors, IL-1 receptor antagonist and cortisol, mild increase in rectal temperature	(87)
		0.4 ng/kg IV	Healthy human volunteers (male, female)	Anxiety, depressed mood, sickness symptoms	Increase in circulating proinflammatory cytokines and cortisol higher in females than males	(90)

(Continued)

TABLE 1 | Continued

PAMP/metabolite	Main receptor	Dose	Species (sex)	Behavioral effects	Additional effects	Reference
		1.0 ng/kg IV	Healthy human volunteers (male)	Sickness symptoms	Microglial activation throughout the brain, increased circulating levels of proinflammatory cytokines	(91)
FK565, MDP, LPS	NOD1, NOD2, TLR4	3, 3, 0.1 mg/kg IP	C57BL/6 mice (male)	NOD agonists alone without effect, synergism with LPS in eliciting sickness	Hypothermia, upregulated levels of proinflammatory cytokines in plasma (proteins) and brain (mRNA), increased circulating corticosterone levels	(10)
Poly I:C	TLR3	6 mg/kg IP	Sprague–Dawley rats (male)	Reduced locomotor activity (6 h), anxiety-like behavior (24 h), reduced saccharin preference (24–72 h)	Decreased body weight gain (24 h), molecular changes in frontal cortex and hippocampus: increased proinflammatory cytokine and IDO expression (mRNA, 6 h), reduced BDNF and TrkB expression (mRNA, 6, 24, 48 h), increased tryptophan (6, 24, 48 h), and kynurenine (24, 48 h) levels	(174)
		2, 6, 12 mg/kg IP	C57BL/6 mice (female)	Dose-dependent acute sickness observed in OFT (4, 8, 12 h) and burrowing (6, 10, 26 h)	Upregulation of proinflammatory cytokines in plasma (protein) and brain (mRNA), biphasic core body temperature change	(96)
		12 mg/kg IP	C57BL/6J mice (male)	Deficit in contextual memory consolidation (24 h)	Diminished BDNF mRNA expression (4 h)	(176)
4-EPS		30 mg/kg IP for 3 weeks	C57BL/6N mice	Increased anxiety and startle reflex	Increase in 4-EPS levels in response to maternal immune activation by Poly I:C	(57)
SCFAs	GPR41	25 mM sodium propionate, 40 mM sodium butyrate plus 67.5 mM sodium acetate in drinking water for 7 weeks	BDF1 mice overexpressing α-synuclein	Motor deficits	α-Synuclein-mediated neuroinflammation	(82)
	GPR43	25 mM sodium propionate, 40 mM sodium butyrate plus 67.5 mM sodium acetate in drinking water for 4 weeks	Germ-free C57BL/6 mice (male and female)		Normalization of microglia density, morphology and immaturity (altered in germ-free mice)	(26)
Sodium butyrate	GPR41	1 g/kg by oral gavage for 3 days	Germ-free C57BL/6J mice (male)	Normalization of blood–brain barrier permeability which is enhanced in germ-free mice	Normalization of occludin expression in frontal cortex which is decreased in germ-free mice, increase of histone acetylation in brain lysates	(28)
	GPR43	1.2 g/kg IP in single injection or for 4 weeks	C57BL/6J mice	Antidepressant-like effect	Increase of histone acetylation in hippocampus	(83)
		1.2 g/kg IP	Aged (24 months) Wistar rats (male)	Rescue of aging-associated memory impairment		(84)
Propionic acid	GPR41	4 µl of 0.26 M solution, ICV	Adolescent (41 days) Long–Evans rats (male)	Restricted behavioral interest in a specific object, impaired social behavior, impaired reversal in T-maze task	Neuroinflammatory response	(85)
	GPR43	4 µl of 0.26 M solution ICV for 8 days	Long-Evans rats	Increase of locomotor activity	Change in molecular phospholipid species in blood and brain	(86)

BDNF, brain-derived neurotrophic factor; 4-EPS, 4-ethyl phenol sulfate; FSL-1; fibroblast-stimulating lipopeptide-1; ICV, intracerebroventricular; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; IP, intraperitoneal; IV, intravenous; LTA, lipoteichoic acid; LPS, lipopolysaccharide; MALP-2, macrophage-activating lipopeptide-2; MDP, muramyl dipeptide; 1-MT, 1-methyltryptophan; NOD, nucleotide-binding and oligomerization domain; OFT, open field test; PAMP, pathogen-associated molecular pattern; poly I:C, polyinosinic:polycytidylic acid; POMC, proopiomelanocortin; SCFA, short-chain fatty acid; TLR, Toll-like receptor; TNF, tumor necrosis factor; TrkB, tropomyosin-related kinase B.

built by a cellular layer that controls the movement of molecules and cells and closely interacts with neighboring immune and other cells that provide functional support to the barrier (56, 58). In the present context, it is particularly worth noting that SCFAs play not only a role in the gut epithelial barrier but also in the development and maintenance of the BBB. This implication has been disclosed in germ-free mice in which increased BBB permeability is associated with reduced expression of the tight junction proteins occludin and claudin-5 in frontal cortex, striatum, and hippocampus (28). A decrease in the expression of these tight junction proteins in the murine hippocampus, but not amygdala, prefrontal cortex, and hypothalamus, has likewise been found after antibiotic-induced disruption of the gut microbiota (54). Re-colonization of the intestine of germ-free adult mice with a normal gut microbiota normalizes BBB permeability and upregulates the expression of tight junction proteins, an effect that is reproduced by butyric acid (28). The microbial control of BBB development and function has very likely a bearing on gut–brain and particularly immune–brain signaling, because the transfer of immune-relevant factors (e.g., cytokines, chemokines, PGs) and even immune cells across the BBB depends on the functional status of the barrier and its regulatory mechanisms (14, 59). Given that the BBB is essential for brain development, function, and homeostasis, the control of BBB permeability is probably an important mechanism whereby the gut microbiota controls brain activity and behavior.

Immune Signaling *via* the Vagus Nerve

As alluded to before, immune signaling from the gut to the brain can also take a neuronal route, particularly *via* the vagus nerve. Microbial as well as immune factors appear to alter the excitability and activity of both enteric sensory as well as vagal afferent neurons, which appear to be connected with each other *via* junctions involving nicotinic acetylcholine receptors (60, 61). One such factor is polysaccharide A derived from *Bacteroides fragilis* which stimulates sensory neurons of the myenteric plexus (62) while components of *Lactobacillus rhamnosus* (JB-1) have a similar stimulant effect on vagal afferent neurons (63). Such microbe-driven neuronal processes are likely to participate in the vagus nerve-dependent effects of probiotics on brain function and behavior (64, 65).

The role of the abdominal vagus in transmitting microbial and immune messages to the brainstem is related both to the proximity of vagal afferent nerve fibers to immunologically relevant structures in the abdominal cavity (11, 16) and to the sensitivity of these nerve fibers to messengers derived from the microbiota and immune system. It has been shown, for instance, that IP administered LPS is primarily transported to the liver where it induces the release of interleukin (IL)-1β from Kupffer cells (macrophage-like cells to screen blood and lymph) (11, 16). The cytokine, in turn, is thought to excite afferent nerve fibers in the hepatic branch of the vagus nerve or to enhance their afferent signaling (11, 66). In addition, the abdominal vagus is associated with paraganglia and connective tissue containing macrophages and dendritic cells that respond to IP administration of LPS with synthesis of IL-1β (67, 68). The abdominal paraganglia of the vagus nerve contain glomus-like cells that have IL-1 receptors (69), are innervated by vagal afferents (70), and appear to act as chemosensory accessory cells (16). Furthermore, vagal afferents innervate abdominal lymph nodes that represent another interface with the visceral immune system (16). Unlike endocrine signaling *via* the bloodstream, sensory neurons are, thus, in a position to enable rapid propagation of immune signals to the brain.

The sensitivity of vagal afferent nerve fibers to PAMPs such as LPS and proinflammatory cytokines has been corroborated by electrophysiological recordings and c-Fos mapping in the central projection areas of sensory neurons. In addition, it has been shown that both vagal and spinal afferent neurons do not only respond to these microbial and immune messengers but can also, under their influence, undergo sensitization to other stimulants. For instance, both LPS and tumor necrosis factor-α (TNF- α) are capable of directly activating vagal afferent neurons in culture (71). In addition, LPS can stimulate sensory neurons *via* activation of the transient receptor potential ankyrin-1 (TRPA1) ion channel (72) and sensitize afferent fibers in mesenteric nerves to serotonin, bradykinin, and gut distension, an effect in which mast cells and cyclooxygenase-2 play a role (73). In a similar manner, IL-1β is able to increase action potential firing in vagal afferents (74, 75), to induce c-Fos expression in the nucleus tractus solitarii, the central projection area of vagal afferent nerve fibers in the medullary brainstem (76), and to sensitize vagal afferent pathways to gastric acid (77). The expression of IL-1 receptors by nodose ganglion cells makes it likely that the cytokine is capable of exciting vagal afferents by a direct action on the axons, although PGs acting *via* EP_3 receptors and cholecystokinin acting *via* CCK_1 receptors have also been implicated (75, 78). Spinal afferent neurons supplying the murine colon are also responsive to proinflammatory cytokines, such as IL-1β and TNF-α, and the mechanical hypersensitivity of mouse colonic nerve fibers evoked by TNF-α is inhibited by a TRPA1 blocker (79).

IMPACT OF IMMUNE STRESS SIGNALING FROM THE PERIPHERY ON BRAIN FUNCTION AND BEHAVIOR

As discussed before, microbial and immune messages originating from the visceral system reach the brain either by an endocrine or neuronal route, the BBB being an important checkpoint for those messengers that arrive *via* the bloodstream. Sensitization of CNS pathways as well as long-term alterations in brain circuitry, connectivity, and activity are ultimately responsible for the mental disturbances in which immune activation and chronic inflammatory disease appear to play a role. The impact of MAMPs and PAMPs, particularly LPS, on the brain *via* particular immune pathways has been most extensively studied in this respect, although a contribution of other factors, such as 4-EPS (57), spermidine (80), and SCFAs is also emerging (**Table 1**). Apart from disturbances of brain function and behavior, PAMPs acting *via* the pattern recognition receptor (PRR) TLR4 (such as LPS) also seem to contribute to the pathogenesis of cerebrovascular disease (81). Specifically, Gram-negative bacteria of the gut microbiota and TLR4 activation stimulate the formation of cerebral cavernous malformations (CCMs) that are risk factors for stroke and

seizure. Activation of TLR4 by LPS accelerates CCM formation in mice, which in turn is prevented by genetic or pharmacological blockade of TLR4 signaling (81).

Short chain fatty acids can enter the brain through uptake by monocarboxylate transporters at the BBB (53). In the brain, SCFAs support the maturation and function of microglial cells which are the resident macrophages of the CNS (26). In a transgenic mouse model of Parkinson's disease, however, SCFAs trigger a microglia-dependent immune response, enhance α-synuclein aggregation, and elicit movement disturbances (82). Injected systemically to mice, butyrate induces an antidepressant-like behavioral response which is associated with an increased expression of brain-derived neurotrophic factor (BDNF) (83). Butyrate is also able to ameliorate the memory decline that develops in aging rats (84), while administration of propionate to rodents has been shown to evoke behavioral abnormalities reminiscent of autism spectrum disorder (85, 86). These findings indicate that microbiota-derived signaling molecules can have both beneficial and deleterious effects on brain function and behavior, the outcome depending very likely on both microbe and host factors.

While most information on the cerebral impact of PAMP/MAMP-evoked immune stimulation has been derived from animal studies, select microbial metabolites, such as LPS, have also been tested in humans. For instance, intravenous LPS injection in healthy human volunteers increases the circulating levels of IL-6, IL-10, TNF-α, soluble TNF receptor, IL-1 receptor antagonist, and cortisol, which is associated with enhanced body temperature, anxiety, negative mood, decreased memory performance, and hyperalgesia (87–90). While these effects are similar to those observed in rodents, the potency of LPS in terms of dose per body weight is >100 times higher in humans (88). Mechanistic studies have shown that the sickness response elicited by intravenous LPS injection in healthy male humans is associated with microglial activation throughout the brain as observed by positron emission tomography (91).

Table 1 summarizes a number of studies in which the effects of PAMPs, MAMPs, and some other microbial metabolites on behavior and related molecular changes have been investigated in rodents and humans. In judging the relevance of these effects it is important to take account of the doses studied and the species, strain, and sex of the subjects tested. Males and females differ in both innate and adaptive immune responses (92) and these sex differences also extend to PAMP/MAMP reactions. For instance, macrophages of male mice express higher levels of TLR4 on their cell surface than those of females, which may explain why male mice respond to LPS with formation of more IL-6 than females (93). The additive effect of LPS and muramyl dipeptide (MDP) to attenuate locomotion is likewise more pronounced in female than male rats (94). Similar observations have been made in humans, given that women react to LPS with enhanced release of proinflammatory cytokines, cortisol, and prolactin compared to males (90). Despite these sex differences, men and women do not differ in LPS-evoked anxiety, mood depression, and sickness, which points to compensatory mechanisms that balance the cerebral impact of the exaggerated immune response in women (90). Sex differences may also influence the pharmacokinetics and pharmacodynamics of immune responses to microbial metabolites (95), and the molecular targets and mechanisms of action of PAMPs/MAMPs may considerably differ with dose (10, 14, 96, 97). This is true for LPS that at the lower dose range induces various dimensions of the sickness reponse as well as depression-like behavior (10, 14) whereas, at a higher dose range, it causes septic shock.

Cytokines As Mediators of LPS-Induced Effects on the Brain

Immune stress signaling across the BBB evokes a neuroinflammatory reaction in the CNS, which contributes to the behavioral disturbances associated with peripheral immune activation. The underlying processes have been most extensively studied with LPS, a PAMP known to target a variety of immune and other cells *via* stimulation of TLR4. At doses <1 mg/kg, LPS reproducibly evokes acute sickness which may evolve into depression-like behavior about 24 h after its injection (13, 98, 99). Chronic exposure to LPS for 8 weeks exerts similar behavioral effects (100). There is some evidence that LPS may induce neuroinflammation also by a mechanism involving NOD-like receptor pyrin domain-containing protein-3 (NLRP3) inflammasome activation and in this way cause long-term deficits in affective and cognitive behavior (101). Although the NLRP3 inflammasome inhibitor Ac-Tyr-Val-Ala-Asp-chloromethylketone prevents the LPS-induced effects on brain and behavior (101), the high dose of LPS used (5 mg/kg) may have caused sepsis-like effects that limit the interpretation of the findings.

Through activation of TLR4, peripheral administration of LPS leads to increased expression of proinflammatory cytokines in the periphery and brain (10, 102, 103). Among these proinflammatory cytokines, IL-1β and TNF-α are considered to be the predominant mediators of LPS-induced sickness behavior, while other cytokines, such as IL-6 and IFN-γ, are thought to primarily amplify the effects of IL-1β and TNF-α (14, 104). Circulating TNFα and IL-1β interact with their receptors on cerebral endothelial cells (CECs), induce the production of cytokines and other secondary messengers, such as PG and nitric oxide (NO), and thereby modulate CNS function and behavior (105–107). Proinflammatory cytokines can also access the brain *via* structures that lack a BBB, such as the circumventricular organs and the choroid plexus, and thus alter brain function (108). Furthermore, monocyte chemoattractants such as monocyte chemoattractant protein-1 (MCP-1/CCL2), which can be expressed by circumventricular organs or activated microglia in response to proinflammatory cytokines, can promote monocyte migration into the brain (103).

