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de Looff, Margot

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Margot de Looff

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Promotores
Prof. dr. F.A.E. Kruyt
Prof. dr. S. de Jong

Beoordelingscommissie
Prof. dr. J.H.M. van den Berg
Prof. dr. A. Gorman
Prof. dr. R. Gosens

Paranimfen

Gerda de Vries
Wouter de Looff

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Chapter 1

General introduction

General introduction

Non-small cell lung cancer Cancer is currently the second cause of death after cardiovascular diseases worldwide. However, it is predicted to become the primary cause of death in the near future [1]. It is expected that globally the burden of cancer rises to 18.1 million new cases and 9.6 million deaths [1]. Lung cancer is the major cause of total cancer related deaths (18.4%) and has a 5 year survival rate of only 18.3% [1,2]. Non-small cell lung cancer (NSCLC) is the most prevalent type, accounting for 85% of lung cancer cases, of which lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LSCC) are the most prevailing subtypes [3]. The remaining 15% of the lung cancers consist of small cell lung cancer (SCLC).

Current treatment options depend on the stage and genetic profile of the cancer and include surgery, radiotherapy, chemotherapy, targeted therapies and/or immune checkpoint inhibitors. Intensive research to biological drivers of NSCLC and the technological developments during the past decade, including next generation sequencing and the different -omics platforms, have resulted in the discovery and application of targeted therapies. Numerous activating mutations in oncogenic drivers have been found in NSCLC, of which *KRAS* (25%), epidermal growth factor receptor (*EGFR*) (17%) and anaplastic lymphoma kinase (*ALK*) (7%) are the three most frequent occurring aberrations [4]. Targeted therapies for patient with mutant EGFR and ALK are successfully applied [5–7]. For other genetic abnormalities new strategies are being developed, of which some are currently under investigation in clinical trials [4]. In spite of the successful application of targeted therapies the overall survival rate of lung cancer remains low compared to other cancer types [2].

Apoptotic TRAIL signalling An interesting therapeutic biological agent for various tumours, including NSCLC, is the tumour-necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL). TRAIL induces selective apoptosis in tumour cells by pro-apoptotic/canonical signalling that is induced via TRAIL-receptor 1 (-R1/DR4) and/or TRAIL-R2 (DR5), but leaves normal cells unharmed [8–10]. Three other TRAIL receptors have been identified: the TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and the soluble Osteoprotegerin (OPG). These three receptors fail to trigger apoptotic responses and are believed to function as decoy receptors that sequester TRAIL, although TRAIL-R4 was reported to modulate T cell cytotoxicity towards cancer cells [11,12]. However, the exact function(s) of the decoy receptors need to be further explored [13]. Activation of TRAIL-R1 and TRAIL-R2 by TRAIL induces homo- or hetero-trimerization and subsequent recruitment of Fas-Associated protein with Death Domain (FADD) and pro-caspase 8 at the intracellular death effector domains (DED) of the receptors (Fig.1). At that instant Caspase 8 is cleaved and activated, and triggers the so-called death receptor, or extrinsic apoptotic pathway, by cleaving downstream substrates including

caspase 3 and 7 (Fig. 1). Co-activation of the intrinsic or mitochondrial apoptotic pathway occurs simultaneously via caspase 8-dependent cleavage of Bid into truncated (t)Bid, resulting in outer mitochondrial membrane permeabilization and the release of cytochrome C into the cytosol (Fig. 1). Next, a complex of cytochrome C, caspase-9 and Apaf-1 is assembled, called the apoptosome, which activates caspase-3 and induces apoptosis [12]. This mitochondrial apoptotic mechanism is also activated by DNA damaging agents, including chemotherapeutics.

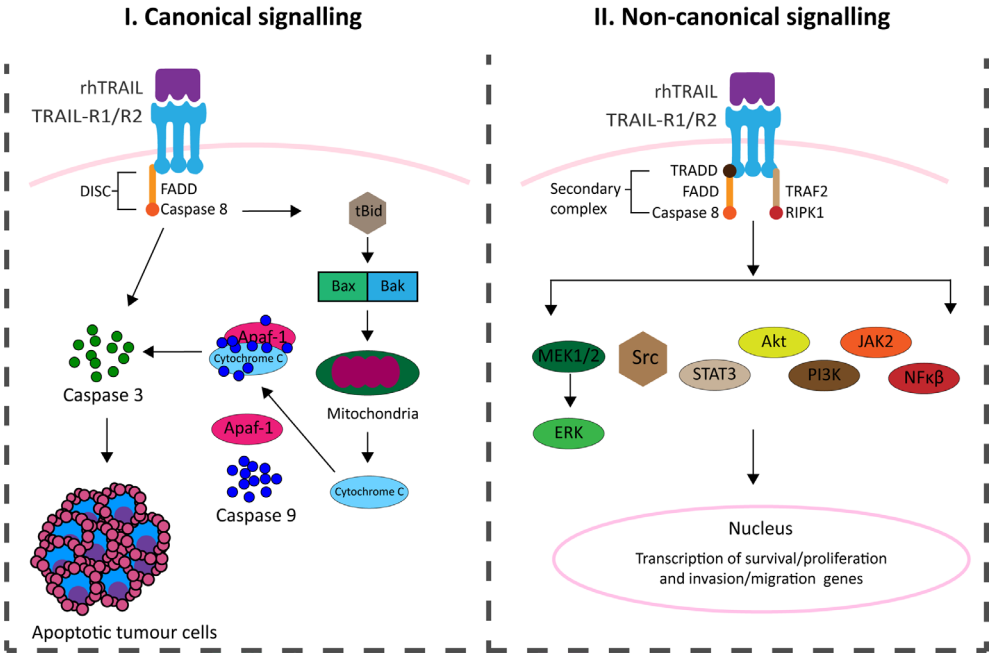

Figure 1. The mechanisms of (non-)canonical TRAIL signalling. I. Canonical TRAIL signalling *Upon binding of TRAIL to TRAIL-R1 and/or TRAIL-R2 the receptors form homo- or hetero-trimers. FADD and pro-caspase 8 are subsequently recruited to the intracellular DED of the receptors. Caspase 8 is cleaved, activated and activates caspase 3 and 7 by cleavage. Simultaneously, caspase 8-dependent cleavage of Bid into truncated (t)Bid occurs, resulting in outer mitochondrial membrane permeabilization and the release of cytochrome C into the cytosol and a complex of cytochrome C, caspase-9 and Apaf-1 is assembled, called the apoptosome, which activates caspase-3 and induces apoptosis.* ***II Non-canonical TRAIL signalling*** *For activation of non-canonical signalling the secondary complex is formed, consisting of TRADD, FADD, caspase-8, TRAF2 and RIPK1. Subsequently, various downstream factors can be activated, including MEK1/2, Src, STAT3, Akt, PI3K, JAK2 and NFκβ, that induce transcription of genes involved in cell survival, proliferation and invasion.*

Pro-tumorigenic TRAIL signalling In spite of the strong anti-tumour potential of TRAIL receptor agonists, intrinsic and acquired resistance to TRAIL driven cell death is frequently observed in *in vitro* and *in vivo* preclinical models and likely contributes to the limited efficacy of TRAIL receptor agonists in clinical studies [14–16]. Moreover, TRAIL receptor

activation in apoptosis resistant cells can result in pro-tumorigenic and even metastasis-promoting activities by activation of pro-inflammatory, pro-survival and invasion pathways [17,18]. Activation of these so-called non-canonical signalling pathways include the formation of the secondary signalling complex initiated at TRAIL-R1 and/or -R2. The secondary complex consists of the Receptor-interacting serine/threonine protein kinase 1 (RIPK1), TNF receptor associated factor 2 (TRAF2), the TNF receptor type 1 associated death domain (TRADD), FADD and caspase 8 [17,19]. Subsequently, various downstream pathways are activated, such as IkB-NFκβ, MAPK-ERK, STAT3, Phosphatidylinositide 3-Kinases (PI3K), Akt, JAK2 and Src [20]. Previously we demonstrated that TRAIL-R2 can activate Src via RIPK1 in TRAIL apoptosis resistant NSCLC cells and stimulates tumour cell migration and invasion [20]. Src is a non-receptor tyrosine kinase and a known proto-oncogene that is often overexpressed and hyperactivated in various cancer types including NSCLC [21–23]. Src is activated by stimulation of plasma membrane receptors and once activated it induces various pathways regulating tumorigenesis, proliferation and migration [24]. Inhibition of Src activity reduces migration, invasion and proliferation in diverse cancer types [20,25–27]. Thus, activation of Src by TRAIL receptor agonists may be an important mediator of pro-tumorigenic signalling.

On top of intrinsic cellular TRAIL resistance, the tumour microenvironment (TME) plays an important role in determining therapeutic efficacy, aside from its role in stimulating the development and progression of cancer [28,29]. The TME consists of cellular and non-cellular components, such as immune cells, stromal cells, fibroblasts and the extra cellular matrix (ECM). The composition of the TME is dynamic, depending on the stage of the tumour, and can have anti- or pro-tumorigenic consequences. An anti-tumorigenic TME is associated with secretion of pro-inflammatory cytokines and the presence of normal fibroblasts (NFs), M1-like macrophages, dendritic cells (DCs), cytotoxic $CD8^+$ T cells and natural killer cells (NK). A pro-tumorigenic TME, on the other hand, is linked with the secretion of immune suppressive cytokines and the presence of M2-like macrophages, cancer associated fibroblasts (CAFs) and regulatory T (T-reg) cells [30]. Dynamic (in)direct interactions between the TME and tumour cells, as well as modifications of the TME support tumorigenesis. For example, tumour cells can secrete pro-inflammatory cytokines after (in)direct interactions with immune cells, thereby creating a pro-tumorigenic niche that further enhances tumour development and metastasis [31–36]. Interestingly, Src has been associated with modulation of cytokine production by tumour cells such that it creates a pro-tumorigenic environment (Liu et al, 2014). A variety of mechanisms and factors that are activated by the TME can protect tumour cells from therapeutic interventions [30,37,38]. For instance, tumour cells can evade immunosurveillance by inhibiting T cell activation via the upregulation of immune checkpoint proteins, like the cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein ligand 1 (PD-L1). Immune checkpoint proteins function as a brake for T cell activation and their subsequent anti-tumour effector functions. In late

stage NSCLC patients, immune checkpoint inhibitors are successful in the clinic as they are proven save and induce effective immune responses that can increase patient survival [39,40]. The efficacy of immune checkpoint inhibitors in untreated and early stage NSCLC patients are very encouraging [4]. Presently, PD-1/PDL-1 inhibitors Nivolumab, Pembrolizumab and Atezolizumab, and Ipilimumab, a CTLA-4 inhibitor, are approved for first and second line therapies [41]. The field of immune checkpoint inhibitors has rapidly developed the last decade and promising results have been accomplished [41,42]. Unfortunately, serious challenges still need to be overcome to further improve the therapeutic efficacy of immune checkpoint inhibitors and targeted therapies, such as the relative limited number of eligible patients as well as the frequency of patients that respond, even when patients are selected based on biomarkers [41,42].

2D and 3D *in vitro* cancer models In oncology research and drug development the translatability of pre-clinical drug responsiveness studies into successful clinical trials is a great hurdle, as only 8% of the promising pre-clinical (animal) studies also have a favourable outcome in clinical studies [43]. Nowadays, two-dimensional (2D) cancer cell models are mostly used in oncology research. However, 2D models do not represent the actual *in vivo* three-dimensional (3D) tumour and lack a TME. The TME includes immune and stromal cells, vascular and ECM. Both the 3D structure and the TME are important factors affecting tumorigenesis, metastasis and therapeutic responsiveness. 3D models, by culturing tumour cells as spheroids and organoids, are being employed more often. Additionally, including components of the TME in cancer cell culture models will likely increase the translatability of *in vitro* findings to the clinic [44]. To allow tumour cells to grow in a 3D fashion, biological matrices, like Matrigel, are successfully used to culture and support the cells in 3D growth. Yet, such matrices are from rodent origin and include unknown growth factors and cytokines which concentrations differ per batch, complicating reproducibility and affecting cell growth [45]. To overcome these issues, synthetic matrices are developed and explored for application of 3D cancer models that also allow inclusion of cells from the TME.

As may be clear from the above, for TRAIL agonists to be clinically applicable and effective against cancer, including NSCLC, the intracellular and extrinsic mechanisms that determine outcome, either pro- or anti-tumorigenic signalling, need to be elucidated in greater detail. Moreover, to augment the translation from bench to bedside, the current pre-clinical models need to be improved to represent the *in vivo* situation better in order to be more predictive for clinical outcome.

The scope of this thesis

As outlined above, there is a great need to improve the overall survival of NSCLC patients and TRAIL receptor agonists remain promising cancer therapeutic agents that need to be

further examined. The central aim of this thesis is to unravel in greater detail the mechanisms involved in TRAIL apoptosis resistance and non-canonical pro-tumorigenic signalling in NSCLC cancer models. We focused on the possible role of Src herein, as this non-receptor tyrosine kinase might play a key role as a regulator and/ or effector of TRAIL receptor non-canonical signalling in resistant NSCLC cells, including as a modulator of immune cell activity via the production of cytokines. Finally, we investigated the application of the synthetic matrix ArtiCYT for 3D cell culturing compared to widely used Matrigel and analysed the applicability of this synthetic matrix for drug responsiveness studies. We expect that a deeper insight in the way Src contributes to TRAIL-dependent anti-tumorigenic signalling will provide clues for strategies to potentiate TRAIL therapy for NSCLC and that the application of a synthetic matrix will improve the translation from pre-clinical to clinical studies.
In **chapter 2** we extensively reviewed the impact of the TME on endogenous TRAIL/ TRAIL-R signalling and on exogenously applied therapeutic TRAIL. Reciprocal interactions between the TME and tumour cells are discussed and how they affect the outcome of TRAIL signalling, either anti- or pro-tumorigenic. Furthermore, the effects of different components of the TME on TRAIL sensitivity were highlighted, including the role of immune effector cells, neutrophils, macrophages and non-hematopoietic stromal cells, and of biochemical and biophysical TME components like mechanical stress, acidity, hypoxia and glucose deprivation.

In **chapter 3** we investigated the role of Src in TRAIL induced signalling in NSCLC cells. The effect of rhTRAIL treatment on Src phosphorylation and activation was studied in apoptosis sensitive (H460) and resistant (A549) NSCLC cells by western blotting. The consequences of Src inhibition on TRAIL induced apoptosis were examined by using pharmacological Src inhibitors, Src knockdown by short hairpin(sh)RNA and knockout by CRISPR/CAS9 technology. Since we previously found that TRAIL activates Src via RIPK1, we examined in more detail which components of the DISC and secondary complex, namely TRADD, TRAF2, FADD and caspase-8, are involved in Src activation by using siRNA approaches. To identify possible downstream mediators of TRAIL-dependent Src signalling, proteins interacting with Src in untreated and rhTRAIL exposed NSCLC cells were studied by Src co-immunoprecipitation (IP)-liquid chromatography (LC)-mass spectrometry (MS). Identified mediators were functionally characterized using western blotting, immunofluorescence microscopy and clonogenic assays.

In **chapter 4** we studied the role of TRAIL treatment and/or Src on the secretion of proteins from TRAIL resistant A549 NSCLC cells, particularly to identify possible immune modulatory factors. Therefore, conditioned medium (CM) was harvested from A549 cells after TRAIL treatment and/or Src depletion and effects on monocyte differentiation, macrophages polarization and CD8+ T cell activation were examined. To examine monocytes differentiation

and macrophage polarization flow cytometry was used to determine M0 (CD11b, CD68), M1 (CD80, CD86) and M2 (CD163, CD206) marker expression. $CD8^+$ T cell activation was detected by examining human T-activator CD3/CD28 Dynabeads stimulated proliferation of T-cells in absence of presence of CM. Importantly, we determined the composition of the secretomes in the different CM by cytokine arrays and mass spectrometry analyses to study Src and TRAIL dependent differences in the composition of the secretomes and in relation to the functional effects.

In **Chapter 5** we investigated the applicability of a synthetic matrix, ArtiCYT, for three-dimensional (3D) spheroid cancer cell (co-)cultures and NSCLC drug responsiveness studies and compared it with an often-used biological matrix (Matrigel). The ArtiCYT composition was optimized for spheroid growth of seven different human breast, lung and ovarian cancer cell lines. The applicability of ArtiCYT for both high throughput screening (HTS) and high content analysis (HCA) was tested by determining sensitivity to the chemotherapeutic drug cisplatin and rhTRAIL. MTS assays were used for HTS and the Operetta high content imaging system was used for HCA The same analyses were used to study the effect of a "feeder" layer of human fibroblasts on rhTRAIL sensitivity of spheroids in ArtiCYT compared to results in Matrigel.

In **Chapter 6** the findings in this thesis were summarized and discussed in a general discussion, including future perspectives of this work.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424. doi:10.3322/caac.21492
2. Cancer Statistics Review, 1975-2015 - SEER Statistics. https://seer.cancer.gov/archive/csr/1975_2015/. Accessed June 22, 2020.
3. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. *Nature*. 2018;553(7689):446-454. doi:10.1038/nature25183
4. Hirsch FR, Scagliotti G V., Mulshine JL, et al. Lung cancer: current therapies and new targeted treatments. *Lancet*. 2017;389(10066):299-311. doi:10.1016/S0140-6736(16)30958-8
5. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. 2020;70(1):7-30. doi:10.3322/caac.21590
6. Oberndorfer F, Müllauer L. Molecular pathology of lung cancer: current status and perspectives. *Curr Opin Oncol*. 2018;30(2):69-76. doi:10.1097/CCO.0000000000000429
7. Lemjabbar-Alaoui H, Hassan OUI, Yang YW, Buchanan P. Lung cancer: Biology and treatment options. *Biochim Biophys Acta - Rev Cancer*. 2015;1856(2):189-210. doi:10.1016/j.bbcan.2015.08.002
8. Ashkenazi A, Holland P, Eckhardt SG. Ligand-Based Targeting of Apoptosis in Cancer: The Potential of Recombinant Human Apoptosis Ligand 2/Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand (rhApo2L/TRAIL). *J Clin Oncol*. 2008;26(21):3621-3630. doi:10.1200/JCO.2007.15.7198

9. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem*. 1996;271(22):12687-12690. http://www.ncbi.nlm.nih.gov/pubmed/8663110. Accessed February 27, 2019.
10. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 1995;3:673-682. doi:10.1016/1074-7613(95)90057-8
11. O'Leary L, van der Sloot AM, Reis CR, et al. Decoy receptors block TRAIL sensitivity at a supracellular level: the role of stromal cells in controlling tumour TRAIL sensitivity. *Oncogene*. 2016;35(10):1261-1270. doi:10.1038/onc.2015.180
12. Gonzalvez F, Ashkenazi a. New insights into apoptosis signaling by Apo2L/TRAIL. *Oncogene*. 2010;29(34):4752-4765. doi:10.1038/onc.2010.221
13. Tawfik D, Groth C, Gundlach JP, et al. TRAIL-receptor 4 modulates γδ T cell-cytotoxicity toward cancer cells. *Front Immunol*. 2019;10(AUG):2044. doi:10.3389/fimmu.2019.02044
14. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: Decisions between life and death. *Int J Biochem Cell Biol*. 2007;39:1462-1475. doi:10.1016/j.biocel.2007.02.007
15. Lemke J, von Karstedt S, Zinngrebe J, Walczak H. Getting TRAIL back on track for cancer therapy. *Cell Death Differ*. 2014;21(9):1350-1364. doi:10.1038/cdd.2014.81
16. Holland PM. Death receptor agonist therapies for cancer, which is the right TRAIL? *Cytokine Growth Factor Rev*. 2014;25(2):185-193. doi:10.1016/j.cytogfr.2013.12.009
17. Azijli K, Weyhenmeyer B, Peters GJ, de Jong S, Kruyt F a E. Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ*. 2013;20(7):858-868. doi:10.1038/cdd.2013.28
18. Kretz A-L, Trauzold A, Hillenbrand A, et al. TRAILblazing Strategies for Cancer Treatment. *Cancers (Basel)*. 2019;11(4):456. doi:10.3390/cancers11040456
19. Varfolomeev E, Maecker H, Sharp D, et al. Molecular Determinants of Kinase Pathway Activation by Apo2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand. *J Biol Chem*. 2005;280(49):40599-40608. doi:10.1074/jbc.M509560200
20. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells. *J Cell Sci*. 2012:4651-4661. doi:10.1242/jcs.109587
21. Guarino M. Src signaling in cancer invasion. *J Cell Physiol*. 2010;223(November 2009):14-26. doi:10.1002/jcp.22011
22. Rothschild S, Gautschi O, Haura E, Johnson F. Src inhibitors in lung cancer: Current status and future directions. *Clin Lung Cancer*. 2010;11(4):238-242. doi:10.3816/CLC.2010.n.030
23. Giaccone G, Zucali PA. Src as a potential therapeutic target in non-small-cell lung cancer. *Ann Oncol*. 2008;19(7):1219-1223. doi:10.1093/ANNONC/MDN048
24. Jin W. Regulation of Src family kinases during colorectal cancer development and its clinical implications. *Cancers (Basel)*. 2020;12(5). doi:10.3390/cancers12051339
25. Lai YH, Lin SY, Wu YS, Chen HW, Chen JJW. AC-93253 iodide, a novel Src inhibitor, suppresses NSCLC progression by modulating multiple Src-related signaling pathways. *J Hematol Oncol*. 2017;10(1). doi:10.1186/s13045-017-0539-3
26. De Toni EN, Kuntzen C, Gerbes AL, et al. P60-c-src suppresses apoptosis through inhibition of caspase 8 activation in hepatoma cells, but not in primary hepatocytes. *J Hepatol*. 2007;46(4):682-691. doi:10.1016/j.jhep.2006.10.010
27. Zhang XHF, Wang Q, Gerald W, et al. Latent Bone Metastasis in Breast Cancer Tied to Src-Dependent Survival Signals. *Cancer Cell*. 2009;16(1):67-78. doi:10.1016/j.ccr.2009.05.017
28. Hanahan D, Weinberg R a. Hallmarks of cancer: The next generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013
29. Wang M, Zhao J, Zhang L, et al. Role of tumor microenvironment in tumorigenesis. *J Cancer*. 2017;8(5):761-773. doi:10.7150/jca.17648
30. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med*. 2013;19(11):1423-1437. doi:10.1038/nm.3394
31. Hartwig T, Montinaro A, von Karstedt S, et al. The TRAIL-Induced Cancer Secretome Promotes a Tumor-Supportive Immune Microenvironment via CCR2. *Mol Cell*. 2017;65(4):730-742.e5.

doi:10.1016/j.molcel.2017.01.021
32. Zhou D-H, Trauzold A, Röder C, Pan G, Zheng C, Kalthoff H. The potential molecular mechanism of overexpression of uPA, IL-8, MMP-7 and MMP-9 induced by TRAIL in pancreatic cancer cell. *Hepatobiliary Pancreat Dis Int*. 2008;7(2):201-209. http://www.ncbi.nlm.nih.gov/pubmed/18397859. Accessed February 27, 2019.
33. Pretti MAM, Bernardes SS, da Cruz JGV, Boroni M, Possik PA. Extracellular vesicle-mediated crosstalk between melanoma and the immune system: Impact on tumor progression and therapy response. *J Leukoc Biol*. 2020. doi:10.1002/JLB.3MR0320-644R
34. Duan Q, Zhang H, Zheng J, Zhang L. Turning Cold into Hot: Firing up the Tumor Microenvironment. *Trends in Cancer*. 2020;6:605-618. doi:10.1016/j.trecan.2020.02.022
35. Chen F, Zhuang X, Lin L, et al. New horizons in tumor microenvironment biology: challenges and opportunities. *BMC Med*. 2015;13:45. doi:10.1186/s12916-015-0278-7
36. Gorczynski RM. IL-17 Signaling in the Tumor Microenvironment. In: *Advances in Experimental Medicine and Biology*. Vol 1240. Springer; 2020:47-58. doi:10.1007/978-3-030-38315-2_4
37. Roma-Rodrigues C, Mendes R, Baptista P V., Fernandes AR. Targeting tumor microenvironment for cancer therapy. *Int J Mol Sci*. 2019;20(4). doi:10.3390/ijms20040840
38. Giraldo NA, Sanchez-Salas R, Peske JD, et al. The clinical role of the TME in solid cancer. *Br J Cancer*. 2019;120(1):45-53. doi:10.1038/s41416-018-0327-z
39. Mencoboni M, Ceppi M, Bruzzone M, et al. Effectiveness and safety of immune checkpoint inhibitors for patients with advanced non small-cell lung cancer in real-world: Review and meta-analysis. *Cancers (Basel)*. 2021;13(6):1-22. doi:10.3390/cancers13061388
40. Zullo L, Rossi G, Dellepiane C, et al. Safety and efficacy of immune checkpoint inhibitors in non-small-cell lung cancer: Focus on challenging populations. *Immunotherapy*. 2021;13(6):509-525. doi:10.2217/imt-2020-0226
41. Ruiz-Cordero R, Devine WP. Targeted Therapy and Checkpoint Immunotherapy in Lung Cancer. *Surg Pathol Clin*. 2020;13(1):17-33. doi:10.1016/J.PATH.2019.11.002
42. Huang Z, Su W, Lu T, et al. First-Line Immune-Checkpoint Inhibitors in Non-Small Cell Lung Cancer: Current Landscape and Future Progress. *Front Pharmacol*. 2020;11:1591. doi:10.3389/fphar.2020.578091
43. Mak IW, Evaniew N, Ghert M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res*. 2014;6(2):114-118. http://www.ncbi.nlm.nih.gov/pubmed/24489990. Accessed August 21, 2019.
44. de Looff M, de Jong S, Kruyt FAE. Multiple Interactions Between Cancer Cells and the Tumor Microenvironment Modulate TRAIL Signaling: Implications for TRAIL Receptor Targeted Therapy. *Front Immunol*. 2019;10:1530. doi:10.3389/fimmu.2019.01530
45. Gelain F, Bottai D, Vescovi A, Zhang S. Designer Self-Assembling Peptide Nanofiber Scaffolds for Adult Mouse Neural Stem Cell 3-Dimensional Cultures. Herman C, ed. *PLoS One*. 2006;1(1):e119. doi:10.1371/journal.pone.0000119

Chapter 2

Multiple interactions between cancer cells and the tumour microenvironment modulate TRAIL signalling:

Implications for TRAIL receptor targeted therapy

Margot de Looff, Steven de Jong and Frank A. E. Kruyt

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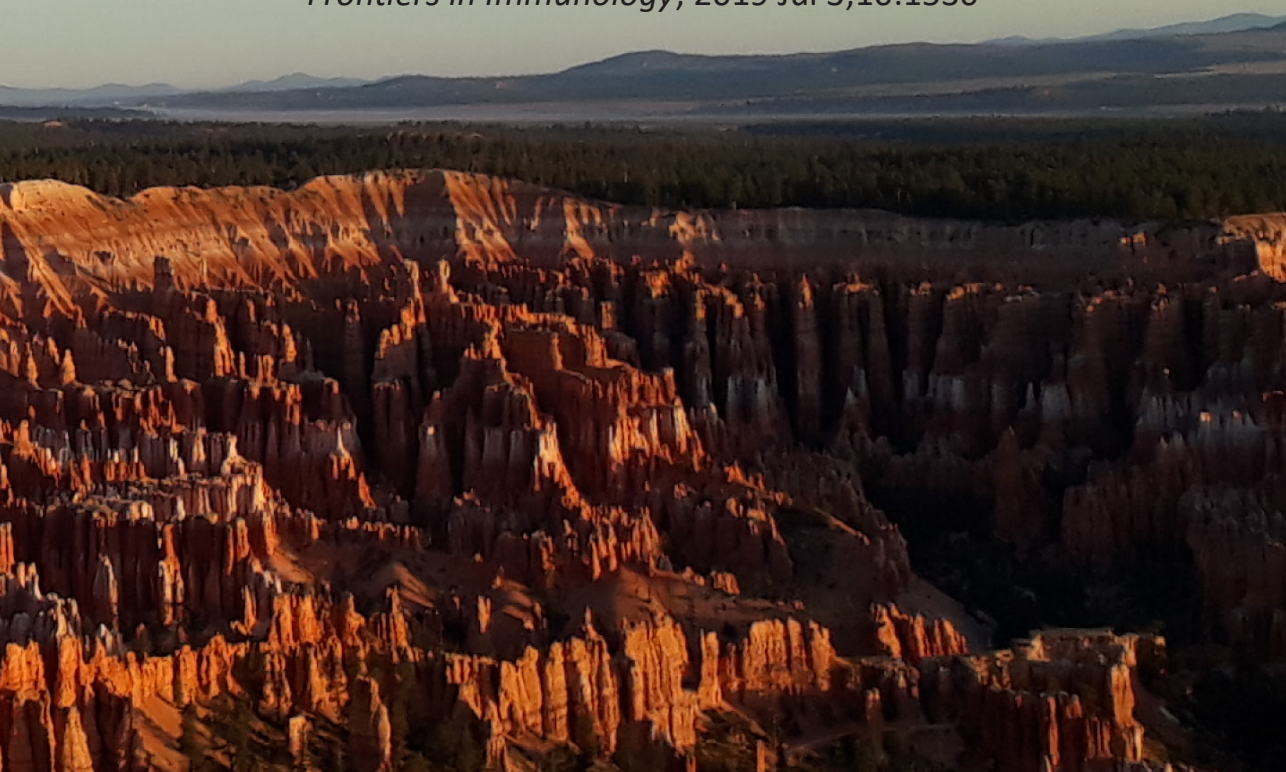

Abstract

Tumour necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) signalling is far more complex than initially anticipated and can lead to either anti- or pro-tumorigenic effects, hampering the successful clinical use of therapeutic TRAIL receptor agonists. Cell autonomous resistance mechanisms have been identified in addition to paracrine factors that can modulate apoptosis sensitivity. The tumour microenvironment (TME), consisting of cellular and non-cellular components, is a source for multiple signals that are able to modulate TRAIL signalling in tumour and stromal cells. Particularly immune effector cells, also part of the TME, employ the TRAIL/TRAIL-R system whereby cell surface expressed TRAIL can activate apoptosis via TRAIL receptors on tumour cells, which is part of tumour immune surveillance. In this review we aim to dissect the impact of the TME on signalling induced by endogenous and exogenous/therapeutic TRAIL, thereby distinguishing different components of the TME such as immune effector cells, neutrophils, macrophages and non-hematopoietic stromal cells. In addition, also non-cellular biochemical and biophysical properties of the TME are considered including mechanical stress, acidity, hypoxia and glucose deprivation. Available literature thus far indicates that tumour-TME interactions are complex and often bidirectional leading to tumour-enhancing or tumour-reducing effects in a tumour model- and tumour type-dependent fashion. Multiple signals originating from different components of the TME simultaneously affect TRAIL receptor signalling. We conclude that in order to unleash the full clinical potential of TRAIL receptor agonists it will be necessary to increase our understanding of the contribution of different TME components on outcome of therapeutic TRAIL receptor activation in order to identify the most critical mechanism responsible for resistance, allowing the design of effective combination treatments.

Introduction

TRAIL receptors (TRAIL-Rs) are able to selectively induce apoptosis in cancer cells and are considered promising therapeutic targets. However, in clinical studies the efficacy of TRAIL-R agonists has been rather disappointing thus far. Novel formulations of TRAIL-R agonists able to more efficiently cluster and activate TRAIL-Rs have been developed and may lead to better therapeutic response [1]. However, the fact that TRAIL signalling is more complex than initially thought hampers the successful use of these receptor agonists. For example, TRAIL signalling was found to have pro-tumorigenic effects in apoptosis resistant tumour cells leading to unwanted stimulation of proliferation and metastatic spread [2,3]. In addition to cell autonomous mechanisms responsible for TRAIL resistance and non-canonical signalling also cell extrinsic signals have been identified that modulate the TRAIL pathway.

Tumours resemble organs as they contain heterogeneous (tumour)cell populations with distinct differentiation status and cellular functions such as blood vessels, immune cells and fibroblasts. The non-cancer cell compartment of a tumour is known as the TME and has been irrefutably demonstrated to play a key role in tumorigenesis, tumour progression and therapeutic efficacy [4–6]. Importantly, dynamic changes in the TME accompany tumour progression and therapeutic resistance and strategies aimed at reprogramming the cellular TME towards an antitumour state provide a promising therapeutic approach.

In this review we focus on the impact of the TME on the outcome of TRAIL signalling in tumour cells. This includes endogenous TRAIL/TRAIL-R signalling being part of immune effector cell functioning and tumour immune surveillance as well as modulation of therapeutic efficacy of TRAIL-R agonists by bidirectional tumour/stroma cell signalling and specific biochemical and biophysical properties characteristic of the TME. The nature and implications of these pleiotropic interactions are highlighted and consequences for the efficacy of TRAIL-based therapy are discussed.

TRAIL receptor targeted cancer therapy

TRAIL receptor agonists have been developed to induce apoptosis selectively in tumour cells, while preserving normal cells [7–9]. Depending on their binding characteristics these agonists bind to both, or either one of the apoptosis-inducing receptors TRAIL-R1 and -R2. These receptors share a death effector domain required for ligand-induced formation of the death-inducing signalling complex (DISC) consisting of FAS-associated death domain (FADD) and procaspase-8, leading to caspase-8 activation and subsequent caspases-dependent apoptosis. This so called extrinsic apoptotic pathway is connected to the intrinsic or mitochondrial apoptotic pathway via the BCL2-family member BID. Caspase-8-dependent BID cleavage produces truncated (t)BID that via interactions with proapoptotic BAX and

BAK disrupt mitochondrial membranes resulting in the release of proapoptotic factors such as cytochrome C, a co-factor for apoptosome formation resulting in activation of initiator procaspase-9 [10]. TRAIL-R agonists are usually designed to have reduced binding affinity for decoy TRAIL-Rs, named TRAIL-R3 and TRAIL-R4. These membrane receptors have a TRAIL-binding domain but lack cytoplasmic domains required for apoptosis activation, whereas a fifth TRAIL binding protein named Osteoprotegerin (OPG) is soluble and also able to sequester TRAIL, thus suppressing TRAIL-R1/R2-dependent apoptosis [11].

In spite of the anticipated powerful therapeutic potential of TRAIL-R agonists, apoptosis resistance is often encountered in cell culture and *in vivo* cancer models, providing an explanation for disappointing results in clinical studies [12–14]. Importantly, TRAIL-Rs were found to induce non-canonical signalling involving activation of pro-inflammatory, pro-survival and proliferation pathways leading to pro-tumorigenic and even metastasis-promoting effects [2,3]. Non-canonical signalling is predominantly mediated by TRAIL-R1 and -R2 and involves the formation of a secondary signalling complex consisting of among others, receptor-interacting serine/threonine protein kinase 1(RIPK1), TNF receptor associated factor 2 (TRAF2) and TNF receptor associated death domain (TRADD) [2,15]. Subsequently, this signalling complex is able to activate various pro-tumorigenic pathways including IκB/ NF-κB, MAPK/ERK, STAT3, PI3K, Akt, JAK2 and Src.

