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Chemotherapy-induced IL-34 enhances immunosuppression by tumor-associated macrophages and mediates survival of chemoresistant lung cancer cells

Muhammad Baghdadi[1#], Haruka Wada[1#], Sayaka Nakanishi[1], Hirotake Abe[1], Nanumi Han[1], Wira Eka Putra[1], Daisuke Endo[2], Hidemichi Watari[2], Noriaki Sakuragi[2], Yasuhiro Hida[3], Kichizo Kaga[3], Yohei Miyagi[4], Tomoyuki Yokose[5], Atsushi Takano[6,7], Yataro Daigo[6,7], Ken-ichiro Seino[1*]

[1]Division of Immunobiology, Institute for Genetic Medicine, Hokkaido University, Kita-15 Nishi-7, Sapporo 060-0815, Japan

[2]Department of Obstetrics and Gynecology, Graduate School of Medicine, Hokkaido University, Kita-15 Nishi-7, Sapporo, 060-8648, Japan

[3]Department of Cardiovascular and Thoracic Surgery, Graduate School of Medicine, Hokkaido University, Kita-15, Nishi-7, Sapporo 060-8648, Japan

[4] Molecular Pathology and Genetics Division, Kanagawa Cancer Center, Yokohama 241-0815, Japan

[5]Department of Pathology, Kanagawa Cancer Center, Yokohama 241-8515, Japan

[6]Department of Medical Oncology and Cancer Center, Shiga University of Medical Science, Otsu 520-2192, Japan

[7]Center for Antibody and Vaccine, Research Hospital, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

[#] These authors contributed equally to this work

[*]Correspondence:

Ken-ichiro Seino, MD, PhD

Division of Immunobiology, Institute for Genetic Medicine, Hokkaido University

Kita-15, Nishi-7, Sapporo 060-0815, Japan

Tel: 81-11-706-5532, Fax: 81-11-706-7545

E-mail: seino@igm.hokudai.ac.jp

Running title

An importance of IL-34 in cancer chemoresistance

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Abstract

The ability of tumor cells to escape immune destruction and their acquired resistance to chemotherapy are major obstacles to effective cancer therapy. Although immune checkpoint therapies such as anti-PD1 address these issues in part, clinical responses remain limited to a subpopulation of patients. In this report, we identified interleukin-34 (IL-34) produced by cancer cells as a driver of chemoresistance. In particular, we found that IL-34 modulated the functions of tumor-associated macrophages to enhance local immunosuppression and to promote the survival of chemoresistant cancer cells by activating AKT signaling. Targeting IL-34 in chemoresistant tumors resulted in a remarkable inhibition of tumor growth when accompanied with chemotherapy. Our results define a pathogenic role for IL-34 in mediating immunosuppression and chemoresistance and identify it as a tractable target for anticancer therapy.

Introduction

Although recent progress in cancer research has helped to develop new selective treatments based on increased understanding of cancer biology, chemotherapy is still one of the most common treatment options for many cancers (1). Chemotherapy is used to induce cell death in tumors and augment immune responses to cancer. However, many cancer patients experience recurrence and ultimately death because of chemoresistance and chemotherapy-induced immunosuppression. In the past few decades, several studies have underlined intrinsic mechanisms that occur inside cancer cells and contribute to chemoresistance such as the induction of anti-apoptotic regulators, ABC transporters, aberrant NF-κB activities and DNA damage machinery (2, 3). Additionally, recent advances in cancer immunology have suggested that the therapeutic resistance of tumors also relies importantly on extrinsic mechanisms represented by the cross talk between tumor cells and other cellular components of the tumor microenvironment (TME), in particular immune cells (4). Indeed, understanding the complex interaction between cancer and immune system has provided unique therapeutic opportunities to improve the clinical benefit of chemotherapy; for example, combining chemotherapy with antibodies that target immune-checkpoint molecules such as programmed cell death-1 (PD-1) in advanced non-small lung cancer patients (5). In spite of its promising results, clinical responses are still limited to a subpopulation of patients (6), and identifying new therapeutic targets is critically needed.

Tumor-associated macrophages (TAMs) constitute the dominant myeloid cell population in many tumors and play critical roles in multiple aspects of TME including therapeutic resistance (7-9). Importantly, increased frequency of pro-tumorigenic TAMs following chemotherapy is a hallmark of developing chemoresistance and correlate with poor clinical outcomes (7-9). In particular, M2-polarized TAMs drive

multiple pro-tumorigenic processes, including immunosuppression, angiogenesis, metastasis, tumor survival and cancer cell stemness (7-12). Consistent with this, targeting of macrophages by colony stimulating factor 1 receptor (CSF1R) inhibitors or blocking monoclonal antibodies showed promising effects to improve chemotherapeutic responses (13, 14). However, considering the critical roles of macrophages in homeostasis and innate immunity, there are concerns about side effects that may arise from extended depletion of these cells at the systemic level. Alternatively, targeting factors induced by chemotherapy and affect macrophage polarization may have the potential to improve the clinical outcome of cytotoxic chemotherapy with fewer side effects. Accordingly, further investigations are needed to identify mechanisms that drive macrophage polarization under chemotherapeutic conditions.

To identify such mechanisms, we developed a model of chemoresistance in lung cancer cells, the major leading cause of cancer deaths in the world and frequently accompanied with chemoresistance (15). Using this model, we identified IL-34 as a novel factor that contributes to immunosuppression and survival of chemoresistant cancer cells in chemotherapy-treated TME. In this report, we described the role of IL-34 as a molecular driver of chemoresistance, and discuss the significance of these basic findings on clinical applications in cancer patients.

Materials and Methods

Cell culture

All cell lines were obtained in 2013 as follows. Lung (A549, H1299), breast (MCF7, MDA-MB-231, MDA-MB-436, T47D), liver (Hep3B, HepG2) and colon (HCT116, SW480) cancer cell lines were obtained from the American Tissue Culture Collection (ATCC). KFr and TuoM-1 cell lines were provided by Prof. Yoshihiro Kikuchi (National Defense Medical College, Saitama, Japan) and Prof. Junzo Kigawa (Tottori University, School of Medicine), respectively. OVISE and OVTOKO cell lines were obtained from JCRB (Japanese Collection of Research Bio resources, Osaka, Japan). All cell lines were cultured at 37℃ with 5% CO_2 in an appropriate culture medium.

Generation of chemoresistant cells

Lung cancer cells were exposed to stepwise increasing concentrations of standard chemotherapies including doxorubicin (0.01–1 μM) or cisplatin (0.01–0.1 μM). In each cycle, cells were treated with cytotoxic agent for 72 hours, and then drug was washed and cells were allowed to recover. After reaching maximal concentrations, chemoresistant cells were exposed to maximal toxic concentrations at regular intervals to maintain their drug resistance. In experiments that utilize supernatants of cell culture, chemoresistant cells were washed 5 times with sterilized PBS, and cultured in media without doxorubicin. The supernatants were harvested 72 hours later and passed through 0.2 μm filter.

Human monocyte culture

Purified human monocytes (Miltenyi Biotec 130-050-201) were stimulated with supernatants of chemosensitive or chemoresistant cell culture (50% of culture medium) on day 0, 3 and 6. For experiments using neutralizing antibodies,

supernatants were pre-treated with 10 μg/ml of α-M-CSF (Peprotech 500-P44), α-IL-34 (R&D 578416) or a matching isotype control (Biolegend 910801 or R&D MAB002). For experiments using selective inhibitors, supernatants were pre-treated with GW2580 (25 nM, Abcam 142096), LY294002 (10 μM, CST 9901) or DMSO.