Peripheral administration of LPS, IL-1β, or TNF-α induces cytokine expression within the brain and leads to the full spectrum of sickness behavior (109, 110). However, when both IL-1β and TNF-α are blocked, LPS-induced sickness is attenuated (104, 111). IL-1β and TNF-α have been demonstrated to exert their behavioral effects *via* activation of the IL-1 receptor of type I (111) and TNF receptor of type 1 (112), respectively. Both receptors are expressed on neurons and other cell types of the CNS and have been proposed to impact on behavior ultimately through direct effects on neuronal activity (104). However, type-1

TNF receptor signaling on astrocytes has been shown to modify hippocampal excitatory synapses and impair contextual learning/memory through an astrocyte–neuron signaling cascade involving presynaptic *N*-methyl-D-aspartate (NMDA)-type glutamate receptors containing the NR2B subunit (113). On the neuronal level, prolonged exposure to TNF-α is able to inhibit long-term potentiation in hippocampal slices (114). In hippocampal neuronal cultures, TNF-α is able to evoke neuronal excitation through activation of sphingomyelin phosphodiesterase 3 and production of ceramide, an intracellular signaling molecule that leads to NMDA receptor-mediated calcium influx (115). This process enhances the insertion of NMDA receptors containing NR1 subunits into the cell membrane and increases the rate and amplitude of NMDA-receptor-mediated calcium bursts. In addition, the mitogen-activated protein kinase (MAPK) signaling pathway is stimulated by TNF-α, given that inhibition of c-jun N-terminal kinase blocks TNF-α-induced sickness (116). The MAPK pathway may also be responsible for the effect of TNF-α to induce depression-like behavior at intracerebroventricular (ICV) doses that are too low to induce signs of sickness (117, 118).

The transduction mechanisms operated by IL-1β in the CNS comprise the MAPK pathway (119, 120) and inhibition of neuronal long-term potentiation by inhibiting calcium channels (121, 122) but also induction of neuronal hyperexcitation *via* formation of ceramide (123). Moreover, activation of the IL-1 receptor stimulates the mTOR pathway and leads to synaptic defects through increased levels of the epigenetic regulator methyl-CpG binding protein 2 (124). Some of the adverse effects of IL-1β in the brain may also result from its ability to impair long-distance signaling of BDNF by attenuating retrograde endosome trafficking (125). Unlike IL-1β, acute ICV injection of IL-6 does not induce sickness behavior, although it is able to induce fever and activate the HPA axis (126). In addition, IL-6 is required for the manifestation of a full sickness response, and genetic deletion of IL-6 blunts the sickness response to LPS and IL-1β (127). The differences in the behavioral effects of IL-1β and IL-6 have been attributed to the apparent inability of IL-6 to stimulate ceramide synthesis (104) although IL-6 is able to activate similar signaling pathways as IL-1β and TNF-α, leading to a reinforcement of proinflammatory cytokine formation. There is also information that the induction of IL-6 by LPS may differ from that of other cytokines. Thus, while LPS causes an early stimulation of nuclear factor κB (NF-κB), activation of the transduction factor NF-IL-6, which contributes to the expression of IL-6 (128), reaches its peak only 8 h post-treatment (129). An involvement of NF-IL-6 in the delayed inflammatory and behavioral response to LPS has been confirmed by genetic deletion of NF-IL-6, the response being reversed 24 h after LPS treatment (130).

When used as a treatment for cancer or hepatitis C, IFN-α induces signs of sickness (fatigue, decreased motivation, reduced appetite, altered sleep) in nearly all patients within the first week, later followed by the development of symptoms of major depression (sadness, decreased mood, anhedonia, impaired cognitive function) in 30–50% of the patients (131). Analysis of potential vulnerability factors has shown that the patients at risk to develop major depression experience a threefold higher increase of circulating ACTH and cortisol levels in response to the first administration of IFN-α than resilient patients (17). Peripheral (132) and central (133) administration of IFN-α to mice causes depression-like behavior (133), and long-term administration of LPS to rats produces a specific cytokine response in the brain characterized by increased IL-1β and IFN-γ levels (100). Inoculation of mice with Bacille Calmette–Guérin, an attenuated form of *Mycobacterium bovis*, induces depression-like behavior, an effect that is absent in IFN-γ receptor knockout mice while an acute episode of sickness behavior persists (134).

Behavioral Effects of Bacterial PAMPs and MAMPs Other Than LPS

In its effect on the brain, LPS is joined by many bacterial, viral, and fungal PAMPs and MAMPs (**Table 1**). Peptidoglycan, for instance, is a cell wall constituent of many bacteria that has been demonstrated to stimulate the innate immune system and modulate behavior. While peptidoglycan is a TLR2 agonist, its fragments γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and MDP activate the intracellular nucleotide-binding and oligomerization domain (NOD) innate immune receptors NOD1 and NOD2, respectively (135). Furthermore, the family of antibacterial pattern recognition molecules termed peptidoglycan recognition proteins (PGRPs) typically binds the muramyl pentapeptide, or tetrapeptide fragment of peptidoglycan (136). In a mechanistic perspective, it is relevant to know that TLR2 can heterodimerize with TLR1 and TLR6, forming TLR2/1 and TLR2/6 heterodimers, respectively. IP injection of the TLR2/6 agonists macrophage-activating lipopeptide-2 or the synthetic analog fibroblast-stimulating lipopeptide-1 induces cytokine release and sickness behavior in rats (137). Similarly, ICV injection of the TLR2/1 agonist Pam3CSK4 evokes sickness which can be attenuated by NF-κB or COX inhibition with indomethacin (138). In addition, TLR2/1 activation causes hypothalamic inflammation and microglia activation, increases physical contacts between microglia and proopiomelanocortin (POMC) neurons, and stimulates the activity of POMC neurons (138). In line with these hypothalamic effects, Pam3CSK4-induced anorexia can be reversed by ICV administration of a melanocortin receptor 3/4 antagonist (138).

Exposure to peptidoglycan early in life, either during pregnancy or postnatally, has adverse effects on the brain. Intravenous injection of peptidoglycan into pregnant dams traverses the placenta and reaches the fetal brain where it causes marked neuronal proliferation through TLR2/6 agonism (139). This fetal neuroproliferative response which involves the neuronal nuclear transcription factor FoxG1 is associated with abnormal cognitive behavior in the pups following birth (139). Exposure of the postnatal mouse brain to the TLR2/1 agonist Pam3CSK4 or TLR2/6 agonist fibroblast-stimulating lipopeptide-1 has a life-long effect on learning and memory, spatial memory being impaired by the TLR2/1 agonist only, while contextual and cued fear learning in adult mice is enhanced by both agonists (140). Peptidoglycan derived from the gut microbiota can also translocate into the brain and activate cerebral PRRs (141). Specifically, the levels of peptidoglycan in the developing brain parallel the postnatal microbial colonization of the gut, while

peptidoglycan-sensing molecules (TLR2, NODs, PGRPs) are expressed during early postnatal brain development and altered in response to perturbation of the gut microbiota (141). Knockout of the PGRP 2 is associated with enhanced sociability and alterations in the expression of the autism risk gene *c-Met*, the synaptogenesis marker *Synaptophysin* and *Bdnf* (141). Germ-free mice are likewise more sociable as reported by the same laboratory (142), whereas Desbonnet et al. (143) find decreased sociability in germ-free mice. Besides acting on the brain, peptidoglycan derived from the gut microbiota also impacts on the systemic immune system, given that it stimulates and primes bone marrow-derived neutrophils through NOD1 (144).

Lipoteichoic acid is a cell wall component of Gram-positive bacteria, which activates primarily TLR2 and in this way causes peripheral immune activation and initiates neuroinflammatory processes in the mouse brain that are associated with downregulation of BBB components and activation of the HPA axis, although emotional behavior is not affected (97). Many commercial LTA preparations are potentially contaminated with LPS, in which case the biological effects of LTA preparations can in part be attributed to the presence of LPS or a particular interaction between TLR2 and TLR4 activation (97). Such positive or negative interactions between different PAMPs in their effects on immune system and brain are of pathophysiological relevance because bacteria are usually equipped with a multitude of PAMPs. NOD agonists, for instance, evoke only mild immune stimulation on their own but synergize with LPS and lead to aggravated cytokine expression and sickness behavior (10). In the colon, however, the interaction of NOD and TLR4 is antagonistic, with NOD2 activation attenuating TLR4-dependent cytokine production and experimental colitis (145).

While activation of PRRs induces behavioral disturbances, inactivation of these receptors can also cause behavioral deficits. Thus, TLR4 knockout mice exhibit reduced novelty-seeking and social interaction in an approach-avoidance conflict situation, a deficit that can be reversed by administration of GABA into the nucleus accumbens shell which appears to be hyperactivated in response to behavioral testing of TLR4 knockout mice (146). TLR2 knockout mice display reduced exploratory behavior, impaired spatial learning, and enhanced contextual and cued fear learning (140). While Park et al. (147) likewise report cognitive impairment in TLR2 knockout mice, they also observe schizophrenia-like symptoms, such as hyperlocomotion, anxiolytic-like behavior, prepulse inhibition deficits, and social withdrawal. These behavioral perturbations and the associated biochemical alterations (increased p-Akt and p-GSK-3α/β expression) are reversed by antipsychotic drug administration (147). Double deletion of the *Tlr2* and *Tlr4* genes is associated with decreased exploratory behavior and impaired performance in a visual discrimination reversal task (148).

Behavioral Effects of Viral PAMPs

While research on the impact of immune stress on the brain has thus far been focused on bacterial immune stimulants, interest in the contribution of viral immune stimulants is also increasing. It is well recognized that psychological stress can adversely influence the outbreak and recurrence of Herpes simplex virus, human immunodeficiency virus (HIV), and hepatitis C virus (HCV) infections (149, 150) *via* activation of the HPA axis and sympathetic adrenomedullary system (151, 152). Vice versa, HIV seropositive patients may suffer from many neuropsychiatric disorders, in particular major depression and dementia (153). Like HCV (154), HIV can cross the BBB simply by infecting macrophages that migrate to the brain where the virus leads to neurotoxin-mediated neuronal loss and synaptic damage (155, 156).

The PAMPs presented by viruses are DNA and RNA molecules as well as surface glycoproteins (157, 158). Nucleic acids of viral origin are recognized by TLR3, TLR7, TLR8, and TLR9 in endosomal compartments, as well as by RNA helicase retinoic acid-inducible gene-I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) found in the cytosol. Receptor stimulation leads to a proinflammatory immune response by activating a battery of transcription factors followed by the production of type I IFNs and other cytokines including IL-1β, IL-6, TNF-α, and IFN-γ, depending on the kind of viral infection (157, 159–161). Furthermore, sensing of viral nucleic acids by RIG-I and other PRRs leads to inflammasome formation and activation of caspase-1, resulting in the production of IL-1β and IL-18, enhancement of the antiviral response of the immune system and pyroptosis (162, 163). The cytokines formed in response to viral infection signal to the brain, evoke sickness behavior and cause other perturbations of brain function and behavior. The effect of the influenza virus to depress food intake in mice, for instance, is attenuated by an IL-1 receptor antagonist (164). In humans, the plasma levels of IL-1β are elevated in patients suffering from post-viral depression when compared to patients who do not develop post-viral depression (165).

Polyinosinic:polycytidylic acid (Poly I:C) is a synthetic PAMP that is widely used to study virus-evoked stimulation of the innate immune system, as it specifically mimics dsRNA molecules occurring during the replication of most viruses except negative-strand RNA viruses (166). Like dsRNA, Poly I:C binds to TLR3 in endosomal compartments (160, 167) and, to a much lesser extent, to cytosolic RIG-I as well as MDA5 if the molecule contains more than 2,000 base pairs (168). Systemic Poly I:C administration to rodents induces expression and release of proinflammatory cytokines, especially type I IFNs, IL-6, IL-1β, and TNF-α (96, 160), the magnitude of effect being considerably smaller than that evoked by the TLR4 agonist LPS (169). Since Poly I:C is rapidly degraded by RNases (170), its prolonged neuroinflammatory and behavioral effects are mediated by the cytokines it induces (171, 172).

Poly I:C given to adult rodents mimics the acute phase of a viral infection, alters a variety of brain functions and gives rise to a sequence of behavioral alterations (**Table 1**). Within 4 h, Poly I:C causes fever and attenuates activity, followed by anxiety-like behavior and anhedonic depression-like behavior that can be observed during the period 24–72 h post-treatment (96, 173, 174). In addition, an impairment of working memory and a deficit in contextual fear memory consolidation become manifest (175, 176). These behavioral perturbations are associated with disruption of the BBB, enhanced expression of indoleamine 2,3-dioxygenase (IDO) in hippocampus and

frontal cortex, suppression of hippocampal neurogenesis and decreased expression of BDNF in hippocampus and cortex (173, 174, 176, 177). In line with the common notion that systemic infection can impair cognitive function, administration of Poly I:C over several days increases amyloid-β_{1-42} levels in hippocampal tissues, paralleled by a deficit in hippocampus-dependent learning tasks (178). Further analysis has revealed that peripheral immune activation by Poly I:C causes dendritic spine loss and impairs dendritic spine formation associated with learning (179). The resulting deficit in multiple learning tasks in mice is mediated by cells derived from peripheral monocytes and a TNF-α-dependent mechanism but does not require microglial function in the CNS (179). Furthermore, systemic Poly I:C amplifies brain pathology in the ME7 model of prion disease and accelerates progression of neurodegeneration (180). In confirmation of the hypothesis that a viral infection during pregnancy is a risk factor for particular neuropsychiatric diseases in the offspring (181), Poly I:C administration to pregnant rodents has been found to evoke a schizophrenia-like phenotype (182–184) as well as depression-like phenotype (185) in the adult offspring, changes that are associated with hippocampal synaptic deficits in the absence of microglial alterations (186).

Signaling Molecules in the Brain Affected by Peripheral Immune Stress

IFN-γ is a strong inducer of the tryptophan-degrading enzyme IDO (187) which has gained center stage as a factor in cytokine-induced mood disorders. Cytokine-induced activation of IDO causes augmented conversion of tryptophan to kynurenine. While kynurenine itself is inactive, its metabolites, 3-hydroxykynurenine and quinolinic acid exert neurotoxic effects through NMDA receptor agonism and generation of oxidative radicals (188). By contrast, kynurenic acid, another kynurenine metabolite, is neuroprotective, acting as an antagonist of NMDA and α7 nicotinic acetylcholine receptors (188). Depressed patients display in fact a reduction of kynurenic acid/3-hydroxykynurenine and/or kynurenic acid/quinolinic acid ratios (189). Kynurenine levels rise not only in response to proinflammatory cytokines but also in response to corticosteroids, given that multiple mRNA transcripts of IDO are differentially expressed in response to different immune stimulants and corticosteroids (190). Importantly, the IDO antagonist 1-methyl-D,L-tryptophan is able to block LPS-induced depression-like behavior, while the levels of proinflammatory cytokines and sickness behavior remain unchanged (191). In a similar vein, the NMDA receptor antagonist ketamine abrogates LPS-induced depression-like behavior without affecting the sickness response (192). Ketamine is able to induce rapid antidepressant effects in patients with treatment-refractory depression, highlighting the potential of targeting glutamatergic neurotransmission as a treatment option for depressive disorders (193).