TRAIL resistance has been often regarded as a tumour-autonomous property and various apoptosis resistance mechanisms have been identified such as absence of caspase-8 or elevated expression of various apoptosis blocking proteins including cellular FLICE-like inhibitory protein (cFLIP), X-linked inhibitor of apoptosis proteins (XIAPs), antiapoptotic BCL-2 family members, which have been extensively reviewed elsewhere [11,16,17]. However, cell extrinsic signals derived from the TME can also modulate TRAIL apoptotic signalling. Current evidence for such interactions and consequences for therapy are discussed below.

The tumour microenvironment

The TME consists of cellular components including various myeloid and lymphoid cells, fibroblasts and endothelial cells that via direct interactions or biochemical cues (auto-, para- and endocrine signalling) communicate with tumour cells. In addition, a non-cellular TME can be distinguished consisting of extracellular matrix (ECM), mechanical pressure and tumorigenic conditions like acidity, hypoglycaemia and hypoxia that impact tumour behaviour [18]. The fate of a tumour is dependent on dynamic properties of the TME ranging from anti- to pro-tumorigenic. The anti-tumorigenic TME encompasses normal fibroblasts (NF), dendritic cells (DCs), natural killer (NK) cells, cytotoxic T cells and M1-activated tumour-associated macrophages (TAMs) involving the activity of proinflammatory cytokines. The pro-tumorigenic TME, on the other hand, is associated with immune suppressive effects of

M2-activated TAMs involving production of anti-inflammatory cytokines, myeloid-derived suppressor cells (MDSC), regulatory T (Treg) cells and B cells, cancer-associated fibroblasts (CAFs) producing aberrant ECM, and TIE2-expressing monocytes and mast cells with angiogenesis stimulatory activity. Similar to TAMs, neutrophils and T helper (Th) cells can have both pro- and anti-tumorigenic activity depending on tumour and immune context. For a comprehensive review of the cellular TME and impact on tumour progression and tumour cell dissemination see Quail and Joyce, 2013[18].

Regulation of endogenous TRAIL by the TME

Physiological role of TRAIL TRAIL has been identified as a key mediator of the innate immune response including a role in tumour immune surveillance. Endogenous TRAIL, encoded by the *TNFSF10* gene, is a 281 amino acid (aa), 33 kDa type II transmembrane protein with a small intracellular domain of 17 aa [19,20]. The extracellular domain of TRAIL can be cleaved by cysteine proteases to produce soluble TRAIL (~20 kDa). TRAIL and TRAIL-Rs are expressed in various tissues including immunogenic organs like spleen and thymus. Indeed, a variety of innate and adaptive immune cells express TRAIL such as monocytes, macrophages, DCs, NK cells and cytotoxic T cells (CTLs) [21,22]. TRAIL and TRAIL-R expression is regulated by a variety of factors depending on the cellular context. For example, IFNs can activate transcription of *TRAIL* via the IRF1/STAT3 complex. Furthermore, *TRAIL* and *TRAIL-R* transcription is regulated by stress-induced factors like nuclear factor of activated T-cells (NFAT), Forkhead Box (FOX) proteins, NF-κB, C/EBP homologous protein, activator protein 1 (AP1) and p53 in both immune and transformed cells [23,24].TRAIL signalling can regulate adaptive immune cells by removing aberrantly activated T effector cells maintaining T cell homeostasis. For example CD8+ T cell memory expansion is regulated by CD4+ T helper (Th1) cells via TRAIL dependent apoptosis [25].

Besides being a cytotoxic effector of immune cells in infectious diseases, TRAIL expressing immune cells also play a role in tumour suppression, although not in a consistent way [21,26]. For example, in mice having only one Trail-R, *Trail-R* knockout had no effect on incidence of spontaneous tumour development in siblings obtained from hybrid *APC-/-* (intestinal adenomas) or *p53-/-* (lymphomas) mice [27], whereas siblings of *Trail-/-* and *P53+/-* mice developed more sarcomas and lymphomas [28]. Further, monitoring carcinogen-induced tumorigenesis in *Trail-/-* mice vs controls demonstrated a tumour suppressive effect of Trail [29]. Intriguingly, in this study no differences were detected on primary tumour formation, however, Trail-R deficient mice showed enhanced metastatic spread to lymph nodes suggesting particularly Trail-mediated suppressive effects on disseminating tumour cells [30]. Thus, the TRAIL/TRAIL-R system is predominantly part of immune effector cell functioning and has variable effects on tumour progression and may differentially impact distinct stages of tumour development.

Immune effector cells Immune effector cells from both the innate and adaptive immune system are part of the TME and elicit both pro- and anti-tumorigenic responses. Various immune effector cells, described in more detail below, express TRAIL allowing them to bind and activate TRAIL-Rs on tumour cells.
NK cells are the main effector cells from the innate immune response and eliminate aberrant tumour cells by granule release (perforin/granzyme) dependent toxicity and via membrane receptor interactions involving FasL, TNFα and TRAIL depending on their differentiation and activation status [31,32]. In syngeneic cancer mice models activation of NK cells by IL12 resulted in IFNγ production, which was essential for further activation and augmenting TRAIL surface expression responsible for anti-metastatic activity in TRAIL sensitive tumours [32,33]. Depletion of each single component of the NK cell-IFNγ-TRAIL axis promoted tumour growth in a chemical-induced murine sarcoma model illustrating its importance in antitumour responses [34]. Moreover, activated NK cells employ membrane bound TRAIL, but not soluble TRAIL, to support their cytotoxicity against neuroblastoma cells, which are normally resistant to soluble TRAIL [35].

Cytotoxic T-cells (CTLs) are the main effector cells of the acquired immune response and also make use of the TRAIL/TRAIL-R system to induce apoptosis in target cells. For example, expression of TRAIL on CTLs can be enhanced by T-cell receptor-mediated interaction with TRAIL-R-expressing human non-small cell lung cancer (NSCLC) cells. IFNα significantly enhanced TRAIL expression on CTLs and effectively triggered apoptosis in TRAIL sensitive NSCLC cells *in vitro*. Antitumour activity was also seen in immune–deficient mice implanted with TRAIL sensitive NSCLC cells in which intratumoural injection of autologous activated CTLs resulted in TRAIL-R2-dependent tumour cell death [36].

DCs play a role in both innate and adaptive immune responses by communicating to both immune effector cells and presenting antigens to T cells. Cytotoxic DCs can be activated by IFNα or IFNγ displaying antitumour activity adopting the TRAIL/TRAIL-R system [37,38].

The activity of the immune effector cells can be counteracted by immunosuppressive Tregs. Tregs secrete a range of soluble factors such as TGFβ, IL10, and IL35, which can suppress effector T cell expansion and cytokine secretion (IFNγ, TNFα) [39]. Tumour infiltrating Tregs repress antitumour immune responses by inhibiting the cytotoxic activity of CTLs, NK cells and DCs. In rodent colon cancer models Tregs were able to inhibit cell death induced by TRAIL expressing DCs. Innate immune response activation by Mycobacterium Bacillus Calmette-Guérin (BCG) combined with cyclophosphamide treatment depleted Tregs and potentiated DC-induced tumour cell killing [40]. Orthotopic implantation of TRAIL resistant murine pancreatic cancer cells in either WT or TRAIL knockout mice resulted in smaller tumours in TRAIL knockout mice. Tumour growth in WT mice was associated with increased tumour

infiltrating $CD4^+$ Treg cells that was further enhanced by treating mice with recombinant TRAIL, which also enhanced tumour growth. Although the underlying mechanism of TRAIL-dependent tumour infiltration was not addressed, it is likely that TRAIL in the context of resistant tumour cells potentiated the immune suppressive effects of Treg cells resulting in enhanced tumour growth [41]. Notably, in mice Tregs can also directly eliminate CTLs via TRAIL/TRAIL-R2-mediated apoptosis [42]. On the other hand, CTLs can produce cytokines that increase the sensitivity of tumour cells for TRAIL. Upon T-cell receptor activation CD8+ CTLs produced soluble IFNγ and TNFα, which increased the susceptibility of neuroblastoma cells for TRAIL-induced caspase-8 activation [43].

Tumour cells can counteract the activity of immune effector cells by inhibiting TRAIL-induced apoptotic signalling. For example, follicular lymphomas expressing CD40, an important co-stimulatory receptor able to interact with ligand expressed on germinal center $CD4^+$ T-cell subpopulations, protected from TRAIL-induced apoptosis by CTLs. CD40 activation induced NF-κB leading to upregulation of antiapoptotic cFLIP and Bcl-XL [44,45].

Antitumour activity of TRAIL expressing DCs was reduced by apoptotic tumour lysate derived from TRAIL sensitive murine lymphomas. TRAIL expression could be partially restored both *in vitro* and *in vivo* upon stimulation by IL15, or LPS leading to prolonged TRAIL expression on DCs and antitumour activity. On the other hand, while stimulating DCs, IL15 inactivated STAT3 in lymphoma cells resulting in TRAIL resistance that could be neutralized by combined treatment with the STAT3 inhibitor Cucurbitacin I leading to an overall effective therapeutic response [46].

Activated TRAIL-Rs on tumour cells can also trigger a counterattack and create an immune suppressive TME. TRAIL resistant human colon cancer cells were found to release micro vesicles containing FAS and TRAIL that were also detectable in plasma from patients. These micro vesicles could eliminate CTLs thus providing an immune escape mechanism [47]. Endogenous cell surface TRAIL on multiple myeloma cells could eliminate osteoclasts and prevent bone formation thereby facilitating metastatic lesions [48,49]. TRAIL-resistant gastric carcinoma cells from primary and metastatic patients expressed TRAIL and TRAIL-Rs, including TRAIL-R4. Interestingly, tumour infiltrating lymphocytes (TIL) from patients with a primary tumour hardly expressed TRAIL/TRAIL-Rs, whereas those from metastatic patients showed high levels and displayed apoptosis. This suggests that in metastatic lesions tumour cells can evade immune surveillance by inducing TRAIL- mediated cell death of TILs [50]. Similarly TRAIL expressing colorectal cancer (CRC) cells in patient samples were linked with apoptosis induction in tumour infiltrating $CD8^+$ T-cells via TRAIL-R1 providing an immune escape mechanism [51].

Taken together, the TRAIL-dependent immune effector function can be potentiated by various cytokines that can be counteracted by Tregs. Particularly TRAIL resistant tumours, but also sensitive tumour cells can respond by expressing or secreting factors that inhibit immune effector cell-induced apoptosis or even eradicate immune cells by TRAIL/TRAIL-R dependent mechanisms.

Neutrophils and macrophages Neutrophils are an essential part of the innate immune system and are the most abundant leukocytes in the blood. Similar to monocytes that can differentiate into macrophages, neutrophils possess phagocytic activity. They can migrate to sides of acute inflammation as well as tumours where they can have both tumour suppressive and supportive functions [52]. Neutrophils and monocytes both express TRAIL and target TRAIL-R expressing tumour cells. *In vitro* experiments showed that IFNα exposure of neutrophils/monocytes led to increased release of soluble TRAIL resulting in apoptosis activation in TRAIL sensitive chronic myeloid leukaemia (CML) cells. Additionally, IFNα protected both neutrophils and monocytes from leucine-zipper TRAIL and soluble rhTRAIL induced apoptosis, which may be related to absence or low levels of TRAIL-R1/R2 and high TRAIL-R3 expression. Furthermore, melanoma patients treated with IFNα showed increased soluble TRAIL serum levels indicating *in vivo* relevance of this anti-tumorigenic mechanism [53]. This mechanism provides an explanation for treatment efficacy of IFNα in CML and melanoma patients.

Peritumoral administration of granulocyte colony-stimulating factor (G-CSF) has been reported to suppress murine mammary adenocarcinoma progression in mice, which was not seen upon *in vitro* exposure of tumour cells to this cytokine. G-CSF appeared to increase the number of infiltrating neutrophils accompanied with upregulation of death-inducing proteins including TRAIL providing an explanation for antitumour activity [54].

In oesophageal squamous cell carcinoma (ESCC) the presence of IL17 producing cells was associated with a favourable prognosis. IL17 stimulated ESCC-dependent secretion of neutrophil-attracting chemokines and, moreover, enhanced their immune effector function also characterized by u TRAIL [55].

Another study found that cathepsin E expressed on immune cells can cleave and activate endogenous cell surface TRAIL on prostate and melanoma cells and enhance macrophage infiltration leading to antitumour activity. Cathepsin E reduced murine melanoma growth in mice, when compared to tumour growth in cathepsin E knockout mice. This was accompanied with increased tumour infiltration of activated macrophages and apoptosis activation in tumour cells [56].

Macrophages were reported to secrete matrix metallopeptidase 12 (MMP12) and stimulate TRAIL-dependent apoptosis in tumour cells. MMP12 activity could be mimicked by a recombinant C-terminal domain peptide, named SR20, that could induce TRAIL-mediated apoptosis both in oncogenic mutated KRAS and WT murine and human NSCLC cells as demonstrated *in vitro* and in orthotopically implanted mice and a KRAS-induced murine mouse model. In addition to protein cleavage activity, SR20 translocated to the nucleus of these cells leading to transcriptional upregulation of *TRAIL* and *TRAIL-R1* mRNA and downregulation of antiapoptotic proteins that was responsible for the observed tumour cell death [57].

Conversely, in addition to the TRAIL-dependent antitumour activity of neutrophils and macrophages also pro-tumorigenic activity has been demonstrated involving the cooperative action of various cytokines produced by tumour cells and different immune cells. For example, in murine hepatocarcinoma and melanoma mice models IL35 was found to polarize neutrophils into a pro-tumorigenic N2 state and enhance tumour infiltration that was accompanied by downregulation of TRAIL expression. This involved the concerted action of various cytokines and immune cells cumulating in IL6/IL1β/IL17/ G-CSF induced STAT3-dependent downregulation of TRAIL expression on neutrophils and simultaneous upregulation of MMP9 together resulting in immune suppression and a proangiogenic state [58,59].

The MUC5AC glycoprotein expressed on pancreatic cancer cells was required for tumour growth *in vivo* by suppressing antitumour effects of neutrophils. MUC5AC was found to suppress tumour secretion of the neutrophil attractant IL8 and, moreover, MUC5AC blocked TRAIL-R mediated apoptosis of tumour cells via an as yet unknown mechanism [60].

Summarizing, macrophages and neutrophils can eliminate tumour cells via the TRAIL/ TRAIL-R system that can be potentiated or suppressed by various mechanisms involving administrated or tumour-derived cytokines as well as tumour or immune cell expressed activators or suppressors.

Non-hematopoietic stromal cells Expression of TRAIL on stromal cells has been demonstrated to be a favourable characteristic for patient survival [61]. Immunohistochemistry (IHC) studies of patient tissue arrays demonstrated that increased TRAIL expression in the epithelium and connective tissues of prostate and ovarian cancer is associated with elongated recurrence free survival and favourable overall survival, respectively. This effect was independent of decreased TRAIL-R expression and increased cFLIP-L expression in tumour cells [62,63].
Mesenchymal stem cells (MSC) are derived from bone marrow and can differentiate into various cell types including osteoblasts and adipocytes and home to tumours making part of

the tumour stroma. *Ex vivo* exposure of human MSCs to TNFα increased TRAIL expression. Subsequent infusion of these hMSC in mice implanted with MDA-MB-231 breast cancer cells inhibited tumour growth. Furthermore, co-culturing these hMSC with several cancer cell lines resulted in apoptosis induction. Interestingly, these dying tumour cells released DNA that acted as damage-associated molecular patterns (DAMPs) that via a TLR3-dependent NF-κB feed forward loop further increased TRAIL expression on hMSC, thereby potentiating their antitumour activity [64]. In follow up work TNFα-activated hMSC were also found to produce IFNβ in response to released DNA/RNA from apoptotic breast cancer cells further enhancing TRAIL expression and potentiation of antitumour activity [65]. Accordingly, this feedforward loop of TRAIL-induced apoptosis was not seen in apoptosis resistant breast cancer cells. Moreover, CAFs isolated from breast cancer patients showed a similar increase in TRAIL and IFNβ upon exposure to DNA.

Thus, bidirectional signalling between TRAIL sensitive tumour cells and stromal cells can create a tumour-suppressive TME.

Modulation of exogenous TRAIL receptor agonist activity and the TME

From a therapeutic standpoint pharmacological administration of TRAIL-R agonists aims to mimic the function of TRAIL-expressing immune effector cells. In this part the impact of different components of the TME on the efficacy of exogenously administrated TRAIL is highlighted.

Stromal cells Stromal cells express TRAIL decoy receptors and create a sink for administrated recombinant TRAIL leading to suppression of antitumour activity. OPG is predominantly secreted by osteoblasts and functions as a paracrine survival factor in bone marrow TME and has been implicated in TRAIL resistance. OPG protected prostate cancer and multiple myeloma cells against TRAIL-mediated cell death [66,67]. Bone marrow stromal cells from breast cancer patients also produced sufficient OPG levels to decrease TRAIL sensitivity of breast cancer cells providing a mechanism for the occurrence of metastatic lesions in the bone [68]. OPG production can be enhanced by cytokines. For example, IL1β increased OPG expression in both TRAIL sensitive MDA-MB231 and resistant MCF7 cells. Gene silencing of OPG enhanced apoptosis in MDA-MB231 cells, but not in MCF7 cells, and a positive correlation was found between OPG levels and TRAIL sensitivity [69]. TRAIL variants have been developed with reduced affinity for decoy receptors and demonstrated superior antitumour activity in the presence of OPG producing cells or recombinant OPG [70]. Similarly, the expression of TRAIL-R3/R4 on CAFs decreased the efficacy of TRAIL induced apoptosis in tumour cells that could be bypassed by developed TRAIL variants with reduced binding activity to these decoy receptors [71].

Epidermal growth factor (EGF) could protect TRAIL sensitive HEK293 and MDA-MB-231 tumour cells for apoptosis involving Akt activation and inhibition of mitochondrial apoptosis [72]. On the other hand, in non-transformed MCF10A human breast epithelial cells EGF sensitized for TRAIL-induced apoptosis that was counteracted by TGFβ involving reduction of DISC formation and activation of cytoprotective autophagy [73]. The differential activity of EGF in normal and tumour cells illustrates differential wiring of TRAIL signalling upon oncogenic transformation.

Cytokines exogenously added or produced by either tumour or stromal cells can inhibit TRAIL-induced apoptosis in tumour cells. TRAIL-sensitive ovarian cancer cells reverted to resistant cells by exposure to IL8 that was associated with downregulation of TRAIL-Rs [74]. IL8 produced by tumour cells or recombinant IL8 also was shown to suppress TRAIL-induced apoptosis in prostate cancer cells by up-regulation of the anti-apoptotic proteins cFLIP(S) and cFLIP(L) in a CXCR2 and NF-*κ*B-dependent way. TRAIL as well as chemotherapy could enhance IL8 expression leading to apoptosis resistance and a CXCR2 antagonist sensitized for TRAIL providing a therapeutic strategy [75]. Primary cancer cells from breast, colon and lung carcinomas produce IL4 that protected tumour cells for TRAIL-induced apoptosis by increasing expression of a number of anti-apoptotic proteins including cFLIP, Bcl-XL and Bcl-2 [76]. Metastatic melanoma cells endogenously express proinflammatory TNFα and IL6 leading to constitutive NF-*κ*B, STAT3 and COX2 expression. Neutralizing antibodies against these cytokines and genetic or pharmacological inhibition of the downstream pathways resulted in sensitization for exogenous TRAIL-induced apoptosis [77]. TLR4 ligation on human lung cancer cells and associated NF-*κ*B activation reduced apoptotic effects of TRAIL and, in addition, promoted the production of immunosuppressive cytokines TGFβ and IL8 together with proangiogenic VEGF [78].

Ovarian cancer (OC) is commonly associated with peritoneal ascites production and provides a unique TME for this tumour type. A proportion of ascites samples taken from OC patients could mediate resistance towards TRAIL-induced apoptosis in a panel of OC cell lines *in vitro* [79]. Further research showed that malignant ascites leads to activation of PI3K, Akt, ERK1/2 and ELK1 and up-regulating cFLIP(s) and Mcl-1 and inhibition of TRAIL-induced caspase-dependent apoptosis [80].

Co-culturing of multiple myeloma cells and HS5 stromal cells attenuated TRAIL-induced cell death involving soluble factors produced by the stromal cells. Antiapoptotic cFLIP was identified as a mediator for resistance as silencing its expression increased TRAIL sensitivity [81]. In a follow up study stromal-mediated resistance was found to involve NF-*κ*B-dependent cFLIP expression that could be prevented by the proteasome inhibitor bortezomib that restored TRAIL sensitivity in tumour cells without affecting stromal HS5 cells [82]. Myoblasts

secreting platelet-derived growth factor BB(PDGF-BB) indirectly affected TRAIL sensitivity by activating Hedgehog (Hh) signalling in cholangiocarcinomas (CCA) thereby shifting the cells towards TRAIL resistance. Inhibition of Hh signalling by cyclopamine increased apoptosis in CCA cells *in vitro* and in a syngeneic RAT CCA model resulting in tumour suppression [83]. Co-culturing of Wnt producing rat embryonic fibroblasts protected TRAIL sensitive human pre-B leukaemia cells for TRAIL-induced apoptosis. Although the precise mechanism was not fully elucidated inhibition of MEK1/ERK1/2 and NF-κB signalling sensitized for TRAIL [84].

TRAIL resistant colon cancer cell lines were sensitized for exogenously administrated TRAIL by combined exposure with IFNγ and TNFα through down-regulation of Bcl-XL. Evidence for a similar resistance mechanism was provided in a murine CT26 colon carcinoma mice model. In this model, tumour infiltrating macrophages, NK cells and T cells secreting IFNγ and TNFα and expressing TRAIL were responsible for suppression of lung metastases since neutralizing TRAIL antibodies blocked antitumour activity leading to increased lung metastases. Moreover, it was shown that adoptive transfer of tumour-specific CD8+ CTLs producing IFNγ and TNFα together with recombinant TRAIL/agonistic mAb therapy effectively induced apoptosis in CT26 tumour cells in mice, whereas TRAIL alone was ineffective, indicating cooperative activity between tumour infiltrating immune cells and TRAIL therapy [85].

Taken together, stromal cells can produce various factors that in a paracrine way suppress or enhance the therapeutic efficacy of TRAIL-R agonists.

TME remodelling Application of exogenous TRAIL may also impact the TME by targeting specific cellular components. In contrast to the dogma that normal cells are refractory to the death inducing effect of TRAIL Liguori *et al* reported susceptibility of monocytes and macrophages for TRAIL-induced apoptosis [86]. *In vitro*, human monocytes and macrophages expressed TRAIL-R1/R2 and underwent apoptosis after TRAIL exposure, whereas neutrophils and lymphocytes expressing mainly decoy TRAIL-R3 did not. Furthermore, in murine fibrosarcoma implanted mice, TRAIL-R expressing monocytes were sensitive for TRAIL. Interestingly, particularly infiltrating TAMs but not normal tissue resident macrophages expressed functional TRAIL receptors. TAMs that have pro-tumorigenic activity in this syngeneic mouse model were sensitive to TRAIL-induced apoptosis resulting in significant decreases in circulating monocytes and infiltrating TAMs and a concomitant reduction in tumour growth and metastasis [86].

TRAIL resistant murine hepatocellular carcinoma (HEPA-1-6) cells *in vitro*, became sensitive for intra-tumoral injection of TRAIL after implantation in mice. Analyses of tumour infiltrating immune cells revealed that TRAIL injections decreased the numbers of Trail-R positive Tregs, whereas levels of $CD8^+$ CTLs increased. Thus, TRAIL treatment appears to deplete Tregs by

apoptosis thereby potentiating $CD8^+$ CTLs-dependent antitumour responses, in addition to the direct apoptotic effects on HEPA-1-6 tumours [87].

In acute myeloid leukaemia HL-60 cells TRAIL triggered apoptosis. However, in the surviving fraction an increase in monocyte maturation markers was observed, requiring TRAIL-R1 and caspases activation. In normal monocytes TRAIL also was able to induce expression of CD14 and CD11b maturation markers associated with enhanced phagocytic capacity and antitumour activity. Accordingly, TRAIL therapy has dual anti-neoplastic activity by directly killing tumour cells and enhancing monocyte/macrophage activity [88].

TRAIL could stimulate the production of pro-inflammatory cytokines IL1β, IL6 and TNFα in a NF-kB-dependent way in human and murine macrophages *in vitro*. Similarly, TRAIL was able to stimulate pro-inflammatory cytokine expression in TAMs in tumours derived from TRAIL sensitive H460 NSCLC cells in nude mice, but not in peritoneal macrophages that was related to high miR-146 expression in the latter leading to silencing of cytokine expression. Moreover, co-cultures of H460 and TRAIL-stimulated TAMs showed that cytokines produced by TAMs potentiate the TRAIL-dependent killing of H460 cells [89].

Tumour vasculature has been reported to be sensitive for TRAIL-induced apoptosis via TRAIL-R2. In different murine tumour models tumour-associated endothelial cells expressed TRAIL-R2 and were sensitive for the killing effect of TRAIL resulting in tumour starvation, even when tumour cells were TRAIL resistant. IHC demonstrated TRAIL-R2 expression in NSCLC patient vasculature and therefore TRAIL-induced collapse of tumour blood vessels was proposed as an alternative or complementary therapeutic strategy [90]. Another favourable effect of TRAIL on the TME was reported by downregulating OPG production in MSCs, fibroblasts and endothelial cells by TRAIL-mediated inhibition of p38/MAPK activation [91].

These findings illustrate that the antitumour activity of TRAIL can be directly enhanced by simultaneously potentiating the antitumour effect of stromal cells or by suppressing the pro-tumorigenic activity of stromal cells leading to an overall therapeutic benefit.

Contrary to above findings, tumour cells were found to create a pro-tumorigenic niche involving TRAIL signalling via indirect means. In TRAIL resistant NSCLC cells TRAIL exposure triggered the secretion of immune-suppressive cytokines IL8, CXCL1, CXCL5 and CCL2 in a FADD- and caspase-8-dependent way. Particularly CCL2 was found to induce monocyte polarization into myeloid-derived suppressor cells (MDSCs) and generated M2 macrophages. Orthotopic implanted TRAIL resistant A549 NSCLC and murine LL3 cells with FADD deleted showed reduced tumour growth compared to implanted WT tumour cells. This was associated with decreased cytokine production including CCL2 leading to reduced tumour

infiltration of tumour supporting monocytes and MDSCs [92].
TRAIL treatment of resistant human pancreatic cells resulted in enhanced invasion *in vitro* and metastatic spread in orthotopically implanted nude mice. TRAIL-induced NF-κB activation stimulated production of pro-inflammatory cytokines IL8 and MCP1/CCL2, proteases MMP7 and MMP9 and urokinase-type plasminogen activator (uPA) that were responsible for pro-inflammatory effects and metastatic spread [93,94]. More recently, the chemokine CCL20 was identified as a TRAIL/ NF-κB inducible target gene in resistant pancreatic cancer cells that indirectly modulated TRAIL resistance in mice by recruiting peripheral blood mononuclear cells (PBMCs), which further increased TRAIL resistance of CCL20-producing pancreatic cancer cells [95]. Thus, in pancreatic cancer TRAIL had unfavourable effects by stimulating pro-inflammatory cytokines production leading to enhanced metastasis and TRAIL resistance.

A positive feedback loop between tumour cells and macrophages was identified in promoting growth and survival of colon cancer cells. Macrophages producing IL1β could stimulate growth of colon cancer cells by activating GSK3β /Wnt signalling [96]. IL1β production by macrophages was induced by tumour cells and resulted in protection of colon cancer HCT116 cells from TRAIL-induced apoptosis. IL1β-mediated TRAIL resistance involved activation of NF-κB and GSK3β/ Wnt pathways leading to stabilization of the EMT transcription factor Snail [97].

TRAIL treatment was also shown to enhance pro-inflammatory cytokine and chemokine production, including IL6, IL8, MCP1, CXCL1 and MIF, in various cancer cell types independent from TRAIL sensitivity although cytokines levels were higher in resistant cancer cells. MCP1 promoted chemotaxis of THP-1 monocytes and IL8 recruited neutrophils that may enhance tumour growth. Mechanistic studies revealed that caspase-8 was required for both apoptosis activation and cytokine production, although non-cleaved procaspase-8 was responsible for cytokine production by functioning as a scaffold for formation of a FADD-Caspase-8-RIPK1-TAK1 signalling complex and subsequent activation of the MEK/ERK pathway and cytokine production [98].

Taken together, depending on the experimental model and tumour type the overall antitumour effect of exogenous TRAIL is modulated by paracrine effects elicited either by direct activation of TRAIL-Rs on tumour cells or indirectly by activation of TRAIL-Rs on stromal cells, particularly immune cells. These effects can have either positive or negative impact on antitumour activity or even have tumour promoting effects.

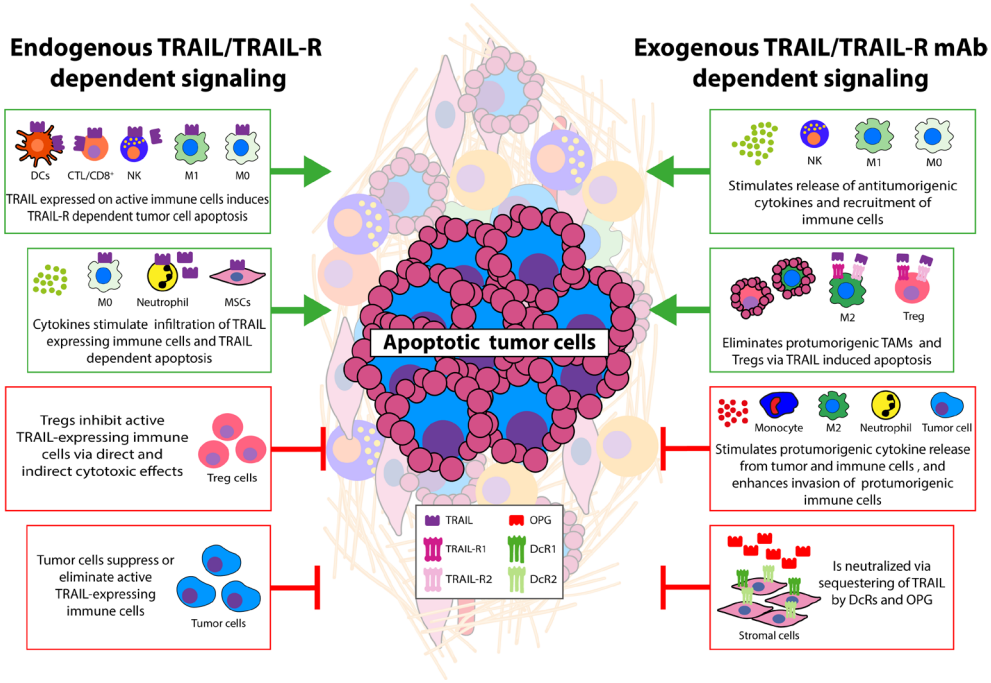

Figure 1. Main interactions between tumour and cellular TME that modulate TRAIL signalling *Schematic overview of effects of cellular TME – tumour interactions on TRAIL/TRAIL-R signalling. Green boxes depict anti-tumorigenic TME interactions. This can be achieved by endogenous TRAIL expression on activated immune effector cells leading to TRAIL-R dependent apoptosis in tumour cells. TRAIL-expressing neutrophils and macrophages can also eliminate tumour cells via TRAIL-Rs and cytokines can enhance infiltration of these cells potentiating tumour killing. Other stromal cell types expressing TRAIL may also display antitumour activity. Exogenous recombinant TRAIL, or TRAIL-R agonistic antibodies can induce cell death in tumour cells and immune suppressive cells (TAMs, Tregs) resulting in enhanced numbers of CTLs and increased phagocytic capacity of neutrophils/ monocytes/ macrophages. TRAIL-induced cell death of tumour endothelial cells has also been demonstrated (not depicted). Exogenous TRAIL can stimulate release of cytokines able to further increase TRAIL/TRAIL-R levels on immune effector cells. Together these events potentiate antitumour activity via the TRAIL/ TRAIL-R system. Red boxes depict pro-tumorigenic interactions. Endogenous TRAIL/TRAIL-R expression on often resistant tumour cells can induce TRAIL-driven cell death in antitumour TILs, CTLs. Cytokines can down-regulate expression of TRAIL on immune effector and neutrophils/macrophages. Decoy TRAIL-R3/R4 and OPG expression on stromal and tumour cells can sequester exogenously added TRAIL/ TRAIL-R agonists and protect against apoptosis. Resistant tumour cells stimulated with TRAIL secrete cytokines that recruit immune suppressive cells and/or induce a suppressive phenotype in tumour infiltrated cells. Cytokines derived from tumour and stromal cells can increase tumour TRAIL resistance by enhancing antiapoptotic signalling or even stimulate metastasis. Although often studied separately, it is anticipated that TME-dependent modulation of endogenous and exogenous TRAIL activity will occur simultaneously. See text for details.*

Biochemical and biophysical properties of the TME

Besides cellular compounds, the TME is also characterized by hypoxia, increased acidity and aberrant tissue stiffness involving alterations in ECM as well as aberrant interstitial pressure. Consequences for TRAIL signalling are exemplified below.

The ECM The ECM is a collection of different macromolecules that are assembled in a three-dimensional structure with unique biochemical and biomechanical properties regulating cell growth, survival, motility and differentiation. The ECM provides cells with a scaffold and regulates hydration and pH as well as the availability of growth factors and cytokines [99]. Cell-ECM interactions play an important role in tumour development and maintenance, and degradation of the ECM is associated with metastatic spread of tumour cells. ECM-cell adhesion signalling predominantly involves interactions between cell surface integrins and fibronectin. Genetic and pharmacological targeting of Integrin-β and downstream signals such as Src, Talin, PI3K and MAPK sensitized both apoptosis resistant and sensitive tumour cells for TRAIL-induced apoptosis *in vitro* by increasing TRAIL-R levels and reducing the threshold for mitochondrial apoptosis [100]. These findings also provide a mechanistic rationale for adherent tumour cells being more resistant to TRAIL than disseminating cells. On the other hand, loss of the epithelial adhesion protein E-cadherin, a key characteristic of cells undergoing EMT, has been linked with TRAIL resistance. EMT induction in lung cancer cells resulted in TRAIL resistance and silencing of E-cadherin also inhibited apoptosis activation. Mechanistically, E-cadherin was found to bind to ligated TRAIL-R1and/or TRAIL-R2 and augment their clustering and coupling to the actin cytoskeleton resulting in efficient DISC assembly and caspase-8 activation. Although elevated levels of E-cadherin in a panel of tumour cells correlated with TRAIL sensitivity, ectopic overexpression of E-cadherin in TRAIL resistant tumour cells did not lead to sensitization indicating context dependency [101].