Humanized mouse model

NOD-SCID-*IL2rg*$^{-/-}$ mice (females, 6 weeks old) were irradiated as previously described (2.5 G) (16, 17) and transferred after 24 hours with 1 x 10^5 human bone marrow cells from one healthy donor (Stem cell technologies, Catalog 70001.4, Lot 626184005). No information regarding the HLA type of donor were available. 3 weeks later, mice were inoculated subcutaneously with 1 x 10^6 of tumor cells, and treated with doxorubicin (intraperitoneal injection, 10 mg/kg/mice, 4 doses / 1 dose per week) when tumors size reached 5 mm. Mice were maintained in a temperature-controlled, pathogen-free room at the Institute for Genetic Medicine, Hokkaido University, and treated with humane care according to animal procedures approved by Animal Care Committee of Hokkaido University (Approval No. 14-0171).

Immunohistochemical analysis

Primary lung cancer samples were obtained earlier with informed consent from Kanagawa Cancer Center. Formalin-fixed and paraffin-embedded tissues were stained with α-IL-34 (Abcam 101443) according to the manufacturer's protocol. Three independent investigators assessed IL-34 positivity without prior knowledge of clinicopathologic data. IL-34 staining was mostly homogenous in cytoplasm, and thus was semiquantitatively evaluated as absent, weak or high. All clinical materials mentioned were approved by individual institutional ethics committees.

Statistics

The significance of differences between two independent subjects' groups were determined by Student's t test or two-sample t test with Welch's correction. Differences among three or more subjects were determined by one-way ANOVA. *P*-value <0.05 was considered to be statistically significant.

Results

Supernatants of chemoresistant cells enhance monocytes differentiation into immunosuppressive M2-polarized macrophages

Monocytes are recruited into tumors by factors secreted in TME and serve as the cellular resource of TAMs (18). Increased frequencies of monocytes and its differentiation into pro-tumorigenic M2-polarized TAMs have been considered as a hallmark of enhanced chemoresistance in tumors (7-9). Consistent with this, we found that frequencies of monocytes but not other myeloid subsets in total peripheral blood leukocytes (PBLs) of healthy donors have increased when stimulated with supernatants of doxorubicin-resistant A549 (A549-DR) and cisplatin-resistant H1299 (H1299-CR) cells compared to chemosensitive counterparts (A549-DS and H1299-CS) (Fig. 1A and Supplementary Fig. S1 and S2A). Next, to evaluate the effects of chemoresistant cells-derived factors on monocytes differentiation, we stimulated purified $CD14^+$ monocytes with supernatants of chemosensitive or chemoresistant cell culture. In both cases, monocytes differentiated into spindle adherent cells (Fig. 1B) that exhibit M2-polarized MΦ characteristics as evidenced by high expression of the pan macrophage marker CD68 (Fig. 1C and Supplementary Fig. S2B) and CD163 which serves as a biomarker of M2-polarized macrophages activated toward tumor-promoting and immunosuppressive phenotype (19) (Fig. 1D and Supplementary Fig. S2B). Importantly, CD68 and CD163 expression levels were remarkably higher in monocytes stimulated with supernatants of A549-DR (A549-DR-MΦ) compared to A549-DS (A549-DS-MΦ) (Fig. 1C and D and Supplementary Fig. S2B). Furthermore, A549-DR-MΦ exhibit decreased levels of MHC (class I and II) and costimulatory molecules such as CD80 and CD86, and enhanced levels of the T cell inhibitory molecule B7-H1 (PD-L1) compared to A549-DS-MΦ (Supplementary Fig. S2C and S2D), indicating an enhanced

immunosuppressive phenotype of macrophages when stimulated with supernatants of chemoresistant cells. Consistent with this enhancement, A549-DR-MΦ showed increased expression levels of immunosuppressive factors such as *IL10* and *TGFβ* (Fig. 1E) and potent capability to suppress T cell response compared to A549-DS-MΦ (Fig. 1F). Together, these results suggest that soluble factors in the supernatants of chemoresistant A549 and H1299 cells mediate monocytes differentiation into M2-polarized macrophages with an enhanced immunosuppressive phenotype.

chemoresistant cells-derived IL-34 enhances monocytes differentiation into M2-polarized macrophages

Macrophages acquire their phenotype in response to various signals present within individual microenvironments (20). In tumors, chemotherapy induces inflammatory responses that enrich TME with factors that repolarize myeloid cells into different phenotype (21). Consistent with this, we found that chemoresistant cells showed enhanced levels of several cytokines and chemokines compared to chemosensitive counterparts (Supplementary Fig. S2E). To clarify the importance of these factors on TAMs phenotype, we first started with the neutralization of M-CSF, since CSF1R-mediated signaling is critical for the recruitment and differentiation of TAMs (13, 14). Unexpectedly, M-CSF blockade did not efficiently decrease expression levels of CD68 and CD163 induced by A549-DR and H1299-CR supernatants (Fig. 2A and B and Supplementary Fig. S2G). On the other hand, a specific inhibitor for CSF1R (GW2580) was effective to abolish these effects (data not shown); suggesting that chemoresistant cells produce a factor that can bind to CSF1R and affect monocyte differentiation rather than M-CSF. The existence of a second ligand for CSF1R has been discovered recently by functional screening of the extracellular

proteome and designated as interleukin-34 (IL-34) (22). IL-34 supports survival of monocytes and macrophages, and induces monocytes differentiation into M2-polarized macrophages with potent immunosuppressive properties (23). Interestingly, ELISA measurement showed that IL-34 was produced by chemoresistant A549 and H1299 cells but not their chemosensitive counterparts (Fig. 2C and Supplementary Fig. S2F). Furthermore, IL-34 blockade was effective to reduce expression levels of CD68 and CD163 induced in monocytes by supernatants of chemoresistant A549 and H1299 cells (Fig. 2D and E and Supplementary Fig. S2G), indicating that effects of these supernatants depend largely on IL-34. Additionally, pretreatment of A549-DR supernatants with α-IL-34 significantly decreased *IL10* and *TGFβ* expression levels in A549-DR-MΦ (Fig. 2F), and impaired the immunosuppressive capabilities of A549-DR-MΦ to suppress T cell response (Fig. 2G) compared to α-M-CSF. Together, these results suggest that IL-34 produced by chemoresistant cells is involved in the enhancement of the immunosuppressive phenotype of TAMs. Thus, we focused in next experiments on IL-34 as a modulator of TME under chemotherapeutic conditions.

IL-34 production is controlled by NF-κB and mediates an autocrine pathway in chemoresistant cells

First, we asked about the mechanism that controls IL-34 production in chemoresistant cells. Previous studies have suggested the involvement of transcription factor NF-κB in IL-34 production (24, 25). Thus, we hypothesized that NF-κB activation by chemotherapy may contribute to IL-34 production in chemoresistant cells (26). As expected, NF-κB activation was significantly stronger in A549-DR than A549-DS cells (Fig. 3A and B). Furthermore, A549-DR cells but not their chemosensitive counterparts showed active NF-κB under steady state (Fig. 3A

and B). Following stimulation with chemotherapy, NF-κB activities in A549-DS cells increased in a time-dependent manner but remained almost constant in A549-DR cells at the time course of treatment (Fig. 3A and B). Next, we utilized a specific inhibitor of NF-κB and examined its effect on IL-34 production. We found that treatment of A549-DR cells with NF-κB inhibitor resulted in decreased *IL34* mRNA levels in a dose-dependent manner (Fig. 3C), suggesting that constitutive activation of NF-κB is required for IL-34 production in chemoresistant cells. Additionally, survival of doxorubicin-treated A549-DR cells was also decreased in correlation with *IL34* expression (data not shown). Thus, we asked if IL-34 has an autocrine effect, which in turn requires the expression of CSF1R in cancer cells. Previous studies have shown that CSF1R expression can be detected in lung and breast cancer cell lines in addition to tumor cells from patients (27, 28). Consistent with this, we found that CSF1R is weakly expressed at mRNA but not protein level in A549-DS cells (Fig. 3D and E). On the other hand, CSF1R expression is increased at mRNA level and can be detected at protein level in A549-DR cells (Fig. 3D and E). Furthermore, IL-34 blockade resulted in decreased survival of doxorubicin-treated A549-DR cells (Fig. 3F), suggesting that IL-34 mediates an autocrine signaling pathway which enhances survival of chemoresistant cells.

