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Selective EGF-Receptor Inhibition in CD4$^+$ T Cells Induces Anergy and Limits Atherosclerosis

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ABSTRACT

BACKGROUND Several epidermal growth factor receptor (EGFR) inhibitors have been successfully developed for the treatment of cancer, limiting tumor growth and metastasis. EGFR is also expressed by leukocytes, but little is known about its role in the modulation of the immune response.

OBJECTIVES The aim of this study was to determine whether EGFR expressed on CD4$^+$ T cells is functional and to address the consequences of EGFR inhibition in atherosclerosis, a T cell-mediated vascular chronic inflammatory disease.

METHODS The authors used EGFR tyrosine kinase inhibitors (AG-1478, erlotinib) and chimeric *Ldlr*$^{-/-}$*Cd4-Cre/Egfr*$^{lox/lox}$ mouse with a specific deletion of EGFR in CD4$^+$ T cells.

RESULTS Mouse CD4$^+$ T cells expressed EGFR, and the EGFR tyrosine kinase inhibitor AG-1478 blocked in vitro T cell proliferation and Th1/Th2 cytokine production. In vivo, treatment of *Ldlr*$^{-/-}$ mice with the EGFR inhibitor erlotinib induced T cell anergy, reduced T cell infiltration within atherosclerotic lesions, and protected against atherosclerosis development and progression. Selective deletion of EGFR in CD4$^+$ T cells resulted in decreased T cell proliferation and activation both in vitro and in vivo, as well as reduced interferon-γ, interleukin-4, and interleukin-2 production. Atherosclerotic lesion size was reduced by 2-fold in irradiated *Ldlr*$^{-/-}$ mice reconstituted with bone marrow from *Cd4-Cre/Egfr*$^{lox/lox}$ mice, compared to *Cd4-Cre/Egfr*$^{+/+}$ chimeric mice, after 4, 6, and 12 weeks of high-fat diet, associated with marked reduction in T cell infiltration in atherosclerotic plaques. Human blood T cells expressed EGFR and EGFR inhibition reduced T cell proliferation both in vitro and in vivo.

CONCLUSIONS EGFR blockade induced T cell anergy in vitro and in vivo and reduced atherosclerosis development. Targeting EGFR may be a novel strategy to combat atherosclerosis. (J Am Coll Cardiol 2018;71:160–72) © 2018 by the American College of Cardiology Foundation.

Epidermal growth factor receptor (EGFR) is a cell membrane-bound receptor with tyrosine kinase activity involved in the control of major signaling pathways, including cell survival, proliferation, and migration. EGFR overexpression, autocrine ligand stimulation, or constitutively active receptor mutants (1,2) can lead to dysregulation of this fine-tuned signaling system, resulting in a variety of pathophysiological disorders and promoting cancer development. Six EGFR ligands have been

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described, including epidermal growth factor (EGF), Heparin Binding-EGF, amphiregulin, and transforming growth factor-α. Extracellular ligand binding causes dimerization of EGFR, which becomes autophosphorylated at distinct tyrosine residues. In addition, EGFR could be transactivated in the absence of a specific ligand through G protein-coupled receptor activation (3).

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EGFR has been extensively explored in cancer. Human and experimental studies have shown that EGFR activation on tumor cells ultimately leads to cell proliferation, invasion, and migration, as well as promoting angiogenesis and inhibiting apoptosis (4). Targeting of EGFR by either neutralizing monoclonal antibodies or small-molecule tyrosine kinase inhibitors (TKIs) have been shown during the past 10 years to be a successful therapeutic strategy in cancer setting (5,6). However, EGFR expression and function have been poorly investigated in nontumoral cells. Some investigators have described expression in circulating leukocytes (7,8), but little is known about EGFR's role in modulation of the immune response.

Atherosclerosis is an inflammatory disease driven by innate and adaptive immunity, in which $CD4^+$ T cells play a pathogenic role. Interestingly, EGFR ligands, including heparin-binding EGF, have been detected in human atherosclerotic plaques (9). The aim of this study was to ascertain the expression of EGFR in human and mouse $CD4^+$ T cells and to investigate the effects of EGFR blockade on $CD4^+$ T cell functions using pharmacological inhibitors and cell-specific genetic deletion in mouse models of atherosclerosis.