Other components of the ECM were also found to regulate TRAIL sensitivity. The elastin microfibril interface-located protein 2 (EMILIN2), a member of the family of ECM glycoproteins, can bind to TRAIL-R1 and to a lesser extent TRAIL-R2 to induce receptor clustering and co-localization in lipid rafts subsequently activating apoptosis [102]. The CCN family of integrin-binding matricellular proteins have pleiotropic functions including regulation of cell proliferation and survival. In prostate cancer cells CCN1 was reported to support cell adhesion via integrins and heparan sulphate proteoglycans (HSPG) and promote growth. However, CCN1 also led to sensitization to TRAIL-induced apoptosis that was dependent of CCN1 interactions with integrins and HSPG receptor Syndecan-4 and activation of protein kinase C [103]. Thus, the ECM can modulate TRAIL-R functioning and affect the outcome of TRAIL exposure.

Mechanical stress Tumour cells experience elevated mechanical stress as a result from multiple factors including increased interstitial fluid pressure by aberrant vasculatures and lack of functional lymphatic vessels in tumours. Moreover, these cells experience solid stress as a result of tumour cell proliferation, aberrant ECM production and an altered TME. Together these factors result in enhanced mechanical stress in tumours that has been associated with decreased efficacy of anti-cancer treatment [104].

A limited number of studies examined the role of mechanical stress on TRAIL sensitivity. Elevated pressure on hepatoma Hep3B cells was reported to sensitize for TRAIL-induced apoptosis by suppressing ERK1/2 activation resulting in decreased Bad phosphorylation and enhanced mitochondrial apoptosis [105]. Similarly, elevated atmospheric pressure on H460 NSCLC cells enhanced TRAIL-dependent apoptosis associated with up-regulation of TRAIL-R2 and potentiation of caspase-8, accompanied by enhanced c-FLIP degradation and reduced expression of XIAP and the antiapoptotic protein Survivin. Enhancement of TRAIL-induced apoptosis was also detected in additional cancer cell lines, whereas normal fibroblast remained TRAIL resistant [106]. The mechanism transmitting pressure-induced TRAIL sensitization has not been elucidated yet.

Interestingly, in xenograft mice models TRAIL treatment rapidly reduced interstitial fluid pressure in TRAIL sensitive tumours, but not in TRAIL resistant ones that was accompanied by less condensed tumours. TRAIL treatment was associated with changes in the TME including stromal widening, macrophage infiltration and better vascular perfusion, which also increased the efficacy of chemotherapy efficacy [107]. Another study in larynx carcinoma HEP2 cells showed a correlation between stiffness and inhibition of TRAIL-induced apoptosis. Actinomycin D treatment reduced cellular stiffness that was linked with F-actin depolarization and susceptibility for TRAIL dependent apoptosis involving decreased Bcl-2 expression [108].

Indirect evidence for a suppressive effect of mechanical stress on TRAIL-mediated apoptosis was provided by Cho *et al*. [109]. Lung tumour stroma is enriched for fibronectin, which is a multi-modular protein able to stretch by partially unfolding under mechanical pressure. This stress can be experimentally mimicked by administration of recombinant type III domain of fibronectin (FnIII-1c). FnIII-1c reduced TRAIL sensitivity in H460 lung cancer cells. Inhibition of TRAIL induced caspase-8-dependent apoptosis was mediated by PI3K/Akt pathway activation via increased binding of αvβ5 integrin to its ligand vitronectin, a plasma protein and ECM factor [109].
Together, few studies thus far showed mostly a positive effect of mechanical stress on TRAIL-induced apoptosis, although clearly more research is required.

Hypoxia Hypoxia is a common condition of the TME and has been reported to affect TRAIL signalling by a variety of mechanisms leading to either enhanced or reduced TRAIL sensitivity. Most studies found that a hypoxic TME diminishes TRAIL sensitivity by a diversity of mechanisms. For example, hypoxia could block mitochondrial apoptosis by upregulating anti-apoptotic proteins or downregulating pro-apoptotic members of the BCL-2 family [110]. More recently, hypoxia was shown to stimulate mitochondrial autophagy resulting in impairment of the mitochondrial amplification loop by reducing mitochondrial release of pro-apoptotic factors such as SMAC. Exogenous substitution by SMAC mimetics or inhibition of XIAP restored TRAIL induced apoptosis under hypoxic conditions [111]. The hypoxia-inducible transcription factor HIF1α was found to be essential for hypoxia-dependent inhibition of TRAIL-induced apoptosis in a number of cancer cell lines *in vitro* [112]. Expression of the cellular prion protein (PrPc) was shown to be enhanced by HIF1α under hypoxia and to mediate TRAIL resistance in colon cancer cells *in vitro* and *in vivo* and may involve enhanced Akt and Bcl-2 activity [113]. Another study linked HIF1α as a major mediator of enhanced TRAIL-R4 production, but not other TRAIL-Rs, at the cell surface of colon cancer cells [114]. HIF2α that has been less well studied in context of TRAIL signalling had a protective effect on TRAIL-induced apoptosis in most pancreatic cancer cell lines tested by transcriptionally enhancing the expression of antiapoptotic protein Survivin. Accordingly, the Survivin inhibitor YM155 sensitized for TRAIL apoptosis under hypoxia [115].

Although most studies demonstrated a decrease in TRAIL-driven cell death under hypoxic conditions, several studies reported pro-apoptotic activity. For example, hypoxia increased TRAIL-induced apoptosis in DU-145 and LNCaP prostate cancer cells accompanied by enhanced activation of caspase-8 and -3 but not caspase-9 [116]. In breast cancer cells hypoxia increased TRAIL-R2 expression via JNK and C-Jun resulting in increased TRAIL sensitivity [117]. Others found that hypoxia decreased PKCε levels in a HIF1α dependent way leading to sensitization for TRAIL [118]. The underlying cause of the differential effects of hypoxia on TRAIL sensitivity is unclear, but may be related to the degree in which the cancer cell line tested is dependent on activation of the mitochondrial amplification loop (type-II cells).

Extracellular pH

High dependency of tumour cells on glucose to fuel aerobic glycolysis, known as the Warburg effect, provides energy and biosynthetic metabolites required for growth. Hypoxic conditions favouring anaerobic glycolysis in tumour cells further enhances the production of extracellular lactate and is a main cause of an acidic pH in the TME [119]. TRAIL was found to induce cell death in a pH-dependent manner. At low pH (6.6) TRAIL-induced apoptosis was augmented in prostate carcinoma and colorectal carcinoma characterized by increased tBID/BAX interactions, cytochrome C release and caspase activation [120]. In gastric carcinoma cells low pH resulted in upregulation of TRAIL-R1 and –R2 gene and protein expression

and increased proapoptotic activity of TRAIL via TRAIL-R2 [121]. Others reported that an acidic extracellular pH of 6.5 enhanced TRAIL-induced cell death in colon carcinoma and hepatocarcinoma cell lines by switching to activation of a caspases- and RIPK1-dependent necroptosis [122].

Glucose Tumour cells suffer usually from glucose deprivation and hypoglycaemic conditions, which can impact TRAIL signalling. TRAIL sensitivity was increased in a glucose deprived environment in a variety of cancer cell lines [123,124]. Glucose deprivation enhanced TRAIL sensitivity by increasing DISC formation and potentiation of mitochondrial depolarization and cytochrome c release and subsequent caspase activation [116,123,124]. In addition, glucose deprivation also increased ceramide levels leading to inhibition of Akt and reduced cFLIP levels leading to enhanced TRAIL sensitivity [124].

In mantle cell lymphoma cells chronic glucose deprivation resulted in a switch from aerobic glycolysis to oxidative phosphorylation thereby maintaining ATP production that was accompanied by reduced sensitivity towards TRAIL induced apoptosis. Glucose-free conditions led to decreased surface expression levels of TRAIL-R1/R2, impaired DISC formation, increased levels of Bcl-2 and XIAP, decreased levels of Bax and cytosolic cytochrome c. Conversely, 2-deoxyglucose that inhibits glycolysis and caused a reduction in ATP levels sensitized for TRAIL-induced apoptosis by potentiating DISC-dependent caspase-8 apoptosis as a result of a general decrease in mRNA translation including antiapoptotic proteins such as cFLIP. This study indicated differences between chronic and temporally glycolysis inhibition on TRAIL signalling likely related to mitochondrial functioning and intrinsic apoptosis [125]. In a follow up study by the same group the balance between the Akt and AMPK and downstream regulation of mTORC1 was proposed to be instrumental in modulating protein translation and the equilibrium between pro- and anti-apoptotic Bcl2 family members [126].

Taken together, mechanical stress, hypoxia, pH and glucose availability all have a direct effect on tumour cell apoptosis sensitivity for TRAIL. The impact of these conditions on stromal cells, and their indirect effects on tumour cells have not been examined as yet.

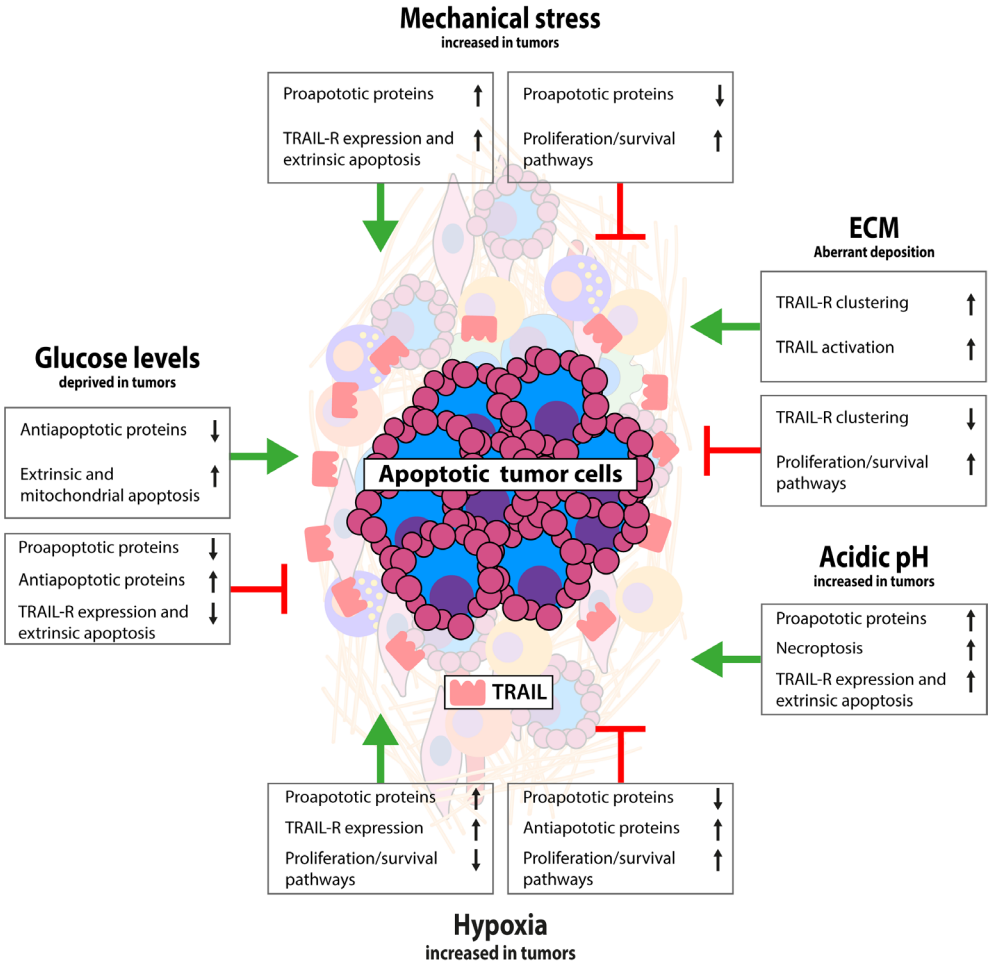

Figure 2. Interactions between tumour and non-cellular TME that modulate TRAIL signalling *Schematic representation of the impact of tumour specific biochemical and biophysical conditions on TRAIL/TRAIL-R signalling. Limited data available thus far indicate mostly a TRAIL sensitizing effect for mechanical stress and acidic pH, resistance by hypoxia and both sensitizing and resistance by ECM and low glucose. Interaction of tumour cells with ECM activates antiapoptotic signalling via integrin signalling, although conversely loss of E-cadherin has been linked with TRAIL resistance. Mechanical stress as a result of external pressure can sensitize for apoptosis and exogenous TRAIL in apoptosis sensitive tumours can reduce interstitial fluid pressure having favourable antitumour effects. Hypoxia and low pH are mostly associated with apoptosis resistance by stimulating antiapoptotic pathways and suppressing mitochondrial apoptosis. Glucose deprivation has been linked with both TRAIL sensitization and resistance, likely depending on chronic or temporal glucose deprived conditions. See text for more details.*

Conclusions and future directions

To unleash the full clinical potential of TRAIL receptor agonists we need to unravel the complexity of TRAIL signalling pathways in order to effectively bypass apoptosis resistance.

As illustrated here, the TME plays an important role in modulating the efficacy of both the endogenous TRAIL/TRAIL-R system mostly used by immune cells as well as of exogenously administrated therapeutic TRAIL receptor agonists. This modulation is complex involving a multi-component TME and a variety of often bidirectional signals that regulate TRAIL-driven apoptosis at distinct cellular and molecular levels. In this context the tumour model and experimental conditions used are of key importance, giving rise to different outcomes of TRAIL/TRAIL-R signalling, being either tumour promoting or suppressive effects. The use of syngeneic mouse models with a fully active immune system appears most valuable to dissect TME-cancer interactions, although obviously the TRAIL/TRAIL-R system in humans is not identical to that in mice.

The cellular TME, consisting of among others immune effector cells, immune-suppressive Tregs, neutrophils, macrophages and non-hematopoietic stromal cells, is able to enhance or reduce the antitumour activity of TRAIL-expressing immune effector cells as well as of exogenous TRAIL (see also Figure 1). In response to TRAIL-R activation tumour and stromal cells can initiate feed forward mechanisms or launch a counterattack leading to suppression of antitumour activity. Interestingly, therapeutic TRAIL can also remodel the TME by, for example, eliminating tumour-infiltrating macrophages, Tregs or tumour endothelial cells resulting in additional antitumour activity. Conversely, TRAIL-R stimulation of particularly apoptosis resistant cancer cells can have pro-tumorigenic effects, illustrated by enhanced cytokine secretion and attraction of immune suppressive cells. Although the underlying mechanisms need further clarification, regulation of endogenous and exogenous TRAIL sensitivity by tumour - stroma cell interactions frequently involve regulation of TRAIL/TRAIL-R levels including decoy receptors and OPG, and NF-κB-dependent regulation of intracellular pro- and antiapoptotic factors such as Bcl2 family members and IAPs together with secretion of cytokines in feedforward or feedback loops. In some studies, the efficacy of therapeutic TRAIL could be potentiated by inhibiting these antiapoptotic regulators. In addition, sequestering of TRAIL by upregulation of decoy receptors can be minimized by the use of designed recombinant TRAIL variants or TRAIL-R1 or -R2 agonistic antibodies that have strongly reduced binding affinity for decoy TRAIL-Rs. Furthermore, to achieve specific targeting of selected tumour or stromal cells, bi-functional TRAIL-R1 or -R2 agonistic antibodies or TRAIL fusion proteins have been developed containing a cell-specific binding moiety in addition to a TRAIL-R binding part. For example, a bi-specific melanoma-associated chondroitin sulphate proteoglycan (MCSP) – DR5 (TRAIL-R2) antibody has been produced that combines high affinity binding to melanoma cells with strong apoptosis-inducing potential [127]. Similarly, bi-specific antibodies have been developed that allow targeted delivery of TRAIL to surface antigens of T cells to enhance their tumoricidal activity. Moreover, bi-specific antibodies combining PD-L1 immune checkpoint inhibition with TRAIL-induced cell death could counteract an immune suppressive TME and augment T cell

activation [128]. Notably, the small molecule ONC201, currently evaluated in clinical studies, targets multiple pathways in tumour cells and includes upregulation of *TRAIL* and *TRAIL-R2* transcription. Within tumours ONC201 prompted activation and accumulation of T-cells ($CD3^+$, $CD4^+$ and $CD8^+$) and NK cells thereby selectively potentiating their antitumour activity that involves the TRAIL/TRAIL-R system [129].

The impact of the non-cellular TME on TRAIL signalling in cancer cells is less well studied. Thus far, studies demonstrated either TRAIL-dependent tumour suppressive or enhancing effects of mechanical stress, hypoxia, acidic pH and glucose shortage, whereas effects on stromal cells have not been explored until now (see also Figure 2). To appreciate the importance of signals derived from these TME components and to develop targeted strategies, it is essential to gain more insight in these poorly studied underlying mechanisms.

To conclude, in order to improve clinical benefit of TRAIL-R agonists the impact of various components of the TME need to be delineated using appropriate cancer models, which will guide the development of better therapeutic strategies.

Conflict of interest

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

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References

1. de Miguel D, Lemke J, Anel A, Walczak H, Martinez-Lostao L. Onto better TRAILs for cancer treatment. *Cell Death Differ*. 2016;23(5):733-747. doi:10.1038/cdd.2015.174
2. Azijli K, Weyhenmeyer B, Peters GJ, de Jong S, Kruyt FAE. Non-canonical kinase signalling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ*. 2013;20(7):858-868. doi:10.1038/cdd.2013.28
3. von Karstedt S, Montinaro A, Walczak H. Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. *Nat Rev Cancer*. 2017;17(6):352-366. doi:10.1038/nrc.2017.28
4. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013
5. Chen F, Zhuang X, Lin L, et al. New horizons in tumour microenvironment biology: challenges and opportunities. *BMC Med*. 2015;13(1):45. doi:10.1186/s12916-015-0278-7
6. Wang M, Zhao J, Zhang L, et al. Role of tumour microenvironment in tumourigenesis. *J Cancer*.

2017;8(5):761-773. doi:10.7150/jca.17648
7. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumour necrosis factor cytokine family. *J Biol Chem*. 1996;271(22):12687-12690. http://www.ncbi.nlm.nih.gov/pubmed/8663110.
8. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 1995;3(6):673-682. http://www.ncbi.nlm.nih.gov/pubmed/8777713.
9. Ashkenazi A, Holland P, Eckhardt SG. Ligand-Based Targeting of Apoptosis in Cancer: The Potential of Recombinant Human Apoptosis Ligand 2/Tumour Necrosis Factor–Related Apoptosis-Inducing Ligand (rhApo2L/TRAIL). *J Clin Oncol*. 2008;26(21):3621-3630. doi:10.1200/JCO.2007.15.7198
10. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ*. 2003;10(1):66-75. doi:10.1038/sj.cdd.4401187
11. Lemke J, von Karstedt S, Zinngrebe J, Walczak H. Getting TRAIL back on track for cancer therapy. *Cell Death Differ*. 2014;21(9):1350-1364. doi:10.1038/cdd.2014.81
12. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: Decisions between life and death. *Int J Biochem Cell Biol*. 2007;39(7-8):1462-1475. doi:10.1016/j.biocel.2007.02.007
13. Stegehuis JH, de Wilt LHAM, de Vries EGE, Groen HJ, de Jong S, Kruyt FAE. TRAIL receptor targeting therapies for non-small cell lung cancer: Current status and perspectives. *Drug Resist Updat*. 2010;13(1-2):2-15. doi:10.1016/j.drup.2009.11.001
14. Holland PM. Death receptor agonist therapies for cancer, which is the right TRAIL? *Cytokine Growth Factor Rev*. 2014;25(2):185-193. doi:10.1016/j.cytogfr.2013.12.009
15. Varfolomeev E, Maecker H, Sharp D, et al. Molecular Determinants of Kinase Pathway Activation by Apo2 Ligand/Tumour Necrosis Factor-related Apoptosis-inducing Ligand. *J Biol Chem*. 2005;280(49):40599-40608. doi:10.1074/jbc.M509560200
16. Trivedi R, Mishra DP. Trailing TRAIL Resistance: Novel Targets for TRAIL Sensitization in Cancer Cells. *Front Oncol*. 2015;5:69. doi:10.3389/fonc.2015.00069
17. Pennarun B, Meijer A, de Vries EGE, Kleibeuker JH, Kruyt F, de Jong S. Playing the DISC: Turning on TRAIL death receptor-mediated apoptosis in cancer. *Biochim Biophys Acta - Rev Cancer*. 2010;1805(2):123-140. doi:10.1016/j.bbcan.2009.11.004
18. Quail D, Joyce J. Microenvironmental regulation of tumour progression and metastasis. *Nat Med*. 2013;19(11):1423-1437. doi:10.1038/nm.3394.Microenvironmental
19. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 1995;3:673-682. doi:10.1016/1074-7613(95)90057-8
20. Mariani SM, Krammer PH. Differential regulation of TRAIL and CD95 ligand in transformed cells of the T and B lymphocyte lineage. *Eur J Immunol*. 1998;28(3):973-982. doi:10.1002/(SICI)1521-4141(199803)28:03<973::AID-IMMU973>3.0.CO;2-T
21. Schaefer U, Voloshanenko O, Willen D, Walczak H. TRAIL: a multifunctional cytokine. *Front Biosci*. 2007;12:3813-3824. http://www.ncbi.nlm.nih.gov/pubmed/17485341.
22. Shepard BD, Badley AD. The Biology of TRAIL and the Role of TRAIL-Based Therapeutics in Infectious Diseases. *Antiinfect Agents Med Chem*. 2009;8(2):87-101. doi:10.2174/187152109787846060
23. Allen JE, El-Deiry WS. Regulation of the human TRAIL gene. *Cancer Biol Ther*. 2012;13(12):1143-1151. doi:10.4161/cbt.21354
24. van Roosmalen I a. M, Quax WJ, Kruyt F a. E. Two death-inducing human TRAIL receptors to target in cancer: Similar or distinct regulation and function? *Biochem Pharmacol*. 2014;91(4):447-456. doi:10.1016/j.bcp.2014.08.010
25. Janssen EM, Droin NM, Lemmens EE, et al. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*. 2005;434(7029):88-93. doi:10.1038/nature03337
26. Falschlehner C, Schaefer U, Walczak H. Following TRAIL's path in the immune system. *Immunology*. 2009;127(2):145-154. doi:10.1111/j.1365-2567.2009.03058.x
27. Yue HH, Diehl GE, Winoto A. Loss of TRAIL-R does not affect thymic or intestinal tumour development in p53 and adenomatous polyposis coli mutant mice. *Cell Death Differ*.

2005;12(1):94-97. doi:10.1038/sj.cdd.4401523
28. Zerafa N, Westwood JA, Cretney E, et al. Cutting edge: TRAIL deficiency accelerates hematological malignancies. *J Immunol*. 2005;175(9):5586-5590. http://www.ncbi.nlm.nih.gov/pubmed/16237043.
29. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ. Increased susceptibility to tumour initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol*. 2002;168(3):1356-1361. http://www.ncbi.nlm.nih.gov/pubmed/11801676.
30. Grosse-Wilde A, Voloshanenko O, Lawrence Bailey S, et al. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumour development. *J Clin Invest*. 2008;118(1):100-110. doi:10.1172/JCI33061
31. Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B. Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. *J Exp Med*. 1998;188(12):2375-2380. http://www.ncbi.nlm.nih.gov/pubmed/9858524.
32. Smyth MJ, Cretney E, Takeda K, et al. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumour metastasis. *J Exp Med*. 2001;193(6):661-670. http://www.ncbi.nlm.nih.gov/pubmed/11257133.
33. Takeda K, Hayakawa Y, Smyth MJ, et al. Involvement of tumour necrosis factor-related apoptosis-inducing ligand in surveillance of tumour metastasis by liver natural killer cells. *Nat Med*. 2001;7(1):94-100. doi:10.1038/83416
34. Takeda K, Smyth MJ, Cretney E, et al. Critical role for tumour necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumour development. *J Exp Med*. 2002;195(2):161-169. http://www.ncbi.nlm.nih.gov/pubmed/11805143.
35. Sheard MA, Asgharzadeh S, Liu Y, et al. Membrane-bound TRAIL supplements natural killer cell cytotoxicity against neuroblastoma cells. *J Immunother*. 2013;36(5):319-329. doi:10.1097/CJI.0b013e31829b4493
36. Dorothée G, Vergnon I, Menez J, et al. Tumour-infiltrating CD4+ T lymphocytes express APO2 ligand (APO2L)/TRAIL upon specific stimulation with autologous lung carcinoma cells: role of IFN-alpha on APO2L/TRAIL expression and -mediated cytotoxicity. *J Immunol*. 2002;169(2):809-817. http://www.ncbi.nlm.nih.gov/pubmed/12097384.
37. Fanger NA, Maliszewski CR, Schooley K, Griffith TS. Human dendritic cells mediate cellular apoptosis via tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Exp Med*. 1999;190(8):1155-1164. http://www.ncbi.nlm.nih.gov/pubmed/10523613.
38. Leplina OY, Tyrinova T V., Tikhonova MA, Ostanin AA, Chernykh ER. Interferon alpha induces generation of semi-mature dendritic cells with high pro-inflammatory and cytotoxic potential. *Cytokine*. 2015;71(1):1-7. doi:10.1016/j.cyto.2014.07.258
39. Zhao H, Liao X, Kang Y. Tregs: Where We Are and What Comes Next? *Front Immunol*. 2017;8:1578. doi:10.3389/fimmu.2017.01578
40. Roux S, Apetoh L, Chalmin F, et al. CD4+CD25+ Tregs control the TRAIL-dependent cytotoxicity of tumour-infiltrating DCs in rodent models of colon cancer. *J Clin Invest*. 2008;118(11):3751-3761. doi:10.1172/JCI35890
41. Beyer K, Normann L, Sendler M, et al. TRAIL Promotes Tumour Growth in a Syngeneic Murine Orthotopic Pancreatic Cancer Model and Affects the Host Immune Response. *Pancreas*. 2016;45(3):401-408. doi:10.1097/MPA.0000000000000469
42. Ren X, Ye F, Jiang Z, Chu Y, Xiong S, Wang Y. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4+CD25+ regulatory T cells. *Cell Death Differ*. 2007;14(12):2076-2084. doi:10.1038/sj.cdd.4402220
43. De Geer A, Carlson L-M, Kogner P, Levitskaya J. Soluble factors released by activated cytotoxic T lymphocytes interfere with death receptor pathways in neuroblastoma. *Cancer Immunol Immunother*. 2008;57(5):731-743. doi:10.1007/s00262-007-0412-2
44. Nuutinen U, Ropponen A, Eeva J, et al. The effect of microenvironmental CD40 signals on TRAIL- and drug-induced apoptosis in follicular lymphoma cells. *Scand J Immunol*. 2009;70(6):565-573. doi:10.1111/j.1365-3083.2009.02330.x
45. Travert M, Ame-Thomas P, Pangault C, et al. CD40 ligand protects from TRAIL-induced apoptosis

in follicular lymphomas through NF-kappaB activation and up-regulation of c-FLIP and Bcl-xL. *J Immunol*. 2008;181(2):1001-1011. http://www.ncbi.nlm.nih.gov/pubmed/18606651.

46. Kumar Hira S, Mondal I, Bhattacharya D, Manna PP. Downregulation of endogenous STAT3 augments tumouricidal activity of interleukin 15 activated dendritic cell against lymphoma and leukemia via TRAIL. *Exp Cell Res*. 2014;327(2):192-208. doi:10.1016/j.yexcr.2014.08.012
47. Huber V, Fais S, Iero M, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology*. 2005;128(7):1796-1804. http://www.ncbi.nlm.nih.gov/pubmed/15940614.
48. Tinhofer I, Biedermann R, Krismer M, Crazzolara R, Greil R. A role of TRAIL in killing osteoblasts by myeloma cells. *FASEB J*. 2006;20(6):759-761. doi:10.1096/fj.05-4329fje
49. Yen M-L, Tsai H-F, Wu Y-Y, Hwa H-L, Lee B-H, Hsu P-N. TNF-related apoptosis-inducing ligand (TRAIL) induces osteoclast differentiation from monocyte/macrophage lineage precursor cells. *Mol Immunol*. 2008;45(8):2205-2213. doi:10.1016/j.molimm.2007.12.003
50. Koyama S, Koike N, Adachi S. Expression of TNF-related apoptosis-inducing ligand (TRAIL) and its receptors in gastric carcinoma and tumour-infiltrating lymphocytes: a possible mechanism of immune evasion of the tumour. *J Cancer Res Clin Oncol*. 2002;128(2):73-79. doi:10.1007/s004320100292
51. Grimm M, Kim M, Rosenwald A, et al. Tumour-mediated TRAIL-Receptor expression indicates effective apoptotic depletion of infiltrating CD8+ immune cells in clinical colorectal cancer. *Eur J Cancer*. 2010;46(12):2314-2323. doi:10.1016/j.ejca.2010.05.025
52. Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. *Blood*. 2008;112(4):935-945. doi:10.1182/blood-2007-12-077917
53. Tecchio C, Huber V, Scapini P, et al. IFN -stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. *Blood*. 2004;103(10):3837-3844. doi:10.1182/blood-2003-08-2806
54. Marino J, Furmento VA, Zotta E, Roguin LP. Peritumoural administration of granulocyte colony-stimulating factor induces an apoptotic response on a murine mammary adenocarcinoma. *Cancer Biol Ther*. 2009;8(18):1737-1743. http://www.ncbi.nlm.nih.gov/pubmed/19633419.
55. Chen C-L, Wang Y, Huang C-Y, et al. IL-17 induces antitumour immunity by promoting beneficial neutrophil recruitment and activation in esophageal squamous cell carcinoma. *Oncoimmunology*. 2018;7(1):e1373234. doi:10.1080/2162402X.2017.1373234
56. Kawakubo T, Okamoto K, Iwata J, et al. Cathepsin E Prevents Tumour Growth and Metastasis by Catalyzing the Proteolytic Release of Soluble TRAIL from Tumour Cell Surface. *Cancer Res*. 2007;67(22):10869-10878. doi:10.1158/0008-5472.CAN-07-2048
57. Dandachi N, Kelly NJ, Wood JP, et al. Macrophage Elastase Induces TRAIL-mediated Tumour Cell Death through Its Carboxy-Terminal Domain. *Am J Respir Crit Care Med*. 2017;196(3):353-363. doi:10.1164/rccm.201606-1150OC
58. Zou J-M, Qin J, Li Y-C, et al. IL-35 induces N2 phenotype of neutrophils to promote tumour growth. *Oncotarget*. 2017;8(20):33501-33514. doi:10.18632/oncotarget.16819
59. Yan B, Wei J-J, Yuan Y, et al. IL-6 Cooperates with G-CSF To Induce Protumour Function of Neutrophils in Bone Marrow by Enhancing STAT3 Activation. *J Immunol*. 2013;190(11):5882-5893. doi:10.4049/jimmunol.1201881
60. HOSHI H, SAWADA T, UCHIDA M, et al. MUC5AC protects pancreatic cancer cells from TRAIL-induced death pathways. *Int J Oncol*. 2013;42(3):887-893. doi:10.3892/ijo.2013.1760
61. Labovsky V, Martinez LM, Davies KM, et al. Association Between Ligands and Receptors Related to the Progression of Early Breast Cancer in Tumour Epithelial and Stromal Cells. *Clin Breast Cancer*. 2015;15(1):e13-e21. doi:10.1016/j.clbc.2014.05.006
62. Anees M, Horak P, El-Gazzar A, et al. Recurrence-free survival in prostate cancer is related to increased stromal TRAIL expression. *Cancer*. 2011;117(6):1172-1182. doi:10.1002/cncr.25504
63. Horak P, Pils D, Kaider A, et al. Perturbation of the Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand Cascade in Ovarian Cancer: Overexpression of FLIPL and Deregulation of the Functional Receptors DR4 and DR5. *Clin Cancer Res*. 2005;11(24):8585-8591. doi:10.1158/1078-0432.CCR-05-1276
64. Lee RH, Yoon N, Reneau JC, Prockop DJ. Preactivation of Human MSCs with TNF-α Enhances Tumour-Suppressive Activity. *Cell Stem Cell*. 2012;11(6):825-835. doi:10.1016/J.