IL-34 enhances survival of chemoresistant cells via CSF1R-mediated AKT signaling

Next, we investigated the molecular mechanism by which IL-34 enhances survival of chemoresistant cells. Previous report suggested the ability of IL-34 to bind CSF1R with high affinity and induce strong phosphorylation of CSF1R and downstream mediators (29). Transphosphorylation of tyrosine residues in the cytoplasmic domain of CSF1R could be observed in A549-DR^{Mock} cells (Fig. 4A). On the other hand,

CSF1R phosphorylation was significantly reduced in A549-DR cells deficient for IL-34 (Fig. 4A), which suggests a functional relevance for IL-34 in this autocrine loop. Next, we compared A549-DR^{Mock} cell survival when pretreated with various kinases inhibitors that act downstream of CSF1R (30). Among these inhibitors, LY294002 (an inhibitor for PI3K/AKT) was capable to significantly reduce survival of doxorubicin-treated A549-DR^{Mock} cells, indicating that survival of these cells is AKT-dependent (Supplementary Fig. S3). AKT acts as a key signaling molecule that links between oncogenic receptors and many essential pro-survival cellular functions (31). Consistent with this, A549-DR^{Mock} cells showed increased levels of phospho-AKT compared to A549-DS cells (Fig. 4B). On the other hand, AKT phosphorylation was significantly reduced in A549-DR$^{\Delta IL\text{-}34}$ and comparable to A549-DS cells (Fig. 4B). Furthermore, AKT phosphorylation was decreased in A549-DR^{Mock} cells when treated with α-IL-34 in a dose-dependent manner (Fig. 4C), which consequently sensitized A549-DR^{Mock} cells to doxorubicin treatment (Fig. 4E). On the other hand, stimulating A549-DR$^{\Delta IL\text{-}34}$ cells with recombinant IL-34 (rIL-34) enhanced AKT phosphorylation in a dose-dependent manner (Fig. 4D), and increased viability of doxorubicin-treated A549-DR$^{\Delta IL\text{-}34}$ cells (Fig. 4F). Finally, we compared the ability of M-CSF and IL-34 to induce AKT activation in chemoresistant cells. Levels of phospho-AKT increased gradually in M-CSF-stimulated A549-DR$^{\Delta IL\text{-}34}$ cells in a time-dependent manner to reach a peak 60 minutes after stimulation and then start to drop (Fig. 4G and H). On the other hand, IL-34 potently induced AKT phosphorylation, reaching its peak 30–60 minutes after stimulation, and peak duration was significantly elongated compared to M-CSF (Fig. 4G and H). Together, these results suggest that IL-34 enhances survival of chemoresistant cells by modulating AKT signal downstream of CSF1R.

IL-34 enhances the immunosuppressive function of TAMs through C/EBPβ-mediated mechanism

Depending on various signals present within individual microenvironments, marked changes in the activity and gene expression programs in macrophages can occur and determine macrophage phenotype and function (32). TAMs are often considered to be synonymous with M2-phenotype characterized with unique transcriptional profile (32, 33). Consistent with this, stimulation of monocytes with supernatants of lung cancer cell culture induced the expression of transcriptional factors (TFs) responsible for M2-polarization such as interferon regulating factor 4 (IRF4), signal transducer and activator of transcription 6 (STAT6) and CCAAT/enhancer-binding protein β (C/EBPβ) (Supplementary Fig. S4A). As suggested above, A549-DR-MΦ showed enhanced immunosuppressive phenotype in an IL-34-dependent manner. However, since A549-DS-MΦ and A549-DR-MΦ showed comparable expression levels of M2 phenotype-related TFs, we hypothesized that IL-34-mediated signaling may affect TAMs function by modulating the activation of certain TFs downstream of CSF1R. To confirm this hypothesis, we first compared the phosphorylation levels of CSF1R in monocytes stimulated with supernatants of A549-DS, A549-DR^{Mock} or A549-DR$^{\Delta IL\text{-}34}$ cells. We found that CSF1R phosphorylation was stronger in macrophages stimulated with supernatants of IL-34-producing A549-DR^{Mock} compared to non-producing A549-DS or A549-DR$^{\Delta IL\text{-}34}$ cells (Fig. 5A). The enhancement of CSF1R phosphorylation in A549-DR^{Mock}-MΦ has a consequent effect on increased activation of AKT signaling (Fig. 5B), which interestingly correlated with a remarkable increase in phosphorylation levels of C/EBPβ compared to other TFs (Fig. 5C and Supplementary Fig. S4B). C/EBPβ, a hallmark of M2-polarized macrophages, belongs to bZIP family of TFs that play essential roles in myeloid development and macrophage activation (32). In particular, C/EBPβ has been shown

to be essential for the expression of immunosuppressive cytokines such as IL-10 and Arginase-1 (ARG1) in M2-polarized macrophages (34). Consistent with this, we found that C/EBPβ activation is accompanied with increased expression levels of *IL10* and *ARG1* in A549-DR^{Mock}-MΦ (Fig. 5D). C/EBPβ specifically regulates M2-associated genes under the control of another bZIP family transcription factor, known as cAMP-responsive element-binding protein (CREB) (35). Furthermore, CREB activities are enhanced upon the activation of PI3K/AKT signaling pathway (36). From these backgrounds, we focused on C/EBPβ as a possible candidate that acts downstream of CSF1R and contributes to IL-34 effects on TAMs function. First, we examined the phosphorylation status of CSF1R, AKT and C/EBPβ in monocytes stimulated with A549-DR^{Mock} supernatants when pretreated with α-IL-34 and/or a selective pharmacologic inhibitor of PI3K/AKT (LY294002). As expected, α-IL-34 has a similar effect to LY294002 on reducing AKT phosphorylation, which in turn has impact on C/EBPβ phosphorylation in A549-DR^{Mock}-MΦ (Fig. 5E). Importantly, α-IL-34 or LY294002 has similar effects on reducing *IL10* and *ARG1* expression levels in A549-DR^{Mock}-MΦ in correlation with decreased C/EBPβ phosphorylation (Fig. 5F). On the other hand, adding rIL-34 to A549-DR$^{\Delta IL\text{-}34}$ supernatants resulted in a remarkable enhancement of AKT and C/EBPβ phosphorylation (Fig. 5G), and a consequent increase in *IL10* and *ARG1* expression levels (Fig. 5H). Importantly, these effects were abolished by LY294002 (Fig. 5G and H), demonstrating the importance of IL-34-induced activation of AKT signaling on the regulation of C/EBPβ function. Collectively, these results suggest a possible mechanism by which IL-34 enhances the immunosuppressive phenotype of TAMs by activating M2-regulating factors such as C/EBPβ.