The sickness behavior evoked by immune stimulation comprises anorexia, a response in which IL-18 plays a role through inhibition of type III GABAergic neurons in the bed nucleus of the stria terminalis (194). Secretion of the "satiety hormone" leptin in response to LPS also contributes to inflammation-induced anorexia. Thus, leptin deficiency attenuates LPS-induced anorexia, and further analysis has shown that both the PI3K and STAT3 signaling pathways in leptin receptor-expressing cells are required for the acute hypophagic response to LPS (195). In addition, leptin is involved in cytokine-induced depression, as ICV leptin administration induces depression-like behavior, while leptin antagonism attenuates particular components of this behavioral response (196). Apart from cytokines and leptin, PGs also mediate some aspects of the sickness in response to immune activation. PGE_2, for instance, has been implicated in inflammation-induced feelings of malaise and discomfort (197). An analogous behavioral readout in mice, LPS-induced conditioned place aversion, is mediated through MyD88-dependent activation of CECs, resulting in cyclooxygenase-1 (COX-1)-dependent PGE_2 synthesis. By activating EP_1 receptors on striatal neurons, PGE_2 inhibits a motivational circuitry through GABA-mediated inhibition of dopaminergic neurons (197).

Cytokines induce a wide range of neurochemical changes in the brain including altered function of monoamine, glutamate, and neuropeptide systems as well as deficits in growth factors such as BDNF (198). Neuropeptide Y (NPY) signaling *via* Y2 and Y4 receptors has in particular been implicated in the short-and long-term behavioral effects of peripheral immune challenge. Thus, Y2 receptor knockout mice are particularly sensitive to the effects of LPS-evoked immune stress to attenuate locomotion and social interaction and to increase anxiety-like behavior, while the LPS-induced rise of circulating corticosterone is suppressed by Y2 receptor knockout (8, 98, 99). Furthermore, knockout of Y2 and Y4 receptors unmasks the ability of a single LPS injection to cause a delayed and prolonged increase in depression-like behavior, which indicates that NPY signaling conveys resilience to some of the adverse effects of immune stress on the brain (98). In addition, combined deletion of NPY and PYY aggravates and prolongs the weight loss caused by Bacille Calmette–Guérin, which attests to an important role of NPY and PYY in orchestrating homeostatic reactions to infection and immune stimulation (199). Changes in serotoninergic and glutamatergic systems seem to be prominently involved in cytokine-induced mood and cognitive symptoms, while the dopamine system has been implicated in symptoms such as fatigue, decreased motivation and altered appetite (200). Cytokine-induced activation of the enzyme GTP cyclohydrolase I (GTP-CH1) is an important mechanism whereby immune activation affects dopamine and serotonin synthesis. Thus, GTP-CH1 activation inhibits the formation of tetrahydrobiopterin which is an essential co-factor of dopamine and serotonin biosynthesis (201). In addition, RNA-Seq studies reveal wide ranging changes of the microglial transcriptome associated with depression-like behavior recorded 7 days after peripheral immune activation by Bacille Calmette–Guérin (202).

Emerging evidence indicates that bioactive lipids, including eicosanoids, endocannabinoids, and specialized pro-resolving mediators (resolvins, maresins, protectins, lipoxins) participate in the regulation of the neuroinflammatory, cerebral and behavioral consequences of immune activation and inflammation (203). PGs are very likely involved in the development of sickness and

depression-like behavior induced by peripheral immune challenge. In particular, PGE_2 is synthesized in the brain in response to a variety of immune signals such as LPS or cytokines, and its administration has been found to evoke sickness behavior through stimulation of EP_2 receptors (204). Conditional deletion of EP_2 receptors in myeloid linage cells blunts the brain microglial response to systemic LPS injection, which attests to a role of EP_2 receptor stimulation in immune–brain signaling (205). Chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTH2) is a PG D_2 receptor that is also involved in the behavioral effects of LPS (206, 207). Inhibition of PG synthesis by nonsteroidal antiinflammatory drugs attenuates the sickness and depression-like behavior induced by LPS, Bacille Calmette–Guérin and interferon-α-2b (204, 208–211). Cyclooxygenase-2 inhibitors, particularly celecoxib, have the potential to ameliorate depression in humans, although a meta-analysis of the pertinent clinical trials points out that the subgroup of patients who could benefit from such therapy has not yet been identified (212).

Endocannabinoids such as 2-arachidonoylglycerol and anandamide constitute another class of lipid mediators that have an impact at several levels of the immune–brain axis (213). Apart from their inhibitory effect on inflammation in the GI tract (5, 213, 214), endocannabinoids influence the responsiveness of vagal and spinal afferent neurons and modify neuronal as well as microglial activity in the CNS. Activation of cannabinoid CB1 receptors which are expressed by visceral afferent neurons in the vagus nerve (215) blocks the effect of TNF to amplify vagal afferent responsiveness (216). In the brain, endocannabinoids appear to protect from BBB dysfunction and neuroinflammatory processes under conditions of immune challenge. Such a homeostatic role is, for instance, deduced from the finding that hydrolysis of the endocannabinoid 2-arachidonoylglycerol by monoacylglycerol lipase generates neuroinflammatory PGs in response to peripheral LPS administration (217, 218). Prevention of the hydrolysis of the endocannabinoid anandamide by an inhibitor of fatty acid amide hydrolyase likewise attenuates the expression of pro- and antiinflammatory cytokines in the frontal cortex and hippocampus of rats following peripheral stimulation of TLR3 (poly I:C) or TLR4 (LPS) and inhibits the LPS-induced anhedonia, but not sickness response (219, 220). Moreover, deletion of CB2 receptors exacerbates, while CB2 receptor agonism attenuates, stress-induced neuroinflammatory responses in the brain (221). These observations are in line with the emerging concept that the endocannbinoid system in the brain operates at the intersection between stress, immune activation, neuroinflammation, emotionality, and neuropsychiatric disease (222, 223).

Specialized pro-resolving mediators, such as resolvins, maresins, protectins, and lipoxins, play not only a role in the resolution of inflammation but also emerge as regulators of neuroinflammatory processes and their impact on brain function and behavior (203, 224). The antiinflammatory effect of resolvins appears to involve neurons, given that the ability of resolvin D1 (RvD1) to attenuate zymosan-evoked peritonitis in mice depends on the vagus nerve, since vagotomy increases the severity of inflammation (224). This implication of the vagus nerve appears to be mediated by netrin-1, an axonal guidance molecule (224). Both RvD1 and resolvin E1 (RvE1) are able to decrease the LPS-evoked expression of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) in microglia cells *in vitro* (225). The underlying mechanisms differ, however, as RvE1 regulates the NFκB signaling pathway and RvD1 acts via microRNA expression (225). *In vivo* experiments demonstrate that RvD1 and RvD2 counteract the depressogenic effect of LPS *via* the mammalian target of rapamycin complex 1 signaling pathway, an effect in which the medial prefrontal cortex and dentate gyrus are of particular relevance (226). A similar antidepressant effect of RvD1 and RvD2 has been observed in the chronic unpredictable stress model (227). The ability of other specialized pro-resolving mediators such as lipoxins, protectins and maresins to regulate neuroinflammatory processes and behavioral reactions to immune stimulation has not yet been systematically investigated. Systemic administration of lipoxin A4 to mice reduces anxiety-related behavior (228), and treatment of rats with an analog of lipoxin A4 has been reported to attenuate neuroinflammation in a rat model of ischemic stroke and to promote sensorimotor recovery (229). Docosahexaenoic acid is a major n-3 polyunsaturated fatty acid present in the brain, and acute ICV infusion of unesterified docosahexaenoic acid protects from LPS-evoked neuroinflammation, an action that involves conversion to specialized pro-resolving mediators such as neuroprotectin D1 (230).

CYTOKINE HYPOTHESIS OF DEPRESSION

The changes in emotional-affective behavior seen in experimental studies of immune stress and in therapeutic trials of interferon therapy are consistent with the cytokine hypothesis of depression. Following its first formulation as macrophage theory of depression by Smith (231), proinflammatory cytokines as well as microglial activation and neuroinflammation have been demonstrated to occur not only in depression but also in other psychiatric disorders such as schizophrenia and bipolar disorder (232–235). In identifying the precise Research Domain Criteria driven by inflammation it has been recognized that inflammatory processes represent pivotal factors in the psychopathology of symptom dimensions shared by different psychiatric conditions (200). In testing the therapeutic potential of antiinflammatory agents in depressed patients (236), the TNF-α blocker infliximab has been found to improve symptoms only in patients with high inflammatory markers, while patients with low levels of peripheral inflammation experience rather negative effects (237). A similar response pattern has been reported for the treatment with omega-3 fatty acids (238). Low levels of TLR3 in peripheral blood mononuclear cells of depressed patients also predict therapeutic efficacy of antidepressant drugs while high expression of this PRR is associated with treatment failure (239). These clinical observations are in part supported by experimental findings that low cytokine concentrations have a positive influence on learning, memory, and synaptic plasticity (240), that meninges-derived T-cells exert beneficial effects on learning behavior through IL-4 and BDNF expression (241), and that adaptive immunity of the

meninges supports cerebral circuits underlying social behavior through IFN-γ (242).

IMPLICATIONS OF THE GUT MICROBIOTA IN MOOD DISORDERS

The cytokine hypothesis of depression has been extended by an increasing number of observations to suggest that the gut microbiota contributes to the pathogenesis of depression, given that depressed patients may present with a change in the microbial community structure, a disruption of the intestinal barrier and increased endotoxinemia (243). Depression-associated changes in the gut microbial community have been observed at the phylum level, with either a decrease (244, 245) or increase (246) in the relative abundance of *Bacteroidetes*. A decrease in *Bacteroidetes* has also been observed in other disorders of the gut–brain axis such as obesity and irritable bowel syndrome (IBS) (247, 248), although comorbid depression in IBS patients is not associated with particular alterations in the microbial profile (249). A causal relationship between gut microbiota and depression has been inferred from the observations that transplantation of fecal microbiota from depressed patients to germ-free or germ-depleted rodents induces a depression-like phenotype in the animals (245, 250). This transfer of a depression-like pathology is accompanied by an increase in the kynurenine/tryptophan ratio and other inflammation markers (250) and an altered profile of metabolites involved in carbohydrate and amino acid metabolism (245).

Given the impact of stress on mood disorders, stress-induced alterations in the commensal microbiota community, GI barrier and GI physiology are increasingly recognized to be relevant to stress-evoked immune activation and behavioral disturbances (251). It has long been known that stress enhances the permeability of the GI mucosa (19), and there is now increasing evidence that a dysfunctional intestinal barrier enables microbiota-driven immune activation to impact on the brain (252). Stress-evoked alterations of the microbial community toward a composition favoring immune stimulation may exacerbate the adverse influence of gut leakiness on the immune–brain interaction. Stress exposure not only disrupts the community structure of the gut microbiota (253) but also induces translocation of, for instance, *Lactobacillus* spp. to the spleen and primes the innate immune system for enhanced reactivity (254).

Antibiotic-induced depletion of the intestinal microbiota blocks stress-induced bacterial translocation and, consequently, TLR activation and neuroinflammation in the brain (255). Antibiotic treatment as well as neutralization of LPS also blunts the formation of the inflammasome-dependent cytokines IL-1β and IL-18 in response to stress, while inflammasome-independent cytokines are not affected (251). In addition, the depressogenic effect of LPS is attenuated in germ-free mice (256). Peripheral administration of the IL-6 receptor antibody MR16-1 counteracts the effect of psychosocial stress to disturb the gut microbiota and to evoke depression-like behavior along alterations in dendritic spine density and synaptic protein expression (257). Taken together with many other findings (252), it is thus emerging that gut microbiota and gut leakiness represent an important interface in the impact of stress on mood disorders.

IMPACT OF INTESTINAL INFLAMMATION ON BRAIN AND BEHAVIOR

Ulcerative colitis and Crohn's disease, the two major manifestations of IBD, are associated with an increased risk of mental disorders and can be exacerbated by stress. The disease impairs the quality of life not only in terms of GI symptoms, discomfort, and pain but also in terms of psychiatric comorbidities. Several mental disorders, including major depression, panic, and generalized anxiety, are more common in IBD patients than in community controls (258). Moreover, the psychiatric comorbidities can affect disease prognosis and response to treatment, as Crohns' disease patients with major depression respond poorly to infliximab (259). In addition, stressful life events are risk factors for IBD development, exacerbation, and relapse (260). Some of the relationships between GI inflammation and emotional-affective disorders can be studied in experimental models of IBD in rodents. Colitis induced by dextran sulfate sodium (DSS) is immunologically similar to ulcerative colitis, while some immune processes occurring in trinitrobenzene sulfonic acid (TNBS)-induced colitis are reminiscent of those seen in Crohn's disease (261). This range of chemically induced models of colonic inflammation is complemented by immunologically provoked paradigms of IBD. Thus, mice deficient in the antiinflammatory cytokine IL-10 (IL-10$^{-/-}$) spontaneously develop a mild, patchy form of colitis which is highly dependent on the composition of the gut microbiota, given that the commensal *Akkermansia muciniphila* is able to induce colitis in IL-10$^{-/-}$ mice (262).

Cerebral and Behavioral Disturbances in Experimental Models of Inflammatory Bowel Disease (IBD)

Various experimental models of GI inflammation are associated with alterations in brain function and behavior (**Table 2**). While IL-10$^{-/-}$ mice have been little studied in this respect, DSS-treated animals exhibit a number of behavioral alterations associated with intestinal inflammation, which have been linked to changes in gut–brain signaling (**Table 2**). For instance, mice with DSS-induced colitis are more anxious and less socially interactive than control mice, and these disturbances occur in parallel with increased circulating IL-6, IL-18, and NPY levels as well as with altered *Npy*, *Bdnf*, *Cox-2*, and *Mineralocorticoid receptor* gene expression in the brain, which points to an involvement of inflammatory and stress mechanisms in the behavioral perturbations (263). Moreover, DSS-treated mice display deficits in novel object recognition memory, which can be prevented by a probiotic (*Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052) and, thus, may involve a major influence of the GI microbiota on behavioral disturbances during colitis (264).

Besides enhanced anxiety, DSS-treated rodents also display aggravated depression-like behavior, a relationship that appears to be sex dependent (33, 265). The increase in anxiety- and

depression-like behavior accompanying DSS-evoked colitis in rats is associated with increased firing rates of colonic afferents, which suggests that altered neuronal signaling is a major factor responsible for the colitis-induced behavioral changes (265). A similar conclusion can be drawn in mice in which DSS-evoked colitis is accompanied by increased anxiety-like behavior only if the vagus nerves are intact (64). In addition, DSS-evoked colitis alters the activity of central neurons. At the height of inflammation, stress-induced c-Fos expression, a marker of neuronal activation, is blunted throughout the corticolimbic system depending on housing conditions (266). Moreover, DSS-treated mice exhibit altered stress-associated behavior, increases in brain IL-6 and growth-regulated oncogene-α levels and brain region-specific changes in *corticotropin-releasing factor (Crf)*, *Bdnf*, and *Npy* gene expression (267). In view of the emerging role of NPY in mediating stress resilience and treating post-traumatic stress disorder (268, 269) it appears worth investigating whether pharmacological manipulation of the NPY system has therapeutic effects in animal models of IBD which are sensitive to stress exposure.