STEM.2012.10.001
65. Yoon N, Park MS, Shigemoto T, Peltier G, Lee RH. Activated human mesenchymal stem/stromal cells suppress metastatic features of MDA-MB-231 cells by secreting IFN-β. *Cell Death Dis*. 2016;7(4):e2191. doi:10.1038/cddis.2016.90
66. Nyambo R, Cross N, Lippitt J, et al. Human Bone Marrow Stromal Cells Protect Prostate Cancer Cells From TRAIL-Induced Apoptosis. *J Bone Miner Res*. 2004;19(10):1712-1721. doi:10.1359/JBMR.040703
67. Locklin RM, Croucher PI, Russell RGG, Edwards CM. Agonists of TRAIL death receptors induce myeloma cell apoptosis that is not prevented by cells of the bone marrow microenvironment. *Leukemia*. 2007;21(4):805-812. doi:10.1038/sj.leu.2404518
68. Neville-Webbe HL, Cross NA, Eaton CL, et al. Osteoprotegerin (OPG) produced by bone marrow stromal cells protects breast cancer cells from TRAIL-induced apoptosis. *Breast Cancer Res Treat*. 2004;86(3):269-279. http://www.ncbi.nlm.nih.gov/pubmed/15567943.
69. Rachner TD, Benad P, Rauner M, et al. Osteoprotegerin production by breast cancer cells is suppressed by dexamethasone and confers resistance against TRAIL-induced apoptosis. *J Cell Biochem*. 2009;108(1):106-116. doi:10.1002/jcb.22232
70. Bosman MCJ, Reis CR, Schuringa JJ, Vellenga E, Quax WJ. Decreased Affinity of Recombinant Human Tumour Necrosis Factor-related Apoptosis-inducing Ligand (rhTRAIL) D269H/E195R to Osteoprotegerin (OPG) Overcomes TRAIL Resistance Mediated by the Bone Microenvironment. *J Biol Chem*. 2014;289(2):1071-1078. doi:10.1074/jbc.M113.491589
71. O'Leary L, van der Sloot a M, Reis CR, et al. Decoy receptors block TRAIL sensitivity at a supracellular level: the role of stromal cells in controlling tumour TRAIL sensitivity. *Oncogene*. 2015;(September 2014):1-10. doi:10.1038/onc.2015.180
72. Gibson EM, Henson ES, Haney N, Villanueva J, Gibson SB. Epidermal growth factor protects epithelial-derived cells from tumour necrosis factor-related apoptosis-inducing ligand-induced apoptosis by inhibiting cytochrome c release. *Cancer Res*. 2002;62(2):488-496. http://www.ncbi.nlm.nih.gov/pubmed/11809700.
73. Cano-González A, López-Rivas A. Opposing roles of TGF-β and EGF in the regulation of TRAIL-induced apoptosis in human breast epithelial cells. *Biochim Biophys Acta - Mol Cell Res*. 2016;1863(8):2104-2114. doi:10.1016/j.bbamcr.2016.05.011
74. Abdollahi T, Robertson NM, Abdollahi A, Litwack G. Identification of interleukin 8 as an inhibitor of tumour necrosis factor-related apoptosis-inducing ligand-induced apoptosis in the ovarian carcinoma cell line OVCAR3. *Cancer Res*. 2003;63(15):4521-4526. http://www.ncbi.nlm.nih.gov/pubmed/12907626.
75. Waugh DJJ, Wilson C. The interleukin-8 pathway in cancer. *Clin Cancer Res*. 2008;14(21):6735-6741. doi:10.1158/1078-0432.CCR-07-4843
76. Todaro M, Lombardo Y, Francipane MG, et al. Apoptosis resistance in epithelial tumours is mediated by tumour-cell-derived interleukin-4. *Cell Death Differ*. 2008;15(4):762-772. doi:10.1038/sj.cdd.4402305
77. Ivanov VN, Partridge MA, Huang SXL, Hei TK. Suppression of the proinflammatory response of metastatic melanoma cells increases TRAIL-induced apoptosis. *J Cell Biochem*. 2011;112(2):463-475. doi:10.1002/jcb.22934
78. He W, Liu Q, Wang L, Chen W, Li N, Cao X. TLR4 signalling promotes immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. *Mol Immunol*. 2007;44(11):2850-2859. doi:10.1016/j.molimm.2007.01.022
79. Lane D, Matte I, Rancourt C, Piché A. The prosurvival activity of ascites against TRAIL is associated with a shorter disease-free interval in patients with ovarian cancer. *J Ovarian Res*. 2010;3(1):1. doi:10.1186/1757-2215-3-1
80. Goncharenko-Khaider N, Matte I, Lane D, Rancourt C, Piché A. Ovarian cancer ascites increase Mcl-1 expression in tumour cells through ERK1/2-Elk-1 signalling to attenuate TRAIL-induced apoptosis. *Mol Cancer*. 2012;11(1):84. doi:10.1186/1476-4598-11-84
81. Perez LE, Parquet N, Shain K, et al. Bone marrow stroma confers resistance to Apo2 ligand/TRAIL in multiple myeloma in part by regulating c-FLIP. *J Immunol*. 2008;180(3):1545-1555. http://www.ncbi.nlm.nih.gov/pubmed/18209050.
82. Perez LE, Parquet N, Meads M, Anasetti C, Dalton W. Bortezomib restores stroma-mediated

APO2L/TRAIL apoptosis resistance in multiple myeloma. *Eur J Haematol*. 2010;84(3):212-222. doi:10.1111/j.1600-0609.2009.01381.x

83. Fingas CD, Bronk SF, Werneburg NW, et al. Myofibroblast-derived PDGF-BB promotes hedgehog survival signalling in cholangiocarcinoma cells. *Hepatology*. 2011;54(6):2076-2088. doi:10.1002/hep.24588
84. Doubravská L, Šímová Š, Cermak L, Valenta T, Kořínek V, Anděra L. Wnt-expressing rat embryonic fibroblasts suppress Apo2L/TRAIL-induced apoptosis of human leukemia cells. *Apoptosis*. 2008;13(4):573-587. doi:10.1007/s10495-008-0191-z
85. Liu F, Hu X, Zimmerman M, et al. TNFα cooperates with IFN-γ to repress Bcl-xL expression to sensitize metastatic colon carcinoma cells to TRAIL-mediated apoptosis. *PLoS One*. 2011;6(1):e16241. doi:10.1371/journal.pone.0016241
86. Liguori M, Buracchi C, Pasqualini F, et al. Functional TRAIL receptors in monocytes and tumour-associated macrophages: a possible targeting pathway in the tumour microenvironment. *Oncotarget*. 2016;7(27). doi:10.18632/oncotarget.9340
87. Diao Z, Shi J, Zhu J, et al. TRAIL suppresses tumour growth in mice by inducing tumour-infiltrating CD4+CD25+ Treg apoptosis. *Cancer Immunol Immunother*. 2013;62(4):653-663. doi:10.1007/s00262-012-1370-x
88. Secchiero P, Gonelli A, Mirandola P, et al. Tumour necrosis factor-related apoptosis-inducing ligand induces monocytic maturation of leukemic and normal myeloid precursors through a caspase-dependent pathway. *Blood*. 2002;100(7):2421-2429. doi:10.1182/blood-2002-01-0047
89. Gao J, Wang D, Liu D, et al. Tumour necrosis factor-related apoptosis-inducing ligand induces the expression of proinflammatory cytokines in macrophages and re-educates tumour-associated macrophages to an antitumour phenotype. *Mol Biol Cell*. 2015;26(18):3178-3189. doi:10.1091/mbc.E15-04-0209
90. Wilson NS, Yang A, Yang B, et al. Proapoptotic Activation of Death Receptor 5 on Tumour Endothelial Cells Disrupts the Vasculature and Reduces Tumour Growth. *Cancer Cell*. 2012;22(1):80-90. doi:10.1016/j.ccr.2012.05.014
91. Corallini F, Celeghini C, Rimondi E, et al. Trail down-regulates the release of osteoprotegerin (OPG) by primary stromal cells. *J Cell Physiol*. 2011;226(9):2279-2286. doi:10.1002/jcp.22564
92. Hartwig T, Montinaro A, von Karstedt S, et al. The TRAIL-Induced Cancer Secretome Promotes a Tumour-Supportive Immune Microenvironment via CCR2. *Mol Cell*. 2017;65(4):730-742.e5. doi:10.1016/j.molcel.2017.01.021
93. Trauzold A, Siegmund D, Schniewind B, et al. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. *Oncogene*. 2006;25(56):7434-7439. doi:10.1038/sj.onc.1209719
94. Zhou D-H, Trauzold A, Röder C, Pan G, Zheng C, Kalthoff H. The potential molecular mechanism of overexpression of uPA, IL-8, MMP-7 and MMP-9 induced by TRAIL in pancreatic cancer cell. *Hepatobiliary Pancreat Dis Int*. 2008;7(2):201-209. http://www.ncbi.nlm.nih.gov/pubmed/18397859.
95. Geismann C, Grohmann F, Dreher A, et al. Role of CCL20 mediated immune cell recruitment in NF-κB mediated TRAIL resistance of pancreatic cancer. *Biochim Biophys Acta - Mol Cell Res*. 2017;1864(5):782-796. doi:10.1016/j.bbamcr.2017.02.005
96. Kaler P, Augenlicht L, Klampfer L. Macrophage-derived IL-1β stimulates Wnt signalling and growth of colon cancer cells: a crosstalk interrupted by vitamin D3. *Oncogene*. 2009;28(44):3892-3902. doi:10.1038/onc.2009.247
97. Kaler P, Galea V, Augenlicht L, Klampfer L. Tumour Associated Macrophages Protect Colon Cancer Cells from TRAIL-Induced Apoptosis through IL-1β- Dependent Stabilization of Snail in Tumour Cells. Jin D-Y, ed. *PLoS One*. 2010;5(7):e11700. doi:10.1371/journal.pone.0011700
98. Henry CM, Martin SJ. Caspase-8 Acts in a Non-enzymatic Role as a Scaffold for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation. *Mol Cell*. 2017;65(4):715-729.e5. doi:10.1016/j.molcel.2017.01.022
99. Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. *EMBO Rep*. 2014;15(12):1243-1253. doi:10.15252/embr.201439246
100. Phipps LE, Hino S, Muschel RJ. Targeting Cell Spreading: A Method of Sensitizing Metastatic Tumour Cells to TRAIL-Induced Apoptosis. *Mol Cancer Res*. 2011;9(3):249-258.

doi:10.1158/1541-7786.MCR-11-0021
101. Lu M, Marsters S, Ye X, Luis E, Gonzalez L, Ashkenazi A. E-Cadherin Couples Death Receptors to the Cytoskeleton to Regulate Apoptosis. *Mol Cell*. 2014;54(6):987-998. doi:10.1016/j.molcel.2014.04.029
102. Mongiat M, Ligresti G, Marastoni S, Lorenzon E, Doliana R, Colombatti A. Regulation of the Extrinsic Apoptotic Pathway by the Extracellular Matrix Glycoprotein EMILIN2. *Mol Cell Biol*. 2007;27(20):7176-7187. doi:10.1128/MCB.00696-07
103. Franzen CA, Chen C-C, Todorovic V, Juric V, Monzon RI, Lau LF. Matrix Protein CCN1 Is Critical for Prostate Carcinoma Cell Proliferation and TRAIL-Induced Apoptosis. *Mol Cancer Res*. 2009;7(7):1045-1055. doi:10.1158/1541-7786.MCR-09-0017
104. Ariffin AB, Forde PF, Jahangeer S, Soden DM, Hinchion J. Releasing Pressure in Tumours: What Do We Know So Far and Where Do We Go from Here? A Review. *Cancer Res*. 2014;74(10):2655-2662. doi:10.1158/0008-5472.CAN-13-3696
105. Hong E, Lee E, Kim J, Kwon D, Lim Y. Elevated pressure enhanced TRAIL-induced apoptosis in hepatocellular carcinoma cells via ERK1/2-inactivation. *Cell Mol Biol Lett*. 2015;20(4):535-548. doi:10.1515/cmble-2015-0030
106. Oh S, Kwon D, Lee HJ, Kim J, Lee E. Role of elevated pressure in TRAIL-induced apoptosis in human lung carcinoma cells. *Apoptosis*. 2010;15(12):1517-1528. doi:10.1007/s10495-010-0525-5
107. Hylander BL, Sen A, Beachy SH, et al. Tumour priming by Apo2L/TRAIL reduces interstitial fluid pressure and enhances efficacy of liposomal gemcitabine in a patient derived xenograft tumour model. *J Control Release*. 2015;217:160-169. doi:10.1016/j.jconrel.2015.08.047
108. Targosz-Korecka M, Biedron R, Szczygiel AM, Brzezinka G, Szczerbinski J, Zuk A. Stiffness changes of tumour HEp2 cells correlates with the inhibition and release of TRAIL-induced apoptosis pathways. *J Mol Recognit*. 2012;25(5):299-308. doi:10.1002/jmr.2192
109. Cho C, Horzempa C, Jones D, McKeown-Longo PJ. The fibronectin III-1 domain activates a PI3-Kinase/Akt signalling pathway leading to αvβ5 integrin activation and TRAIL resistance in human lung cancer cells. *BMC Cancer*. 2016;16(1):574. doi:10.1186/s12885-016-2621-6
110. Han SH, Kim M, Park K, Kim T-H, Seol D-W. Blockade of processing/activation of caspase-3 by hypoxia. *Biochem Biophys Res Commun*. 2008;375(4):684-688. doi:10.1016/j.bbrc.2008.08.091
111. Knoll G, Bittner S, Kurz M, Jantsch J, Ehrenschwender M. Hypoxia regulates TRAIL sensitivity of colorectal cancer cells through mitochondrial autophagy. *Oncotarget*. 2016;7(27):41488-41504. doi:10.18632/oncotarget.9206
112. Jeong J-K, Moon M-H, Seo J-S, Seol J-W, Park S-Y, Lee Y-J. Hypoxia inducing factor-1α regulates tumour necrosis factor-related apoptosis-inducing ligand sensitivity in tumour cells exposed to hypoxia. *Biochem Biophys Res Commun*. 2010;399(3):379-383. doi:10.1016/j.bbrc.2010.07.082
113. Park J-Y, Jeong J-K, Lee J-H, et al. Induction of cellular prion protein (PrPc) under hypoxia inhibits apoptosis caused by TRAIL treatment. *Oncotarget*. 2015;6(7):5342-5353. doi:10.18632/oncotarget.3028
114. Pei G-T, Wu C-W, Lin W-W. Hypoxia-induced decoy receptor 2 gene expression is regulated via a hypoxia-inducible factor 1α-mediated mechanism. *Biochem Biophys Res Commun*. 2010;391(2):1274-1279. doi:10.1016/j.bbrc.2009.12.058
115. Harashima N, Takenaga K, Akimoto M, et al. HIF-2α dictates the susceptibility of pancreatic cancer cells to TRAIL by regulating survivin expression. *Oncotarget*. 2017;8(26):42887-42900. doi:10.18632/oncotarget.17157
116. Lee YJ, Moon M-S, Kwon SJ, Rhee JG. Hypoxia and low glucose differentially augments TRAIL-induced apoptotic death. *Mol Cell Biochem*. 2005;270(1-2):89-97. http://www.ncbi.nlm.nih.gov/pubmed/15792357.
117. Hong S-E, Kim CS, An S, et al. TRAIL restores DCA/metformin-mediated cell death in hypoxia. *Biochem Biophys Res Commun*. 2016;478(3):1389-1395. doi:10.1016/j.bbrc.2016.08.134
118. Gobbi G, Masselli E, Micheloni C, et al. Hypoxia-induced down-modulation of PKCepsilon promotes trail-mediated apoptosis of tumour cells. *Int J Oncol*. 2010;37(3):719-729. http://www.ncbi.nlm.nih.gov/pubmed/20664941.

119. Kato Y, Ozawa S, Miyamoto C, et al. Acidic extracellular microenvironment and cancer. *Cancer Cell Int*. 2013;13(1):89. doi:10.1186/1475-2867-13-89
120. Lee YJ, Song JJ, Kim JH, Kim H-RC, Song YK. Low extracellular pH augments TRAIL-induced apoptotic death through the mitochondria-mediated caspase signal transduction pathway. *Exp Cell Res*. 2004;293(1):129-143. doi:10.1016/J.YEXCR.2003.09.015
121. Hong R, Han SI. Extracellular acidity enhances tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis via DR5 in gastric cancer cells. *Korean J Physiol Pharmacol*. 2018;22(5):513-523. doi:10.4196/kjpp.2018.22.5.513
122. Meurette O, Rebillard A, Huc L, et al. TRAIL Induces Receptor-Interacting Protein 1–Dependent and Caspase-Dependent Necrosis-Like Cell Death under Acidic Extracellular Conditions. *Cancer Res*. 2007;67(1):218-226. doi:10.1158/0008-5472.CAN-06-1610
123. Muñoz-Pinedo C, Ruiz-Ruiz C, Ruiz de Almodóvar C, Palacios C, López-Rivas A. Inhibition of glucose metabolism sensitizes tumour cells to death receptor-triggered apoptosis through enhancement of death-inducing signalling complex formation and apical procaspase-8 processing. *J Biol Chem*. 2003;278(15):12759-12768. doi:10.1074/jbc.M212392200
124. Nam SY, Amoscato AA, Lee YJ. Low glucose-enhanced TRAIL cytotoxicity is mediated through the ceramide–Akt–FLIP pathway. *Oncogene*. 2002;21(3):337-346. doi:10.1038/sj.onc.1205068
125. Robinson GL, Dinsdale D, MacFarlane M, Cain K. Switching from aerobic glycolysis to oxidative phosphorylation modulates the sensitivity of mantle cell lymphoma cells to TRAIL. *Oncogene*. 2012;31(48):4996-5006. doi:10.1038/onc.2012.13
126. MacFarlane M, Robinson GL, Cain K. Glucose—a sweet way to die. *Cell Cycle*. 2012;11(21):3919-3925. doi:10.4161/cc.21804
127. He Y, Hendriks D, van Ginkel R, Samplonius D, Bremer E, Helfrich W. Melanoma-Directed Activation of Apoptosis Using a Bispecific Antibody Directed at MCSP and TRAIL Receptor-2/Death Receptor-5. *J Invest Dermatol*. 2016;136(2):541-544. doi:10.1016/J.JID.2015.11.009
128. de Bruyn M, Wei Y, Wiersma VR, et al. Cell Surface Delivery of TRAIL Strongly Augments the Tumouricidal Activity of T Cells. *Clin Cancer Res*. 2011;17(17):5626-5637. doi:10.1158/1078-0432.CCR-11-0303
129. Wagner J, Kline CL, Zhou L, et al. Dose intensification of TRAIL-inducing ONC201 inhibits metastasis and promotes intratumoural NK cell recruitment. *J Clin Invest*. 2018;128(6):2325-2338. doi:10.1172/JCI96711

Chapter 3

TRAIL-induces Src mediated MEK/ERK, SMAD3 and β-catenin signalling in apoptosis resistant NSCLC cells

Margot de Looff, Win Sen Heng, Steven de Jong

and Frank A. E. Kruyt

Abstract

Non-small cell lung cancer (NSCLC) patients require better treatments to improve prognosis. Tumour-necrosis factor related apoptosis-inducing ligand (TRAIL) receptors (TRAIL-R1 and -R2) are appealing therapeutic targets to eradicate tumours specifically via caspase-dependent apoptosis. However, resistance is often observed and TRAIL-R activation can even activate pro-tumorigenic non-canonical signalling pathways. Previously, we found that TRAIL-induced RIPK1-Src-STAT3 signalling was involved in NSCLC cell migration and invasion *in vitro*. In the present study, the contribution of the non-receptor tyrosine kinase Src in TRAIL signalling in NSCLC cell lines was further examined. TRAIL sensitive H460 and resistant A549 NSCLC cells showed distinct time-dependent rhTRAIL-induced Src phosphorylation patterns with early activation in A549 cells. Pharmacological Src inhibition as well as shRNA knockdown or CRISPR/CAS9-dependent knockout of Src expression did not alter sensitivity to rhTRAIL-induced apoptosis in both cell lines. Silencing of secondary complex proteins showed that TRADD, but not TRAF2, FADD nor caspase-8, was required for Src activation in A549 cells. Possible mediators of Src-dependent rhTRAIL signalling were identified by Src co-IP-LC-mass spectrometric analyses. In A549 cells the number of Src-interacting proteins increased after rhTRAIL treatment, whereas protein numbers decreased in H460 cells. Various tumorigenic proteins were found in complex with Src in rhTRAIL treated A549 cells, including components of the RAF-MEK1/2-ERK, Wnt and SMAD3 signalling pathways. Functional analyses showed that Src mediated phosphorylation of MEK1/2 and ERK, prevented phosphorylation of SMAD3 and was required for nuclear translocation of ERK and β-catenin in A549 cells. Clonogenic growth of both Src proficient and deficient A549 cells was not affected by rhTRAIL exposure, although Src depletion and MEK1/2 inhibition reduced colony size and numbers significantly. In conclusion, rhTRAIL-induced and Src dependent MEK/ERK, SMAD3 and β-catenin signalling may contribute to the known pro-tumorigenic effects of rhTRAIL in resistant NSCLC cells. However, this needs to be further examined, as well as the potential therapeutic implications of targeting these pathways when combined with TRAIL receptor agonists.

Introduction

Non-small cell lung cancer (NSCLC) is the most prevalent lung cancer type, accounting for approximately 85% of all lung cancers [1]. The average 5-year survival rate is 18-20% [2]. Surgery combined with (neo)adjuvant chemotherapy or radiotherapy is currently the main treatment for early-stage lung cancer. Tyrosine kinase inhibitors and, more recently, immune checkpoint inhibitors have been successfully used in subgroups of patients with advanced disease, however, novel treatment strategies are still needed to improve overall prognosis of NSCLC [3–5].

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors have been identified as promising therapeutic targets based on their tumour-selective apoptosis-inducing activity [6–8]. TRAIL receptor 1 (-R1) and TRAIL-R2 induce apoptosis via their intracellular death effector domains. These domains subsequently recruit the Fas-Associated protein with Death Domain (FADD) and pro-caspase 8 to establish the death-inducing signalling complex (DISC) where caspase 8 is cleaved and activated. Subsequent effector caspase activation results in irreversible apoptosis. This pathway, also known as the death receptor/extrinsic apoptotic pathway, often involves simultaneous caspase 8-dependent cleavage of Bid, leading to cytochrome C release from mitochondria, apoptosome assembly and activation of caspase 9 and further downstream caspase activation, also known as the intrinsic/mitochondrial apoptotic pathway [9].

Various TRAIL receptor agonists have been developed and assessed. However, intrinsic and acquired resistance have been observed frequently in both *in vitro* and *in vivo* preclinical models [10,11]. Therapeutic targeting of TRAIL receptors has thus far shown limited efficacy in clinical studies, although currently new studies with novel and perhaps better TRAIL formulations are under evaluation [11–14]. Besides resistance towards apoptosis, TRAIL receptor activation can induce unwanted pro-tumorigenic and even metastasis-promoting effects by activation of non-apoptotic signalling pathways [14,15]. These non-canonical pathways involve the formation of a secondary signalling complex consisting of the receptor-interacting serine/threonine protein kinase 1 (RIPK1), TNF receptor associated factor 2 (TRAF2), the TNF receptor type 1 associated death domain (TRADD), FADD and caspase 8 [15,16]. Previously, we reported TRAIL-dependent activation of the RIPK1-Src-STAT3 pathway in resistant NSCLC cells that contributed to tumour cell migration and invasion [17].

Src is often overexpressed or hyper-activated in cancer and known to be involved in oncogenic processes like cell proliferation, survival and metastatic spread [18–20]. Src comprises different functional domains, including two Src homology (SH) domains and a catalytic domain. Its activity is mainly regulated by two phosphorylation sites, the positive regulatory auto-phosphorylation at Tyr418 in the catalytic domain and the negative regulatory

Tyr530 phosphorylation at the C-terminal part [21,22]. Elevated Src expression and activation has been reported in the majority of lung cancers, especially in NSCLC [20,23]. Activated Src can phosphorylate and thereby inactivate caspase 8 resulting in TRAIL resistance [24,25]. Furthermore, Src activation has been implicated in Akt activation after TRAIL treatment in breast cancer cells and inhibition of Src sensitized hepatocellular carcinoma cells for TRAIL-induced apoptosis [26,27]. In the current study, we examined the possible role of Src and underlying mechanisms in modulating apoptotic and pro-tumorigenic TRAIL signalling in NSCLC cells.

Material and methods

Cell lines A549 and H460 cells were obtained from the ATCC and cultured in RPMI 1640 (Gibco, Waltham, USA) with 10% FBS (Bodinco, Alkmaar, The Netherlands). Cells were maintained in a humidified 5% CO2 atmosphere at 37˚C. The cell lines were tested annually for authenticity by short tandem repeat profiling DNA fingerprinting (Baseclear, Leiden, The Netherlands) and for mycoplasm by PCR.

Reagents Human rhTRAIL was obtained from Peprotech Inc. (London, UK). Pharmacological inhibiters used were Dasatinib (Axon Medchem, Groningen, The Netherlands), Selumetinib (AZD6244, Axon Medchem) and SIS3 (Cayman chemical, Ellsworth, USA).

Modulation of Src expression Short interfering (si)RNAs against TRADD (SR305738 Origene, Rockville, USA), FADD (SR305777 Origene), TRAF2 (SR304927 Origene) and caspase 8 (sc-29930 Santa Cruz, Dallas, USA) were transfected in cells with Oligofectamine reagent (ThermoFisher, Waltham, USA) according manufactures protocol. In short, $3.5*10^5$ cells were plated in a 6 wells plate and incubated overnight. Subsequently, cells were washed with PBS and 800 µl optimum medium (Gibco) was added, prior to adding short interfering (si)RNA transfection mix. Transfection mix was prepared: 3 µl Oligofectamine was dissolved in 12 µl Opti-MEM per well and incubated for 10 min at RT. Next, 185 µl with 1 µM siRNA was added and the transfection mix was incubated for 20 min at RT. In each well 200 µl transfection mix was added dropwise to the cells and incubated for 4 h at 37°C followed by addition of 500 µl medium with 30% FCS. Short hairpin RNA silenced Src in A549 cells were described previously [17]. Src gene knockout (A549-Src KO) and empty vector control (A549-Src ctrl) cells were generated by CRISPR-Cas9 technology. crRNAs were designed using https://benchling.com. DNA oligonucleotides for Src exon 4: GTCCTTCAAGAAAGGCGAG (guide 1) and exon 5: AGCCCAAGGATGCCAGCCAG (guide 2) were ordered from IDT (Leuven, Belgium) and cloned into pSpCas9(BB)-2A-GFP(PX458) (Addgene Teddington, UK), according to the protocol of Ann Ran *et al.* [28]. After transformation in bacteria (One Shot™ TOP10 Chemically Competent E. coli; Thermo Fisher Scientific, Bleiswijk, Netherlands), successful cloning was validated by

sequencing. The Src KO and empty vector (Src ctrl) constructs were transfected in A549 cells with a FuGENE® HD reagent-DNA ratio of 3:1 according to manufacturer's protocol. Briefly, $2,5*10^5$ A549 cells per well (6-well plate) were incubated overnight for transfection. After 48 h of transfection, GFP positive cells were single cell sorted with the MoFlo cell sorter (Beckman Coulter, Brea, USA). Clonal cultures were evaluated for A549-Src KO by western blot and a representative clone was selected.

MTT cell viability assay $1*10^4$ cells in 100 µl medium per well were plated in a 96-wells plate (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) and incubated overnight. 100 µl medium with or without rhTRAIL was added for 24 or 48 h yielding a total volume of 200 µl per well. Next, 20 µl of 5 mg/ml Thiazolyl Blue Tetrazolium Bromide (MTT; Sigma-Aldrich, Saint Louis, USA) solution in PBS was added per well and incubated at 37˚C for 3 h. The plates were centrifuged at 900 rpm for 15 min without brake. The formazan crystals were dissolved using 200 µl dimethyl sulfoxide (DMSO; Merck, Burlington, USA) and absorbance was measured at 520 nm (Biorad, Hercules, USA).

Western blot analysis Cells, treated as indicated, were washed twice with ice-cold PBS and lysed with M-Per (ThermoFisher) including 100x Halt phosphatase and protease inhibitors (ThermoScientific, Waltham, USA) for 1.15 h and centrifuged at 14.000 rpm at 4°C for 10 min. Protein concentrations were determined by Bradford protein assay [29]. 20 µg of protein per sample was loaded and separated on 8-12% SDS–PAGE gels and electro blotted onto polyvinylidenedifluoride (PVDF) membranes (Immobilon-P PVDF membrane 0.45µm; Merck-Millipore, Burlington, USA). Subsequently, membranes were blocked for 1 hr at RT in 5% albumine bovine fraction V (Thermo Scientific, pH 7.0) washed in TBS + 0.05% Tween with pH 8.0 (TBS-T) and incubated overnight at 4°C with the primary antibodies diluted in TBS-T. The membranes were washed thrice for 5 min in TBS-T and incubated with Horseradish peroxidase secondary antibodies (DAKO, Santa Clara, USA) for 1 hr at RT. After incubation, the membrane was washed twice for 5 min in TBS-T, followed by 5 min washing in TBS. Subsequently the blots were incubated with the chemiluminescent Lumi-Light (Roche, Basel, Switzerland) and bands visualized with the Chemidoc imaging system (BioRad). The following primary antibodies were used. From Cell Signaling Technologies (Danvers, USA): Src (#2109), pSrc tyr416 (#2101), pSrc tyr527 (#2105), Caspase 8 (#9746), TRADD (#3684), TRAF2 (#4724), FADD (#2782), Erk1/2 (#9102), pErk thr202/tyr204 (#9106), MEK1 (#2352), MEK2 (#9147), pMEK1/2 Ser217/221 (#9154), SMAD3 (#9523) and Lamin A/C (#4777). Other antibodies used: β-actin (MP biomedicals), GAPDH (Ab128915; Abcam, Cambridge, UK), pSMAD3 (Ab52903, Abcam) and β-catenin (BD biosciences, Franklin Lakes, USA). All antibodies used were dissolved in 5% albumine bovine fraction V (Thermo Scientific, pH 7.0) in TBS-T.

Immunoprecipitation The cells were grown in a T165 flask until 60-70% confluency followed by treatment as indicated. The cells were washed twice with ice cold PBS and scraped in cold NP-40 buffer (50 mM Tris pH 7.7, 150 mM NaCl and 0.5% v/v Igepal) containing 100x Halt phosphatase and protease inhibitors (ThermoFisher). Bradford assay was performed to determine protein concentration and samples were stored on ice, or at -20°C until use. A beads-antibody complex was prepared prior the actual immunoprecipitation. For each condition: 1,5 mg of superparamagnetic Dynabeads protein G (ThermoFisher) were isolated and 10 µg Src antibody (clone GD11; Sigma Aldrich) diluted in 200 µl PBS + 0.1% Tween-20 (PBS-T) (#P1379; Sigma-Aldrich) was added. The beads-antibody solution was rotated for 10 min at RT, and washed once in 200 µl PBS-T. Subsequently, the complex was washed twice in 200 µl conjugation Buffer (20 mM Sodium Phosphate, 0.15M NaCl pH 7-9), resuspended in 250 µl 5 mM BS^3 (Life technologies, Carlsbad, USA) and incubated for 60 min at RT with rotation. The cross-linking reaction was quenched by adding 12.5 µl quenching buffer (1M Tris HCl pH 7.5) for 15 min with rotation at RT. The Src antibody-beads complexes were washed thrice with 200 µl PBS-T and 1.5 mg antibody-beads were mixed with 500 µg protein per sample. Binding of the beads-antibody-protein complex was allowed for 1 hr under rotation at 4°C. The beads-antibody-protein complexes were washed thrice with 200 µl washing buffer (citrate-phosphate buffer, pH 5.0) and the beads-antibody complexes were mixed with 20µl elution buffer (0.1 M citrate; pH 2) including 6x SDS loading buffer. The mixture was heated for 10 min at 70^{o}C and proteins were separated by 8-12% SDS page gel for western blot analyses.

Mass spectrometry Src was co-immunoprecipitated as described above in control and 1 hr rhTRAIL exposed A549 and H460 cells. A549-Src-KO cells served as a non-specific binding control The protein mixtures were loaded on a RunBlue 1 mm*10 well 8%- Bis-Tris – gel (Expedeon, Cambridge, UK) after heating the mixture at 70^{o}C for 10 min. Whole gel processing procedure was performed as described previously [30]. LC-MS/MS was performed by the Ultimate 3000 HPLC system (Thermo Scientific) coupled online to a Q-Exactive-Plus mass spectrometer with a NanoFlex source (Thermo Scientific).

Data processing The mass spectrometry data was processed as described previously [31]. In short, the PEAKS 8.0 (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) software was applied to the spectra generated by the Q-exactive plus mass spectrometer to search against a Human Protein database (SwissProt containing 20197 entries) using fixed modification carbamidomethylation of cysteine and the variable post translational modifications oxidation of methionine with a maximum of 5 posttranslational modifications per peptide at a parent mass error tolerance of 10 ppm and a fragment mass tolerance of 0.03 Da. False discovery rate was set at 0.1% and at least 2 unique peptides per protein should be present. Proteins were corrected for background/contamination by eliminating proteins present

in IgG1, IgG2b and A549-Src KO-Src IP samples and the fold change (FC) and Log2(FC) of the remaining proteins were calculated. To identify proteins of interest, a STRING network analysis was performed as well as a KEGG pathways analysis.

Immunofluorescence microscopy In 96 wells plates 5000 cells per well were seeded and incubated overnight. The cells were treated as indicated and fixed with 100 µl 2% paraformaldehyde for 15 min. Subsequently, cells were washed twice with PBS, permeabilized with PBS + 0.5% Tween for 10 min, washed twice with 100 µl PBS + 0.5% Tween, blocked for 1 hr in 2% BSA + 0.1% Tween20 + 1:50 normal goat serum (DAKO) in PBS and washed twice with PBS + 0.5% Tween. The primary antibodies diluted in blocking buffer were added and incubated for 1 hr. Primary antibodies used were: Src (#2109; Cell Signaling Technologies), β-catenin (BD biosciences), Mouse IgG (#610153; BD biosciences) and Rabbit IgG (Cell Signaling Technologies). Secondary antibodies: Alexa488 IgG (Invitrogen), Alexa 568 (Invitrogen). After washing twice with 100 µl PBS + 0.5% Tween the secondary antibody diluted 1:200 in blocking buffer was added and incubated for 1 hr. Subsequently, cells were washed twice with PBS + 0.5% Tween followed by incubation with 2 mg/ml DAPI (Sigma) for 10 min, washed twice with PBS and stored at 4°C until analysis. Pictures were taken with the EVOS digital colour fluorescence microscope (Invitrogen, Carlsbad, USA) and analysed.

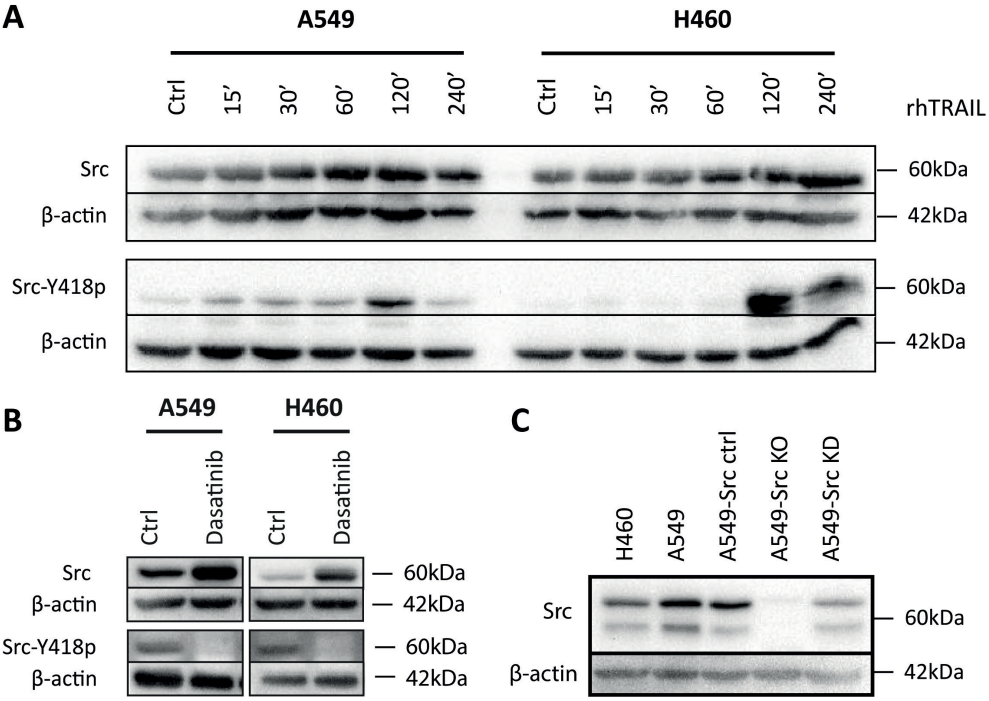
Clonogenic assay For colony formation, 200 cells per well were seeded in a 6 wells plate and rhTRAIL (50 ng/ml), selumetinib (Selu; 0.1 µM) or SIS3 (3 µM) were added after overnight incubation. After 10 days cells were fixed with methanol for 15 min, stained with 0.1% crystal violet solution and washed thoroughly with water and plates were dried. Colony numbers and size were analysed using the Vspot spectrum (AID, Strasbourg, Germany) and the colony counter plugin from ImageJ with a minimum-maximum size ($Pixel^2$) 50-100000, circularity 0.8-1 and a minimum area of 20.