IL-34 enhances immunosuppression by TAMs and attenuates antitumor effects of chemotherapy

Next, we evaluated the significance of IL-34 on the attenuation of chemotherapeutic effects of doxorubicin in a humanized NOD-SCID-*IL2rg*$^{-/-}$ mouse model (16, 17). In *vivo* treatment with doxorubicin had little impact on A549-DR^{Mock} tumor growth compared to A549-DS (Fig. 6A and B). Notably, tumor growth of A549-DR$^{\Delta IL\text{-}34}$ tumors was significantly suppressed compared to IL-34-producing A549-DR^{Mock} tumors (Fig. 6A and B). Increased frequencies of M2-polarized pro-tumorigenic TAMs have been considered as a hallmark of enhanced chemoresistance in tumors (7-9). Consistent with this, A549-DR^{Mock} tumors were highly infiltrated with CD68^{+}CD163^{+} M2-polarized TAMs (Fig. 6C and D), which correlate with suppression of cytotoxic CD8^{+} T cell response (Fig. 6E and F and Supplementary Fig. S5A and S5B), in addition to increased Treg to Th1 cells ratio compared to IL-34-non-producing tumors (Supplementary Fig. S5C, S5D and S5E), which indicates the importance of IL-34-modified TAMs in regulating immune tolerance within TME. Additionally, we have also compared tumor growth in normal (non-humanized) NOD-SCID-*IL2rg*$^{-/-}$ mice. As shown in Supplementary Fig. S5F, doxorubicin had suppressed growth of A549-DS and A549-DR$^{\Delta IL\text{-}34}$ tumors which can be explained by the direct effect of doxorubicin on A549 cells. On the other hand, doxorubicin had little impact on A549-DR^{Mock} tumor growth due to the resistant nature of these tumors (as enhanced by autocrine signaling of IL-34). Adding lymphocytes in humanized NOD-SCID-*IL2rg*$^{-/-}$ mice resulted in more suppression of tumor growth in A549-DS and A549-DR$^{\Delta IL\text{-}34}$ tumors but not in A549-DR^{Mock} tumors due to the enhanced immunosuppression in these tumors, which suggests that suppression of tumor growth in this model is a collective action of the direct effect of doxorubicin on A549 cells in addition to antitumor immune response. It is worth to be mentioned here

that the HLA type-matching between tumor and immune cells was not confirmed in this model due to the lack of information regarding the HLA type of donor. Thus, it is unknown whether interactions and phenotypes are antigen/MHC-dependent. A recent report has suggested a potent role of IL-34 in suppressing allogeneic immune responses against transplants (37). Thus, IL-34 produced by chemoresistant tumors in this model may similarly suppress anti-tumor T cell responses within TME.

In addition to immunosuppression, TAMs release various factors that contribute to tumor progression and chemoresistance (7-9). Consistent with this, we found that $CD68^{+}CD163^{+}$ TAMs isolated from A549-DR^{Mock} tumors showed enhanced expression levels of immunosuppressive cytokines such as *IL10* and *TGFβ*, factors that provide critical survival signals to cancer cells such as *IL6*, *TNFα* and *EGF*, in addition to factors that enhance angiogenesis such as *VEGF* and modulate matrix components of TME to facilitate tumor progression and invasion such as *MMP9* and *MMP13* (Fig. 6G). Importantly, this enhancement was abolished in $CD68^{+}CD163^{+}$ TAMs isolated from A549-$DR^{\Delta IL\text{-}34}$ tumors (Fig. 6G). Collectively, these results suggest that IL-34 has significant impact on enhancing the pro-tumorigenic properties of TAMs as characterized by increased ability to potently suppress antitumor immune response and provide cancer cells with various factors necessary to survive under chemotherapeutic conditions.

IL-34 expression in primary human lung cancers correlates with poor prognosis

Finally, we asked whether IL-34 expression could be detected in primary human lung cancers. Recent protocols suggest the application of chemotherapy as an adjuvant treatment after surgical resection of tumors. Since the majority of lung cancer patients in our institute are treated with chemotherapy after the surgical removal of tumors, it was extremely difficult to obtain samples from lung cancer

patients before and after chemotherapy. Thus, we chose to evaluate IL-34 expression at mRNA and protein levels in primary lung cancer tissues removed by surgical procedures. This cohort comprises data from lung cancer patients without any preselection. 45% of samples were obtained from Japanese women over 60 years old without smoking history, and 77.4% of cases were diagnosed with stage I of non-small lung cancers with 5 years of follow-up period (Supplementary table S1 and S2). RT-qPCR analysis showed detectable levels of *IL34* mRNA in primary lung cancer tissues compared with normal lung tissues (Fig. 7A). More importantly, immunohistochemical staining revealed that IL-34 could be also detected at protein levels in cancer tissues but not in adjacent normal tissues (Fig. 7B), and high expression of IL-34 significantly correlates with poor prognosis (Fig. 7C and Supplementary Fig. S6A), suggesting that IL-34 expression may serve as an important prognostic biomarker in lung cancer patients. Additionally, *IL34* and *CSF1R* expression, which can be detected at low levels in primary lung adenocarcinoma cells, were induced after exposure to doxorubicin (Fig. 7D) or cisplatin (Fig. 7E). Thus, IL-34 may also serve as a biomarker to monitor the development and progression of chemoresistance in cancer patients treated with chemotherapy.

Finally, we examined *IL34* and *CSF1R* expression in various cancer cell lines including breast, liver, colon and ovary cancers. Quantitative PCR analysis showed detectable levels of *IL34* and *CSF1R* mRNA in different cancer cells with some variations (Supplementary Fig. S6B and S6C). Importantly, *IL34* and *CSF1R* expression levels were higher in a cisplatin-resistant ovarian cancer cell line compared to its chemosensitive counterpart (Supplementary Fig. S6D). Together, these observations suggest that IL-34 and CSF1R may characterize a population of chemoresistant cancer cells in various types of cancer.

Discussion

Recent progress in understanding the relation between macrophage function and therapeutic resistance has helped to improve new therapeutic strategies based on the characteristics of TME (7-9). In this report, we provide evidence that chemotherapy-treated TME adopt a novel strategy to develop chemoresistance and suppress antitumor immunosurveillance by triggering IL-34 production in tumor cells (Supplementary Fig. S6E).

IL-34 is a newly discovered cytokine, which shares a common receptor (CSF1R) with M-CSF (22). Although both cytokines mediate monocytes/macrophages survival and proliferation, IL-34 binds to CSF-1R with higher affinity and induces stronger tyrosine phosphorylation of CSF-1R and downstream mediators (29). IL-34 plays important roles in the pathogenicity of diseases associated with chronic inflammation such as viral infections and inflammatory bowel disease (38, 39). In cancer, IL-34 was found to promote tumor progression and metastatic process of osteosarcoma via promotion of angiogenesis and macrophage recruitment (40). Emerging evidence indicates that chemotherapy treatment increases the proportion of M2-polarized pro-tumorigenic TAMs, which in turn limit the efficacy of chemotherapy (7-9). Additionally, chemotherapy induces NF-κB activation, which plays critical roles in the development and progression of cancer chemoresistance via intrinsic mechanisms such as induction of anti-apoptotic genes expression, and extrinsic mechanisms such as the expression of several cytokines and chemokines that modulate the interaction between tumor cells and other cellular components of TME (41, 42). Importantly, NF-κB activation is linked to IL-34 production following stimulation with proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β (43). As shown in this study, chronic exposure of lung cancer cells to cytotoxic agents results in enhanced NF-κB activation, which in turn induced IL-34 expression in

chemoresistant cells. Thus, we expected that IL-34 play critical roles in the modulation of TME under chemotherapeutic conditions.