METHODS

ANIMALS. Experiments were conducted according to the guidelines formulated by the European Community for experimental animal use (L358-86/609EEC) and were approved by the ethics committee of INSERM and the French Ministry of Agriculture (agreement A75-15-32). To generate a cell-specific knockout of *Egfr* in $CD4^+$ T cells, we crossbred mice carrying a *Cd4Cre* allele with mice carrying a floxed *Egfr* allele. All animals have been backcrossed more than 10 generations on *C57bl/6* background. Ten-week-old male C57BL/6 *Ldlr*$^{-/-}$ mice were put on a high-fat diet for 8 weeks and were treated orally (daily gavage) with the specific EGFR TKI erlotinib (15 mg/kg/day). For bone marrow transplantation experiments, 10-week-old male *C57bl/6 Ldlr*$^{-/-}$ mice were subjected to medullar aplasia by lethal total body irradiation (9.5 Gy). The mice were repopulated with an intravenous injection of bone marrow cells isolated from femurs and tibias of sex-matched C57BL/6 *Cd4Cre Egfr*$^{+/+}$ mice or *Cd4Cre Egfr*$^{lox/lox}$ littermates. After 4 weeks of recovery, mice were fed a proatherogenic diet containing 15% fat, 1.25% cholesterol, and 0% cholate for 4, 6, and 12 weeks.

EXTENT AND COMPOSITION OF ATHEROSCLEROTIC LESIONS. Plasma cholesterol was measured using a commercial cholesterol kit (Biomérieux, Marcy-l'Étoile, France). The heart was removed, and successive 10-μm transversal sections of aortic sinus were obtained. Lipids were detected using Red Oil staining. The presence of T cells was studied using specific antibodies as previously described (polyclonal anti-CD3, Agilent, Santa Clara, California) (10). *Egfr* was detected in cells and lesions using rabbit polyclonal anti-phospho-Egfr (Cell Signaling, Boston, Massachusetts). For human staining, an anti-EGFR antibody (clone 31G7, AbCys, Paris, France) was used. At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used. Morphometric studies were performed using HistoLab software (Microvisions, Evry, France) (10).

SPLEEN CELL RECOVERY AND PURIFICATION. Spleen cells were purified according to standard protocols as follows. $CD4^+$ T cells were negatively selected using a cocktail of antibody-coated magnetic beads from Miltenyi Biotec (Bergisch Gladbach, Germany) (anti-CD8a, anti-CD11b, anti-CD45R, anti-DX5, and anti-ter 119), according to the manufacturer's instructions, yielding $CD4^+$ cells with >95% purity. $CD11c^+$ cells were positively selected with biotin-conjugated anti-CD11c monoclonal antibody (7D4, BD Pharmingen, Franklin Lakes, New Jersey), streptavidin microbeads (Miltenyi Biotec), followed by 2 consecutive magnetic cell separations using LS columns (Miltenyi Biotec), yielding $CD11c^+$ cells with >80% purity.

$CD4^+$ T CELL CULTURE AND CYTOKINE ASSAYS. Cells were cultured in RPMI-1640 supplemented with GlutaMAX (Thermo Fisher Scientific, Waltham, Massachusetts), 10% fetal calf serum, 0.02 mmol/l β-mercaptoethanol, and antibiotics. For cytokine measurements, $CD4^+$ T cells were cultured at 1×10^5 cells/well for 48 h in anti-CD3-coated microplates (10 μg/ml) or with concanavalin A (10 μg/ml; Sigma-Aldrich, St. Louis, Missouri). In some experiments, $CD4^+$ T cells were stimulated with purified soluble CD3-specific antibody (1 μg/ml; BD

ABBREVIATIONS AND ACRONYMS

EGF = epidermal growth factor
EGFR = epidermal growth factor receptor
IFN = interferon
IL = interleukin
IQR = interquartile range
LDL = low-density lipoprotein
Th = T helper
TKI = tyrosine kinase inhibitor
Treg = regulatory T