Statistics All experiments were performed at least 3 times independently, unless otherwise indicated. False discovery rate (FDR) statistics were performed on the KEGG pathway analysis and significant changes are shown as LOG2(p) fold changes. For the clonogenic assays two way ANOVA statistics were performed on the mean ± SD from 3 independent experiments with $p<0.05$;**$p<0.01$;***$p<0.001$.

Results

Src does not modulate rhTRAIL induced apoptotic signalling in A549 and H460 cells TRAIL resistant A549 and sensitive H460 cells were examined for Src activation after rhTRAIL (50 ng/ml) exposure for various time periods. Different phosphorylation patterns of Src-Y418p (activated Src) were observed in A549 and H460 cells. Src-Y418p was detected at early

timepoints in A549 but not in H460 cells (Fig. 1A). In H460 cells very low basal levels of Src-Y418p were detected with no increases within 60 min of rhTRAIL treatment, whereas high levels of Src-Y418p were detected at later time points that were not seen in A549 cells (Fig. 1A). To investigate whether Src regulates rhTRAIL sensitivity, Src activity was inhibited by Dasatinib or its expression downregulated by either shRNA-dependent silencing (knockdown, KD) or genetic ablation (knockout, KO) using CRISPR/Cas9 gene editing. Dasatinib effectively prevented phosphorylation of Src-Y418p in A549 and H460 cells and Src expression was effectively silenced in A549-Src KD cells and depleted in A549-Src KO cells compared to A549-Src ctrl (Fig. 1B-C). Src inhibition did not significantly affect rhTRAIL sensitivity in rhTRAIL resistant or sensitive cells (Fig. 1D). Also, A549 Src-KD and -KO cells showed no altered rhTRAIL sensitivity as compared to parental A549 and A549-Src ctrl cells (Fig. 1E). Overall, rhTRAIL treatment resulted in differential Src activation in sensitive and resistant NSCLC cells. However, Src was not instrumental for rhTRAIL-mediated apoptosis.



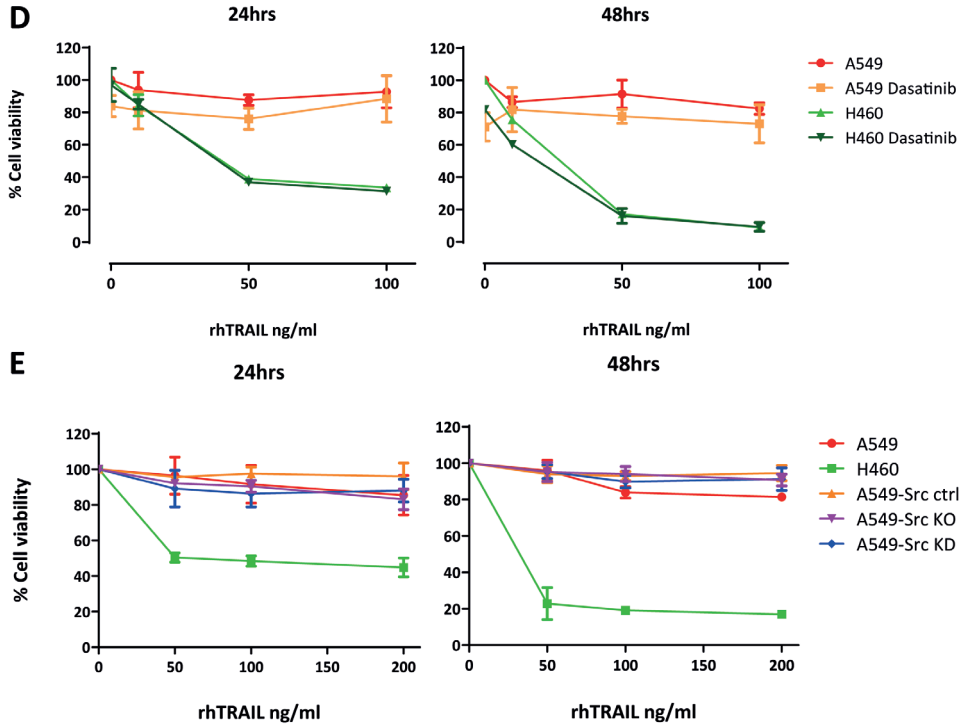


Figure 1. Src is differentially activated in A549 and H460 cells and does not regulate TRAIL induced apoptosis (A) *Western blot analysis of total and phosphorylated Src in A549 and H460 cells after treatment with 50 ng/ml rhTRAIL for the indicated time periods. In resistant A549 cells Src phosphorylation was detected at early time points. In sensitive H460 cells, Src phosphorylation was detected only at later time points.* ***(B)*** *Western blot analysis of total Src and Src-Y418p showing potent Src inhibition after treatment with 1 μM Dasatinib for 24 h.* ***(C)*** *Src expression levels in H460, A549, A549-Src ctrl, CRISPR/CAS9 knockout (A549-Src KO) and short hairpin knockdown (A549-Src KD).****(D)*** *MTT assays measuring cell viability of A549 and H460 cells treated with different concentrations rhTRAIL and in absence or presence of Dasatinib for 24 or 48 h. Data represents the mean from 3 independent experiments ± SD.* ***(E)*** *MTT assays of H460, A549, A549-Src ctrl, A549-Src KD and A549-Src KO cells treated with indicated concentrations rhTRAIL for 24 or 48 h. Data represents the mean from 3 independent experiments ± SD.*

TRADD is required for rhTRAIL mediated Src activation Previously we demonstrated that TRAIL-induced Src activation is mediated by RIPK1 [32]. To further examine the involvement of other DISC and secondary complex components, caspase 8, FADD, TRADD and TRAF2 were silenced with specific siRNAs in A549 cells. Caspase 8, FADD, and TRAF2 silencing did not alter Src activation conclusively (data not shown). However, TRADD silencing delayed Src activation when compared to control cells and resulted in increased caspase 8 cleavage (Fig. 2). Thus, TRADD mediated rhTRAIL-induced Src activation and suppressed caspase 8 cleavage.

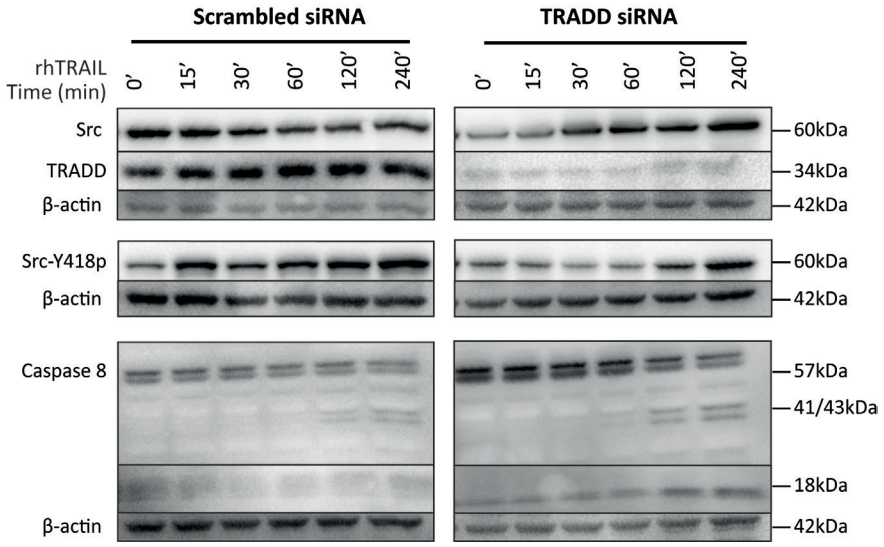


Figure 2. TRADD mediates rhTRAIL-induced Src activation in A549 cells *Protein levels of Src, TRADD, Src-Y418p and caspase 8 in rhTRAIL treated A549 cells transfected with control scrambled siRNA or TRADD siRNA were examined by western blotting, indicating that TRADD is involved in Src activation at early time points.*

Analysis of the Src protein interactome To further elucidate the underlying molecular mechanisms of Src-mediated pro-tumorigenic rhTRAIL signalling, we set out to study the interactome of Src in A549 and H460 cells. Cells were treated for 1 hr with 50 ng/ml rhTRAIL, followed by Src immunoprecipitation and tryptic peptide-based mass-spectrometry to identify possible differential interacting proteins. In untreated A549 cells 314 proteins were found to interact with Src, increasing to a total of 435 proteins after rhTRAIL treatment, of which 181 were newly interacting proteins (Fig. 3A). On the contrary, in H460 cells rhTRAIL treatment resulted in a reduction of Src binding proteins, from 560 proteins to 180 proteins, respectively, with only 21 newly bound proteins (Fig. 3A). In both untreated cell lines, 220 overlapping Src-interacting proteins were found, and 125 overlapping proteins in rhTRAIL treated cells. In rhTRAIL exposed A549 cells 87 unique proteins interacted with Src, compared to only 11 unique proteins in rhTRAIL treated H460 cells. Taken together, substantial differences in the number of Src binding proteins and interactome composition between H460 and A549 cells were found in both untreated and rhTRAIL-treated conditions. Notably, an overall increase in the number of interacting proteins and detection of unique interactors was seen in rhTRAIL-treated A549 cells, whereas in H460 cells protein numbers decreased and only a small number of unique interactors were detected.

KEGG pathway analyses were performed subsequently with all Src interacting proteins to obtain insight in associated biological processes (Fig. 3B). Top 10 significant annotations

were highly similar between the A549 and A549-rhTRAIL Src interactomes, representing predominantly metabolic and biosynthesis pathways, with endocytotic pathways being unique in rhTRAIL treated cells (Fig. 3B). In the H460 and H460-rhTRAIL Src interactomes 4 out of 10 pathways overlapped and most processes were linked with nucleotide and metabolic pathways (Fig. 3B). Opposed to the A549 interactomes, proteins involved in endocytosis were found in complex with Src in untreated H460 cells, which were not detected in H460-rhTRAIL interactomes. Next, proteins showing at least a 2-fold increased or decreased binding to Src after rhTRAIL exposure were selected for further evaluation. In A549 cells interactomes 182 proteins increased and 60 decreased, whereas in H460 cells 21 proteins increased and 60 proteins decreased (Fig. 4A). KEGG pathway analysis revealed that proteins belonging to various pathways, including metabolism and endocytosis, were increased in A549-rhTRAIL and decreased in H460-rhTRAIL Src interactomes (Fig. 4B).

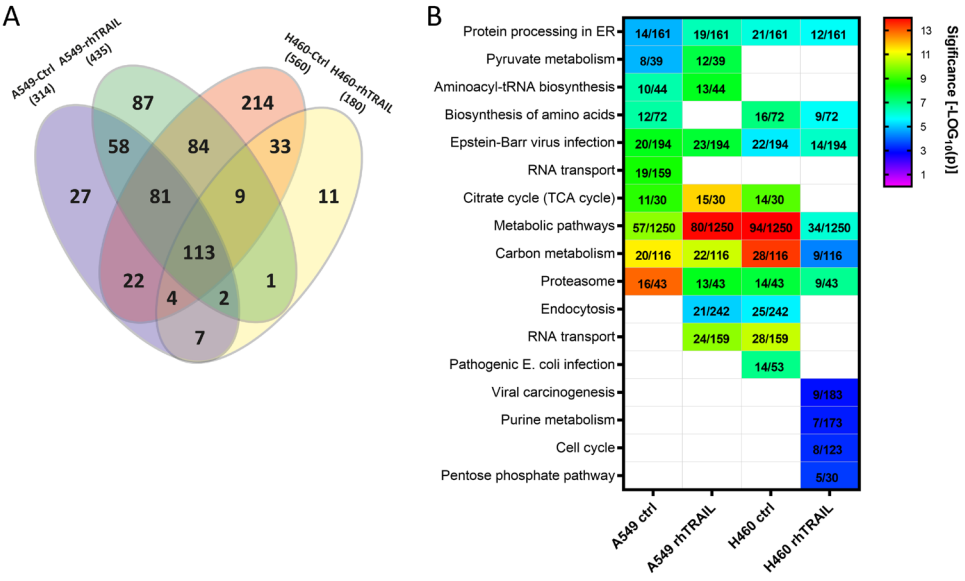


Figure 3. Analyses of Src interactomes in H460 and A549 cells with(out) rhTRAIL treatment
Src co-IP was performed in H460 and A549 cells -/+ rhTRAIL for 1 hr followed by mass-spectrometry (MS) analyses to examine the Src interactomes (n=1). ***(A)*** *Venn diagrams representing proteins found in the Src interactomes of A549-ctrl, A549-rhTRAIL, H460-ctrl and H460-rhTRAIL cells. After rhTRAIL treatment, the number of proteins in the Src interactome of A549 increased and of H460 decreased.* ***(B)*** *Heatmap showing the top 10 most significantly represented KEGG pathways based on analyses of the Src interactomes of A549-ctrl, A549-rhTRAIL, H460-ctrl and H460-rhTRAIL cells. Significant p values from the false discovery rate are depicted with their -log10(p) values by a colour range. The number of proteins present in the interactomes per KEGG pathway is shown.*

To identify the potential interactors that might mediate rhTRAIL pro-tumorigenic signalling, STRING network and KEGG pathway analyses were performed on the top 15% proteins that either increased or decreased at least 2-fold in interactomes obtained after rhTRAIL treatment (Fig. 4A). This selection yielded 75 upregulated proteins and 60 downregulated proteins in A549-rhTRAIL, and 21 upregulated proteins and 87 downregulated proteins in H460-rhTRAIL interactomes. Using string network analysis, in the Src interactome from rhTRAIL treated A549 cells, among others MEK1, MEK2, SMAD3 and β-catenin (Catenin-β1/CTNNB1) were upregulated (Fig. 5A). On the other hand, MEK2, PP2A, β-catenin and Catenin-α1 were downregulated in the H460-rhTRAIL interactome (Fig. 5A). Notably, the phosphatase PP2A was downregulated in both the A549-rhTRAIL and H460-rhTRAIL interactomes, PP2A is a major Src inhibitor as well as a regulator of the Wnt signalling pathway by dephosphorylating Wnt pathway components, which can result in both suppression and stimulation of tumour growth, depending on the cellular context (Fig. 5A) [33,34].

KEGG pathway analysis further confirmed that Src, MEK1, MEK2, SMAD3 and β-catenin were implicated in various cancer-related pathways, being able to interact with Src as well as with each other (Fig. 5B). Such interactions might mediate Src-dependent pro-tumorigenic effects of rhTRAIL exposed A549 cells. MEK1 and MEK2 are part of the RAS mediated RAF-MEK1/2-ERK proliferation and survival pathway, which is often hyperactivated in lung cancers [35,36]. SMAD3 is a mediator of TGF-β signalling and has both tumour suppressive and oncogenic functions [37]. Wnt-β-catenin signalling promotes stemness, tumorigenesis and cancer cell proliferation in various cancers including NSCLC [38–40].

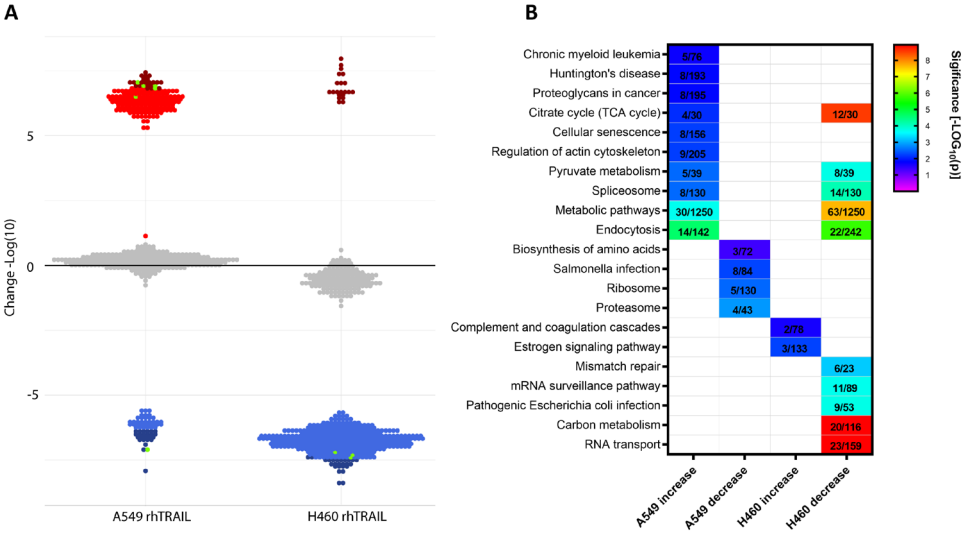


Figure 4. Proteins in the Src interactomes that change 2-fold or more in H460 and A549 cells with(out) rhTRAIL treatment (A) *Dot plots showing proteins in the Src interactomes of A549-rhTRAIL*

and H460-rhTRAIL. Each dot represents a protein with an at least a 2-fold change in abundancy in the corresponding interactome after rhTRAIL exposure; blue (decrease), red (increase) and grey (less than 2-fold change). The proteins indicated in dark blue or red represent the top 15% most fold-changed proteins ***(B)*** *KEGG pathway heatmaps based on the top 15% most differential proteins detected in the Src interactomes (p=0.05) indicated in* ***(A).*** *Significant p values from the false discovery rate are depicted with their LOG2 values by colour. The numbers of proteins representing a KEGG pathway are indicated.*

MEK1/2, SMAD3 and β-catenin as possible mediators of rhTRAIL-induced Src signalling

The interactions of MEK1, MEK2, SMAD3 and β-catenin with Src were confirmed by direct co-IP/western blotting experiments (Fig. 5C). High levels of MEK1 associated with Src were detected in A549 cells independent of rhTRAIL treatment. MEK2 levels, however, were very low in both cell lines (Fig. 5C). An apparent increase in SMAD3 and β-catenin binding to Src was observed in A549-rhTRAIL cells. In contrast, β-catenin interactions decreased in rhTRAIL-treated H460 cells (Fig. 5C).

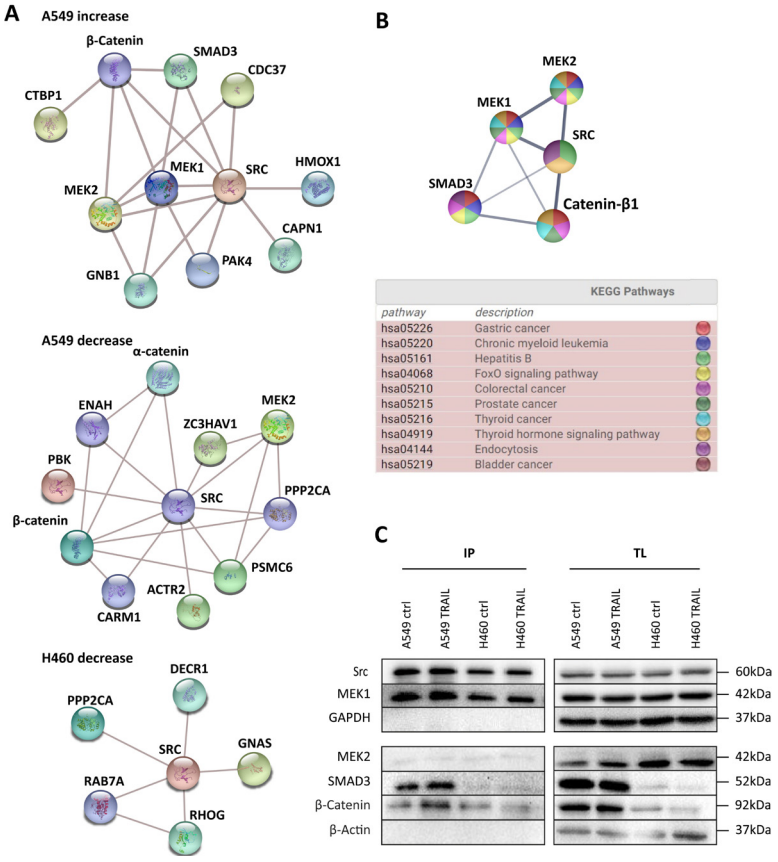


Figure 5. Potential TRAIL-regulated mediators of Src-dependent pro-tumorigenic signalling in A549 cells *String network analysis of the proteins identified in the Src interactomes that* ***(A)*** *increased in A549-rhTRAIL, decreased in A549-rhTRAIL, decreased in H460-rhTRAIL.* ***(B)*** *The Src-MEK1-MEK2-*

SMAD3-β-catenin network and KEGG pathway analyses; top 10 most significant cancer associated pathways are shown. ***(C)*** *Src co-IP experiments to determine interactions with MEK1, MEK2, SMAD3 and β-catenin in A549 cells treatment for 1 hr with 50 ng/ml rhTRAIL as compared to the total lysate (TL).*

To further investigate the involvement of these proteins in Src mediated signalling, possible time dependent effects of rhTRAIL on phosphorylation of MEK1/2, ERK (which is activated by MEK1/2) and SMAD3 (Ser423/425), and the expression of β-catenin were determined in A549, A549-Src ctrl and A549-Src KO cells (Fig. 6A). Basal MEK1/2 phosphorylation levels were higher in A549 and A549-Src ctrl cells compared to Src depleted cells, whereas total MEK1 and MEK2 levels remained mostly unaltered (Fig. 6A). Phospho-MEK1/2 (pMEK1/2) levels increased after 15 min rhTRAIL, particularly in Src proficient cells. Normally, pMEK1/2 phosphorylates and activates Erk which is subsequently translocated to the nucleus. pERK is therefore a functional readout for MEK1/2 activation. After 15 min of rhTRAIL treatment downstream ERK phosphorylation was also stronger in A549 and A549-Src ctrl cells when compared to A549-Src KO cells, while total ERK levels were unaffected (Fig. 6A). β-catenin expression slightly decreased upon rhTRAIL treatment in A549 and A549-Src ctrl cells, whereas lower basal levels were found in Src deficient cells and no clear effect of rhTRAIL on expression was seen (Fig. 6A). Levels of phospho-SMAD3 (pSMAD; Ser423/425) increased strongly upon rhTRAIL treatment in A549-Src KO cells compared to the Src proficient cells (Fig. 6A). Taken together, Src was found to be involved in rhTRAIL-induced MEK1/2 and ERK activation, the regulation of β-catenin protein levels and the suppression of SMAD3-Ser243/425 phosphorylation.

Effect of Src and rhTRAIL on the subcellular localization of pERK, SMAD3 and β-catenin Next, the effects of Src on the cytoplasmic and nuclear localization of the identified downstream proteins were examined by subcellular fractionation of cell lysates and western blotting. rhTRAIL treatment in A549 cells resulted in increased Src levels in the nuclear fraction at later timepoints, concomitant with a decrease in cytoplasmic Src (Fig. 6B; Supplementary fig. 2A). pERK levels increased in the cytosol at early timepoints post rhTRAIL treatment and localized also to the nucleus after 240 min in Src proficient cells, whereas pERK levels overall decreased in Src deficient cells (Fig. 6B, supplementary fig. 2A). Total SMAD3 levels increased in the nuclear fraction of Src deficient cells after rhTRAIL treatment, whereas levels remained mostly constant in Src proficient cells (Fig. 6B; Supplementary fig. 2A). Unfortunately, we were not able to detect pSMAD3 in the nuclear fraction after subcellular fractionation. However, we found an increase in pSMAD (Ser423/425) levels in the total lysates of Src deficient A549 cells after rhTRAIL treatment (Fig. 6A). In general, pSMAD3 is known to translocate to the nucleus, suggesting that nuclear SMAD3 represents pSMAD3 [41,42]. However, this needs to be further substantiated. Nuclear β-catenin levels increased upon rhTRAIL treatment in Src proficient cells, which was less detectable in Src deficient cells (Fig. 6B; Supplementary fig. 2A).

Next, we explored Src and β-catenin localization following rhTRAIL treatment by immunofluorescent microscopy in A549 cells. We found increased co-localisation of Src and β-catenin at the periphery of the nucleus upon rhTRAIL treatment (Fig. 6C, supplementary fig. 2B). Furthermore, an increase in membrane localised β-catenin was seen upon rhTRAIL treatment, that was not observed in Src deficient cells (Fig. 6C; Supplementary fig. 2B). Together, these results showed that Src is required for rhTRAIL induced pERK nuclear translocation and a peri-nuclear localisation of β-catenin in A549 cells.

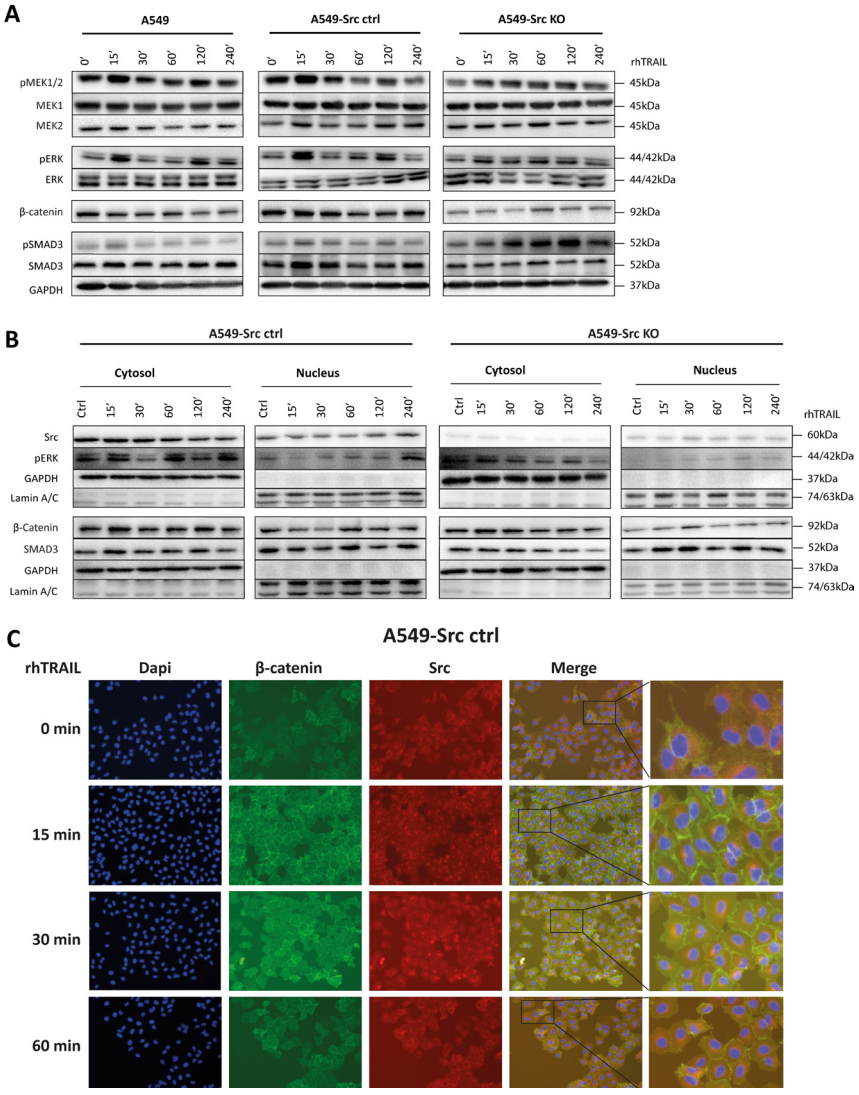


Figure 6. Effect of TRAIL and Src on MEK1/2, ERK, β-catenin and SMAD3 activity in A549 cells ***(A)*** *Western blot analysis showing expression of the indicated (phosphorylated) proteins that were identified as possible mediators of TRAIL-induced Src signalling in A549 cells. Protein expression was*

determined in A549, A549-Src ctrl and A549-Src KO cells treated for indicated time periods with 50 ng/ml rhTRAIL. Src appeared to be involved in the activation of MEK1/2 and ERK, regulation of β-catenin protein levels and the suppression of SMAD30-Ser243/425 phosphorylation. ***(B)*** *Western blot analysis of proteins in cytosolic and nuclear extracts from A549-Src ctrl and A549-Src KO cells after treatment with 50 ng/ml rhTRAIL for the indicated time periods.* ***(C)*** *Immunofluorescent microscopic analyses (40x) of Src and β-catenin in A549-Src ctrl cells after rhTRAIL exposure at the indicated times. DAPI (blue), β-catenin (green) and Src (red) and merged pictures and enlargements.*

Effects of rhTRAIL, Src, MEK1/2 and SMAD3 on clonogenic growth of A549 cells Finally, we investigated the possible effects of Src and the identified downstream proteins MEK1/2 and SMAD3 on clonogenic growth of A549 cells (Figure 7). Src deficiency resulted in significantly reduced average colony size, but had no effect on the number of colonies (Fig. 7). Furthermore, rhTRAIL treatment did not affect colony formation or growth, neither in Src proficient nor deficient cells. SMAD3 inhibition with inhibitor of SMAD3 (SIS3) had no significant effect on colony formation and growth, whereas MEK1/2 inhibition with Selumetinib significantly reduced colony growth although independent of Src status or rhTRAIL treatment (Fig. 7B). In Src KO cells, MEK1/2 inhibition also significantly reduced the number of colonies (Fig. 7A). Thus, independent of rhTRAIL exposure, Src and MEK1/2 both stimulated colony growth in A549 cells, and MEK1/2 also enhanced colony formation in Src KO cells.

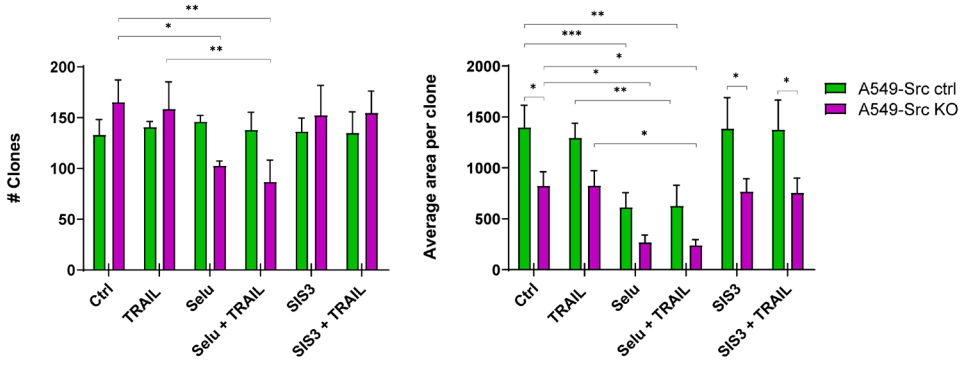


Figure 7. Effects of TRAIL, Src, MEK1/2 and SMAD3 on A549 in clonogenic assays *Clonogenic assays of A549-Src ctrl and -Src-KO cells with(out) rhTRAIL (50ng/ml), selumetinib (Selu, MEK1/2 inhibitor) and specific inhibitor of SMAD3 (SIS3). Colony numbers and size were determined after 10 days by imageJ software analyses. The bar graphs depict* ***(A)*** *the mean number of colonies (clonogenicity) and* ***(B)*** *the mean size of the colonies (cell growth). Data represents the mean from 3 independent experiments ± SD. Statistics were performed with a two-way ANOVA p<0.05;**p<0.01;***p<0.001.*

Discussion

In this study we examined the possible function of Src as a regulator of TRAIL-induced apoptosis and mediator of pro-tumorigenic TRAIL signalling in NSCLC cells. We found different rhTRAIL-induced Src activation profiles in apoptosis sensitive H460 versus resistant A549 cells. The rapid rhTRAIL-dependent Src phosphorylation observed in A549 cells required the

presence of TRADD, a component of the secondary signalling complex. However, Src activity did not regulate sensitivity to rhTRAIL-induced apoptosis in these NSCLC cell lines. Using a Src co-IP/mass-spectrometry approach to find downstream effectors, we identified proteins that bind to Src upon rhTRAIL treatment of A549 cells. MEK1, MEK2, SMAD3 and β-catenin were selected for further analysis. Src activation was found to mediate MEK1/2 and subsequent ERK phosphorylation and SMAD3 ser423/425 phosphorylation, and to regulate β-catenin expression. Activation of these signalling pathways by Src was accompanied by pERK and β-catenin (peri)nuclear translocation. Finally, neither Src expression, nor rhTRAIL exposure could significantly affect colony formation of A549 cells, although the size of the colonies was reduced in Src deficient A549 cells, independent of rhTRAIL treatment. Inhibition of MEK1/2 reduced both the number and size of the colonies, also independently of rhTRAIL. Together these results imply that the identified Src downstream effectors regulate proliferative properties of A549 cells, however, these proteins could not be directly linked with rhTRAIL-induced Src-dependent pro-tumorigenic activity.

Our finding that Src activity does not regulate apoptosis sensitivity in the NSCLC cells is in contrast with several previous reports. For example, Src has been found to inhibit TRAIL induced apoptosis by phosphorylating procaspase 8 at Tyr380 that prevents its activation [24,26]. In addition, Src inhibition in breast cancer, melanoma and hepatocellular carcinoma cells restored or increased sensitivity towards TRAIL induced apoptosis that was associated with increased caspase 8 and caspase 3 activity [26,33,43]. Although, we have to extend our studies to a larger panel of NSCLC cells, it could be that the apoptosis modulatory function of Src is tumour type dependent. The underlying molecular mechanisms that cause the different functioning of Src in regulating TRAIL-induced apoptosis in different tumour types remain to be elucidated.

Previously we found that rhTRAIL via RIPK1, a kinase and component of the secondary complex, is a mediator for TRAIL-induced Src activation [44]. Here we found that TRADD, another component of the secondary complex, is also required for rhTRAIL-dependent Src activation in A549 cells. We did not find involvement of FADD, caspase 8 or TRAF2 in Src activation. Our findings are consistent with the known function of TRADD, which is to recruit RIPK1 to the TRAIL receptors leading to RIPK1 activation and subsequent activation of non-apoptotic signalling cascades by preventing DISC formation and FADD-caspase 8 driven cell death [45].

Src is known to interact with various proteins often resulting in conformational changes and subsequent activation or inactivation of Src, thereby also affecting the ability of Src to interact with other proteins [46–48]. Here, we examined the interactome of Src in A549 and H460 cells to identify possible mediators of pro-tumorigenic rhTRAIL signalling. Interestingly,

the number of identified Src-binding proteins in untreated and rhTRAIL treated H460 cells was inverse to the numbers found in A549 and reflects the differences observed in rhTRAIL-induced Src activation in both NSCLC cell lines. In A549 cells the number of proteins in complex with Src increased after rhTRAIL exposure, likely contributing to pro-tumorigenic signalling, whereas the number of proteins decreased in H460 cells that may reflect lower protein expression during the apoptotic process at least in part due to caspase-dependent protein degradation.
Surprisingly, in the Src interactome we found decreased binding of the Src inhibitor PP2A after rhTRAIL treatment in both sensitive and resistant NSCLC cells. PP2A has been reported to inhibit Src activation by dephosphorylating Src-Tyr416 and, as a consequence, the activation of apoptosis by reducing inhibitory caspase 8-Tyr380 phosphorylation. Furthermore, in resistant cells, TRAIL treatment has been found to induce ubiquitination of PP2A causing its degradation leading to Src-dependent caspase 8 inactivation [33]. PP2A degradation could explain decreased binding of Src and PP2A in rhTRAIL treated A549 cells. However, such a mechanism cannot explain the decrease in Src-PP2A interactions in apoptosis sensitive H460 cells. The Src-PP2A interactions were reduced after 1 hr rhTRAIL treatment, a time point at which irreversible apoptosis is induced in H460 cells. Effector caspase-3 cleaves PP2A at the regulatory A subunit, through which its activity is increased and the apoptotic commitment of the cell is enhanced [49,50]. Cleavage of PP2A might result in decreased interactions with Src and could explain why we found a sudden increase in Src phosphorylation upon 2 h rhTRAIL treatment. Yet, the exact mechanisms and the role of Src, caspase 8 (phosphorylation) and caspase 3 herein need to be further examined.