First, we identified a paracrine effect of IL-34 represented by recruiting high frequencies of M2-polarized TAMs and enhancing its immunosuppressive phenotype (Supplementary Fig. S6E). Chemoresistance is significantly enhanced when chemotherapy increases the proportion of M2-polarized pro-tumorigenic TAMs, which in turn limit the efficacy of chemotherapy (12). Indeed, IL-34 secreted by chemoresistant cells enhanced monocytes differentiation into M2-polarized macrophages in *vitro*. Additionally, in a humanized mouse model, IL-34-producing chemoresistant tumors were infiltrated with increased frequencies of M2-polarized TAMs, which was negatively correlated with the frequencies of tumor-infiltrating cytotoxic $CD8^+$ T cells, indicating the importance of IL-34 in recruiting M2-polarized TAMs with potent abilities to suppress antitumor immune response under chemotherapeutic condition. Furthermore, TAMs showed enhanced expression levels of immunosuppressive and chemo-protective factors in IL-34-producing chemoresistant tumors. Previous reports have suggested that cancer cells produce various factors that affect differentiation and immunosuppressive features of tumor-infiltrating myeloid cells (44). In our experiments, we found that macrophages showed comparable expression levels of factors responsible for transcription control in macrophages such as *IRF4*, *STAT6* and *C/EBPβ* when stimulated with supernatants of chemosensitive or chemoresistant cells. Even though, macrophages showed enhanced phosphorylation level of C/EBPβ when stimulated with supernatants of chemoresistant cells, which was mediated by IL-34-induced activation of CSF1R/AKT signaling pathway. C/EBPβ plays critical roles in the regulation of the immunosuppressive phenotype of myeloid cells including M2 macrophages and myeloid-derived suppressor cells (MDSCs) (32). The activation of C/EBPβ cascade

induces M2 macrophage-specific gene expression and promotes muscle injury repair (35). Similarly, IL-34-induced C/EBPβ activation may help to enhance the immunosuppressive and pro-tumorigenic functions of TAMs in order to protect tumors from immune attack and maintain homeostasis of TME under chemotherapeutic conditions.

The second role of IL-34 in chemoresistance is the unexpected autocrine effect on activating AKT signal pathway downstream of CSF1R in chemoresistant cells (Supplementary Fig. S6E). AKT plays critical roles in chemoresistance to cytotoxic agents such as paclitaxel and cisplatin in human cancers (45-47). AKT also acts downstream of CSF1R to transduce signals from M-CSF and IL-34 (48-50). In addition to myeloid cells, recent reports have suggested that CSF1R mRNA can be detected in some cancers like lung and breast cancer cells (27, 28). In our experiments we found that CSF1R expression is upregulated and can be detected at protein levels in chemoresistant cells. Upon binding to IL-34, CSF1R enhances AKT activation, providing a critical signal that help cancer cells to survive under chemotherapeutic conditions.

In summary, we identify a novel role for IL-34 produced by cancer cells following chemotherapy treatment in the formation of chemo-protective niche in a paracrine manner through the recruitment of M2-polarized TAMs with enhanced pro-tumorigenic phenotype in addition to autocrine effects by enhancing AKT-mediated survival signal downstream of CSF1R, and thus help to maintain chemoresistance in cancer cells, suggesting IL-34 as a promising target to overcome chemoresistance in future therapeutic strategies.

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Figure legends

Figure 1.

Monocytes differentiate into M2-polarized macrophages with enhanced immunosuppressive phenotype when stimulated with supernatants of chemoresistant cells. A, frequencies of $CD14^+CD11b^+$ fraction in PBLs treated for 7 days with media or supernatants of chemosensitive (A549-DS) or chemoresistant (A549-DR) cells as evaluated by FACS (left) and graph (right). Forward scatter (FSC) and side scatter (SSC) parameters were used to detect cell size distribution and perform initial gating to remove debris. B, representative photomicrographs of macrophages differentiated from monocytes cultured for 7 days in the presence of A549-DS or A549-DR supernatants. C and D, FACS analysis for CD68 (C) or CD163 (D) expression in purified $CD14^+$ monocytes cultured in media or in the presence of A549-DS or A549-DR supernatants for 7 days. Large cells were gated based on FSC/SSC. Mean fluorescence intensity (MFI) of monocytes was considered as 1. E, RT-qPCR analysis of various cytokines and chemokines expression in A549-DS-MΦ or A549-DR-MΦ. Data from purified $CD14^+$ monocytes were set as 1. F, evaluation of the immunosuppressive function of macrophages. A549-DS-MΦ or A549-DR-MΦ were co-cultured with autologous CD4 or CD8 T cells stimulated with anti-CD3/28 for 72 hours (ratio 1:5) and IFNγ production was determined by ELISA. Data are presented as mean ± SEM. $^*P < 0.05$.

Figure 2.

Chemoresistant cells-derived IL-34 mediates monocytes differentiation into M2-polarized macrophages with enhanced immunosuppressive properties. A and B, purified $CD14^+$ monocytes were cultured in media or A549-DR supernatants pretreated with α-M-CSF or control Ig (rabbit polyclonal) for 7 days. The expression of

CD68 (A) or CD163 (B) was evaluated on day 7 by FACS (left) and graph (right). MFI of monocytes was considered as 1. Large cells were gated based on FSC/SSC. C, ELISA measurement of IL-34 in the supernatants of chemosensitive or chemoresistant cells as measured in undiluted 72 hours culture. D and E, purified $CD14^+$ monocytes were cultured in media or A549-DR supernatants pretreated with α-M-CSF, α-IL-34 (mouse monoclonal IgG1) or control Ig (rabbit polyclonal and mouse IgG1) for 7 days. CD68 (D) or CD163 (E) expression was evaluated on day 7 by FACS (left) and graph (right). MFI of monocytes was considered as 1. Large cells were gated based on FSC/SSC. F, RT-qPCR analysis of *IL10* and *TGFβ* expression in A549-DR-MΦ. Data from purified $CD14^+$ monocytes were set as 1. G, ELISA measurement of IFNγ production by CD3/CD28-stimulated CD4 or CD8 T cells co-cultured for 72 hours with A549-DR-MΦ (5:1) as indicated. Data are presented as mean ± SEM. $^*P < 0.05$. N.D. not detected.

Figure 3.

NF-κB controls IL-34 production in chemoresistant cells. A, immunoblots for phospho-IKKβ, total-IKKβ, phospho-p65, total-p65 and Actin on lysates from A549-DS or A549-DR cells after exposure to doxorubicin (1 μM) at the indicated times. B, quantification densitometry of immunoblots described in (A). C, PCR analysis of *IL34* mRNA expression (normalized to *Actin*) in A549-DR cells 12 hours after treatment with BAY11-7082 at the indicated doses. D and E, CD115 and Actin expression was confirmed by RT-qPCR (D) or western blot (E) in chemosensitive or chemoresistant A549 cells compared to purified $CD14^+$ monocytes as a positive control. F, cell survival assay of A549-DR cells pretreated with α-IL-34 or control Ig (mouse IgG1) 1 hour before stimulation with doxorubicin (1 μM). Data are presented as mean ± SEM. $^*P < 0.05$.

Figure 4.