Cerebral alterations associated with TNBS-induced colitis have been less thoroughly examined, but a relevant study has shown that TNBS-evoked colitis induces behavioral despair in mice (**Table 2**), which can be mitigated by inhibition of NO synthase (270). Potential effects of TNBS-induced colitis on other behavioral dimensions await to be explored, as TNBS-induced colitis has a number of cerebral effects such as increased excitability of hippocampal slices, altered hippocampal glutamatergic transmission and microglial activation, and lowered seizure threshold (271, 272). These effects appear to be cytokine and microglia dependent, because they are reversed by ICV administration of an anti-TNF-α antibody and minocycline, an inhibitor of microglial activation (273). In line with these findings, a positron emission tomography and *ex vivo* biodistribution study found increased levels of [^{11}C]PBR28, an imaging biomarker of neuroinflammation, in the cerebellum 11 days after induction of colitis, suggesting activation of microglia or infiltration of macrophages in the brain (274).

Intestinal inflammation can also be triggered by parasites, and infection of mice with *Trichuris muris* has been found not only to cause mild to moderate colonic inflammation but also to elicit anxiety-like behavior (275). These effects are associated with increased circulating levels of TNF-α, IFN-γ, kynurenine and kynurenine/tryptophan ratio, and decreased hippocampal expression of *Bdnf* (275). Etanercept and, to a lesser degree, budesonide reduce cytokine and kynurenine levels and normalize behavior (**Table 2**) but do not influence *Bdnf* expression. By contrast, the probiotic *Bifidobacterium longum* NCC3001 normalizes both behavior and hippocampal *Bdnf* expression but does not affect circulating cytokine and kynurenine levels. Moreover, the anxiety-like behavior associated with *Trichuris muris* infection persists after vagotomy (275), unlike the anxiety-like behavior associated with DSS-induced colitis, which is prevented by vagotomy (64). Thus, the pathways and mechanisms underlying the behavioral perturbations in response to experimental colitis depend on the immunological triggers of GI inflammation.

Intestinal Inflammation and Visceral Pain

IBD and experimental colitis are frequently linked to visceral and somatic hyperalgesia (276). Importantly, DSS-induced colitis enhances the spontaneous activity of spinal afferent neurons supplying the rat colon, and reduction of this spontaneous activity by intracolonic administration of lidocaine causes conditioned place preference in DSS-treated but not control rats (265). This finding indicates that colitis gives rise to continuous abdominal discomfort and not just to hyperalgesia toward acute stimuli (265). Continuous firing of afferent neurons from the inflamed gut is also likely to explain the increased expression of c-Fos and the enhanced phosphorylation of p42/44 MAPK in the lumbosacral spinal cord that takes place in mice suffering from DSS-induced colitis in the absence of noxious stimulation (277). Although inflammatory mediators are known to sensitize GI nociceptors, peripheral pain hypersensitivity alone does not explain all pain symptoms in IBD patients as some patients still suffer from pain in remission, and pain is poorly correlated with inflammatory markers (276, 278). Likewise, under conditions of experimental colitis in rodents, visceral hyperalgesia is not always going in parallel with inflammation. For example, DSS-induced colitis in mice leads to chemical and mechanical visceral hyperalgesia that persists for several weeks post-DSS treatment when intestinal inflammation has already subsided (279, 280). Similarly, mechanical visceral hyperalgesia associated with TNBS-induced colitis in rats can still be measured at a time when inflammation has resolved (281). Although a review of GI hyperalgesia mechanisms is beyond the scope of this article, it should not go unnoticed that TRPV1 ion channels play a role in post-inflammatory visceral hyperalgesia, since TRPV1 knockout and TRPV1 blockade prevent the development of mechanical visceral hyperalgesia after colitis (280, 282).

Chronic pain is a major internal stressor and as such a risk factor for the pathogenesis of anxiety and mood disorders (283, 284), a relationship that is also true for patients with Crohn's disease in whom pain is associated with enhanced anxiety (285). TRPV1 desensitization in DSS-treated rats not only suppresses ongoing activity in spinal afferent neurons but also prevents the development of anxiety- and depression-like behavior associated with DSS-induced colitis (265). This observation confirms that nociceptive signaling from the colon is an important trigger for secondary changes in the brain that underlie the emotional-affective disturbances induced by experimental colitis.

Gut Hormone Signaling in Intestinal Inflammation

Apart from immune and inflammatory mediators and nociceptive messages, gut hormones emerge as an important interface between GI inflammation, visceral pain, and perturbations of brain function, and behavior. IBD alters the expression of several GI and circulating gut hormones, including PYY and GLP-1 (39, 286–288), although the data are inconsistent due to heterogeneities in patient selection and assay methodology. Similarly, colonic PYY has been found to be reduced in response to TNBS in rats, while in response to DSS both increased and reduced colonic PYY levels have been reported (289–291). PYY

TABLE 2 | Effects of gastrointestinal inflammation on emotional-affective and cognitive behavior.

Type of inflammation	Experimental design	Species (sex)	Behavioral effects	Additional effects	Reference
DSS-induced colitis	Three 7-day DSS cycles (3.5, 3, 3% w/v in drinking water) with 5-day recovery periods (tap water) in between	AKR mice (male)	Increased anxiety	DSS-induced anxiety prevented by vagotomy and by the probiotic *Bifidobacterium longum* NCC3001 given daily during and after DSS exposure for 14 d	(64)
DSS-induced colitis	11-day exposure to DSS (2% w/v) in drinking water	WT; NPY KO and PYY KO mice on mixed C57BL/6:129/SvJ (1:1) background (male and female)	Increased anxiety (male WT); increased depression-like behavior (female WT)	Decreased anxiety (female NPY KO and PYY KO); decreased depression-like behavior (male PYY KO)	(33)
DSS-induced colitis	7-day exposure to DSS (2% w/v) in drinking water	C57BL/6N mice (male)	Increased anxiety; decreased social interaction	Repeated WAS exposure (7 d) during DSS exposure prevents behavioral deficits	(263)
DSS-induced colitis	5-day exposure to DSS (5% w/v) in drinking water	Sprague-Dawley rats (male)	Increased anxiety; increased depression-like behavior	Resiniferatoxin-induced desensitization of colonic TRPV1 channels reverses behavioral deficits	(265)
DSS-induced colitis	5-day exposure to DSS (3% w/v) in drinking water	C57BL/6 mice (male and female)	Increased anxiety; decreased novel object recognition memory	Probiotics (mixture of *Lactobacillus rhamnosus* R0011 and *L. helveticus* R0052) administered daily for 7 days before and during DSS exposure) prevent behavioral deficits	(264)
TNBS-induced colitis	Single intrarectal administration of TNBS (10 mg in 50% ethanol)	NMRI mice (male)	Increased depression-like behavior	Nitric oxide synthase inhibition ameliorates depression-like behavior	(270)
Trichuris muris infection-induced colitis	Single infection with *Trichuris muris* (300 eggs/mouse)	AKR mice (male)	Increased anxiety-like behavior	Etanercept, budesonide and the probiotic *Bifidobacterium longum* NCC3001 normalize behavior	(275)

DSS, dextran sulfate sodium; KO, knockout; NPY, neuropeptide Y; PYY, peptide YY; TNBS, trinitrobenzene sulfonic acid; TRPV1, transient receptor potential vanilloid-1; WAS, water avoidance stress; WT, wildtype.

can influence not only appetite but also emotional-affective behavior and visceral pain. Genetic deletion of PYY enhances depression-like behavior in mice, whereas anxiety-related behavior stays unaltered (33). Under conditions of DSS-induced colitis, PYY influences emotional-affective behavior in a sex-dependent manner, because anxiety-like behavior is modified only in female PYY knockout mice while depression-like behavior is altered only in male PYY knockout mice (33). Acute IP administration of PYY (3–36) induces schizophrenia-relevant behaviors including a profound impairment of social interaction (292). PYY is also involved in regulation of pain signaling, given that PYY knockout mice are more sensitive to somatic and visceral pain (293). In a wider perspective, these findings point to a potential involvement of PYY in the pain and emotional-affective alterations seen in animal models of colitis and IBD patients alike.

Like PYY, GLP-1 also influences emotional-affective behavior as revealed by the anxiogenic effect after acute administration and antidepressant effect after chronic administration of the GLP-1 analog, exendin-4, in rats (294). Other GLP-1 analogs have been reported to have an analgesic effect in both somatic and visceral pain states: ROSE-010 shows promising results in relieving visceral pain in patients suffering from IBS (295), while liraglutide suppresses the visceral hyperalgesia induced by LPS or water avoidance stress (WAS) in rats (296). In addition, intrathecally administered GLP-1 receptor agonists are able to alleviate pain evoked by peripheral nerve injury, bone cancer, and diabetes in rats and formalin-induced pain in rats and mice without affecting acute nociceptive responses (297). Therefore, PYY, GLP-1, and potentially other gut hormones emerge as targets for the control of GI inflammation, pain, and their relation to brain processes regulating emotion and affect.

Gut Microbiota, Intestinal Inflammation, and Stress Responsivity

An emerging interface between GI immune system, GI inflammation and brain is posed by the gut microbiota and its multitude of signaling molecules. Germ-free mice display an exaggerated neuroendocrine response to restraint stress (298), and there is increasing evidence that the GI microbiota plays a role in the impact of stress on gut and brain (299). Mice lacking any microbiota display enlarged volumes of amygdala, hippocampus, and periaqueductal gray but a decreased volume of the anterior cingulate cortex compared to conventionally colonized mice (300, 301). These findings, together with marked alterations in brain neurochemistry, brain ultrastructure (300, 302, 303), brain microglia (26), and BBB function (28) may explain why germ-free mice display exaggerated stress responsivity (298), hyperactivity (302, 303), and visceral pain hypersensitivity (301). It would appear that the GI microbiota plays an important role in the development of the gut–brain axis, and that its disruption

predisposes to maladaptive stress responsivity and behavioral profile (299, 304).

As discussed above, SCFAs seem to be important messengers of GI bacteria with a beneficial impact on the brain, and such a role may also be attributed to food, prebiotics, and probiotics that facilitate a physiological community structure of the GI microbiota. The prebiotic sialyllactose, for instance, has been shown to prevent the change in colonic microbiota composition, the increase in anxiety-like behavior and the decrease in hippocampal neurogenesis evoked by social disruption stress in mice (305). Fructooligosaccharides and galactosaccharides are likewise able to protect mice from the disruption of the microbiota community structure, the enhancement of anxiety- and depression-like behavior, and the rise of the circulating corticosterone concentration elicited by chronic psychosocial stress (306). The benefical effects of the prebiotics are associated with increased production of SCFAs in the gut (306).

Despite the clinical observations that stress has an adverse influence on IBD, *predictable* chronic WAS fails to modify the severity of DSS-evoked colitis in the mouse, but prevents colitis-evoked sickness behavior anxiety and disruption of social interaction (263). This effect of repeated WAS is associated with a rise of circulating corticosterone, an increase in hypothalamic *Npy* expression and a blockade of the colitis-associated induction of c-Fos in thalamus, hypothalamus, amygdala, and prefrontal cortex (263, 277). Likewise, the ability of colitis to amplify the expression of c-Fos in the lumbosacral spinal cord in response to noxious chemical stimulation of the colon is blunted by repeated WAS (277). In keeping with this finding, repeated WAS fails to aggravate mechanical and thermal hyperalgesia associated with DSS-induced colitis in mice (277) much as it fails to exacerbate visceral hyperalgesia associated with TNBS-induced colitis in rats (307). Taken together, these observations provide an explanation for the resilience effect of predictable chronic stress and show that the immune stress associated with experimental colitis alters not only brain function but also the cerebral processing of psychological stress and its impact on behavior. In addition, the influence of GI inflammation on stress processing in the brain is modified by environmental conditions, given that environmental enrichment appears to improve stress resilience as deduced from region-specific changes in the activity of the central amygdala, hippocampus, and infralimbic cortex (266).

THE CIRCLE COMPLETED: THE STRESSED BRAIN FACILITATES IMMUNE ACTIVATION AND INFLAMMATION IN THE GUT

Adverse and Beneficial Effects of Stress on Intestinal Inflammation

Psychosocial stress is known to trigger disease exacerbation and relapses of IBD as well as IBS, a relationship that has been confirmed in animal models of GI inflammation (308). For instance, restraint stress amplifies the severity of colitis in IL-10$^{-/-}$ mice as revealed by histopathology, increased expression of proinflammatory cytokines, and aggravated loss of body weight (309). Likewise, neonatal maternal separation stress enhances the permeability of the colonic mucosa both in wildtype and IL-10$^{-/-}$ mice, but colitis develops only in IL-10$^{-/-}$ mice (310), which attests to the multifactorial pathogenesis of chronic GI inflammation (311). DSS-treated mice exposed to chronic restraint stress (312), repeated psychological stress (313), repeated WAS (314), or a combination of repeated social defeat stress and overcrowding (315) also present with enhanced colonic inflammation scores, lowered body weight, and increased proinflammatory cytokine levels. Similarly, TNBS-induced colitis is aggravated after exposure to various stressors (316–319), which demonstrates an adverse impact of stress on disease course and severity. However, the outcome of the interaction between stress and GI inflammation is variable, depending on the type and duration of stress, the type of experimental inflammation and the experimental design. Repeated WAS, for example, has been shown to be without effect on acute and chronic DSS-induced colonic inflammation, on the one hand (263, 320), but to reactivate a quiescent chronic inflammation after exposure to DSS, on the other hand (314).

By contrast, there are also studies attesting to an ameliorating effect of stress on colonic inflammation. Cakir et al. (321) and Gülpinar et al. (322) both report that colitis severity is attenuated if animals are exposed to WAS during colitis induction, or if they are subjected to a controllable electric shock prior to colitis induction, respectively. In contrast to other experiments, these two studies used a single acute stressor, which raises the hypothesis that the acutely stressed brain and body are able to suppress inflammation. In fact, high concentrations of glucocorticoids released by the acute stress exposure are very likely responsible for the antiinflammatory action of stress, as deduced from the antagonistic effects of a glucocorticoid receptor antagonist (321, 322) and a CRF receptor antagonist (322). In line with this contention, chemical stimulation of the paraventricular hypothalamic nucleus, the major source of brain CRF, with glutamic acid alleviates TNBS-evoked colitis as measured by reduced colonic damage scores and blunted colonic levels of IL-6 and IL-17 (323).

In some animal studies, chronic stress *per se* has been shown to provoke the development of spontaneous colitis. In mice exposed to chronic subordinate colony housing, the colon displays an increased histological damage score, a higher number of inflammatory cells, and increased cytokine secretion (324, 325). Similar findings have been obtained in mice exposed to repeated social interaction stress, in which body weight loss, increased circulating cytokine levels and signs of colonic damage were observed (326). Changes in both glucocorticoid and cytokine signaling appear to be involved in the disease onset, but the relative importance of peripheral and central mechanisms remains to be investigated. It is not known whether chronic psychological stress may also trigger GI inflammation in humans. There are only some case reports that extensive physical stress such as that experienced by long-distance runners may give rise to reversible ischemic colitis (327, 328).