Differential analyses of the Src interactomes in H460 and A549 in the absence or presence of rhTRAIL allowed us to identify possible mediators of non-apoptotic TRAIL signalling. SMAD3, β-catenin, MEK1 and MEK2 were selected and their interactions with Src were confirmed by co-IPs, although this does not necessarily imply direct protein-protein interactions. Expression analyses provided evidence of rhTRAIL-dependent Src-MEK1/2-ERK, Src-β-catenin and Src-SMAD3 signalling (see also summarizing Fig. 8).
SMAD3, a downstream effector of TGF-β signalling, can have both tumour suppressive and oncogenic functions. Phosphorylation of SMAD3 at Ser423/425 has been associated with tumour suppressive activity [37,51]. Interestingly, in A549 Src-KO cells rhTRAIL treatment increased pSMAD3-Ser423/425 and total SMAD3 levels in the nucleus of A549 Src-KO also increased, the latter possibly representing pSMAD3. However, pharmacological inhibition of pSMAD3 with SIS3, a selective SMAD3 inhibitor that prevents TGF-β induced phosphorylation, did not alter clonogenicity of both Src deficient and proficient A549 cells. SMAD3 is involved in TGF-β induced migration, although in our hands SMAD3 inhibition with SIS3 did not affect the migratory capacity of NSCLC (unpublished data; [52]). TRAIL can induce epithelial-mesenchymal transition (EMT) in various cancer cells, including lung tumour cells,

via amongst others TGF-β/SMAD signalling pathway [53]. Possibly, SMAD3 inhibition prevents TRAIL-induced EMT, which needs further investigation. Hence, our current experiments did not show a pro-tumorigenic function of SMAD3 in rhTRAIL-Src non-canonical signalling.
β-catenin, a downstream effector of Wnt signalling, is known to promote stemness, tumorigenesis and cancer cell proliferation [38,39]. Dephosphorylation of β-Catenin and subsequent protein accumulation is followed by nuclear translocation and transcriptional regulation of target genes. On the other hand, N-terminal phosphorylation of β-catenin results in ubiquitination and degradation of β-catenin. Src via activation of the focal adhesion kinase (FAK) has a pivotal role in the nuclear trans-localisation and activity of β-catenin by lowering β-catenin affinity for membrane localised E-Cadherin [54–57]. rhTRAIL treatment of Src proficient cells resulted in higher basal levels of β-catenin, increased levels in the nucleus, peri-nuclear area as well as at the cell membrane. Although, we could not confirm β-catenin nuclear translocation by immunofluorescence microscopy, we found distinct spots where Src and β-catenin co-localised at the periphery of the nucleus. Src translocation to the nucleus has been previously associated with both increased and decreased tumorigenic effects in various tumour types, among which osteosarcoma, breast cancer pancreatic and acute myeloid leukaemia [58]. Whether perinuclear staining implies ER localization is yet unclear, as well as the possible functional consequences of perinuclear co-localisation. Overall, the role of Src in altering the subcellular localisation of β-catenin remains to be further elucidated.
MEK1 and MEK2 are part of the RAS mediated RAF-MEK1/2-ERK proliferation and survival pathway that is often hyperactivated in lung cancers [35,36]. We found that depletion of Src and simultaneous inhibition of MEK1/2 reduced the clonogenicity of rhTRAIL resistant A549 cells, which was stronger than after MEK1/2 inhibition or Src ablation alone. Simultaneous treatment with pharmacological inhibitors of Src and MEK in NSCLC and ovarian cancer *in vitro* and *in vivo* has shown synergistic anti-tumour effects [59–61]. Further studies are needed to confirm and examine whether and how these proteins are involved in TRAIL-Src non-canonical signalling in resistant NSCLC cells.

Taken together, our results provide deeper insight in the possible mechanisms underlying TRAIL-RIPK1-Src mediated pro-tumorigenic signalling. Src was not instrumental for causing apoptosis resistance in A549 cells, but could modulate MEK1/2, SMAD3 and β-catenin signalling, although their involvement in pro-tumorigenic signalling should be further corroborated. Whether these possible pro-tumorigenic pathways provide therapeutic targets that would increase TRAIL efficacy in resistant NSCLC cells, remain to be investigated.

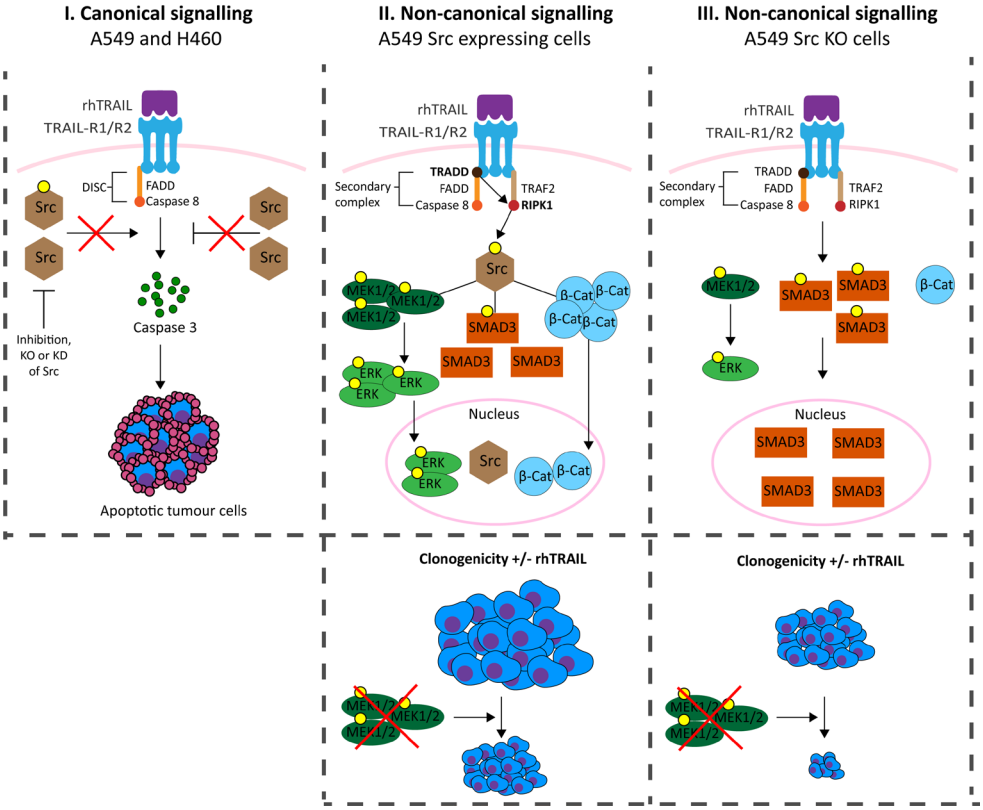


Figure 8. Schematic representations of the identified mechanisms by which Src can mediate TRAIL-dependent signalling involving MEK1/2-ERK, SMAD3 and β-catenin *In TRAIL resistant A549 and sensitive H460 cells I. Canonical signalling was not modulated by Src, as neither inhibition, knockdown nor knockout enhanced TRAIL driven cell death. In II. Non-canonical signalling in A549 Src expressing cells rhTRAIL activated Src by phosphorylation via TRADD-RIPK1. Src activation was persuaded by interactions with MEK1/2, SMAD3 and Catenin-β and subsequent phosphorylation of SMAD3 and MEK1/2. MEK1/2 activated ERK, which was translocated to the nucleus, as well as Catenin-β. Regarding clonogenicity, inhibition of MEK1/2 reduced the size of the clones, independent of rhTRAIL treatment. In III. Non-canonical signalling in A549 Src KO cells rhTRAIL induced phosphorylation of MEK1/2-ERK and SMAD3, yet only SMAD3 was translocated to the nucleus. The clones of Src deficient cells were significantly smaller in size and upon MEK1/2 inhibition their size was further reduced, as well as their number, independently of rhTRAIL exposure.*

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References

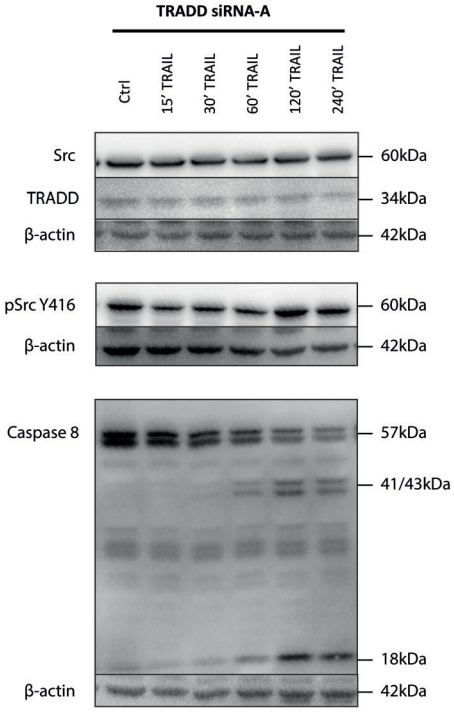
1. Travis WD, Brambilla E, Nicholson AG, et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances since the 2004 Classification. *J Thorac Oncol*. 2015;10(9):1243-1260. doi:10.1097/JTO.0000000000000630
2. Lung and Bronchus Cancer — Cancer Stat Facts. https://seer.cancer.gov/statfacts/html/lungb.html. Accessed June 9, 2020.
3. Osmani L, Askin F, Gabrielson E, Li QK. Current WHO guidelines and the critical role of immunohistochemical markers in the subclassification of non-small cell lung carcinoma (NSCLC): Moving from targeted therapy to immunotherapy. *Semin Cancer Biol*. 2018;52(Pt 1):103-109. doi:10.1016/j.semcancer.2017.11.019
4. Duma N, Santana-Davila R, Molina JR. Non–Small Cell Lung Cancer: Epidemiology, Screening, Diagnosis, and Treatment. *Mayo Clin Proc*. 2019;94(8):1623-1640. doi:10.1016/j.mayocp.2019.01.013
5. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. *Nature*. 2018;553(7689):446-454. doi:10.1038/nature25183
6. Ashkenazi A. Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. *Nat Rev Drug Discov*. 2008;7(12):1001-1012. doi:10.1038/nrd2637
7. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem*. 1996;271(22):12687-12690. http://www.ncbi.nlm.nih.gov/pubmed/8663110.
8. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 1995;3:673-682. doi:10.1016/1074-7613(95)90057-8
9. Gonzalvez F, Ashkenazi a. New insights into apoptosis signaling by Apo2L/TRAIL. *Oncogene*. 2010;29(34):4752-4765. doi:10.1038/onc.2010.221
10. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: Decisions between life and death. *Int J Biochem Cell Biol*. 2007;39(7-8):1462-1475. doi:10.1016/j.biocel.2007.02.007
11. Lemke J, von Karstedt S, Zinngrebe J, Walczak H. Getting TRAIL back on track for cancer therapy. *Cell Death Differ*. 2014;21(9):1350-1364. doi:10.1038/cdd.2014.81
12. Von Karstedt S, Montinaro A, Walczak H. Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. *Nat Rev Cancer*. 2017;17(6):352-366. doi:10.1038/nrc.2017.28
13. de Miguel D, Lemke J, Anel A, Walczak H, Martinez-Lostao L. Onto better TRAILs for cancer treatment. *Cell Death Differ*. 2016;23(5):733-747. doi:10.1038/cdd.2015.174
14. Kretz A-L, Trauzold A, Hillenbrand A, et al. TRAILblazing Strategies for Cancer Treatment. *Cancers (Basel)*. 2019;11(4):456. doi:10.3390/cancers11040456
15. Azijli K, Weyhenmeyer B, Peters GJ, de Jong S, Kruyt F a E. Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ*. 2013;20(7):858-868. doi:10.1038/cdd.2013.28
16. Varfolomeev E, Maecker H, Sharp D, et al. Molecular Determinants of Kinase Pathway Activation by Apo2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand. *J Biol Chem*. 2005;280(49):40599-40608. doi:10.1074/jbc.M509560200
17. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells. *J Cell Sci*. 2012:4651-4661. doi:10.1242/jcs.109587
18. Guarino M. Src signaling in cancer invasion. *J Cell Physiol*. 2010;223(November 2009):14-26. doi:10.1002/jcp.22011
19. Rothschild S, Gautschi O, Haura E, Johnson F. Src inhibitors in lung cancer: Current status and future directions. *Clin Lung Cancer*. 2010;11(4):238-242. doi:10.3816/CLC.2010.n.030
20. Giaccone G, Zucali PA. Src as a potential therapeutic target in non-small-cell lung cancer. *Ann Oncol*. 2008;19(7):1219-1223. doi:10.1093/annonc/mdn048
21. Yeatman TJ. A renaissance for SRC. *Nat Rev Cancer*. 2004;4(6):470-480. doi:10.1038/nrc1366
22. Roskoski R. Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun*. 2005;331(1):1-14. doi:10.1016/j.bbrc.2005.03.012
23. Zhang J, Kalyankrishna S, Wislez M, et al. Src-family kinases are activated in non-small cell

lung cancer and promote the survival of epidermal growth factor receptor-dependent cell lines. *Am J Pathol*. 2007;170(1):366-376. doi:10.2353/ajpath.2007.060706
24. Cursi S, Rufini A, Stagni V, et al. Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *EMBO J*. 2006;25(9):1895-1905. doi:10.1038/sj.emboj.7601085
25. Tsang JLY, Jia SH, Parodo J, et al. Tyrosine phosphorylation of caspase-8 abrogates its apoptotic activity and promotes activation of c-Src. *PLoS One*. 2016;11(4). doi:10.1371/journal.pone.0153946
26. De Toni EN, Kuntzen C, Gerbes AL, et al. P60-c-src suppresses apoptosis through inhibition of caspase 8 activation in hepatoma cells, but not in primary hepatocytes. *J Hepatol*. 2007;46(4):682-691. doi:10.1016/j.jhep.2006.10.010
27. Zhang XHF, Wang Q, Gerald W, et al. Latent Bone Metastasis in Breast Cancer Tied to Src-Dependent Survival Signals. *Cancer Cell*. 2009;16(1):67-78. doi:10.1016/j.ccr.2009.05.017
28. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013;8(11):2281-2308. doi:10.1038/nprot.2013.143
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72(1-2):248-254. doi:10.1016/0003-2697(76)90527-3
30. Piersma SR, Warmoes MO, de Wit M, de Reus I, Knol JC, Jiménez CR. Whole gel processing procedure for GeLC-MS/MS based proteomics. *Proteome Sci*. 2013;11(1):17. doi:10.1186/1477-5956-11-17
31. Zhang J, Xin L, Shan B, et al. PEAKS DB: De novo sequencing assisted database search for sensitive and accurate peptide identification. *Mol Cell Proteomics*. 2012;11(4). doi:10.1074/mcp.M111.010587
32. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3-dependent invasion in resistant non-small cell lung cancer cells. *J Cell Sci*. 2012;125(19):4651-4661. doi:10.1242/jcs.109587
33. Xu J, Xu Z, Zhou J, Zhuang Z, Wang E. Regulation of the Src-PP2A Interaction in Tumor Necrosis Factor (TNF) -related Apoptosis-inducing Ligand (TRAIL) -induced Apoptosis *. 2013;288(46):33263-33271. doi:10.1074/jbc.M113.508093
34. Thompson J, Williams C. Protein Phosphatase 2A in the Regulation of Wnt Signaling, Stem Cells, and Cancer. *Genes (Basel)*. 2018;9(3):121. doi:10.3390/genes9030121
35. Caunt CJ, Sale MJ, Smith PD, Cook SJ. MEK1 and MEK2 inhibitors and cancer therapy: The long and winding road. *Nat Rev Cancer*. 2015;15(10):577-592. doi:10.1038/nrc4000
36. Frémin C, Meloche S. From basic research to clinical development of MEK1/2 inhibitors for cancer therapy. *J Hematol Oncol*. 2010;3:8. doi:10.1186/1756-8722-3-8
37. Millet C, Zhang YE. Roles of Smad3 in TGF-β signaling during carcinogenesis. *Crit Rev Eukaryot Gene Expr*. 2007;17(4):281-293. doi:10.1615/CritRevEukarGeneExpr.v17.i4.30
38. Shang S, Hua F, Hu ZW. The regulation of β-catenin activity and function in cancer: Therapeutic opportunities. *Oncotarget*. 2017;8(20):33972-33989. doi:10.18632/oncotarget.15687
39. Cui C, Zhou X, Zhang W, Qu Y, Ke X. Is β-Catenin a Druggable Target for Cancer Therapy? *Trends Biochem Sci*. 2018;43(8):623-634. doi:10.1016/j.tibs.2018.06.003
40. DJ S. Wnt signaling pathway in non-small cell lung cancer. *J Natl Cancer Inst*. 2014; doi:10.1093/JNCI/DJT356
41. Xu L. Regulation of Smad Activities. *Biochim Biophys Acta*. 2006;1759(11-12):503. doi:10.1016/J.BBAEXP.2006.11.001
42. Hill CS. Nucleocytoplasmic shuttling of Smad proteins. *Cell Res 2009 191*. 2008;19(1):36-46. doi:10.1038/cr.2008.325
43. Phipps LE, Hino S, Muschel RJ. Targeting Cell Spreading: A Method of Sensitizing Metastatic Tumor Cells to TRAIL-Induced Apoptosis. *Mol Cancer Res*. 2011;9(3):249-258. doi:10.1158/1541-7786.MCR-11-0021
44. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. *Kinome Profiling of Non-Canonical TRAIL Signaling Reveals RIP1-Src-STAT3 Dependent Invasion in Resistant Non-Small Cell Lung Cancer Cells*.; 2012. doi:10.1242/jcs.109587
45. Cao X, Pobezinskaya YL, Morgan MJ, Liu Z. The role of TRADD in TRAIL-induced apoptosis and

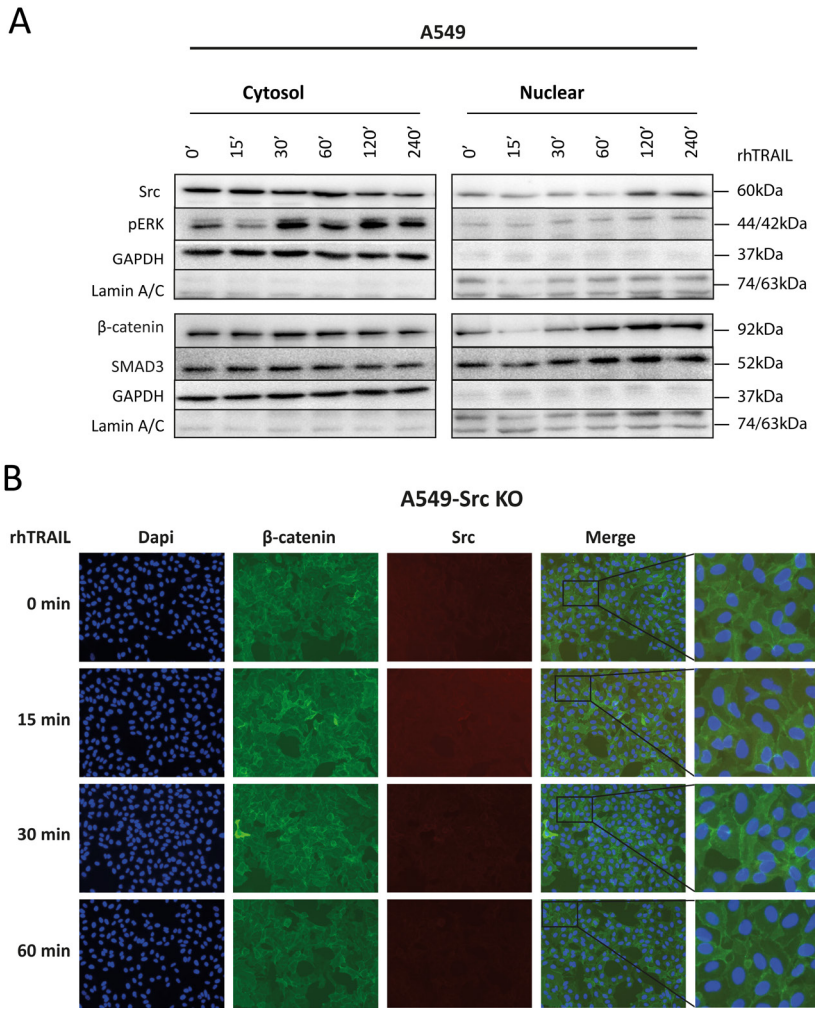
signaling. *FASEB J*. 2011;25(4):1353-1358. doi:10.1096/fj.10-170480

46. Bjorge JD, Jakymiw a, Fujita DJ. Selected glimpses into the activation and function of Src kinase. *Oncogene*. 2000;19(49):5620-5635. doi:10.1038/sj.onc.1203923
47. Thomas JW, Ellis B, Boerner RJ, Knight WB, White GC, Schaller MD. SH2- and SH3-mediated interactions between focal adhesion kinase and Src. *J Biol Chem*. 1998;273(1):577-583. doi:10.1074/jbc.273.1.577
48. Brown MT, Cooper JA. Regulation, substrates and functions of src. *Biochim Biophys Acta - Rev Cancer*. 1996;1287(2-3):121-149. doi:10.1016/0304-419X(96)00003-0
49. Van Hoof C, Goris J. Phosphatases in apoptosis: to be or not to be, PP2A is in the heart of the question. *Biochim Biophys Acta - Mol Cell Res*. 2003;1640(2-3):97-104. doi:10.1016/S0167-4889(03)00029-6
50. Santoro MF, Annand RR, Robertson MM, et al. Regulation of Protein Phosphatase 2A Activity by Caspase-3 during Apoptosis *. *J Biol Chem*. 1998;273(21):13119-13128. doi:10.1074/JBC.273.21.13119
51. Tarasewicz E, Jeruss JS. Phospho-specific Smad3 signaling: Impact on breast oncogenesis. *Cell Cycle*. 2012;11(13):2443-2451. doi:10.4161/cc.20546
52. Kocic J, Bugarski D, Santibanez JF. SMAD3 is essential for transforming growth factor-β1-induced urokinase type plasminogen activator expression and migration in transformed keratinocytes. *Eur J Cancer*. 2012;48(10):1550-1557. doi:10.1016/J.EJCA.2011.06.043
53. Peyre L, Meyer M, Hofman P, Roux J. TRAIL receptor-induced features of epithelial-to-mesenchymal transition increase tumour phenotypic heterogeneity: potential cell survival mechanisms. *Br J Cancer 2020 1241*. 2020;124(1):91-101. doi:10.1038/s41416-020-01177-w
54. Whitehead J, Vignjevic D, Fütterer C, Beaurepaire E, Robine S, Farge E. Mechanical factors activate β-catenin-dependent oncogene expression in APC 1638N/+ mouse colon. *HFSP J*. 2008;2(5):286-294. doi:10.2976/1.2955566
55. Benham-Pyle BW, Sim JY, Hart KC, Pruitt BL, Nelson WJ. Increasing β-catenin/Wnt3A activity levels drive mechanical strain-induced cell cycle progression through mitosis. *Elife*. 2016;5. doi:10.7554/eLife.19799
56. Roura S, Miravet S, Piedra J, García De Herreros A, Duñachl M. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. *J Biol Chem*. 1999;274(51):36734-36740. doi:10.1074/jbc.274.51.36734
57. Gayrard C, Bernaudin C, Déjardin T, Seiler C, Borghi N. Src- and confinement-dependent FAK activation causes E-cadherin relaxation and β-catenin activity. *J Cell Biol*. 2018;217(3):1063-1077. doi:10.1083/jcb.201706013
58. Bagnato G, Leopizzi M, Urciuoli E, Peruzzi B. Nuclear functions of the tyrosine kinase Src. *Int J Mol Sci*. 2020;21(8):2675. doi:10.3390/ijms21082675
59. Simpkins F, Jang K, Yoon H, et al. Dual Src and MEK inhibition decreases ovarian cancer growth and targets tumor initiating stem-like cells. *Clin Cancer Res*. 2018;24(19):4874-4886. doi:10.1158/1078-0432.CCR-17-3697
60. Yuan M, Xu L, Zhang J, et al. SRC and MEK Co-inhibition Synergistically Enhances the Anti-tumor Effect in Both Non-small-cell Lung Cancer (NSCLC) and Erlotinib-Resistant NSCLC. *Front Oncol*. 2019;9(JUN):586. doi:10.3389/fonc.2019.00586
61. Beadnell TC, Mishall KM, Zhou Q, et al. The Mitogen-Activated Protein Kinase Pathway Facilitates Resistance to the Src Inhibitor Dasatinib in Thyroid Cancer. *Mol Cancer Ther*. 2016;15(8):1952-1963. doi:10.1158/1535-7163.MCT-15-0702

Supplementary figures



Supplementary figure 1. TRADD2 is required for early Src phosphorylation after rhTRAIL exposure
Western blot analysis depicting Src, TRADD, Src-Y418p and caspase 8 protein levels in A549 cells in absence or presence of rhTRAIL (50ng/ml) after shRNA-mediated silencing of TRADD, using a different siRNA than used in Fig. 2



Supplementary figure 2. Subcellular localisation of Src, MEK1, MEK2, SMAD3 and β-catenin after rhTRAIL treatment (A) *Western blot analysis showing Src, MEK1, MEK2, SMAD3 and β-catenin protein levels in the cytosol and nucleus from A549 cells after treatment with rhTRAIL (50 ng/ml) for the indicated time periods. Expression patterns were similar as seen in A549-Src ctrl cells (Fig 6B).* ***(B)*** *Immunofluorescent microscopy of A549 Src-KO cells stained with DAPI (blue), β-catenin (green) and Src (red) and merged pictures with enlargements. Staining of Src and β-catenin were determined after 15, 30 and 60 minutes rhTRAIL treatment. There was no increased β-catenin localisation at the plasma membrane and peri-nuclear area as we found for A549-Src control cells (Fig 6C).*

Chapter 4

Effects of TRAIL treatment and Src on cytokine secretion by resistant A549 NSCLC cells and consequences for immune cell activity

Margot de Looff, Steven de Jong and Frank A. E. Kruyt

Abstract

Tumour-necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) is a potential anti-cancer agent that can have unwanted pro-tumorigenic effects in apoptosis resistant tumour cells. These include altered secretion of cytokines that can modulate the activity of immune cells. Previously, we found that Src plays a role in TRAIL-induced non-apoptotic signalling in resistant A549 non-small cell lung cancer (NSCLC) cells. Here, we explored the possible involvement of Src in mediating rhTRAIL-induced changes in the secretome of A549 cells and examined the possible effects on monocyte differentiation, macrophages polarization and $CD8^+$ T cell activation. Conditioned media (CM) was obtained from A549 Src proficient (S+) and deficient (S-) cells upon short (1-2 h, CM-S) or long (24 h, CM-L) exposure time to rhTRAIL (T+) or untreated (T-) cells. The effects of the different CM-S and CM-L on monocytes and differentiated M0 macrophages was determined by flow cytometry analyses of M0 (CD11b, CD68), M1 (CD80, CD86) and M2 (CD163, CD206) marker expression. Our preliminary findings suggest that the different CM-S and CM-L are all able to polarize macrophages, independent from Src status and rhTRAIL treatment. CM-S (S+/T+), when compared to the other 3 CM-S, had some inhibitory effect on $CD8^+$ T cell activation. Cytokine array analyses showed higher cytokine levels in CM-S derived from A549 Src proficient cells compared to the CM-S obtained from A549 Src deficient cells. Increased levels of amongst others GRO-α/β/γ (CXCL-1/2/3) and GRO-α (CXCL1) were found in the CM-S derived from Src proficient cells. The levels of these cytokines were decreased in CM-S from Src deficient cells, particular in CM-S (S-/T+). More in depth analyses of the different CM-S and CM-L by mass-spectrometry identified a number of cytokines involved in immune related biological processes in CM derived from A549 Src proficient cells (CM-S(S+/T-), CM-S(S+/T+) and CM-L(S+/T-), but not in CM-S(S-/T+), CM-L(S+/T+) and CM-L(S-/T+). Taken together, these preliminary findings suggest that A549-derived CM-S and CM-L can modulate both macrophage differentiation and polarization, however, mostly independent of Src expression and rhTRAIL treatment. Furthermore, CM from Src proficient A549 cells seems to reduce CD8+ T cell activation.

Introduction

Lung cancer is the most lethal cancer type in both men and women worldwide [1,2]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers and although prognosis has improved with the development of targeted drugs and immune checkpoint inhibitors, the overall survival rate remains low due to intrinsic and acquired resistance [3,4].

Tumour development and therapy resistance are regulated, amongst others, by the tumour microenvironment (TME) [5,6]. The TME includes extracellular matrix, endothelial cells, fibroblasts and immune cells like macrophages and T cells. Tumour cells and cells from the TME interact directly and indirectly via the secretion of cytokines and chemokines [7]. Macrophages are present in the TME throughout all stages of tumour development and have different activation states, being M0 (resting/undifferentiated macrophages), M1 and M2. Upon cytokine stimulation monocytes differentiate into M0 macrophages, which in turn can polarize into M1 or M2 subtypes. A variety of M1 and M2 subtypes have been characterised by specific markers and gene signatures [8–10]. M1 macrophages have been linked with pro-inflammatory/anti-tumorigenic characteristics whereas M2 macrophages are associated with anti-inflammatory/pro-tumorigenic properties. Tumour-infiltrated M2 macrophages are also referred to as tumour associated macrophages (TAMs) (12–14). Cytotoxic $CD8^+$ T cells are important immune effector cells and elevated levels in tumours have been associated with improved cancer prognosis [14]. The potency of effector $CD8^+$ T cells depends on their ability to infiltrate into the tumour, which requires differentiation and proliferation from naïve T cells into effector cells regulated by specific cytokines and co-stimulatory factors. Cytokines derived from tumour cells and TAMs can prevent and reduce $CD8^+$ T cell activation thereby hampering anti-tumour activity [14,15].

Tumour-necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) is known for its potent and selective induction of cell death via pro-apoptotic TRAIL receptors in a broad range of tumour cell types, including NSCLC. Although the apoptosis effector function of TRAIL has shown promise in preclinical studies, its efficacy in clinical trials thus far is limited due to tumour apoptosis resistance and unwanted non-canonical functions like stimulation of tumour cell migration, invasion and proliferation via various pathways [16,17]. Previously, we identified a TRAIL-induced RIPK1-Src-STAT3 signalling pathway that stimulates pro-tumorigenic activity in resistant NSCLC cells [18]. Src is a non-receptor tyrosine kinase that is often overexpressed or hyper-activated in cancer and is known to stimulate tumour development and metastatic progression [19]. Furthermore, Src has been implicated in regulating tumour infiltrating immune cells via cytokine-mediated crosstalk between cancer and inflammatory cells [18,20].

Tumour intrinsic and TME extrinsic mechanisms can affect the efficacy of TRAIL signalling

and treatment outcome (reviewed in [7]). It has been reported that TRAIL exposure of apoptosis-resistant cancer cells causes a tumour supportive immune-modulatory effect by stimulating the secretion of pro- and anti-inflammatory cytokines [21,22]. Particularly, TRAIL resistant NSCLCs were shown to produce a pro-tumorigenic secretome in which C-C Motif Chemokine Ligand 2 (CCL2) converted monocytes into M2-like macrophages and stimulated their infiltration into the tumour [22]. Others demonstrated that Src activation in both infiltrating immune cells and cancer cells mediated increased cytokine production thus enhancing inflammation and tumour development [23].

In the present study we investigated the possible involvement of Src in TRAIL-dependent modulation of the NSCLC cell secretome and the subsequent impact on immune cell activity. The secretomes of apoptosis resistant Src proficient and deficient A549 cells were analysed by cytokine arrays and mass-spectrometry analysis to identify possible Src- and/or TRAIL-dependent immune modulatory factors. We found that Src and rhTRAIL affected the composition of the secretome of A549 NSCLC cells. Exposing macrophages to all CM-L resulted in a significant decrease in cell population with CD80-CD86 M1 marker expression, whereas an increase in cells with M1 CD86 marker expression alone was detected. No significant effects on macrophage differentiation/polarization or T cell activation were observed for other macrophage markers and CM-S/L.

Material and methods

Cell culture and generation of conditioned media A549 cells were obtained from the ATCC and cultured in RPMI 1640 (Gibco, Waltham, USA) with 10% FBS (Bodinco, Alkmaar, The Netherlands). Cells were maintained in a humidified 5% CO2 atmosphere at 37˚C. The cell lines were regularly tested for their authenticity by short tandem repeat (STR) profiling DNA fingerprinting (Baseclear, Leiden, The Netherlands) and for mycoplasm by PCR. A549 Src knockout (S-) and A549 empty vector control cells (S+) have been generated previously (Chapter 3). To obtain conditioned media (CM) $6*10^6$ cells were seeded in a T75 in RPMI + 10% FCS, unless otherwise stated, and grown overnight to ~60-70% confluency. The cells were untreated (T-), or treated with 50 ng/ml rhTRAIL (T+) (Peprotech Inc., London, UK) for 1-2 h (CM-S) or 24 h (CM-L) in RPMI + 10% FCS. The cells were washed twice with RPMI or RPMI with 0.5% FCS and cultured further in the same medium for 24 h to obtain CM. Cell debris was removed by centrifugation at 211 RCF for 3 min or by filter sterilized with a 0.2 µM filter (Corning, Corning, USA) and CM were used immediately or stored at -20˚C for later use.