IL-34 enhances survival of chemoresistant cells via AKT-mediated mechanism. A, western blot analysis of whole cell lysates from A549-DS, A549-DR^{Mock} or A549-DR$^{\Delta IL\text{-}34}$ cells stained with α-CSF1R (CD115), α-phospho-CSF1R (P-Tyr708 or P-Tyr723) and Actin. Quantification densitometry of detected bands is demonstrated in the upper panel. B, phosphorylation levels of AKT compared between A549-DS, A549-DR^{Mock} or A549-DR$^{\Delta IL\text{-}34}$ cells by immunoblots and densitometric quantification. C, phosphorylation levels of AKT in A549-DR^{Mock} pretreated with indicated doses of α-IL-34 as estimated by immunoblots and densitometric quantification. D, phosphorylation levels of AKT in A549-DR$^{\Delta IL\text{-}34}$ stimulated with indicated doses of rIL-34 as estimated by immunoblots and densitometric quantification. E, cell survival assay of A549-DR^{Mock} cells pretreated with indicated doses of α-IL-34 for 1 hour before stimulation with doxorubicin (1 μM). F, cell survival assay of A549-DR$^{\Delta IL\text{-}34}$ cells stimulated with indicated doses of rIL-34 for 1 hour before stimulation with doxorubicin (1 μM). G, time-course of AKT phosphorylation in A549-DR$^{\Delta IL\text{-}34}$ stimulated with rM-CSF or rIL-34 (1 ng/ml) as estimated by western blot. H, dynamism of AKT phosphorylation induced by rM-CSF or rIL-34 in A549-DR$^{\Delta IL\text{-}34}$ cells as estimated by densitometric quantification of immunoblots showed in (G). Data are presented as mean ± SEM. $^{*}P < 0.05$. N.D. not detected.

Figure 5.

IL-34 modifies TAMs function through C/EBPβ-mediated mechanism. A, phosphorylation levels of CSF1R (P-Tyr708 or P-Tyr723) in A549-DS-MΦ (blue bars), A549-DR^{Mock}-MΦ (red bars) or A549-DR$^{\Delta IL\text{-}34}$-MΦ (green bars) at day 7 of culture as estimated by immunoblots (left) and densitometric quantification (right). B,

phosphorylation or total levels of AKT in macrophages described in (A) as estimated by immunoblots (left) and densitometric quantification (right). C, phosphorylation levels of C/EBPβ in macrophages described in (A) as estimated by immunoblots (left) and densitometric quantification (right). D, RT-qPCR analysis of *IL10* and *ARG1* expression in macrophages described in (A) at day 7 of culture. Data from purified $CD14^+$ monocytes were set as 1. E, phosphorylation levels of AKT or C/EBPβ in A549-DR^{Mock}-MΦ as estimated by immunoblots (left) and densitometric quantification (right). Monocytes were cultured in the presence of A549-DR^{Mock} supernatants pretreated with α-IL-34 and/or LY294002 for 7 days. F, RT-qPCR analysis of *IL10* and *ARG1* expression in macrophages described in (E). Data from purified $CD14^+$ monocytes were set as 1. G, phosphorylation levels of AKT or C/EBPβ in A549-$DR^{\Delta IL\text{-}34}$-MΦ as estimated by immunoblots (left) and densitometric quantification (right). Monocytes were cultured in the presence of A549-$DR^{\Delta IL\text{-}34}$ supernatants after the addition of rIL-34 and/or LY294002 for 7 days. H, RT-qPCR analysis of *IL10* and *ARG1* expression in macrophages described in (G). Data from purified $CD14^+$ monocytes were set as 1. Data are presented as mean ± SEM. * $P < 0.05$.

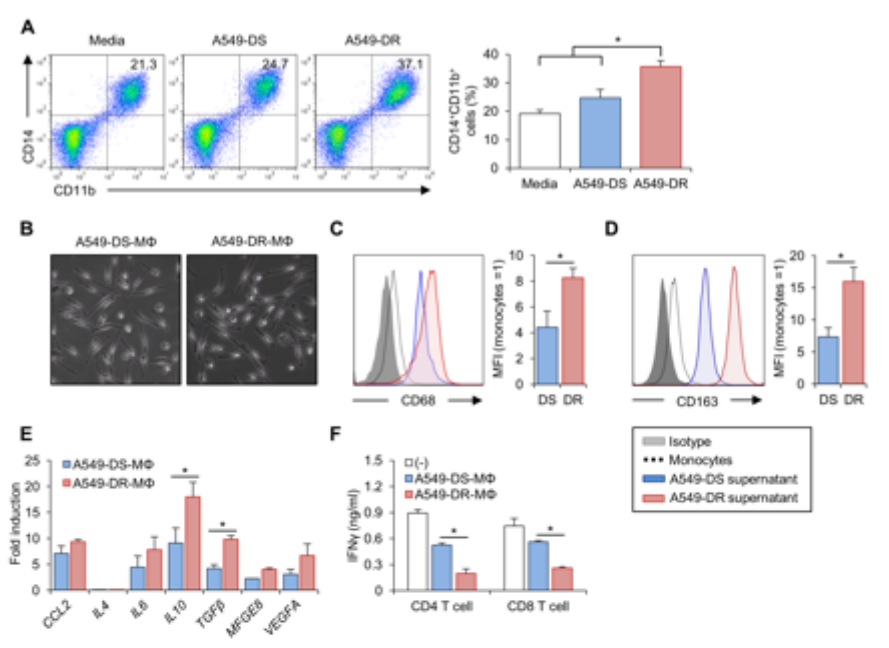
Figure 6.

High infiltration of immunosuppressive TAMs in IL-34-producing chemoresistant tumors. A, tumor growth in humanized NOD-SCID-*IL2rg*$^{-/-}$ mice challenged with A549-DS, A549-DR^{Mock} or A549-$DR^{\Delta IL\text{-}34}$ cells and treated with doxorubicin (DOX) or PBS (n=5 per group). Error bars were removed for better view and showed in (B). B, comparison of tumor size at day 25 between different groups described in (A). C and D, frequencies of $CD68^+CD163^-$ or $CD68^+CD163^+$ TAMs populations as evaluated by FACS (C) or graph (D). E and F, frequencies of $CD8^+CD3^+$ cells in tumor-infiltrating lymphocytes as evaluated by FACS (E) or graph (F). Forward scatter (FSC) and side

scatter (SSC) parameters were used to detect cell size distribution and to perform initial gating to remove debris, and isotype control antibody staining was used to set gates. G, RT-qPCR analysis of various factors expressed in $CD68^{+}CD163^{+}$ TAMs isolated from A549-DS, $A549\text{-}DR^{Mock}$ or $A549\text{-}DR^{\Delta IL\text{-}34}$ tumors. Data are presented as mean ± SEM. $^{*}P < 0.05$.

Figure 7.