Mechanisms Involved in the Impact of Stress on the Gut

Psychosocial stress acts primarily on the brain and disturbs brain function in many ways (329), and these cerebral

perturbations can adversely affect many peripheral organs, including the GI tract (**Figure 2**). Exposure to stress alters not only the activity of the HPA axis and its adrenocortical hormonal system but also that of the parasympathetic nervous system, the sympathetic nervous system, and the sympathetic adrenomedullary hormonal system (**Figure 2**). The HPA axis appears to be the most important stress response system (330) and represents a particularly important interface between stress and the gut, with CRF as a mediator operating both in the brain and GI tract (331). Glucocorticoids released from the adrenal cortex (cortisol in humans, corticosterone in rodents) dampen immune processes and are likely to interfere with immunological processes during stress. In addition, glucocorticoids can also induce non-neuronal catecholamine enzymes (332) which may add to the multiple signaling mechanisms of chronic stress exposure.

Both physical and psychological stressors cause formation of proinflammatory cytokines in the periphery (333), which may be due to "sterile inflammation" (334). In this process, damage-associated molecular pattern molecules, such as heat shock protein-72, uric acid, and ATP activate various PRRs (TLRs, NODs) that stimulate cytokine production (334). Importantly, there are individual differences in the sensitivity of the peripheral immune system that predict vulnerability to social stress (335). Specifically, IL-6 is most highly upregulated in mice that respond to chronic stress with exaggerated social avoidance behavior, whereas IL-6 knockout mice as well as mice treated with an IL-6 monoclonal antibody are resilient to social stress (335). These findings may have a bearing on stress-related psychiatric disorders as patients with treatment-resistant major depression display highly elevated serum levels of IL-6 (335).

In the gut, stress leads to disruption of mucosal tight junctions, which enhances mucosal permeability, facilitates microbial translocation, induces an immune response, and promotes inflammation (19). In addition, stress disrupts the community structure of the gut microbiota (253), which also weakens the mucosal barrier. The paracellular permeability through tight junctions of the GI mucosa is under the control of myosin light chain kinase (MLCK) which is involved in cytoskeletal regulation. MLCK can be activated by cytokines such as TNF-α, which enhances tight junction permeability by actomyosin contraction and reorganization of the tight junction (19). ML-7, a specific MLCK inhibitor, is able to blunt the increase of colonic paracellular permeability and the rise of LPS, ACTH, and corticosterone plasma levels evoked by partial restraint stress in rats (336).

CRF and NPY have proved to be important mediators of stress-related brain–gut interactions because both peptides occur at multiple sites in the gut and brain and affect various functions in both organ systems (32, 331, 337). CRF, for instance, participates in the stress-evoked inhibition of upper GI transit and stimulation of colonic motility (331). NPY serves a proinflammatory role in the gut, while cerebral NPY protects against distinct disturbances in response to immune challenge, enforcing stress resilience both in brain and periphery (337, 338). During restraint stress, fecal pellet output is significantly increased in mice deficient in NPY or the gut hormone PYY, relative to wildtype mice (338, 339). CRF_1 receptor blockade reduces defecation in wildtype and NPY knockout mice but has no effect in PYY knockout mice (338). Endogenous NPY and PYY thus appear to inhibit the colonic motor stimulation evoked by stress, the effect of NPY depending on endogenous CRF acting via CRF_1 receptors (338).

TRANSLATIONAL IMPLICATIONS

The information reviewed here reveals a bidirectional interplay between stress and the immune system, which is particularly obvious in the complex relationship between the GI immune system and the CNS. Psychosomatic medicine has long known that the digestive system is a preferred target of somatoform manifestations of stress. This is not, however, the end of the story. The stress-evoked disturbance of GI function, in which the cross-talk between the gut microbiota and the local immune system is of particular relevance, is signaled back to the CNS and may cause further disturbances of brain function. Thus, the impact of stress on the gut initiates a vicious cycle that is composed of both a brain–gut and gut–brain segment, the two segments being connected in the gut *via* the microbiota-immune network (**Figure 2**). This integrated view is important to consider in order to appreciate the mutual relationship between "stress and immunity" in a broad perspective.

The research we have reviewed here focusses on the impact of peripheral immune stress on the brain, given that a dysbalance of gut microbiota-immune homeostasis is thought to have a bearing on many neuropsychiatric disorders including Parkinson's disease, multiple sclerosis, autism spectrum disorder, anxiety disorders, chronic fatigue syndrome, IBS, major depressive disorder, and cognitive decline (53, 55, 57, 82, 247, 250, 252, 299). Although research involving animal models provides compelling evidence for a causal relationship in an increasing number of instances, most evidence obtained from clinical studies is still of an associative nature. Thus, there is an appreciable gap in the translation of basic research to clinical applications across different microbe and host species. In addition, nutritional, environmental, genetic, epigenetic, and physiological factors will shape the microbiota-immune network in rodents in a quite different manner than that of humans. Furthermore, we still know little about the resilience of this GI network under changes of the external and internal environment, given that a disturbance of microbiota-immune homeostasis represents itself a local stress scenario.

The pathways along which peripheral immune stress is communicated to the brain are multifactorial, comprising both circulating molecules (microbe-derived molecules, immune mediators, gut hormones) and neuronal messengers. Through these signaling systems, several transmitter and neuropeptide systems within the brain are altered, enabling adaptive processes related to stress coping and resilience to take place or, if these measures are exhausted, giving rise to various CNS pathologies. Particular microbe-derived molecules (e.g., SCFAs), immune mediators (e.g., cytokines), and CNS messengers (e.g., neurotrophic factors, NPY) may play a particular role in determining whether inputs from the gut have a beneficial or deleterious effect on the brain. Dissection of the complex information flow

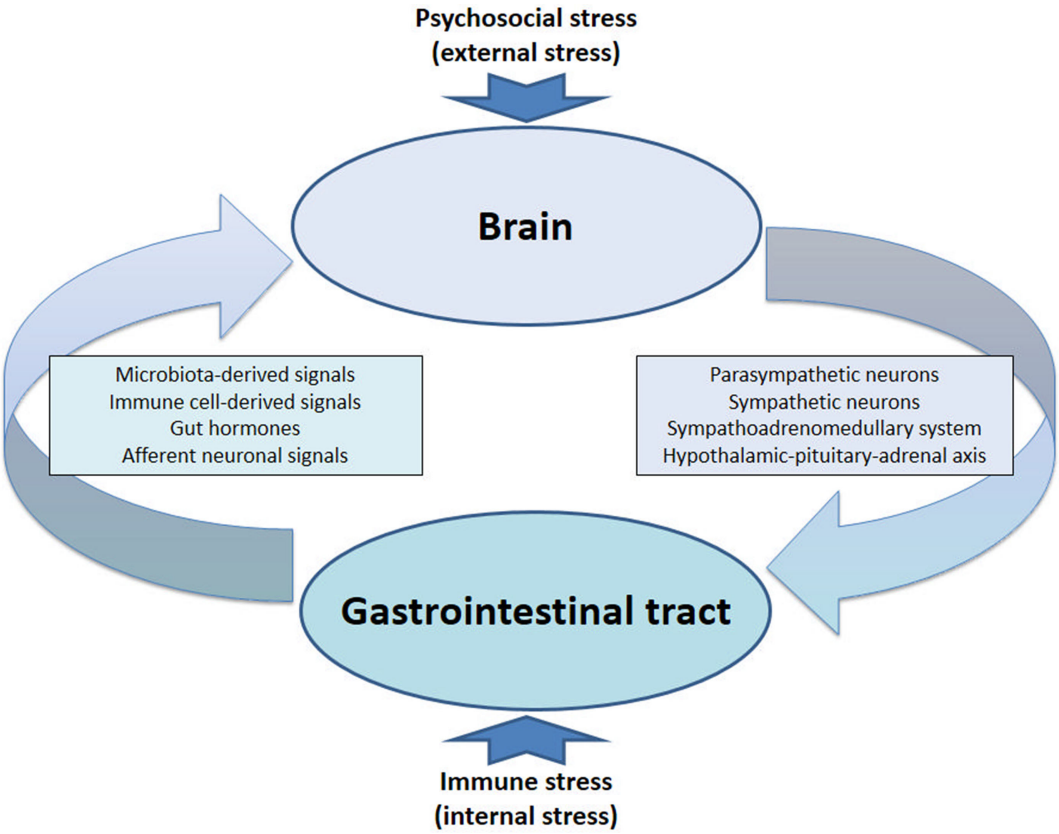

FIGURE 2 | The circle completed: bidirectional communication between gut and brain under conditions of stress. The stressed brain facilitates immune activation and inflammation in the gut, while immune stress in the gut and periphery feeds back to the brain.

from gut to brain will help identifying biomarkers of immune processes that carry a risk to stress the brain and unfold novel opportunities for therapeutic intervention.

AUTHOR CONTRIBUTIONS

PH conceived the article layout, wrote part of the review, integrated the parts written by the coauthors, and approved the final manuscript. AF, AMH, GZ, AJ, and FR assisted in the article layout, wrote part of the review, created the tables and figures, and approved the final manuscript.

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Nutritional Immunity Triggers the Modulation of Iron Metabolism Genes in the Sub-Antarctic Notothenioid *Eleginops maclovinus* in Response to *Piscirickettsia salmonis*

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Martínez D, Oyarzún R, Pontigo JP, Romero A, Yáñez AJ and Vargas-Chacoff L (2017) Nutritional Immunity Triggers the Modulation of Iron Metabolism Genes in the Sub-Antarctic Notothenioid Eleginops maclovinus in Response to Piscirickettsia salmonis. Front. Immunol. 8:1153. doi: 10.3389/fimmu.2017.01153

Iron deprivation is a nutritional immunity mechanism through which fish can limit the amount of iron available to invading bacteria. The aim of this study was to evaluate the modulation of iron metabolism genes in the liver and brain of sub-Antarctic notothenioid *Eleginops maclovinus* challenged with *Piscirickettsia salmonis*. The specimens were inoculated with two *P. salmonis* strains: LF-89 (ATCC® VR-1361™) and Austral-005 (antibiotic resistant). Hepatic and brain samples were collected at intervals over a period of 35 days. Gene expression (by RT-qPCR) of proteins involved in iron storage, transport, and binding were statistically modulated in infected fish when compared with control counterparts. Specifically, the expression profiles of the transferrin and hemopexin genes in the liver, as well as the expression profiles of ferritin-M, ferritin-L, and transferrin in the brain, were similar for both experimental groups. Nevertheless, the remaining genes such as ferritin-H, ceruloplasmin, hepcidin, and haptoglobin presented tissue-specific expression profiles that varied in relation to the injected bacterial strain and sampling time-point. These results suggest that nutritional immunity could be an important immune defense mechanism for *E. maclovinus* against *P. salmonis* injection. This study provides relevant information for understanding iron metabolism of a sub-Antarctic notothenioid fish.

Keywords: ***Eleginops maclovinus*, iron metabolism, notothenioid, nutritional immunity, iron-withholding**

HIGHLIGHTS

Iron deprivation is an innate immunity mechanism through which fish can limit the amount of iron available to invading bacteria.

The proteins involved in storage, transport, and iron binding modulated their expression during the experimental course.

The iron-related immune gene presented tissue-specific expression profiles that varied in relation to the injected bacterial strain (LF-89 or Austral-005) and sampling time-point.

INTRODUCTION

The innate immune system in fish is an essential component in the fight against pathogenic agents. In contrast, the adaptive immune system is limited in fish due to being poikilothermic, having a restricted repertoire of antibodies, and presenting low lymphocyte proliferation, maturation, and memory (1). The cells and molecules of the innate immune system use non-clonal pattern recognition receptors, such as lectins, Toll-like receptors, and NOD-like receptors. These receptors not only recognize molecular structures essential for microorganism survival/pathogenicity (2) but also allow the innate immune response to rapidly respond to control pathogen growth and promote inflammation and adaptive immunity mechanisms (3).

Under inflammation and/or infestation conditions, the innate immune system induces various antimicrobial mechanisms, such as depleting the iron available to pathogens at the systemic and cellular levels (4). This defense mechanism, known as nutritional immunity or iron withholding, consists in the removal of this nutrient from circulation and posterior sequestration within cells (5). Proinflammatory cytokines, such as IL-6, stimulate the transcriptional upregulation of hepcidin, triggering and potentiating the hypoferric response of inflammation (6).

In eukaryotic cells and most prokaryotic organisms, iron is needed for survival and proliferation. This necessity arises as iron is a constituting element of hemoproteins, the iron-sulfur protein (Fe-S), and proteins that use iron in other functional groups to carry out essential functions of cellular metabolism (7). In fish, the iron preferentially crosses the apical membrane of gills and intestine in ferrous state (8) by the divalent metal transporter (9). Once inside the cell, iron can be stored in the cytoplasmic ferritin (i.e., iron that is not needed for immediate use) (10), sent toward the mitochondria (i.e., Fe-S cluster biogenesis or heme synthesis), or be used in other iron pathways (7).

Cellular iron export is regulated by hepcidin and ferroportin, the binding of both proteins leads the internalization and degradation of ferroportin (11). Ferroportin is responsible for iron transfer from the basolateral membrane of the cell to the blood (12), but iron export also depends on the presence of an associated copper, such as hephaestin (implicated in intestinal iron transport) and ceruloplasmin (implicated in the iron export from non-intestinal cells) (13, 14). This ferroxidase allows iron to be carried in the cytoplasmic transferrin and be sent to another tissue (7). Cellular iron uptake can occur through transferrin receptor 1 (TfR1), which is ubiquitously expressed in tissues (15), or through TfR2, a homologous receptor of TfR1 that is found in hepatic duodenal crypt cells and erythroid cells, localizations suggestive of a more specialized role for this receptor in iron metabolism (16).

When faced with an infectious process, the proteins involved in iron homeostasis can limit access to this nutrient. However, microorganisms have developed direct and indirect mechanisms for capturing iron from different sources *in vivo* (17). The direct capture of this element involves the expression of membrane proteins (i.e., receptors) that can directly bond with proteins that transport iron in the host. Iron can also be indirectly captured through the synthesis of hemophores and siderophores, which scavenge and deliver iron to the bacterial membrane (17). In addition to these mechanisms, other pathogenic bacteria can produce proteases that degrade iron-transporter proteins, or hemolysins, which are cytotoxic to erythrocytes and other cell types, promoting the uptake of the heme group (18).

Piscirickettsia salmonis is the etiological agent of Piscirickettsiosis, a disease that causes high mortalities in the aquaculture industry. This bacterium generates a systemic infection characterized by colonization in several organs, including the kidney, liver, spleen, intestine, ovary, gills, and brain (19). In the brain of *Oncorhynchus kisutch*, the infective dose of *P. salmonis* is 100 times higher than that of the liver or kidney, indicating that this tissue might be a preferred replication site for this bacterium (20). Recent *P. salmonis* genome sequencing and annotation has allowed for identifying a set of orthologous genes involved in iron uptake, indicating that this bacterium can obtain iron from different sources, including ferric iron, heme iron, and free Fe^{2+} (21–25).