PBMC isolation, macrophage differentiation and polarization Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (see also Fig.1). Buffy coats from

2 different donors (Sanquin, Groningen, Netherlands), or freshly obtained blood was diluted 1:3 in PBS and 30 ml was added slowly on top of 15 ml Ficoll. The cells were centrifuged for 20 min at 900 RCF without brake at RT, collected carefully and washed thrice in PBS. PBMCs were used immediately, or stored in 90% FCS + 10% DMSO at -80˚C. To isolate monocytes, PBMCs were resuspended RPMI1640 (+ L-glutamine + 25mM HEPES; Gibco) + 1% sodium-pyruvate (Gibco) + 1% non-essential amino acids (Gibco) + 1% penicillin/streptomycin (Gibco) at $2.5*10^6$/ml and $5*10^6$ PBMCs were plated per well in a 6 wells plate. After 4 h in a humidified 5% CO2 atmosphere at 37˚C incubator, allowing monocyte adherence, remaining non-adherent cells were removed by washing twice with RPMI. M0 differentiation was induced by adding 2ml of RPMI + 10% FCS + 1% sodium-pyruvate, 1% non-essential amino acids, 1% penicillin/streptomycin and 20 ng/ml M-CSF (Biolegend, San Diego, USA) to the adhered monocytes for 7 days. The medium was replenished every 2-3 days. Thereafter the macrophages were polarized into either M1 or M2 subtypes for 24 or 48 h after adding either 100 ng/ml LPS (Sigma) and 20 ng/ml IFN-γ (R&D systems, Minneapolis, USA) for M1 subtype, or 20 ng/ml IL-4 (Biolegend) for M2 subtype. M0 macrophages were maintained with freshly added 20 ng/ml M-CSF. To determine the effects of CM on monocytes and M0 macrophages CM-S/L + 10% FCS was added for 24 to 48 h followed by M0/M1/M2 flow cytometric marker analyses (see also Fig. 1A). CM-S, diluted 1:1 with RPMI, with 10% FCS was added to the monocytes immediately upon isolation for 7 days (Fig. 1B).

Flow cytometry Macrophages were washed twice with cold PBS and detached with 1 ml of non-enzymatic cell dissociation solution (Sigma). The cell suspension was centrifuged for 5 min at 400 RCF and washed with PBS + 2% FCS (FACS buffer). To determine M0, M1 and M2 status the cells were stained with CD11b-FITC (1:100; M0), CD68-PeCy7 (1:500, M0), CD86-PE (1:100; M1), CD80-BV421 (1:500; M1), CD163-PerCPcy5 (1:100; M2) and CD206-APC (1:500; M2) and ZombieAqua (1:100) for cell viability (all antibodies were obtained from Biolegend) in 100 µl FACS buffer per sample and incubated for 30 min at 4°C in the dark. Subsequently, the cells were washed with FACS buffer and resuspend in cold PBS-formaldehyde (Sigma) 2% for 10 min, washed twice with FACS buffer and resuspended in 500 µl FACS buffer for further analyses with the LSR-II (BD biosciences). With FLowJo macrophages were selected based on forward and side scatter and subsequently their marker expression was determined. The data was analysed with Graphpad PRISM software.

T-cell isolation and activation PBMCs were thawed and $1*10^8$ cells were resuspended in 1 ml PBS + 3% FCS + 10 mM EDTA. The $CD8^+$ T cells were isolated from the PBMCs by a MagniSort human naïve T cell enrichment kit (ThermoFisher) according manufacturer's protocol. Subsequently, the cells were labelled with CellTrace Violet cell proliferation kit (ThermoFisher) according to manufacturer's protocol to analyse the expansion of T cells. To that end, $1*10^5$ T cells were plated per well in a 96 wells plate in different concentrations CM

+ RPMI 1640 (+ L-glutamine + 25mM HEPES; Gibco) supplemented with 10% FCS. The cells were immediately activated with 100 IE/ml IL-2 and different amounts (0, 1:4, 1:16 or 1:32) of Dynabeads human activator CD3/CD28 (ThermoFisher). After 5 days of proliferation the cells were resuspended carefully and PI (ThermoFisher) was added 1:1000. T cell activation and expansion was measured by FACSverse (BD) flow cytometry. The data was analysed with FlowJo and Graphpad PRISM software.

Cytokine arrays Cells ($5*10^5$) were seeded per well in a 6 wells plate, incubated overnight and untreated or treated with 50 ng/ml rhTRAIL for 1 hr. Subsequently, cells were washed twice with serum free RPMI and incubated for 24 h in 1.5 ml serum free RPMI. CM was taken and centrifuged for 3 min at 211 RCF prior to analyses with the AAH-CYT-5 cytokine antibody array kit (RayBio, Peachtree Corners, USA) according to manufacturer's protocol. Cytokine secretion in the CM was determined with the Chemidoc imaging system (BioRad, Hercules, USA) and quantified by ImageJ.

Mass spectrometry For secretome analyses all CM were collected and ultra-centrifuged with Amicon Ultracel-3 centrifugal filter units (Merck Millipore, Burlington, USA) at 4000 RCF for 1 hr at RT. The protein concentration was determined by Bradford and 500 µg protein per sample was used for mass spectrometry analysis. Protein mixtures were loaded on RunBlue 1 mm*10 well 8%- Bis-Tris –gel (Expedeon, Cambridge, UK) after heating the mixture at 70^{o}C for 10 min. Whole gel processing procedure was performed as described previously [24]. Online chromatography of the extracted tryptic peptides was performed with the Ultimate 3000 HPLC system (Thermo Scientific) coupled online to a Q-Exactive-Plus mass spectrometer with a NanoFlex source (Thermo Scientific) equipped with a stainless-steel emitter. The data were processed as described previously (21). In short, the PEAKS 8.0 (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) software was applied to the spectra generated by the Q-exactive plus mass spectrometer to search against a Human Protein database (SwissProt containing 20197 entries). False discovery rate was set at 0.1% and at least 2 unique peptides per protein should be present. Next the secreted proteins were selected based on their signalling peptide with Phobius and signalP, free online available software. The LOG2 of the fold change was calculated and secreted proteins with a LOG2 fold change of ≥2 were selected for further analysis. Next, GO biological function analysis was performed with STRING 11.0 software.

Statistics False discovery rate (FDR) statistics were performed on the GO biological process analysis and KEGG pathway analysis. Significant changes are shown as LOG2(p) fold changes.

Results

Effects of Src and rhTRAIL on A549 conditioned media and macrophage polarization We started by exploring the possible effects of Src status and/or rhTRAIL exposure on altering the secretome of A549 cells by investigating the effects of the corresponding A549-derived conditioned media (CM) on macrophage polarization (for experimental outline see Fig. 1). CM was obtained from A549 Src proficient (S+) and deficient (S-) cells either untreated (T-) or treated with rhTRAIL (T+) for 1-2 h (short, S) or 24 h (long, L) and their effects on M0 macrophage polarization were investigated (Fig. 1A). The four different CM-S induced M1 and M2 polarization similarly as control polarized macrophages (Fig. 2A). CD80 was expressed in the majority of control M1 macrophages, whereas CD80-CD86 was co-expressed in approximately 60% of the cells cultured in CM-S. Flow cytometric marker expression analyses showed that all CM-L, independent of Src status and rhTRAIL treatment, induced M0 (CD11b, CD68) polarization into M1 (CD80, CD86) and M2 (CD163, CD206) to a similar extent (Fig. 2B). The fraction of cells expressing M0 and M2 markers was similar to control polarized macrophages, whereas M1 polarization was partially less, indicated by lower percentages of CD80/CD86 expression in the CM-L exposed macrophages. Compared to CM-S, CM-L induced CD86 expression in a larger fraction of cells. Together, these experiments indicated that the different CM-S and CM-L polarize macrophages to a similar extent, independent from Src status and TRAIL treatment.

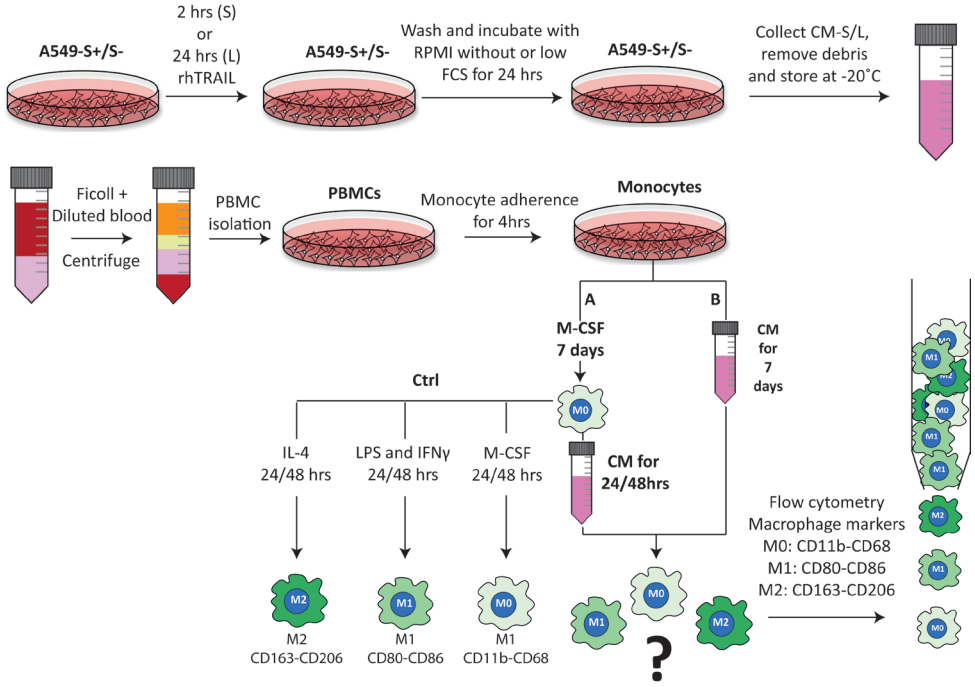


Figure 1. Outline of the experimental design to examine the possible immune modulatory effects of TRAIL treatment and Src status on protein secretion by A549 cells *Graphical outline of the experimental*

design. Src proficient (S+) or deficient (S-) A549 were treated with rhTRAIL for 1-2 (short, S) or 24 h (long, L), washed and incubated with low/no serum containing medium for 24 h and conditioned medium (CM) was collected and stored at -20°C for further use (see methods for more details). PBMCs were isolated from buffy coats by Ficoll gradient centrifugation and monocytes were isolated after adherence for ~4 h. As controls, monocytes were differentiated into M0 macrophages by M-CSF for 7 days and subsequently polarized into M1 or M2 macrophages, or kept as M0, after exposure to the indicated cytokines for 24 to 48 h. To investigate the effect of the four different secretomes on monocytes or M0 macrophages ***A)*** *the different CM were added to M0 macrophages for 24 to 48 h, or* ***B)*** *CM-S added immediately to the isolated monocytes and incubated for 7 days. M0, M1 or M2 macrophage marker expression was determined by flow cytometry (M0: CD11b and CD68; M1: CD80 and CD86; M2: CD163 and CD206).*

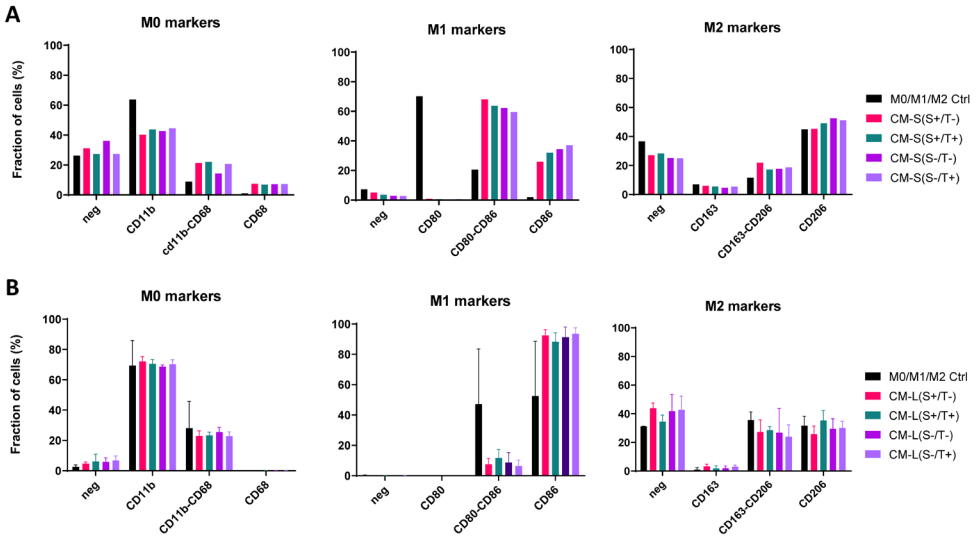


Figure 2. The effect of Src status and TRAIL treatment on A549-derived CM on macrophage polarization *M0 differentiated monocytes were exposed to the different CM derived from A549 cells and M0, M1 and M2 marker expression was determined by flow cytometry. The relative cell fractions of each marker set are indicated. Control polarized M0, M1 and M2 macrophages were included as controls.* ***A)*** *The effects of the indicated CM obtained after 1-2 h rhTRAIL exposure (CM-S(S+/T-), CM-S(S+/T+), CM-S(S-/T-) or CM-S(S-/T+)) on macrophages polarization are shown (N=1). Exposure of the different CM to M0 macrophages resulted in macrophages polarization, although independent from Src status or rhTRAIL treatment.* ***B)*** *The effects of the CM obtained after 24 h rhTRAIL exposure (CM-L(S+/T-), CM-L(S+/T+), CM-L(S-/T-) or CM-L(S-/T+)) on macrophage polarization are shown (N=3). Polarization was compared to M0/M1/M2 controls (N=2).*

Effects of the conditioned media on monocyte differentiation and macrophage polarisation

Next, the possible direct effects of the four different CM-S on monocytes to macrophages differentiation was investigated as well as subsequent macrophage polarization. We continued with CM-S because of the fast response (within 15 min) of A549 cells to rhTRAIL as described in chapter 3, which is also relevant with respect to the short half-life of TRAIL *in vivo*. Isolated monocytes from 2 different donors (Fig. 3A-B) were directly cultured in

2-fold diluted CM-S (CM-$S_{1/2}$) as culturing monocytes in undiluted CM-S resulted in a high amount of cell death (data not shown). All 4 CM-$S_{1/2}$ induced M0 differentiation and M1 and M2 polarization with overall somewhat lower activity of CM-$S_{1/2}$(S+/T-) (Fig. 3). These experiments did not show a major effect of rhTRAIL and Src status on CM dependent monocyte differentiation and polarisation, although this should be further substantiated.

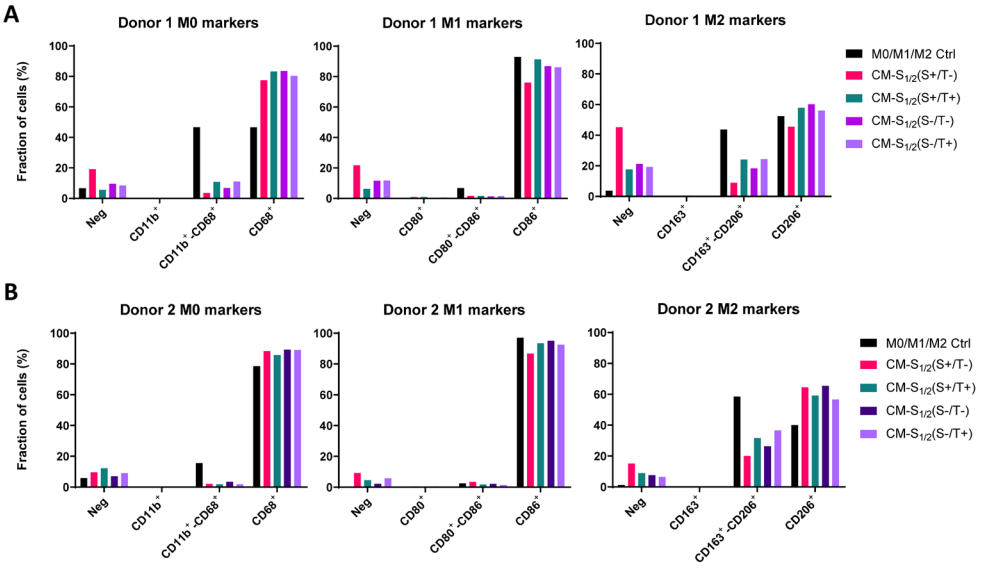


Figure 3. The effect of Src status and TRAIL treatment on A549-derived CM on monocyte differentiation and macrophage polarization *The indicated CM, diluted 1:1 with fresh medium, were tested for their ability to induce monocyte differentiation into M0, and subsequently M1/ M2 macrophage polarization. Monocytes were obtained from* ***A)*** *donor 1 and* ***B)*** *donor 2. M0, M1 and M2 marker expression was determined by flow cytometry and the relative cell fractions of each marker set are indicated (N=1). As controls standard polarized M0, M1 and M2 macrophages are included. The fraction of macrophages expressing M0 markers (CD11b-CD68), M1 markers (CD80-CD86) or M2 markers (CD163-CD206) after culturing monocytes in CM-$S_{1/2}$(S+/T-), CM-$S_{1/2}$(S+/T+), CM-$S_{1/2}$(S-/T-) or CM-$S_{1/2}$(S-/T+) for 7 days.*

Effects of the conditioned media on $CD8^+$ T cell activation The effects of the different CM-S on T cell activation were analysed subsequently. $CD8^+$ T cells were isolated from PBMCs obtained from two different donors and exposed to undiluted, 1:2 diluted (CM-$S_{1/2}$) and 1:4 diluted CM-S (CM-$S_{1/4}$). Their effects on human T-activator CD3/CD28 Dynabeads stimulated proliferation was examined. Prior to this, T cell activation was determined at different dilutions of CD3/CD28 Dynabeads by flow cytometry. Up to six T cell divisions could be detected that was optimal at a 1:4 beads/ cell ratio (Fig. 4A and B). No effects of CM-S were found when the T-cells were cultured in undiluted or 1:2 diluted CM-S (data not shown). T cell proliferation decreased moderately after six divisions when cultured in CM-$S_{1/4}$(S+/

T+) when compared to the CM-$S_{1/4}$(S+/T-). This effect was not seen in CM-$S_{1/4}$(S-/T+) vs CM-$S_{1/4}$(S-/T-) (Fig. 4C). These results suggest that the repression of T cell activation by the rhTRAIL-induced secretome is Src dependent.

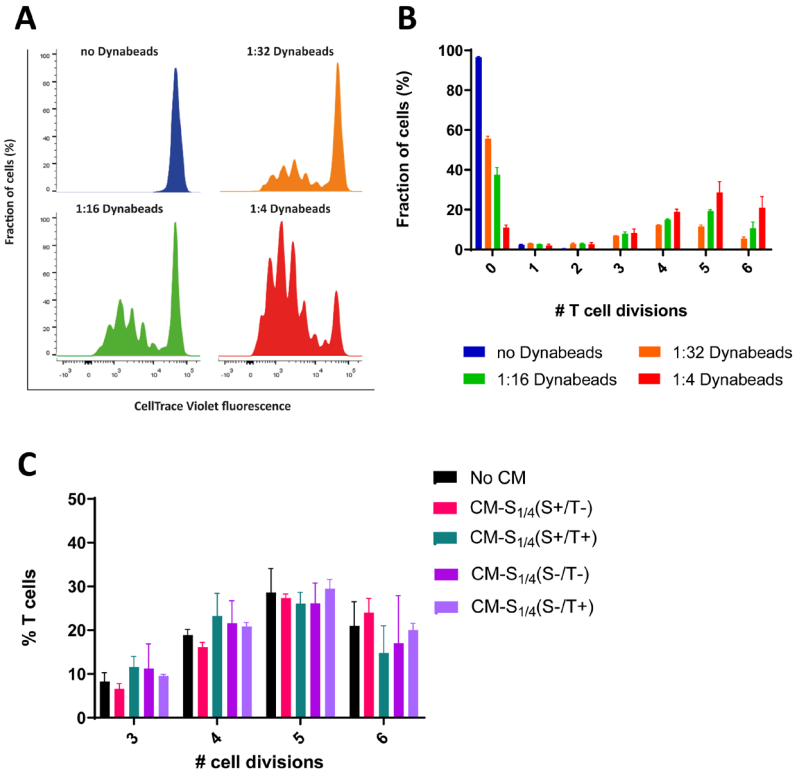
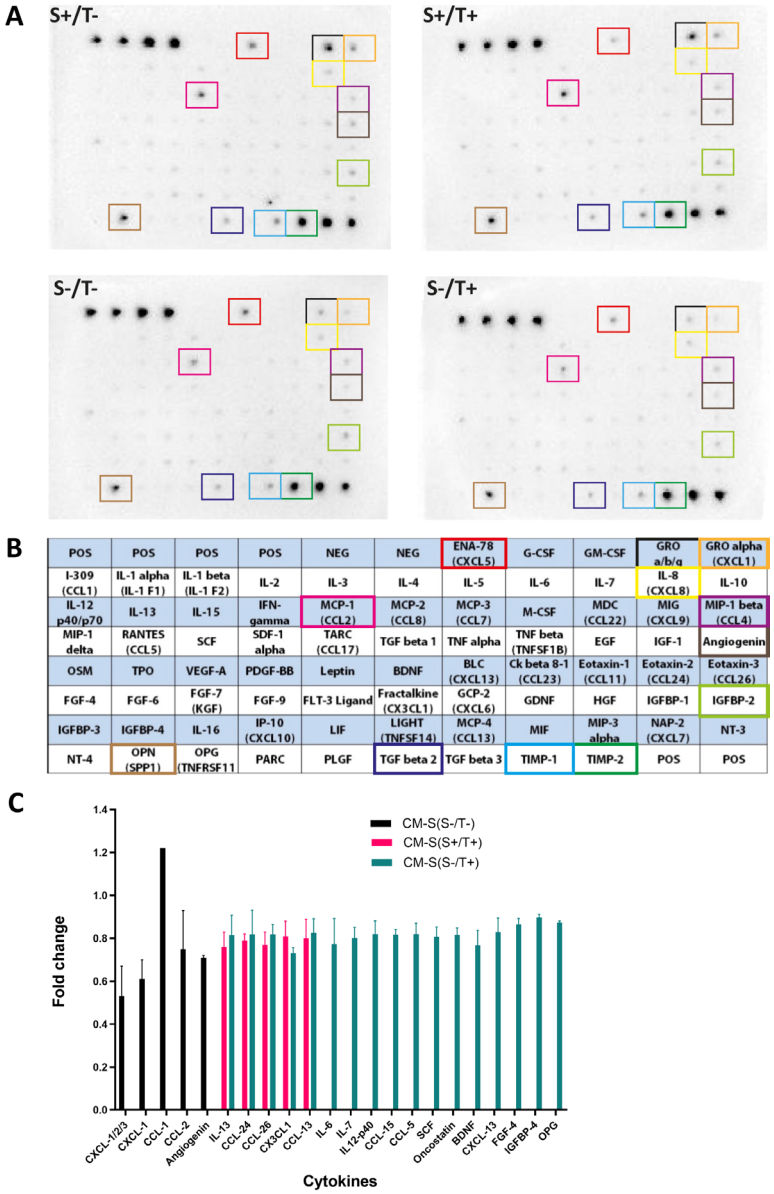


Figure 4. The effects of Src status and TRAIL treatment on A549-derived CM on CD8⁺ T cell activation *The indicated CM were diluted 1:4 in medium and incubated with freshly isolated CD8⁺ T cells obtained from PBMCs in absence/presence of 0, 1:4, 1:16 or 1:32 human activator CD3/CD28 Dynabeads. CellTrace Violet fluorescence was used to determine T cell proliferation.* ***A)*** *Dose dependent effects of CD3/CD28 Dynabeads dilution on T cell proliferation. The number of cell divisions is indicated.* ***B)*** *Bar graph representation of the number of CD8⁺ T cell divisions at different CD3/CD28 Dynabeads dilutions shown in panel A.* ***C)*** *CD8⁺ T cell proliferation upon addition of CM-$S_{1/4}$(S+/T-), CM-$S_{1/4}$(S+/T+), CM-$S_{1/4}$(S-/T-) or CM-$S_{1/4}$(S-/T+) and 1:4 Dynabeads. T cells were isolated from two different donors (N=2); results mean +/- SD.*

Cytokine secretion in the different conditioned media Next, the effect of Src status and rhTRAIL treatment on cytokine secretion in the four different CM-S was investigated using a cytokine array (Fig. 5A; corresponding cytokines are indicated in Fig. 5B). ENA-78 (CXCL5), GROa/b/c, GRO alpha, IL-8 (CXCL8), MCP1 (CCL2), MIP1-beta (CCL4), Angiogenin, IGFBP-2, OPN (SPP1), TGF-beta2, TIMP-1 and TIMP-2 were detected in the four different CM-S (Fig. 5 & Supplementary fig. 1). Quantitative analysis demonstrated that CM-S(S-/T-) compared to CM-S(S+/T-) had decreased levels of CXCL-1/2/3, CXCL-1, CCL-2 and angiogenin, and

increased CCL-1 secretion ranging from 1.4 to 2-fold changes (Fig. 5C). Both CXCL-1/2/3 and CCL-2 are known to mediate polarization of macrophages [26,27]. CCL-13 was decreased in CM-S from both Src proficient and deficient cells after rhTRAIL treatment, which is a known chemoattractant for T cells [28] (Fig. 5C). Together, these findings suggest that particularly Src and to a lesser extent rhTRAIL modulated cytokine secretion by A549 cells.



POS	POS	POS	POS	NEG	NEG	ENA-78 (CXCL5)	G-CSF	GM-CSF	GRO a/b/g	GRO alpha (CXCL1)
I-309 (CCL1)	IL-1 alpha (IL-1 F1)	IL-1 beta (IL-1 F2)	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8 (CXCL8)	IL-10
IL-12 p40/p70	IL-13	IL-15	IFN-gamma	MCP-1 (CCL2)	MCP-2 (CCL8)	MCP-3 (CCL7)	M-CSF	MDC (CCL22)	MIG (CXCL9)	MIP-1 beta (CCL4)
MIP-1 delta	RANTES (CCL5)	SCF	SDF-1 alpha	TARC (CCL17)	TGF beta 1	TNF alpha	TNF beta (TNFSF1B)	EGF	IGF-1	Angiogenin
OSM	TPO	VEGF-A	PDGF-BB	Leptin	BDNF	BLC (CXCL13)	Ck beta 8-1 (CCL23)	Eotaxin-1 (CCL11)	Eotaxin-2 (CCL24)	Eotaxin-3 (CCL26)
FGF-4	FGF-6	FGF-7 (KGF)	FGF-9	FLT-3 Ligand	Fractalkine (CX3CL1)	GCP-2 (CXCL6)	GDNF	HGF	IGFBP-1	IGFBP-2
IGFBP-3	IGFBP-4	IL-16	IP-10 (CXCL10)	LIF	LIGHT (TNFSF14)	MCP-4 (CCL13)	MIF	MIP-3 alpha	NAP-2 (CXCL7)	NT-3
NT-4	OPN (SPP1)	OPG (TNFRSF11	PARC	PLGF	TGF beta 2	TGF beta 3	TIMP-1	TIMP-2	POS	POS

Figure 5. Src and/or rhTRAIL dependent modulation of cytokine expression in CM-S from A549 cells *Cytokine expression in the four different CM-S from A549 were determined by cytokine arrays.* ***A)***

Cytokine array dot blots are shown obtained from CM-S from A549-S+ or A549-S- cells treated for 1 hr with 50 ng/ml rhTRAIL. Most abundant and differentially expressed cytokines are indicated. ***B)*** *Content of the cytokine array with corresponding positions indicated in coloured boxes. Indicated cytokines are, ENA-78 (CXCL5; red), GROα/b/c (black), GRO alpha (orange), IL-8 (CXCL8; yellow), MCP1 (CCL2; pink), MIP1-beta (CCL4; purple), Angiogenin (grey), IGFBP-2 (light green), OPN (SPP1; brown), TGF-beta2 (dark blue), TIMP-1 (light blue) and TIMP-2 (dark green). An independent experiment is also shown in supplementary figure 1.* ***C)*** *Quantitative analysis of cytokine expression (+/- SD) compared to CM-S(S+/T-) (N=2). Hence, Src status and rhTRAIL treatment modulate cytokine secretion by A549 cells.*

Analyses of the secretomes by mass spectrometry We continued by analysing the eight different secretomes in greater detail by mass-spectrometry (MS). In CM-S(S+/T-), CM-S(S+/T+), CM-S(S-/T-) and CM-S(S-/T+), 146, 88, 149 and 106 secreted proteins were identified, respectively (Fig. 6A/B). In CM-L(S+/T-), CM-L(S+/T+), CM-L(S-/T-) and CM-L(S-/T+) samples more secreted proteins were detected resulting in 180, 171, 411 and 376 proteins, respectively (Fig. 6A/B). Most proteins identified in the CM-S(S+/T-), CM-S(S+/T+), CM-S(S-/T-) or CM-S(S-/T+) conditions were overlapping with the same CM-L conditions (Fig. 6A). The number of proteins detected in CM-L (411) from untreated Src deficient cells was higher than that in CM-L from untreated Src proficient cells (180) and this difference remained after rhTRAIL treatment (376 vs 176, respectively) (Fig. 6B). In both CM-S and CM-L, rhTRAIL treatment reduced the number of (unique) secreted proteins, independent of the Src status (Fig. 6B). Several factors identified in the cytokine arrays were also detected by MS analyses, such as TIMP2 in all CM-S conditions, TIMP1 in CM-S(S+/T-) and in all CM-L conditions, and CXCL5 in CM-L(S-/T-) and CM-L(S-/T+). A KEGG pathway analysis was performed on all detected proteins in the individual CM. The top 10 most significantly annotated pathways are depicted in Supplementary fig. 2, but did not show clear differences.

GO analysis was performed on the uniquely detected proteins for each individual CM-S or CM-L condition (Table 1) and the top 10 most significantly annotated processes are depicted in Fig. 6C. In CM-S(S+/T-) the processes were mostly related to bacteria defence responses, whereas in CM-S(S+/T+) immune modulatory processes were found (Fig. 6C). Of note, negative regulation of extrinsic apoptosis signalling pathways (asp) via death domain receptors (DDR) due to expression of HMOX1 and FADD, was seen in CM-S(S+/T+) and might be a factor involved in the TRAIL apoptosis resistance of A549 cells. Comparing CM-S from untreated Src deficient and proficient cells showed an increase in metabolic and developmental processes in Src deficient CM-S. GO analyses of the four different CM-L indicated presence of proteins linked with glycosylation and immune processes in CM-L(S+/T-), metabolic and immune processes in CM-L(S+/T+) and metabolic and catabolic processes involving various lipids in CM-L(S-/T-). In both CM-S(S-/T+) and CM-L(S-/T+) no GO biological processes were found, possibly because of a low number of unique proteins. Overall, these preliminary results suggest Src and rhTRAIL dependent changes in the A549 secretome, of which some can be linked to alterations in immune-related processes.

Table 1. The unique proteins in CM-S and CM-L used for GO Biological processes analysis

CM-S(S+/T-)	CM-S(S+/T+)	CM-S(S-/T-)	CM-S(S-/T+)	CM-L(S+/T-)	CM-L(S+/T+)	CM-L(S-/T-)	CM-L(S-/T+)
CATC	HMOX1	COCH	KTN1	TRFL	DHE3	VTNC	FRAS1
CO4A2	GELS	APRV1	FUMH	BPIB1	ACPH	LTBP4	KTN1
ZA2G	RAB10	MATN3	ALBU	MFGM	IPO9	APOH	DCBD2
LYSC	CLU	B4GA1	RHG01	MUC5B	UBR4	LAMB2	CHSTB
SMOC1	MATR3	FSTL1	RTN4	GALT7	GELS	MATN2	STAT
B2MG	UBR4	FUCO	PRDX4	NNRE	BCAM	CATL2	
GALT7	FADD	ISK5		TIG1	G3PT	DKK1	
LTBP3	SPB4	EPDR1		AMBP	NIT2	MGAT2	
FAT1	SNX1	SRPX		CATC		EFNA1	
AGRG6	ACPH	FBN2		ZA2G		PCDH9	
CSF1		DNS2A		EXT1		SAP3	
TFPI1		SEM4B				ASPH	
VGF		C1R				SGCE	
PROL4		GALNS				RAB10	
DSC1		AMD				B3GA3	
TIMP1		NGAL				PROS	
HSP13		CATL2				RCN1	
PROS		COCA1				ASAH1	
PIP		CAB45				ADAM9	
ERP29		PCP				FUCO	
DCD		MFGM				S39AA	
UGGG1		VLDLR				MPRI	
		LRC15				PLOD3	
		BMP1				NEUR1	
		SAP				FSTL3	
		PPGB				IMPA3	
		IBP2				PPT1	
		DSG4					
		SDC4					
		SEM3C					
		TRFM					
		CYTM					
		CO5A2					
		FSTL5					

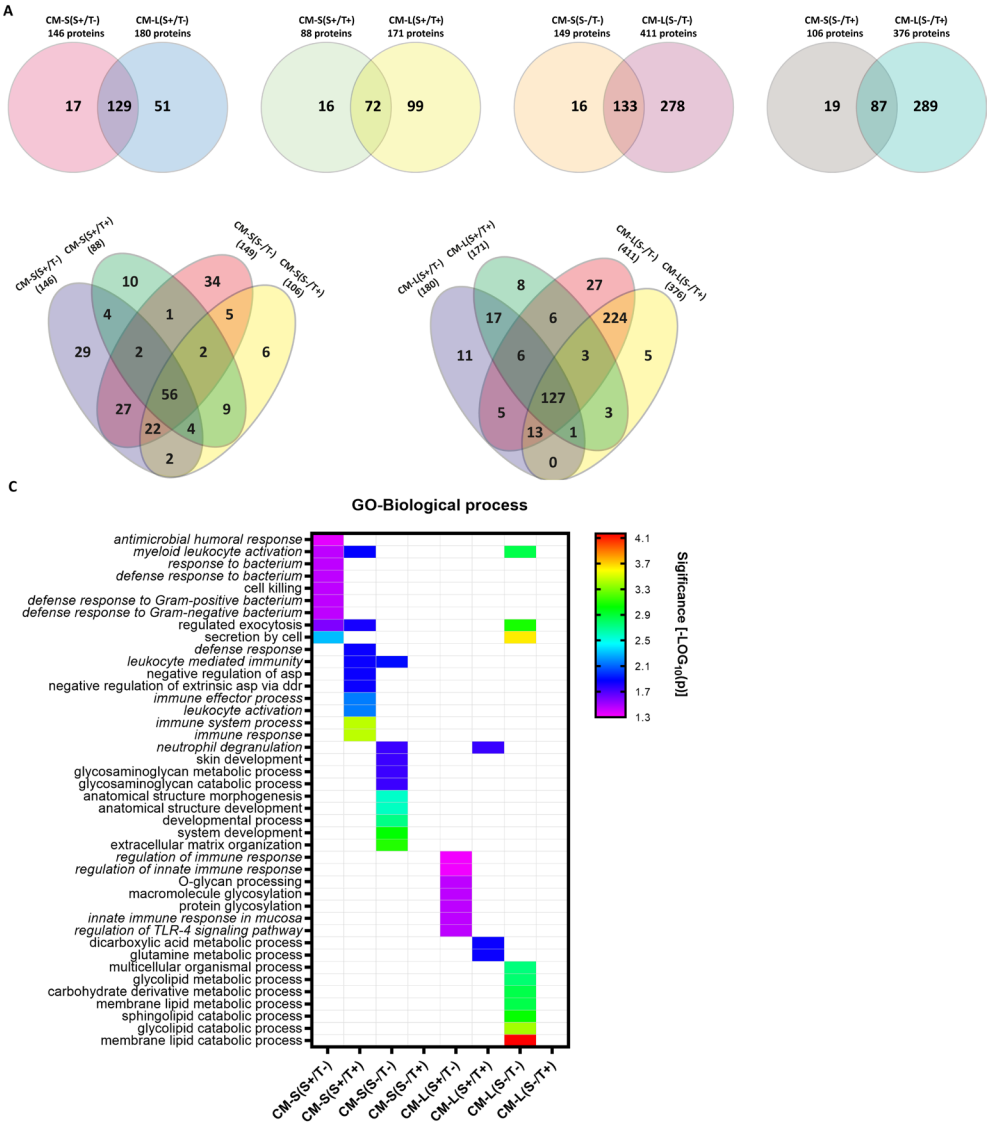


Figure 6. Mass-spectrometry analysis of the CM derived from Src-/+ and rhTRAIL (un)treated A549 cells *Mass spectrometry analysis was performed on CM-S and CM-L from A549-S+ and A549-S- cells after 2 hr (CM-S) or 24 (CM-L) hr 50ng/ml rhTRAIL treatment (N=1). Proteins in the different CM were analysed by LC-MS. A) Venn diagrams indicating the numbers of detected secreted proteins and overlapping proteins in CM-S and CM-L per condition, S+/T-, S+/T+, S-/T- and S-/T+ respectively. B) Venn diagrams of the different CM-S and CM-L. C) GO analyses of the uniquely present proteins in each of the four CM-S or CM-L. Significant differences were determined by the FDR and are shown as the -Log10(p). The top 10 most significantly annotated biological processes are depicted.*

Discussion

In this study we examined the effect of TRAIL treatment and Src status in A549 cells on protein secretion and its subsequent modulatory effects on immune cells. Our data suggest that the different conditioned media (CM-L and CM-S), derived from Src proficient and deficient A549 cells, untreated or treated with rhTRAIL, to a similar extend were able to induce M1 and M2 polarization. Furthermore, CM from rhTRAIL treated Src proficient A549 cells, compared to untreated control, seemed to moderately reduce T cell activation. Cytokine array analyses of Src proficient cells demonstrated increased secretion of several cytokines. More in depth analysis of the secretomes by mass-spectrometry showed effects of both Src status and TRAIL exposure on the number of proteins secreted by NSCLC cells, some of which are known to modulate immune cell activity. Together, the data obtained so far indicate that Src and rhTRAIL affect cytokine production of A549 cells. However, these cytokine alterations did not have strong effects in functional assays on macrophage polarization and T cell activation, indicating that additional *in vitro* and *in vivo* studies are required to gain more insight in Src and TRAIL induced alterations the secretome.