IL-34 is expressed in primary lung cancer tissues and correlates to poor prognosis. A, RT-qPCR analysis of *IL34* expression in primary lung cancer tissues from patients diagnosed with adenocarcinoma (ADC), squamous cell carcinoma (SCC) or small cell lung carcinoma (SCLC), as normalized to *Actin*. B, immunohistochemistry staining of IL-34 in cancer or normal tissues from lung cancer patients. IL-34 staining in cerebral cortex or skin is showed in the lower panel as positive controls. C, Kaplan-Meier analysis of overall survival in 332 lung cancer patients stratified as high (green line, n=83) or weak/absent (blue line, n=249). D and E, RT-qPCR analysis of *IL34* and *CSF1R* expression in primary lung adenocarcinoma cells after exposure to doxorubicin (D) or cisplatin (E) at the indicated doses. Data are presented as mean ± SEM. $^{*}P < 0.05$.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37.1
CD14
CD11b
CD14+CD11b+ cells (%)
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A549-DR
B
A549-DS-MΦ
A549-DR-MΦ
C
CD68
MFI (monocytes =1)
DS
DR
D
CD163
MFI (monocytes =1)
DS
DR
Isotype
Monocytes
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A549-DR supernatant
E
A549-DS-MΦ
A549-DR-MΦ
Fold induction
CCL2
IL4
IL6
IL10
TGFβ
MFGE8
VEGFA
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(-)
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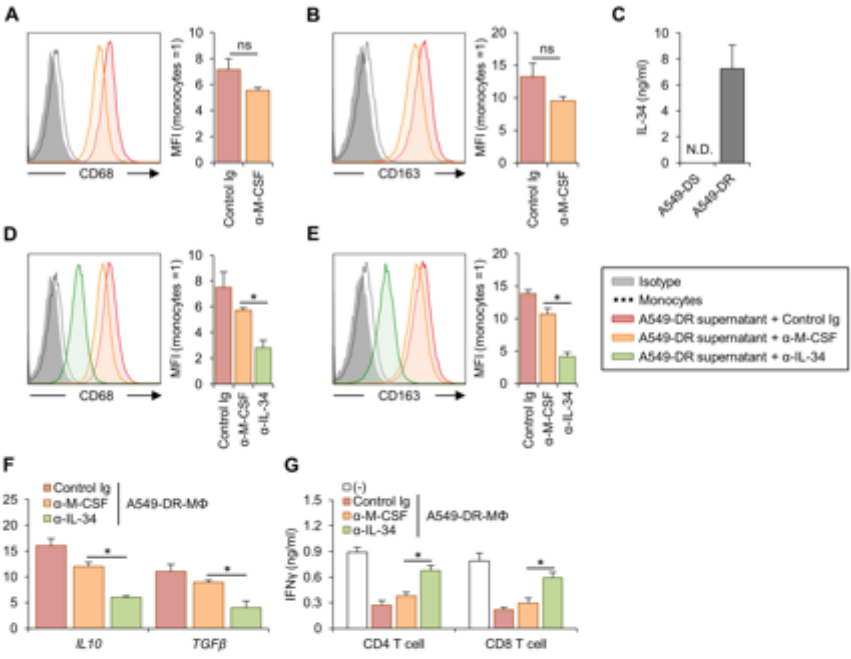
IFNγ (ng/ml)
CD4 T cell
CD8 T cell

Figure 1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IL-34 (ng/ml)
N.D.
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A549-DR
D
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*
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α-IL-34
E
CD163
MFI (monocytes =1)
*
Control Ig
α-M-CSF
α-IL-34
Isotype
Monocytes
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A549-DR supernatant + α-M-CSF
A549-DR supernatant + α-IL-34
F
Control Ig
α-M-CSF
α-IL-34
A549-DR-MΦ
Fold induction
IL10
TGFβ
G
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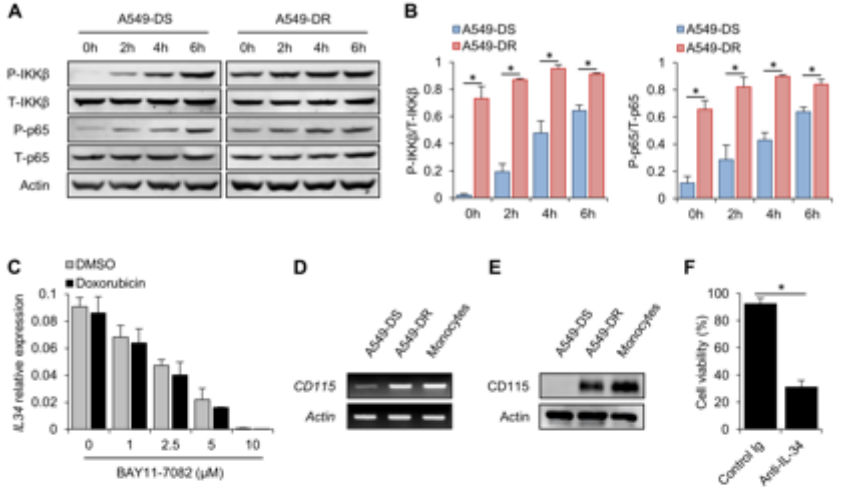
IFNγ (ng/ml)
CD4 T cell
CD8 T cell

Figure 2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A549-DR
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P-p65/T-p65
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C
DMSO
Doxorubicin
IL34 relative expression
BAY11-7082 (μM)
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D
A549-DS
A549-DR
Monocytes
CD115
Actin
E
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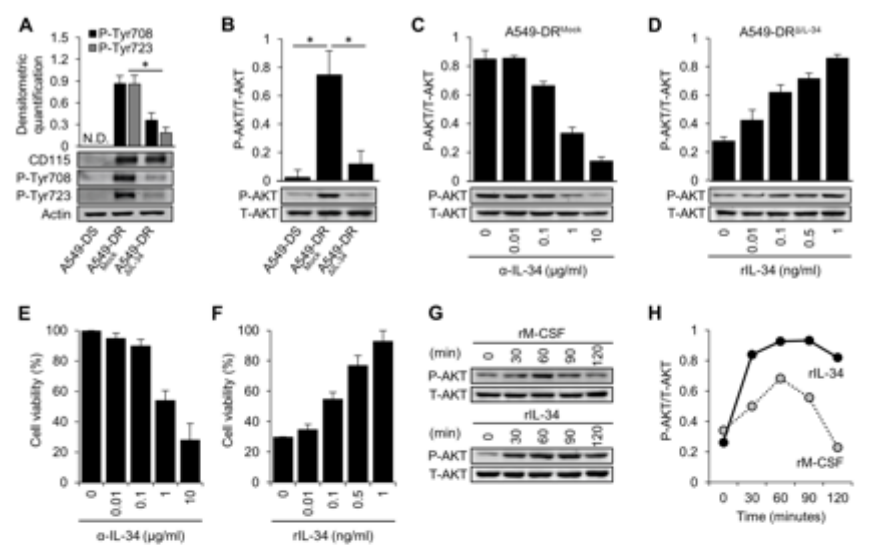
A549-DR
Monocytes
CD115
Actin
F
Cell viability (%)
Control Ig
Anti-IL-34

Figure 3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F
Cell viability (%)
rIL-34 (ng/ml)
G
rM-CSF
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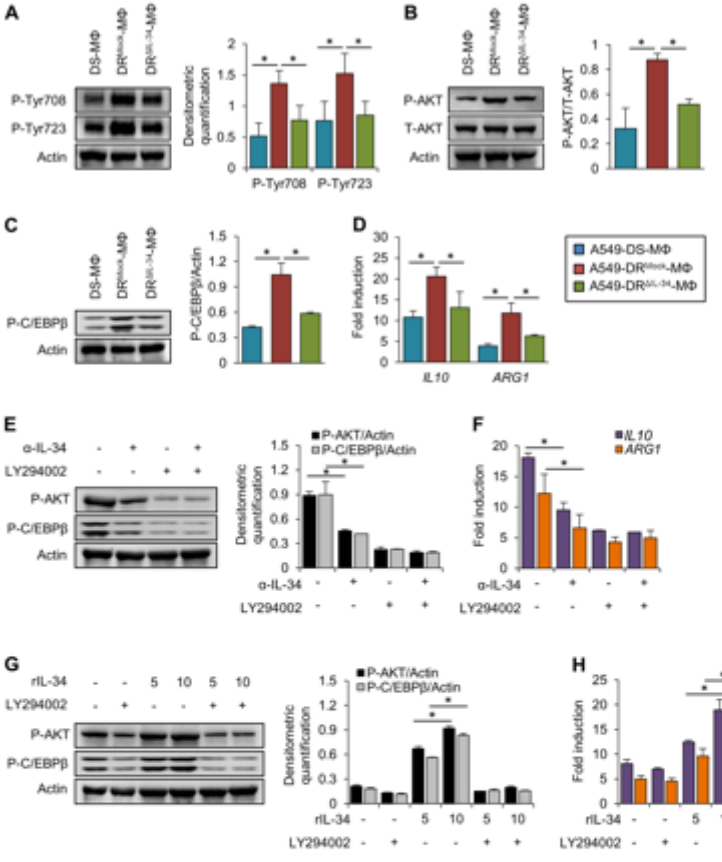
Time (minutes)