Piscirickettsiosis was initially reported as a disease in salmonids (26); however, evidence of this disease exists in other non-salmonid species, such as *Dicentrarchus labrax* (27), *Atractoscion nobilis* (28), *Oreochromis mossambicus*, and *Sarotherodon melanotheron* (29). Furthermore, genomic material (i.e., DNA) of this microorganism has been detected in fish endemic to Chile, including *Eleginops maclovinus*, *Odontesthes regia*, *Sebastes capensis*, and *Salilota australis* (30). The role that these native fish could play in disease transmission, as well as the effects that this bacterium could have on the tissues of these endemic organisms, is unknown. Studies by Vargas-Chacoff et al. (31) group reported increased levels antibodies (IgM) in *E. maclovinus* specimens injected with total proteins of *P. salmonis*, with an activation of the intermediate metabolism of the muscle to supply the energetic demand caused by the injection and the high culture density (32). Additionally, Martínez et al. (33) indicated that the injection of live *P. salmonis* modulates the expression of ferritin-H in liver, spleen and muscle of *E. maclovinus*, suggesting the possible activation of an iron-limiting system.

Eleginops maclovinus (Cuvier, 1830) is a sub-Antarctic notothenioid of the Eleginopsidae (Osteichthyes) family and Notothenioidei suborder. This fish is considered a related species to the Antarctic notothenioids clade (34) and is one of the most eurythermal and euryhaline representatives of this suborder (35, 36). This species habits area associated with salmonid culture centers, subsisting off of unconsumed pellet feed and salmonid excrements (37). The latter suggests an interaction in the natural environment between native and farmed fish, with the consequent transference of microorganisms that presenting different degrees of pathogenicity and resistance to antibiotics. The objective of this study was to evaluate the temporal modulation of iron metabolism genes in liver and brain of *E. maclovinus* challenge with two live strains of *P. salmonis*: LF-89 as reference strain (ATCC® VR-1361™) and Austral-005 strain as an antibiotic resistant.

MATERIALS AND METHODS

Samples

Assessments were conducted with the same specimens and experimental procedures as used in Martínez et al. (33). Briefly, immature fish of *E. maclovinus* (20 ± 5 g body weight) were transferred to the Calfuco Coastal Laboratory facilities of the Faculty of Science (Universidad Austral de Chile, Valdivia, Chile). Fish were acclimated for 4 weeks to the following conditions, as detailed in Vargas-Chacoff et al. (36): 500 L flow-through tanks; 3.1 kg m^{-3} density; 32 psu seawater (1,085 mOsm kg^{-1}); 12:12 h light:dark photoperiod; and 12.0 ± 0.5°C. Fish were fed in proportion to 1% body weight once daily with commercial dry pellets (Skretting Nutrece 100) containing 48% protein, 22% fat, 13.5% carbohydrates, 8% moisture, and 8.5% ash. All experimental protocols complied with guidelines for the use of laboratory animals, as established by the Chilean National Commission for Scientific and Technological Research (CONICYT, Spanish acronym) and the Universidad Austral de Chile.

P. salmonis LF-89 and Austral-005 Strains

Inoculates of *P. salmonis* were donated by the Metabolism and Biotechnology Laboratory (Institute of Biochemistry and Microbiology, Faculty of Sciences, Universidad Austral de Chile). LF-89 (ATCC® VR-1361™) was used as a reference strain (38), and Austral-005 strain was used as an antibiotic-resistant representative (39).

P. salmonis LF-89 and Austral-005 Strains Infection Assays

After acclimation, the fish were randomly distributed among rectangular tanks (100 L) and submitted in the experimental treatments: control (fish injected with only the culture medium); LF-89 (fish injected with LF-89 strain); and Austral-005 (fish injected with Austral-005 strain). Each treatment was performed in duplicate. The fish ($n = 126$) were injected with 100 µL of culture medium (control) or with 100 µL at a concentration of 1×10^8 of live bacteria, and sampled at day 1, 3, 7, 14, 21, 28, and 35 postinjection (dpi). Fish were fasted for 24 h before each sampling, netted and submitted to lethal doses of 2-phenoxyethanol (1 mL L^{-1} water). Over the course of the experiment, fish were maintained following Vargas-Chacoff et al. (36): 3.1 kg m^{-3} density, flow-through system, natural photoperiod (12:12 h light:dark), and temperature (12.0 ± 0.5°C). Fish were fed in proportion to 1% body weight once daily with commercial dry pellets (Skretting Nutrece Defense 100) containing 48% protein, 22% fat, 13.5% carbohydrates, 8% moisture, and 8.5% ash.

Total RNA Extraction

Liver and brain tissues from *E. maclovinus* at different experimental conditions were aseptically extracted and used for total RNA extraction. RNA was extracted from each tissue (50 mg) by homogenization in TRIzol (Ambion) following the manufacturer's instructions. The RNA pellets were dissolved in diethyl pyrocarbonate water and stored at −80°C. Subsequently, the RNA was quantified at 260 nm on a NanoDrop spectrophotometer (NanoDrop Technologies®), and their quality determined by electrophoresis on 1% agarose gel. Total RNA (2 µg) was used as a reverse transcription template to synthesize cDNA, applying MMLV-RT reverse transcriptase (Promega) and the oligo-dT primer (Invitrogen) according to standard procedures.

RT-qPCR Analysis of Gene Expressions

Reactions were carried out on an AriaMx Real-time PCR System (Agilent). cDNA was diluted to 100 ng and used as a RT-qPCR template with reactive Brilliant SYBRGreen qPCR (Stratagene). Primers were designed for ferritin-H, ferritin-M, ferritin-L, ceruloplasmin, transferrin, hepcidin, haptoglobin, hemopexin, and 18s. Reactions were performed, in triplicate, in a total volume of 14 µL, which contained 6 µL SYBRGreen, 2 µL cDNA (100 ng), 1.08 µL of primers mix, and 4.92 µL of PCR-grade water. The applied PCR program was as follows: 95°C for 10 min, followed by 40 cycles at 90°C for 10 s, 60°C for 15 s, and 72°C for 15 s. Melting curve analysis of the amplified products was performed after each PCR to confirm that only one PCR product was amplified and detected. Expression levels were analyzed using the comparative Ct method ($2^{-\Delta\Delta CT}$) (40). The data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the uninfected fish (control). The primers used are listed in **Table 1**.

The PCR products were resolved on 2% agarose gel, purified using the E.Z.N.A Gel Extraction Kit (Omega Biotek), and sequenced by Macrogen Inc. Sequences were identified through BLAST analysis (http://blast.ncbi.nlm.nih.gov) against sequences in the NCBI GenBank database. All data are given in terms of relative expression and are expressed as the mean ± standard error of the mean (SEM). PCR efficiencies were determined by linear regression analysis of sample data using LinRegPCR (41).

Statistical Analyses

Assumptions of variance normality and homogeneity were tested. Data were logarithmically transformed when needed to fulfill conditions for parametric analysis of variance (ANOVA). Gene expression was tested by two-way ANOVA, using the different injections and time as variance factors. ANOVA were followed by a Tukey's *post hoc* test to identify different groups. Differences were considered significant when $P < 0.05$.

RESULTS

Primary Structure of Iron-Related Immune Genes in *E. maclovinus*

Ferritin-M, transferrin, ceruloplasmin, haptoglobin, and hemopexin cDNA fragments were obtained by conventional PCR using *E. maclovinus* liver cDNA as a template and heterologous primers designed from the sequences of other fish species. The PCR products of each gene were purified from 2% agarose gel using the E.Z.N.A Gel Extraction Kit (Omega Biotek) and were sequenced by Macrogen Inc. Sequences were identified through BLAST analysis (http://blast.ncbi.nlm.nih.gov) against the NCBI GenBank. Subsequently, RT-qPCR primers were designed from specific *E. maclovinus* sequences. Partial cDNA coding sequences

TABLE 1 | Primer sequences for nutritional immunity used in the experiments.

Primer	Nucleotide sequences (5′ → 3′)	PCR product size	Efficiency liver (%)	Efficiency brain (%)
Ferritin-H Fw	AGTGGAGGCCCTTGAATGTGC	130	101.8	97.3
Ferritin-H Rv	GTCCAGGTAGTGAGTCTCGATGAA			
Ceruloplasmin Fw	GTTTCCAGCCACCTTTCAGACAGT	104	101.8	102.1
Ceruloplasmin Rv	TCGCCTCCATGCCACCTTTAAT			
Transferrin Fw	AACATCCCCATGGGTCTAATCT	124	105.4	100.5
Transferrin Rv	CACTTCCAGCACACTTTGAACA			
Hepcidin Fw	CCGTATACAAGAGCAAGGCG	100	103.2	96.3
Hepcidin Rv	ATCCGAATGCCTTTGTACAGC			
Haptoglobin Fw	ACTGAGCTAACACCAGCTGTA	137	103.4	98.5
Haptoglobin Rv	CCTGCAGCGTAGATGTCTCCA			
Hemopexin Fw	TGATGCAGCAGTAGACGATCCTT	140	103.4	97.4
Hemopexin Rv	GCTGAGCTGGACCATGAAAGCC			
Ferritin-M Fw	CCCGGCTTCGCTCACTTCTTCAA	107	105.9	101.4
Ferritin-M Rv	TCCTGCAGGAAGATGCGTCCTC			
Ferritin-L Fw	AAGCTGCTGGAATATCAGAACAT	123	103.3	102.4
Ferritin-L Rv	CTTCTGGTAGTCCAGGGAAAA			
Housekeeping (18s) Fw	GTCCGGGAAACCAAAGTC	116	104.9	104.9
Housekeeping (18s) Rv	TTGAGTCAAATTAAGCCGCA			

were obtained and deposited in GenBank under the following accession numbers: MF741822 for ferritin-M, MF741819 for transferrin, MF741824 for ceruloplasmin, MF741821 for haptoglobin, and MF741820 for hemopexin. Ferritin-L was amplified using heterologous primers, and ferritin-H (MF741823) was amplified using published primers (33). The hepcidin sequence was obtained from GenBank under accession number EU221592.

Mortality after Infection with *P. salmonis*

During the experimental period no mortality or changes in behavior were observed.

Expression of Iron-Related Immune Genes

Real-time PCR analyses were used to measure the expression of iron-related immune genes in *E. maclovinus* liver and brain tissues at 1, 3, 7, 14, 21, 28, and 35 dpi with *P. salmonis*. Hepatic ferritin-M expression (**Figure 1A**) significantly increased (5- to 25-fold) at 3, 7, and 14 dpi, with differences in the degree of fold-increase between the LF-89 and Austral-005 groups. In the brain, both strains induced increased ferritin-M expression at 1 and 28 dpi, with statistically significant downregulation at 14 dpi, when compared with the control (**Figure 1B**).

Hepatic ferritin-L expression increased in the Austral-005 group at 3, 7, and 14 dpi. Injection with LF-89 only positively modulated hepatic ferritin-L expression at 28 dpi, with no statistical differences when compared with the control during the other sampling time-points (**Figure 2A**). In contrast to liver expression, ferritin-L was upregulated in the brain at 1 and 28 dpi but downregulated at 14 dpi for both experimental groups (**Figure 2B**).

The expression of ferritin-H in the liver of *E. maclovinus* during *P. salmonis* infection was previously reported elsewhere (33). Specifically, hepatic ferritin-H was upregulated at 3 and 7 dpi, downregulated at 14 dpi, and then upregulated at 21, 28, and 35 dpi by both strains. In the brain, both strains led to significantly increased ferritin-H expression (3.5- to 8.5-fold) at 1 dpi, but drastically decreased expression at 3 and 7 dpi. Following this time-point, specimens injected with Austral-005 showed a steady increase in brain expression from 14 to 35 dpi. In turn, fish injected with LF-89 showed increased ferritin-H expression at 14 and 21 dpi, but brain expression again decreased at 28 and 35 dpi (**Figure 3**).

Transferrin expression was similar in both the LF-89 and Austral-005 groups. Expression peaks of hepatic transferrin were recorded at 3 dpi (30- to 50-fold) and 21 dpi (10-fold) (**Figure 4A**). In the brain, both *P. salmonis* strains modulated transferrin expression, with peaks recorded at 1, 14, and 28 dpi when compared with the control (**Figure 4B**).

Ceruloplasmin was similarly expressed in the liver of both experimental groups, with no differences when compared with the control condition at 1, 3, or 7 dpi. While that the LF-89 group showed increased hepatic ceruloplasmin expression at 14 dpi, the Austral-005 group presented increased expression at 21 dpi (**Figure 5A**). In the brain, only the Austral-005 strain evidenced upregulated ceruloplasmin expression (1, 14, 21, and 35 dpi). The LF-89 group, however, showed no statistical differences when compared with the control during the experiment in brain (**Figure 5B**).

The hepatic expression profile of hepcidin was dependent on the injected bacterial strain. Gene expression was upregulated 1, 14, 21, 28, and 35 dpi in the LF-89 group, whereas hepcidin was upregulated in the Austral-005 group at 1, 21, and 28 dpi. Furthermore, the Austral-005 group presented negative modulation of hepatic hepcidin expression at 14 dpi, when compared with the control group (**Figure 6A**). In the brain, hepcidin was also differentially modulated over time in the Austral-005 group, with significant peak expressions recorded at 1, 3, 14, 28, and 35 dpi. By contrast, the LF-89 group only showed significant brain hepcidin modulation at 14 dpi (**Figure 6B**).

For both the LF-89 and Austral-005 groups, liver expression of hemopexin was significantly upregulated from 1 to 3 dpi before decreasing at 7 and 14 dpi, increasing at 21 and 28 dpi, and finally decreasing to basal levels at 35 dpi (**Figure 7A**). In the brain, hemopexin expression in the LF-89 group only significantly increased, when compared with the control, at 1 and 29 dpi.

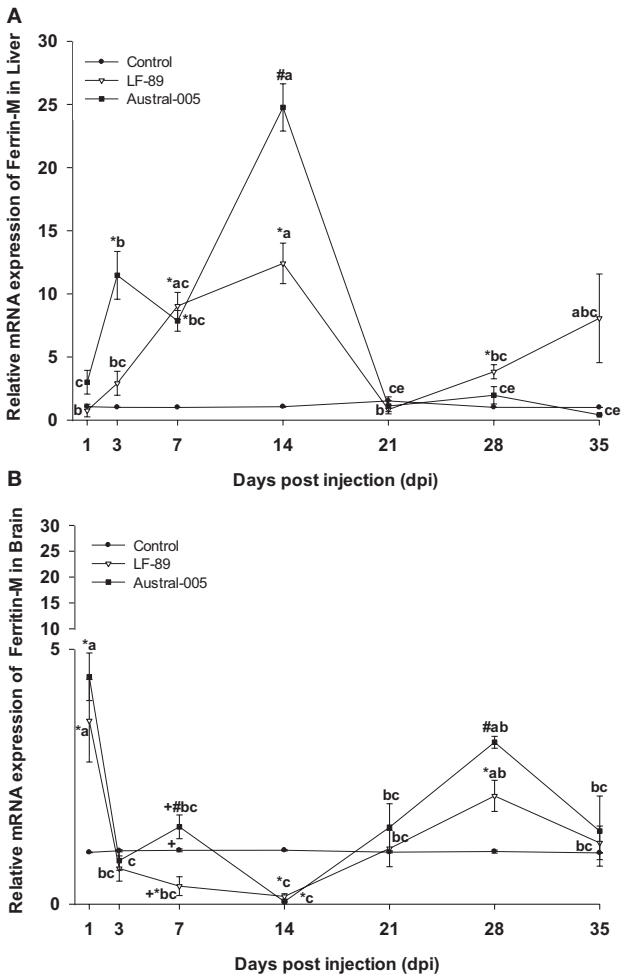

FIGURE 1 | Gene expression of ferritin-M in liver **(A)** and brain **(B)** of *Eleginops maclovinus* specimens injected with *Piscirickettsia salmonis* strain LF-89 (▽) or Austral-005 (■) during 35 days. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using the 18s ribosomal protein as the internal reference gene. Each value is the mean ± SEM ($n = 6$). Different letters indicate statistical differences within the same treatment over time. Symbols indicate statistical differences between different treatments (control, LF-89, or Austral-005) at the same sampling time-point [two-way analysis of variance, $P < 0.05$].