Figure 4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P-AKT
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P-AKT/Actin
P-C/EBPβ/Actin
Densitometric quantification
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ARG1
Fold induction
G
rIL-34
LY294002
P-AKT
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Densitometric quantification
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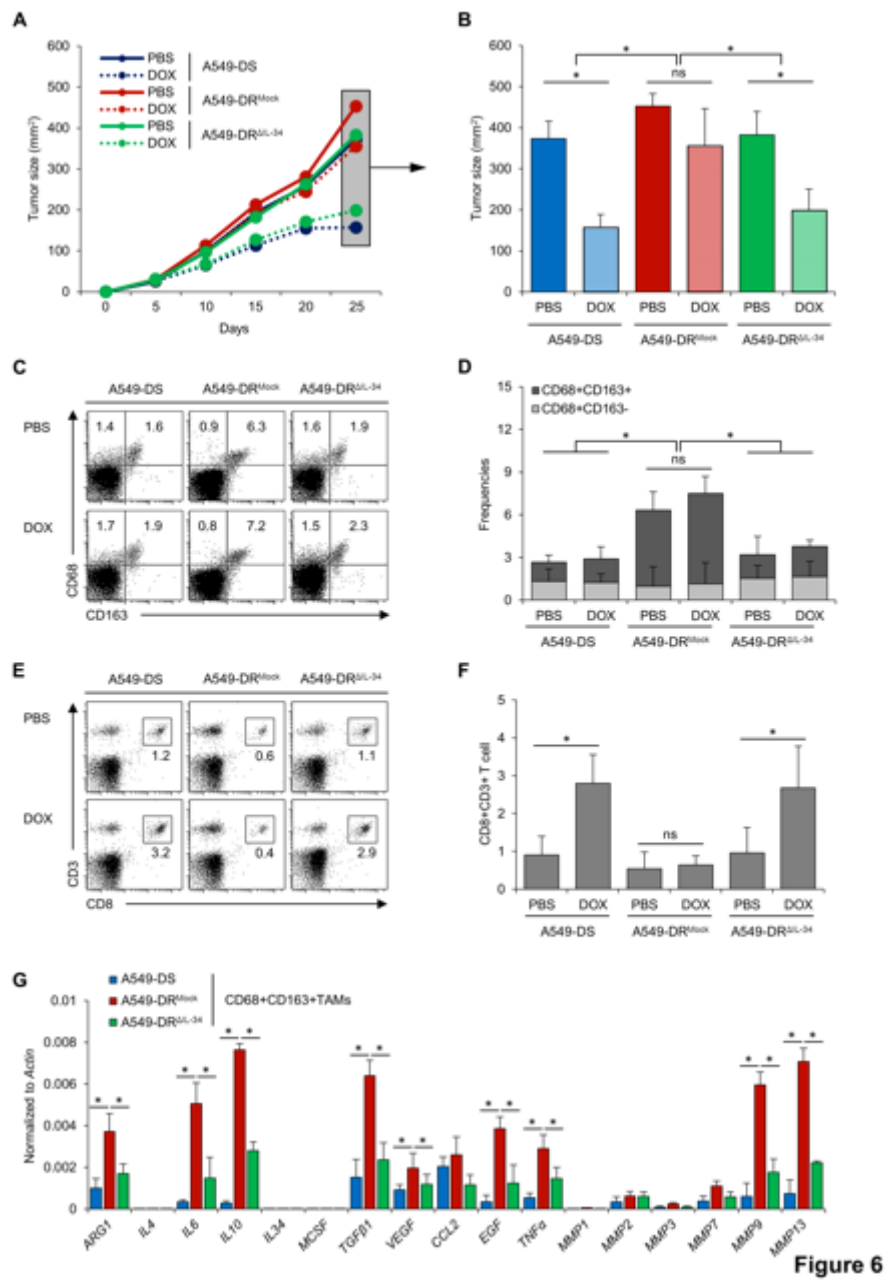
IL10
ARG1
Fold induction

Figure 5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Tumor size (mm³)
Days
B
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CD68
CD163
D
CD68+CD163+
CD68+CD163-
Frequencies
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Il6
Il10
Il34
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TNFα
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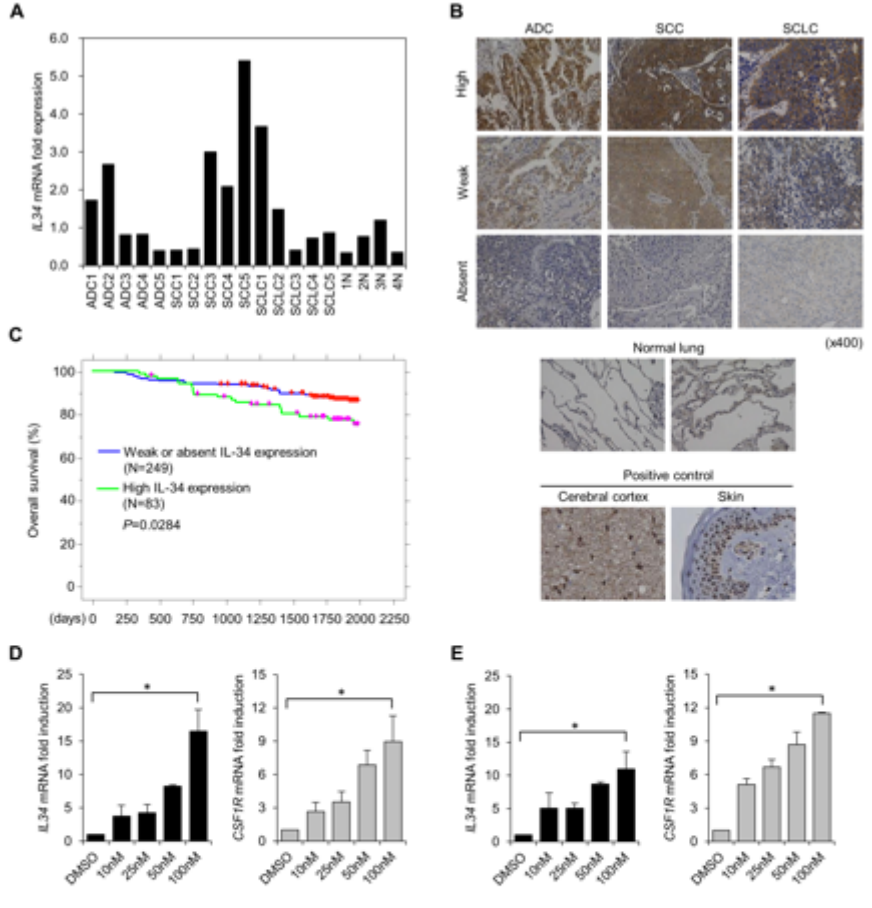
Figure 6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SCC3
SCC4
SCC5
SCLC1
SCLC2
SCLC3
SCLC4
SCLC5
1N
2N
3N
4N
B
ADC
SCC
SCLC
High
Weak
Absent
(x400)
Normal lung
Positive control
Cerebral cortex
Skin
C
Overall survival (%)
100
80
60
40
20
0
Weak or absent IL-34 expression
(N=249)
High IL-34 expression
(N=83)
P=0.0284
(days) 0 250 500 750 1000 1250 1500 1750 2000 2250
D
IL34 mRNA fold induction
CSF1R mRNA fold induction
*
DMSO
10nM
25nM
50nM
100nM
E
IL34 mRNA fold induction
CSF1R mRNA fold induction
*
DMSO
10nM
25nM
50nM
100nM

Figure 7