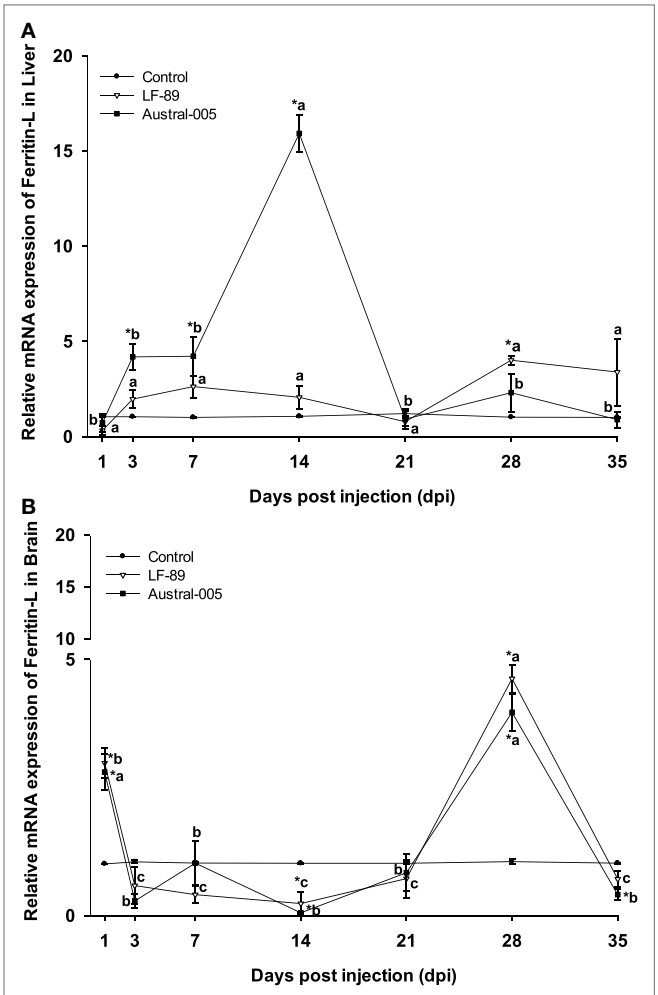

FIGURE 2 | Gene expression of ferritin-L in liver **(A)** and brain **(B)** of *Eleginops maclovinus* specimens injected with *Piscirickettsia salmonis* strain LF-89 (▽) or Austral-005 (■) during 35 days. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using the 18s ribosomal protein as the internal reference gene. Each value is the mean ± SEM ($n = 6$). Different letters indicate statistical differences within the same treatment over time. Symbols indicate statistical differences between different treatments (control, LF-89, or Austral-005) at the same sampling time-point [two-way analysis of variance, $P < 0.05$].

For the Austral-005 group, brain expression of hemopexin was increased at 1, 3, 28, and 35 dpi but presented no differences when compared with the control at 7, 14, and 21 dpi (**Figure 7B**).

Finally, haptoglobin in the liver increased in expression at 21 dpi (10- to 25-fold) for both the LF-89 and Austral-005 groups. Slight increases in expression (sixfold) were observed during the first days post-injection for only the Austral-005 group. In contrast, the LF-89 group presented increased expression at 1 dpi but no differences when compared with the control at 3, 7, and 14 dpi. Hepatic haptoglobin expression in the LF-89 group then steadily increased from 21 to 35 dpi (**Figure 8A**). In the brain, haptoglobin presented no variations in expression for both *P. salmonis* strains during the first days postinjection. However, this gene was then downregulated in the LF-89 and Austral-005 groups, when compared with the control, at 7 and 14 dpi. At 28 and 35 dpi, the Austral-005 group showed drastically increased haptoglobin expression in the brain, whereas the LF-89 group only showed such an increase at 28 dpi (**Figure 8B**).

DISCUSSION

The competition for iron between pathogens and hosts underscores the need to evaluate how iron-related immune genes modulate expression to withhold this nutrient and, consequently, reduce bacterial load (42). In the present study, the partial coding sequences (cDNA) of proteins implicated in *E. maclovinus* iron metabolism were identified, including ferritin-M, transferrin, ceruloplasmin, hemopexin, and haptoglobin. These genes,

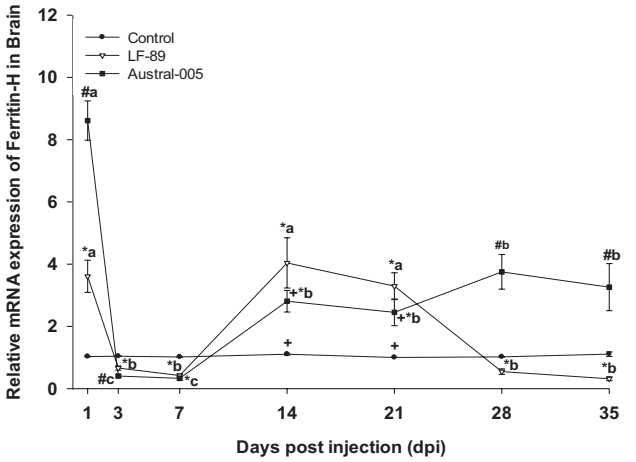

FIGURE 3 | Gene expression of ferritin-H in brain of *Eleginops maclovinus* specimens injected with *Piscirickettsia salmonis* strain LF-89 (▽) or Austral-005 (■) during 35 days. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using the 18s ribosomal protein as the internal reference gene. Each value is the mean ± SEM ($n = 6$). Different letters indicate statistical differences within the same treatment over time. Symbols indicate statistical differences between different treatments (control, LF-89, or Austral-005) at the same sampling time-point [two-way analysis of variance, $P < 0.05$].

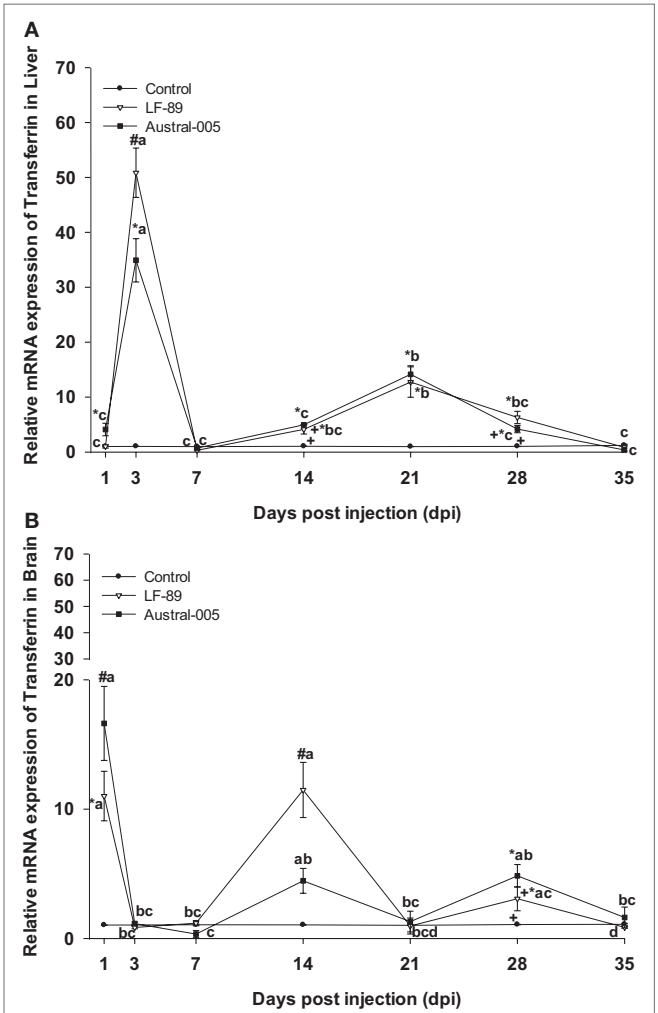

FIGURE 4 | Gene expression of transferrin in liver **(A)** and brain **(B)** of *Eleginops maclovinus* specimens injected with *Piscirickettsia salmonis* strain LF-89 (▽) or Austral-005 (■) during 35 days. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using the 18s ribosomal protein as the internal reference gene. Each value is the mean ± SEM ($n = 6$). Different letters indicate statistical differences within the same treatment over time. Symbols indicate statistical differences between different treatments (control, LF-89, or Austral-005) at the same sampling time-point [two-way analysis of variance, $P < 0.05$].

together with ferritin-H, ferritin-L, and hepcidin were modulated in response to the injection with *P. salmonis*. The expression profiles of the transferrin and hemopexin genes in the liver, as well as the expression profiles of ferritin-M, ferritin-L, and transferrin in the brain, were similar for both experimental groups (i.e., injected with LF-89 or Austral-005 strains). Nevertheless, the remaining genes presented tissue-specific expression profiles that varied in relation to the injected bacterial strain and sampling time-point. It is probable that genetic differences between the bacterial strains would induce the host to modulate certain genes much more than others, both in the liver and in the brain. The Austral-005 strain is antibiotic resistant maintained in the AUSTRAL-SRS medium (39, 43). In turn, LF-89 is the only reference strain originally obtained from *O. kisutch* (38) and it is used worldwide.

The liver regulates iron homeostasis through the synthesis and storage of proteins involved in iron metabolism (44). In the present study, the ferritin-H, ferritin-M, and ferritin-L genes responded to bacterial infection, through a modulation in its expression during the experimental course, with tissue-specific expression profiles. The increased expression of these genes, in the liver and brain, could be consistent with a decrease in serum iron content and the need to increase iron storage so as to limit availability for bacterial growth. This would be in line with existing literature, where the expression of ferritin is downregulated under conditions of iron deficiency and upregulated during iron abundance in *D. labrax* (45). Other studies indicate that in conditions of inflammation and infestation, the expression of iron-related immune genes is upregulated (46–49). Additionally, the synthesis of proteins involved in cellular iron uptake and storage is modulated by cellular iron levels. Under conditions of iron deficiency, iron regulatory proteins actively bind to iron responsive elements and stabilize TfR mRNA, while also decreasing the translation of ferritin mRNA. Conversely, high iron levels decrease iron response element binding activity, leading to efficient ferritin mRNA translation and decreased TfR mRNA stability, thus favoring iron uptake (50).

The hepatic expression profiles for ferritin-M and ferritin-L were similar, increasing during the first 2 weeks of the challenge, particularly in fish injected with the Austral-005 strain. A similar profile expression was reported for ferritin-L subunit in *Salmo salar* (23) and ferritin-H en *E. maclovinus* (33), both challenged with *P. salmonis*. In the brain, iron might be undergoing rapid storage at 1 dpi and release at 14 dpi, as per the results obtained for ferritin-M and -L. However, the expression of both ferritins

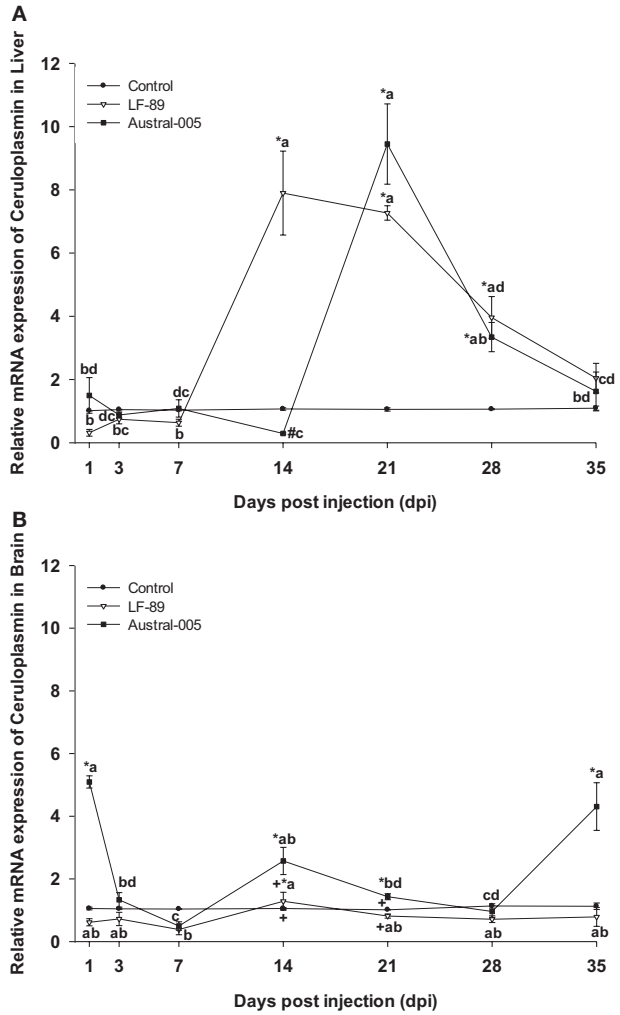

FIGURE 5 | Gene expression of ceruloplasmin in liver **(A)** and brain **(B)** of *Eleginops maclovinus* specimens injected with *Piscirickettsia salmonis* strain LF-89 (▿) or Austral-005 (■) during 35 days. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using the 18s ribosomal protein as the internal reference gene. Each value is the mean ± SEM ($n = 6$). Different letters indicate statistical differences within the same treatment over time. Symbols indicate statistical differences between different treatments (control, LF-89, or Austral-005) at the same sampling time-point [two-way analysis of variance, $P < 0.05$].

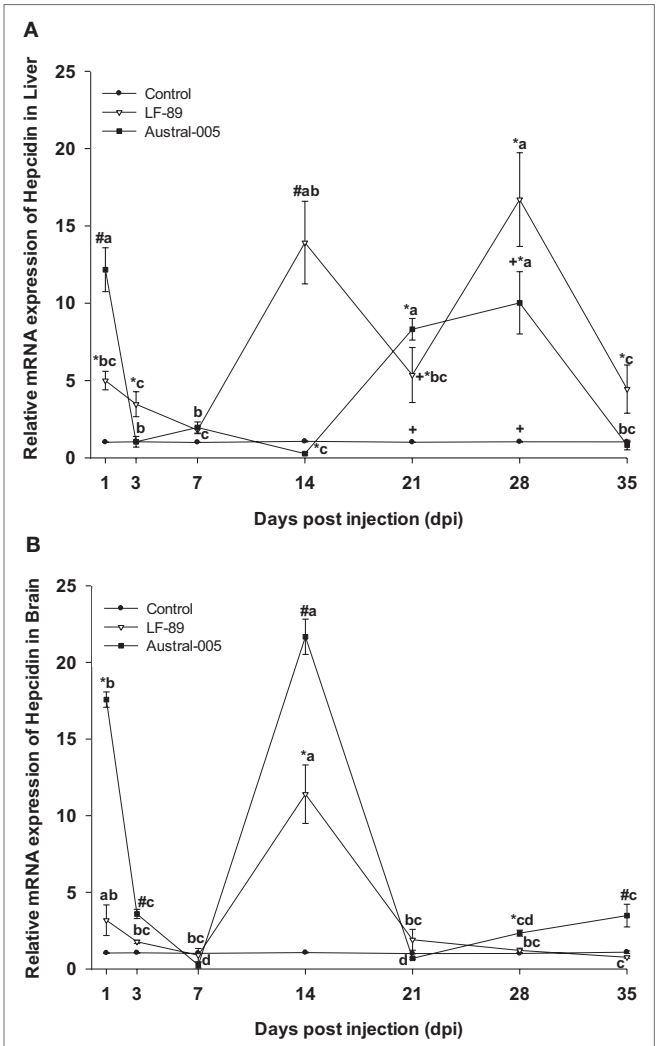

FIGURE 6 | Gene expression of hepcidin in liver **(A)** and brain **(B)** of *Eleginops maclovinus* specimens injected with *Piscirickettsia salmonis* strain LF-89 (▿) or Austral-005 (■) during 35 days. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using the 18s ribosomal protein as the internal reference gene. Each value is the mean ± SEM ($n = 6$). Different letters indicate statistical differences within the same treatment over time. Symbols indicate statistical differences between different treatments (control, LF-89, or Austral-005) at the same sampling time-point [two-way analysis of variance, $P < 0.05$].

increased again at 28 dpi. The expression of ferritin-H in the brain also increased at 1 dpi, fell at 3 and 7 dpi, and finally became generally upregulated during the remainder of the experimental period. It is probable that the different ferritin (H, M, and L) expression patterns are due to the different iron needs of each tissue, as well as the necessity to synthesize one subunit over another as per functional variations. High ferroxidase activity is presented by H-rich ferritins, resulting in more active iron oxidation and sequestration, as well as more pronounced antioxidant functions (51). In turn, L-rich ferritins form more physically stable molecules that may contain a greater amount of iron in the cavity and may have more pronounced iron-storage functions (51). Finally, M-rich ferritins present both the ferroxidase function of ferritin-H and the storage function of ferritin-L (10).

In the present study, the upregulated expression of ferritins (H, M, and L) coincided in some cases with an increased expression of the transferrin gene in the liver and brain. This would indicate that transferrin might be rapidly removing circulating iron through reversible binding with this element. Another mechanism has been reported for mammals, where iron uptake can be independent of transferrin and involve the action of a ferritin secretor able to deliver iron to multiple organs, including the brain. Furthermore, iron uptake is greater when iron is delivered by ferritin-H when compared with ferritin-L (52). On the other

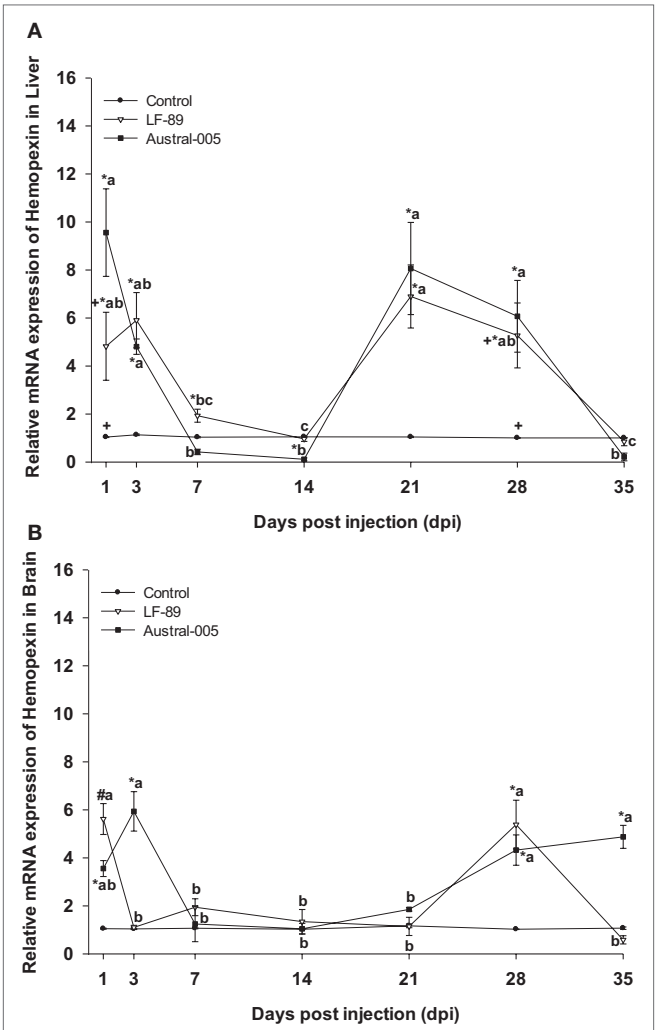

FIGURE 7 | Gene expression of hemopexin in liver **(A)** and brain **(B)** of *Eleginops maclovinus* specimens injected with *Piscirickettsia salmonis* strain LF-89 (▿) or Austral-005 (■) during 35 days. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using the 18s ribosomal protein as the internal reference gene. Each value is the mean ± SEM ($n = 6$). Different letters indicate statistical differences within the same treatment over time. Symbols indicate statistical differences between different treatments (control, LF-89, or Austral-005) at the same sampling time-point [two-way analysis of variance, $P < 0.05$].

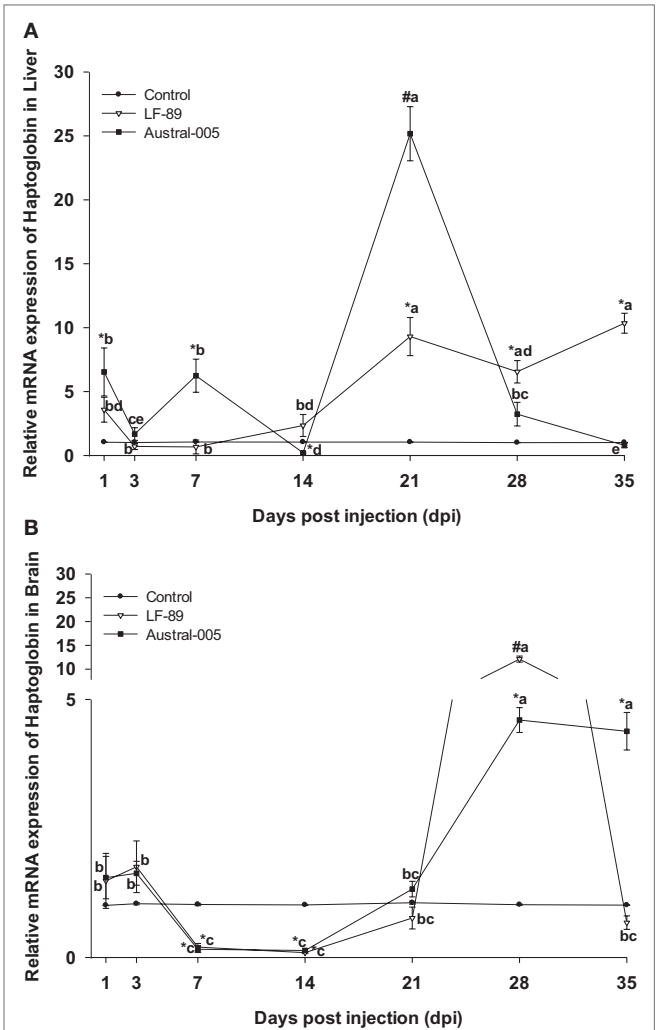

FIGURE 8 | Gene expression of haptoglobin in liver **(A)** and brain **(B)** of *Eleginops maclovinus* specimens injected with *Piscirickettsia salmonis* strain LF-89 (▿) or Austral-005 (▪) during 35 days. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using the 18s ribosomal protein as the internal reference gene. Each value is the mean ± SEM ($n = 6$). Different letters indicate statistical differences within the same treatment over time. Symbols indicate statistical differences between different treatments (control, LF-89, or Austral-005) at the same sampling time-point [two-way analysis of variance, $P < 0.05$].

hand, the transferrin gene expression in the brain of teleost fish is species-specific, detecting in liver, kidney, and stomach of *Salmo salar*, but not in brain (53), compared to *Gadus morhua*, a species in which transferrin is synthesized in the brain (54). These results shown the first instance of transferrin mRNA detection in a fish of Antarctic origin (Notothenioidei), suggesting that local synthesis of this protein could play a role in the immune response of *E. maclovinus*.

Ceruloplasmin, a homologous protein to intestinal hephaestin (7) can be synthetized in the liver (55) and in low amounts spleen, brain, gills, intestine, muscle, skin, and stomach of *Ictalurus punctatus* (56). In the present study, ceruloplasmin expression levels in the liver were greater than those found in the brain. Furthermore, ceruloplasmin brain expression was unchanged, when compared with the control, following injection with the LF-89 strain, and Austral-005 only incremented gene transcription at 1, 14, and 35 dpi. It is possible that genetic differences between the bacterial strains would result in a differential response in brain tissue. Prior studies assessing the modulation of ceruloplasmin in the brain of fish do not exist, but in mammals, cells of the central nervous system synthesize ceruloplasmin as a glycophosphatidylinositol-anchored protein (57). This phenomenon suggests that this isoform interacts with ferroportin to transfer iron from cells to the blood, i.e., cross the blood–brain barrier (57). In the liver,

ceruloplasmin expression was upregulated from 14 to 28 dpi in the LF-89 group and from 21 to 28 dpi in the Austral-005 group. These expression patterns indicate that ceruloplasmin acts as a positive acute-phase protein able to promote the uptake of transferrin iron and subsequent transport toward bone marrow or other precise tissue. Such as function would be in line with findings published for *I. punctatus*, a species that increases the gene expression of ceruloplasmin in the liver after injection with *Edwardsiella ictaluri* (56). In Antarctic notothenioids, ceruloplasmin has been found in the liver and head kidney, thereby suggesting that increased ceruloplasmin expression could prevent the deleterious accumulation of ferrous iron in tissues (58).

In mammals, bonding between hepcidin and ferroportin induces the internalization and posterior degradation of ferroportin, meaning decreased iron export (11). In the currently conducted analyses, the genic expression levels of hepcidin were variable in both the liver and brain, being the expression in liver increased 20-fold higher than the control condition, with elevated expression maintained during nearly the entire experimental period for the LF-89 group and on determined days for the Austral-005 group. In the brain, genic expression of hepcidin differed from that in the liver. In particular, Austral-005 injection resulted in increased gene expression during nearly the entire experimental period, whereas LF-89 injection resulted in increased expression only at 14 dpi. It is possible that the tissue-specific expression profiles obtained for this gene would be due not only to the physiological functions of hepcidin in regulating iron homeostasis (59, 60) but also to inherent antimicrobial properties (61). Ganz and Nemeth (62) indicate that the antibacterial properties of hepcidin could be a result of participating in plasma iron depletion, as corroborated by various studies supporting that genic hepcidin expression is upregulated under inflammatory conditions (63–67). In Antarctic notothenioid fish exists a type of hepcidin composed by four, not eight, cysteine residues, suggesting an adaptive evolution of this gene to a cold climate (68).

Proinflammatory cytokines, such as IL-6, stimulate the transcriptional upregulation of hepcidin, triggering, and enhancing the hypoferric response of inflammation (6). However, prolonged iron uptake could limit the availability of this nutrient to erythroid precursors, which use iron in the heme group (69). This group forms a part of hemoproteins, including myoglobin and hemoglobin. The last belongs to a family of positive acute-phase proteins, whose synthesis is induced by inflammatory cytokines (70–72). In the present study, the genic expression of haptoglobin varied over the course of the experimental period for both analyzed tissues. Hepatic gene expression increased in transcription during the first days postinjection and at 21 dpi of fish injected with the Austral-005 strain. However, fish injected with LF-89 showed increased expression of this gene during the final days of the challenge period. The haptoglobin expression profile in the brain differed from that found in the liver. Particularly, downregulated gene transcription was recorded for both strains at 7 and 14 dpi. At 28 and 35 dpi, the Austral-005 group evidenced increased transcription, whereas the LF-89 group only showed increased transcription at 28 dpi. These results suggest that this gene, as with the other evaluated genes, undergoes tissue-specific up- and downregulation following infection with *P. salmonis*. This positive acute-phase protein might also play a fundamental role in the immune response of *E. maclovinus*, as has been reported in a prior study regarding the upregulation of haptoglobin and serum amyloid A in the liver following bacterial and/or viral stimulation (73).

Uptake of the haptoglobin–hemoglobin complex in mammals occurs by endocytosis through the CD163 receptor, which is exclusively expressed in monocytes/macrophages (70). Formation of this complex under hemorrhagic, hemolytic, and/or cell-injury conditions prevents the negative effects of iron contained in the hemo group (71). Hemopexin, a protein with increased affinity for the free hemo group prevents the negative effects of iron contained within the heme group (74). One homolog to hemopexin, termed warm-temperature acclimation-related 65 kDa protein (wap65), has been identified in teleost fish (75) and cartilaginous fish (76). In the present study, the expression of hemopexin in the liver and brain was positively modulated during the experimental course, probably due to their participation in the immune response against *P. salmonis*. The present findings are in line with that reported for *Misgurnus mizolepis*, a species in which the wap65-2 isoform was found upregulated in the liver and brain following a challenge with *Edwardsiella tarda* (75).

Compared to *Oryzias latipes* and *I. punctatus*, where the transcripts of wap65-2 are restricted to the liver y wap65-1 transcripts can be found in various tissues (77, 78). Orthologous genes exist for hemolysins and respective secretion components in the *P. salmonis* genome, suggesting that this bacterium could acquire iron from host hemo groups (23).

CONCLUSION

This study is the first to provide cDNA sequences for ferritin-M, transferrin, ceruloplasmin, haptoglobin, and hemopexin in the sub-Antarctic notothenioid *E. maclovinus*. The expression of these genes, together with that of ferritin-L and hepcidin, presented transcriptional differences following the challenge with *P. salmonis* LF-89 or Austral-005 strain. Indeed, tissue-specific expression profiles were found dependent on the sampling time-point and injected bacterial strain. These variations in transcript abundances suggest the activation of an innate immune response via iron deprivation, so as to limit bacterial growth. Future studies are needed to complement how nutritional immunity acts in this sub-Antarctic fish at a protein and functional level, as well as to establish differences when compared with mammals, organisms in which nutritional immunity has been widely studied.

ETHICS STATEMENT

All experimental protocols complied with guidelines for the use of laboratory animals, as established by the Chilean National Commission for Scientific and Technological Research (CONICYT, Spanish acronym) and the Universidad Austral de Chile.

AUTHOR CONTRIBUTIONS

DM, LV-C, and AY conception and design of research; DM, RO, JP, and LV-C performed the experiments; DM, RO, AR, and JP analyzed the samples; DM, JP, RO, and LV-C analyzed the data; DM, AR, LV-C, and AY interpreted the results of experiments; DM and RO prepared the figures; DM, AR, LV-C, and AY drafted the manuscript; LV-C and DM edited and revised the manuscript; DM and LV-C approved the final version of manuscript.

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