Inflammation and immune responses can result in reduced or enhanced tumour growth and metastatic spread, depending on tumorigenesis stage (early/late), composition of the TME and the types of cytokines secreted [29]. The effect of TRAIL-induced changes in tumour cell secretomes and consequences on differentiation and polarisation of macrophages have not been well studied. Several studies reported that various cancer cell types, including NSCLC, showed TRAIL-dependent increases of pro-inflammatory cytokines and chemokines. These were able to modify the TME and stimulate monocyte chemotaxis and TAM polarisation, and also enhanced metastatic spread [7,22,30–32]. Our current study mainly extends on a report by Hartwig *et al.* [22] who demonstrated that the secretome of TRAIL treated A549 cells promoted M2 polarization of monocytes [22]. Particularly, we aimed to explore the possible involvement of the previously identified TRAIL receptor/RIPK1/Src pathway on A549 protein secretion and subsequent immune modulatory activity. However, as mentioned above, we did not find evidence that rhTRAIL-induced changes in the secretome affected monocyte polarization to either M1 or M2 phenotypes. It should be noted though, that in the study by Hartwig *et al.* A549 cells were treated with isoleucine zipper TRAIL known to have different TRAIL receptor activation properties than rhTRAIL and, moreover, higher concentrations of the TRAIL receptor agonist were applied, which may also account for different outcomes [22,33].

Src is known to be involved in the production of cytokines in tumour cells via, amongst others, NFκβ, ERK, JNK and STAT3 [34,35]. NFκβ is an important immune modulator and its activation results in cytokine and chemokine production, as well as activation of immune

cells[36]. This makes Src a potential immune modulatory therapeutic target that may lead to increased immune destruction and/or suppression of metastatic spread. Interestingly, inhibition of Src with Dasatinib in CML patients increased the number of immune cells and enhanced their prognosis by reducing relapses [37]. Inhibition of Src with Dasatinib has also been demonstrated to stimulate the cytolytic activity of γδ T cells and subsequent their anti-tumour activity [37]. Furthermore, resistance towards Dasatinib resulted in increased expression of pro-inflammatory and pro-invasive cytokines IL-1β and MMP9 in human thyroid cancer cells [38]. Here, we found that Src proficient A549 cells, when compared to Src deficient cells, had an altered composition of CM-S after rhTRAIL treatment leading to mildly reduced $CD8^+$ T cell activation. Additionally, the secretome analyses showed enhanced expression of CCL2, SERPINB4 and gelsolin which are known to reduce T cell activation [39–41].

Although we did not observe clear effects of rhTRAIL exposure and Src status on A549-derived CM in functional assays, the analyses of the secretomes by cytokine array and mass-spectrometry showed substantial differences. Cytokine array analyses of the CM from Src deficient cells showed decreased levels of especially CXCL-1/2/3 and CCL2. These cytokines are known to stimulate migration and invasion of tumour cells and to enhance angiogenesis, while at the same time stimulating the migration of immune cells towards the tumour resulting in inflammation and pro-tumorigenic effects [42–45]. Furthermore, CXCL-1/2/3 and CCL2 can induce chemoresistance and macrophage polarization [26,27]. CCL2 is also known to reduce $CD8^+$ T cell immune responses and infiltration in tumours [46,47]. Mass spectrometry on the CM-S and CM-L demonstrated that treatment with rhTRAIL in Src proficient cells increased the secretion of factors involved in immune modulation. These include HMOX1, which induces M2 polarisation, as well as SERPINB4 and MATR3, which are involved in biological processes associated with negative regulation of leukocyte activation. Secreted Gelsolin was also detected and has been demonstrated to inactivate $CD4^+$ T and $CD8^+$ T cells in prostate cancer [41]. Src depletion alone increased factors regulating the extracellular matrix. Src depletion combined with rhTRAIL treatment did alter significantly the annotated biological processes, mainly due to detection of low numbers of uniquely expressed proteins. Thus, our mass-spectrometry analyses indicated that short-term rhTRAIL treatment of Src expressing A549 cells alters the secretion of immune modulatory and pro-tumorigenic cytokines, including proteins involved in reducing immune cell effector functions. Although these results need to be further corroborated, the differences detected in secretome composition indicate that TRAIL exposure time and Src dependent changes can have functional consequences, either by directly altering immune cell activity or via modifications of other components of the TME. Further studies are required to determine the effects of TRAIL exposure and Src status on tumour growth and dissemination in additional *in vitro* and *in vivo* models.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424. doi:10.3322/caac.21492
2. Lung and Bronchus Cancer — Cancer Stat Facts. https://seer.cancer.gov/statfacts/html/lungb.html.
3. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. *Nature*. 2018;553(7689):446-454. doi:10.1038/nature25183
4. Hirsch FR, Scagliotti G V., Mulshine JL, et al. Lung cancer: current therapies and new targeted treatments. *Lancet*. 2017;389(10066):299-311. doi:10.1016/S0140-6736(16)30958-8
5. Roma-Rodrigues C, Mendes R, Baptista P V., Fernandes AR. Targeting tumor microenvironment for cancer therapy. *Int J Mol Sci*. 2019;20(4). doi:10.3390/ijms20040840
6. Giraldo NA, Sanchez-Salas R, Peske JD, et al. The clinical role of the TME in solid cancer. *Br J Cancer*. 2019;120(1):45-53. doi:10.1038/s41416-018-0327-z
7. de Looff M, de Jong S, Kruyt FAE. Multiple Interactions Between Cancer Cells and the Tumor Microenvironment Modulate TRAIL Signaling: Implications for TRAIL Receptor Targeted Therapy. *Front Immunol*. 2019;10:1530. doi:10.3389/fimmu.2019.01530
8. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage polarization: Different gene signatures in M1(Lps+) vs. Classically and M2(LPS-) vs. Alternatively activated macrophages. *Front Immunol*. 2019;10. doi:10.3389/fimmu.2019.01084
9. Duluc D, Delneste Y, Tan F, et al. Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood*. 2007;110(13):4319-4330. doi:10.1182/blood-2007-02-072587
10. Roszer T. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators Inflamm*. 2015;2015. doi:10.1155/2015/816460
11. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. 2002;23(11):549-555. doi:10.1016/S1471-4906(02)02302-5
12. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. 2010;22(2):231-237. doi:10.1016/j.coi.2010.01.009
13. Noy R, Pollard JW. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity*. 2014;41(1):49-61. doi:10.1016/j.immuni.2014.06.010
14. Maimela NR, Liu S, Zhang Y. Fates of CD8+ T cells in Tumor Microenvironment. *Comput Struct Biotechnol J*. 2019;17:1-13. doi:10.1016/j.csbj.2018.11.004
15. Farhood B, Najafi M, Mortezaee K. CD8 $^{+}$ cytotoxic T lymphocytes in cancer immunotherapy: A review. *J Cell Physiol*. 2019;234(6):8509-8521. doi:10.1002/jcp.27782
16. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: Decisions between life and death. *Int J Biochem Cell Biol*. 2007;39(7-8):1462-1475. doi:10.1016/j.biocel.2007.02.007
17. Azijli K, Weyhenmeyer B, Peters GJ, de Jong S, Kruyt FAE. Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ*. 2013;20(7):858-868. doi:10.1038/cdd.2013.28
18. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. *Kinome Profiling of Non-Canonical TRAIL Signaling Reveals RIP1-Src-STAT3 Dependent Invasion in Resistant Non-Small Cell Lung Cancer Cells*. *J Cell Sci*. 2012;125(Pt 19):4651-4661. doi:10.1242/jcs.109587
19. Guarino M. Src signaling in cancer invasion. *J Cell Physiol*. 2010;223(November 2009):14-26. doi:10.1002/jcp.22011
20. Liu ST, Pham H, Pandol SJ, Ptasznik A. Src as the link between inflammation and cancer. *Front Physiol*. 2014;4:416. doi:10.3389/fphys.2013.00416
21. Falschlehner C, Schaefer U, Walczak H. Following TRAIL's path in the immune system. *Immunology*. 2009;127(2):145-154. doi:10.1111/j.1365-2567.2009.03058.x
22. Hartwig T, Montinaro A, von Karstedt S, et al. The TRAIL-Induced Cancer Secretome Promotes a Tumor-Supportive Immune Microenvironment via CCR2. *Mol Cell*. 2017;65(4):730-742.e5. doi:10.1016/j.molcel.2017.01.021

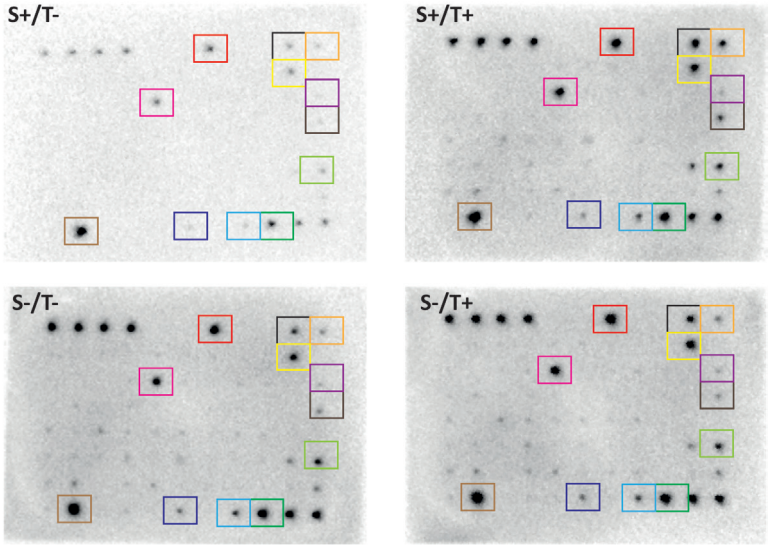
23. Liu ST, Pham H, Pandol SJ, Ptasznik A. Src as the link between inflammation and cancer. *Front Physiol*. 2014;4 JAN. doi:10.3389/fphys.2013.00416
24. Piersma SR, Warmoes MO, de Wit M, de Reus I, Knol JC, Jiménez CR. Whole gel processing procedure for GeLC-MS/MS based proteomics. *Proteome Sci*. 2013;11(1). doi:10.1186/1477-5956-11-17
25. Zhang J, Xin L, Shan B, et al. PEAKS DB: De novo sequencing assisted database search for sensitive and accurate peptide identification. *Mol Cell Proteomics*. 2012;11(4). doi:10.1074/mcp.M111.010587
26. Couderc B, Naour A Le, Prat M, et al. Mesenchymal stromal cells release CXCL1/2/8 and induce chemoresistance and macrophage polarization. *bioRxiv*. December 2018:482513. doi:10.1101/482513
27. Sierra-Filardi E, Nieto C, Domínguez-Soto Á, et al. CCL2 Shapes Macrophage Polarization by GM-CSF and M-CSF: Identification of CCL2/CCR2-Dependent Gene Expression Profile. *J Immunol*. 2014;192(8):3858-3867. doi:10.4049/jimmunol.1302821
28. Mendez-Enriquez E, García-Zepeda EA. The multiple faces of CCL13 in immunity and inflammation. *Inflammopharmacology*. 2013;21(6):397-406. doi:10.1007/s10787-013-0177-5
29. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. *Cell*. 2010;140(6):883-899. doi:10.1016/j.cell.2010.01.025
30. Trauzold a, Siegmund D, Schniewind B, et al. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. *Oncogene*. 2006;25:7434-7439. doi:10.1038/sj.onc.1209719
31. Zhou D-H, Trauzold A, Röder C, Pan G, Zheng C, Kalthoff H. The potential molecular mechanism of overexpression of uPA, IL-8, MMP-7 and MMP-9 induced by TRAIL in pancreatic cancer cell. *Hepatobiliary Pancreat Dis Int*. 2008;7(2):201-209. http://www.ncbi.nlm.nih.gov/pubmed/18397859.
32. Henry CM, Martin SJ. Caspase-8 Acts in a Non-enzymatic Role as a Scaffold for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation. *Mol Cell*. 2017;65(4):715-729.e5. doi:10.1016/j.molcel.2017.01.022
33. Ganten TM, Koschny R, Sykora J, et al. Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. *Clin Cancer Res*. 2006;12(8):2640-2646. doi:10.1158/1078-0432.CCR-05-2635
34. Ramnath RD, Sun J, Bhatia M. Involvement of Src family kinases in substance P-induced chemokine production in mouse pancreatic acinar cells and its significance in acute pancreatitis. *J Pharmacol Exp Ther*. 2009;329(2):418-428. doi:10.1124/jpet.108.148684
35. Ungefroren H, Sebens S, Groth S, Gieseler F, Fändrich F. Differential roles of Src in transforming growth factor-β regulation of growth arrest, epithelial-to-mesenchymal transition and cell migration in pancreatic ductal adenocarcinoma cells. *Int J Oncol*. 2011;38(3):797-805. doi:10.3892/ijo.2011.897
36. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. *Signal Transduct Target Ther*. 2017;2:17023. doi:10.1038/sigtrans.2017.23
37. Rivera-Torres J, José ES. Src tyrosine kinase inhibitors: New perspectives on their immune, antiviral, and senotherapeutic potential. *Front Pharmacol*. 2019;10:1011. doi:10.3389/FPHAR.2019.01011/BIBTEX
38. Kessler BE, Mishall KM, Kellett MD, et al. Resistance to Src inhibition alters the BRAF-mutant tumor secretome to promote an invasive phenotype and therapeutic escape through a FAK>p130Cas>c-Jun signaling axis. *Oncogene*. 2019;38(14):2565-2579. doi:10.1038/s41388-018-0617-1
39. Vitiello PF, Shainheit MG, Allison EM, Adler EP, Kurt RA. Impact of tumor-derived CCL2 on T cell effector function. *Immunol Lett*. 2004;91(2-3):239-245. doi:10.1016/J.IMLET.2003.12.009
40. de Koning PJA, Kummer JA, de Poot SAH, et al. Intracellular Serine Protease Inhibitor SERPINB4 Inhibits Granzyme M-Induced Cell Death. *PLoS One*. 2011;6(8):e22645. doi:10.1371/JOURNAL.PONE.0022645
41. Chen CC, Chiou SH, Yang CL, et al. Secreted gelsolin desensitizes and induces apoptosis of infiltrated lymphocytes in prostate cancer. *Oncotarget*. 2017;8(44):77152. doi:10.18632/

ONCOTARGET.20414

42. Cheng Y, Ma X lei, Wei Y quan, Wei XW. Potential roles and targeted therapy of the CXCLs/CXCR2 axis in cancer and inflammatory diseases. *Biochim Biophys Acta - Rev Cancer*. 2019;1871(2):289-312. doi:10.1016/j.bbcan.2019.01.005
43. Yang C, Yu H, Chen R, et al. CXCL1 stimulates migration and invasion in ER-negative breast cancer cells via activation of the ERK/MMP2/9 signaling axis. *Int J Oncol*. 2019;55(3):684-696. doi:10.3892/ijo.2019.4840
44. Wang N, Liu W, Zheng Y, et al. CXCL1 derived from tumor-associated macrophages promotes breast cancer metastasis via activating NF-κB/SOX4 signaling. *Cell Death Dis*. 2018;9(9):1-18. doi:10.1038/s41419-018-0876-3
45. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002;420(6917):860-867. doi:10.1038/nature01322
46. Sanford DE, Belt BA, Panni RZ, et al. Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: A role for targeting the CCL2/CCR2 axis. *Clin Cancer Res*. 2013;19(13):3404-3415. doi:10.1158/1078-0432.CCR-13-0525
47. Fridlender ZG, Buchlis G, Kapoor V, et al. CCL2 blockade augments cancer immunotherapy. *Cancer Res*. 2010;70(1):109-118. doi:10.1158/0008-5472.CAN-09-2326

Supplementary figures

A

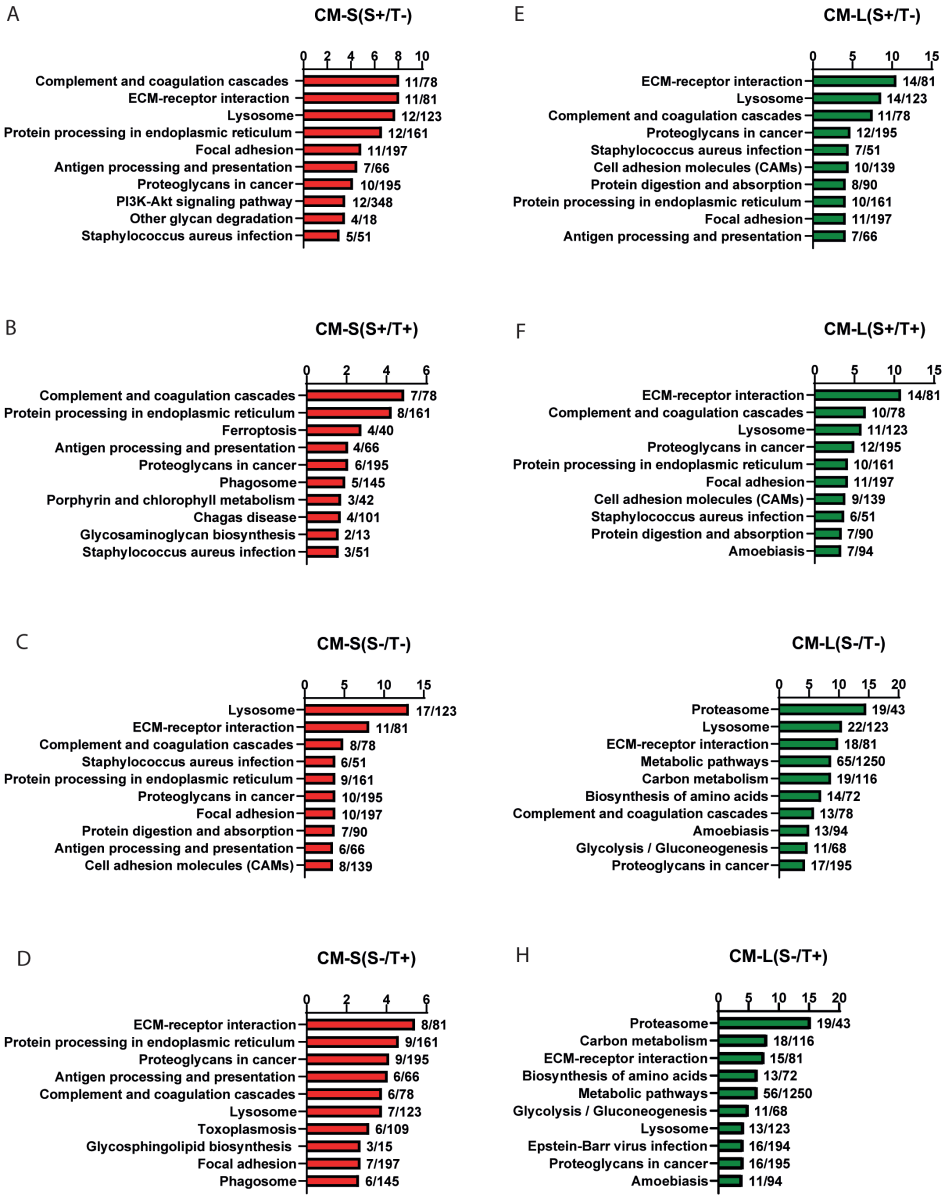


B

POS	POS	POS	POS	NEG	NEG	ENA-78 (CXCL5)	G-CSF	GM-CSF	GRO a/b/g	GRO alpha (CXCL1)
I-309 (CCL1)	IL-1 alpha (IL-1 F1)	IL-1 beta (IL-1 F2)	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8 (CXCL8)	IL-10
IL-12 p40/p70	IL-13	IL-15	IFN-gamma	MCP-1 (CCL2)	MCP-2 (CCL8)	MCP-3 (CCL7)	M-CSF	MDC (CCL22)	MIG (CXCL9)	MIP-1 beta (CCL4)
MIP-1 delta	RANTES (CCL5)	SCF	SDF-1 alpha	TARC (CCL17)	TGF beta 1	TNF alpha	TNF beta (TNFSF1B)	EGF	IGF-1	Angiogenin
OSM	TPO	VEGF-A	PDGF-BB	Leptin	BDNF	BLC (CXCL13)	Ck beta 8-1 (CCL23)	Eotaxin-1 (CCL11)	Eotaxin-2 (CCL24)	Eotaxin-3 (CCL26)
FGF-4	FGF-6	FGF-7 (KGF)	FGF-9	FLT-3 Ligand	Fractalkine (CX3CL1)	GCP-2 (CXCL6)	GDNF	HGF	IGFBP-1	IGFBP-2
IGFBP-3	IGFBP-4	IL-16	IP-10 (CXCL10)	LIF	LIGHT (TNFSF14)	MCP-4 (CCL13)	MIF	MIP-3 alpha	NAP-2 (CXCL7)	NT-3
NT-4	OPN (SPP1)	OPG (TNFRSF11	PARC	PLGF	TGF beta 2	TGF beta 3	TIMP-1	TIMP-2	POS	POS

Supplementary Fig. 1 The effect of Src expression and/or rhTRAIL treatment on cytokine expression in the different CM-S A) *The expression of cytokines in the different CM-S obtained after treating*

A549-S+ or A549-S cells for 1 hr with 50 ng/ml rhTRAIL and incubation in RMPI for 24 h. Depicted are the cytokines that were highly expressed or showed changed expression in either of the CM-S. ***B)*** *The selected cytokines depicted in A).*



Supplementary fig. 2 KEGG pathway analysis on total protein composition of CM-S and CM-L *All secreted proteins present in the CM-S and CM-L determined by mass spectrometry analysis subjected to a KEGG pathway analysis. The top 10 most significantly annotated pathways are depicted for CM-S in* ***A-D)*** *and for CM-L in* ***E-H)***

Chapter 5

ArtiCYT-p:
a novel synthetic extracellular matrix for generating 3D models in cancer research.

Margot de Looff, Gert Jan Meersma, Frank A.E. Kruyt

and Steven de Jong

Abstract

Three-dimensional (3D) *in vitro* cancer models mimic the physiological properties of tumours better than regular 2D cell cultures and are increasingly used in cancer research. 3D cell cultures are often supported by Matrigel that provides extracellular matrix (ECM) to facilitate 3D growth. However, batch-to-batch variations in the composition of Matrigel decrease the reproducibility of experimental conditions and the composition of the matrix is not adjustable to better mimic the tumour microenvironment. To overcome these limitations, synthetic matrices with well-defined chemical structures, containing constituents such as tailored cell signalling activating synthetic peptides and adjustable stiffness, are developed to provide better alternatives. In the present study we investigated the utility of the synthetic peptide-linked matrix, ArtiCYT-p, for 3D cancer cell culturing in comparison to Matrigel. First, we optimized ArtiCYT-p composition for spheroid growth of 8 different breast, lung and ovarian cancer cell lines. Next, we tested the applicability of ArtiCYT-p for both high throughput screening (HTS) and high content analysis (HCA) by determining sensitivity to the chemotherapeutic drug cisplatin and targeted recombinant human TNF-related apoptosis inducing ligand (rhTRAIL). We found that ArtiCYT-p was suitable for use in both HTS and HCA studies and no substantial differences in drug sensitivity were observed between Matrigel and ArtiCYT-p based 3D models. Overall, we conclude that ArtiCYT-p is a potential synthetic substitute for Matrigel for generating 3D cancer cell models that are suitable to perform drug sensitivity studies.

Introduction

The translation of pre-clinical findings into clinically relevant studies is very inefficient. Less than 8% of promising pre-clinical animal drug sensitivity studies are successfully translated into phase III clinical trials [1]. With regard to cancer related drug therapies the translatability is remarkably unsatisfactory, as approximately 85% of the early clinical trials fail despite successful pre-clinical studies [1]. Alternative drug testing models are needed to improve this disparity between bench and clinic.

The use of cancer cell lines in preclinical studies has been indispensable for cancer research. Generally, cancer cells are cultured and employed as 2D monolayer models. 2D cultures evidently do not mimic the multi-layered cellular 3D configuration of tumours and culturing cancer cells as monolayers is known to affect gene expression, cell behaviour and morphology as well as the response to therapeutics when compared to the *in vivo* situation [2,3]. Therefore, a variety of 3D models have been developed for *in vitro* drug testing, such as spheroid, organoid, and cancer-on-chip (co-) culture models [4,5]. 3D spheroid models can be divided into two categories, namely non-scaffold based and scaffold based models, the latter comprising cells cultured in matrices derived from biological or synthetic origins with adjustable stiffness to mimic tumour specific ECM [2,6]. 3D models have been used to study invasion, migration, malignant transformation, angiogenesis, metabolism, hypoxia and drug efficacy, also under co-culture conditions with immune and/or stromal cells [2,3,5,7]. Yet, 2D models remain mostly used for their convenience in high throughput screening (HTS) and high content analyses (HCA), since 3D models require more complicated and laborious culture conditions and may have reproducibility issues [8].

Matrigel is widely used to support 3D cultures and has been employed in a variety of assays to study for example invasion and angiogenesis. In *in vivo* rodent models, Matrigel is often used to promote tumour growth of xenografted cells (9,10,11). Matrigel is a basement membrane extract obtained from Englebreth-Holm-Swarm mouse sarcoma cells [10,11] and is rich in ECM proteins such as laminin, fibronectin, collagen IV, entactin, growth factors and cytokines. Basement membrane proteins play an important role in tumour growth, progression, invasion and migration [12–16]. Despite its successful use, Matrigel lacks high quantities of collagen-I and hyaluronic acid as often found in human tumours *in vivo* [17,18]. Furthermore, the presence of unknown concentrations of growth factors, cytokines and additional proteins is a considerable drawback in the application of Matrigel [19]. These factors greatly affect cellular characteristics including proliferation, differentiation, metastatic properties and drug sensitivity and could reduce reproducibility and controllability. To circumvent these disadvantages, there is increasing interest in the design and development of synthetic bioactive matrices with a well-defined and adjustable composition.

The synthetic matrix ArtiCYT-p is a nanofiber-based hydrogel consisting of self-assembling nanofibers to which ECM-related adhesion-supporting small peptides can be covalently bound facilitating cell adherence and growth. These small peptides are based on basement membrane proteins, among which laminin, fibronectin and collagen IV, that are involved in a variety of biological processes such as cell adherence, migration and differentiation. ArtiCYT-p with covalently-linked synthetic peptides directs the behaviour of cells by influencing various intracellular pathways, depending on the cell type and underlying research question (20–22,23,24). Hence, the composition and ratio of peptides in ArtiCYT-p can be adjusted based on their presence in the tissue specific basement membrane, thereby affecting cellular properties and behaviour [23].

Platinum-based therapies remain a mainstay for the treatment of many cancer types, although resistance is often inevitable. Novel targeted therapies such as human recombinant tumour necrosis factor-related apoptosis-inducing ligand (rhTRAIL) may improve treatment responses. rhTRAIL is a promising therapeutic agent as it induces apoptosis selectively in tumour cells, while sparing normal cells [24–26]. However, resistance has been observed in preclinical models, and therapeutic targeting of TRAIL receptors in early clinical studies showed limited efficacy [27]. The use of 3D cell cultures may provide more realistic models to determine sensitivity to these therapeutics.

Thus far, ArtiCYT-p has not been tested for use in 3D cancer cell cultures and drug sensitivity assays. In the present study, we investigated the utility of ArtiCYT-p in comparison with Matrigel for 3D culturing of 7 human breast, lung and ovarium cancer cell lines. Moreover, ArtiCYT-p 3D and Matrigel 3D cultures were used for cisplatin and rhTRAIL sensitivity testing in HTS and HCA. We conclude that ArtiCYT-p provides a suitable synthetic environment for generating 3D cancer cell models and drug testing.

Material and methods

Cells and regular culturing The human breast cancer cell lines BT474, MDA-MB-361 and MCF-7, human non-small cell cancer (NSCLC) cell lines A549 and H460, human ovarian cancer cell lines OVCAR3 and SKOV3, and the human bone marrow/stromal cell line HS27A were obtained from the ATCC and tested for authenticity by short tandem repeat (STR) profiling DNA fingerprinting (Baseclear, Leiden, The Netherlands). All cells were maintained in RPMI 1640 (Gibco, Waltham, USA) with 10% FCS (Bondinco, Alkmaar, the Netherlands), except for MDA-MB-361 and SKOV3 that were cultured in DMEM high glucose (Gibco) plus 10% FCS, in a humidified 5% CO2 atmosphere at 37˚C. 2D monolayer culturing was performed according to standard protocols. A549 and H460 cells were stably transfected with dTomato fluorescent protein, pRRL-SFFV-dTomato (Addgene, Watertown, USA). To obtain viral

particles, 293T cells were cultured in 75cm^2 flasks until ~50% confluency in DMEM + 10% FCS, 1% L-glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). A549 and H460 were transduced with a lentiviral dTomato vector.

3D cell culturing 3D culturing was accomplished with ArtiCYT (Nano Fiber Matrices BV, Groningen, the Netherlands) without peptides (ArtiCYT-np) or ArtiCYT-p containing a mix of IKVAV (laminin), YIGSR (laminin), RGDS (fibronectin), PHSRN (fibronectin), and DGEA (collagen) peptides (ArtiCYT-p) [21], or with growth factor reduced Matrigel (Corning, Corning, USA). The ArtiCYT-p mixture contained 0.4 mg/ml hyaluronic acid sodium salt (from a 5 mg/ml stock solution), 0.3-0.9% ArtiCYT-p (2.3% w/v stock solution) and the desired number of cells in RPMI with 10% FCS. 60 µl of cell-ArtiCYT-p solution was added per well in a 96 well plate and incubated for 1 hr at RT, followed by addition of 150 µl RPMI 10% FCS medium on top of the solidified matrix. Plates were maintained in an incubator at 37°C. Matrigel was prepared by thawing overnight in the fridge, or for 1-2 h at RT. The desired number of cells was resuspended in RPMI + 10% FCS and mixed 1:1 with cold Matrigel; 60 µl of the cell-Matrigel solution was added per well and allowed to solidify for 1 hr at 37°C before the addition of 150 µl medium. Medium was replaced every 2-3 days and spheroid growth was monitored daily with brightfield microscopy.
For co-culturing experiments 1*10^4 HS27a cells were seeded in 96 well plates as a feeder layer and allowed to adhere overnight. Next, medium was removed and ArtiCYT-p or Matrigel solution including 1500 A549 or H460 cells, prepared as described above, was added on top of the adhered HS27a cells.
To prevent adherence of cancer cells to the bottom of the wells during 3D culturing the plates were coated with Poly 2-hydroxyethyl Methacrylate (polyHEMA) (Sigma Aldrich, Saint Louis, USA) as described previously [28]. In short, 1.5 g polyHEMA, was dissolved in 95 ml molecular biology grade absolute ethanol and 5ml cell culture grade distilled water for 8 h at 65°C. Next, 50 µl of the polyHEMA solution was added per well and the 96-well plates were allowed to dry for 2-3 days. This procedure was repeated once. Before plating the cells, wells were washed with PBS for 5 min. For the agarose coating a sterile 1% agarose (ultra-pure, Sigma Aldrich) solution was prepared in PBS and stored at 60°C. 50 µl was added per well in a 96 well plate and solidified at RT for 30 min. Otherwise, ultra-low attachment (ULA) treated plates (#6055800, Perkin Elmer, Waltham, USA) were used.

High throughput screening Cell viability of the spheroids was assessed by MTS assays for high throughput screening (HTS). Cells were seeded and cultured in ArtiCYT-p or Matrigel as described above and treated with indicated concentrations of cisplatin (1 mg/ml stock concentration, Accord, Welwyn Garden City, UK). Subsequently, medium was removed and 100 µl fresh medium was added followed by addition of 20 µl CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, USA) solution and incubation

for 4 h at 37°C. The supernatant was transferred to a 96 wells plate and absorbance was immediately analysed at 520nm with the iMARK microplate absorbance reader (Biorad, Hercules, USA).

High content analysis Cell imaging, cell viability and cell death analysis of the 3D cultures was performed with the Operetta high content imaging system and Harmony software (version 4.6, PerkinElmer) or ImageJ. Spheroids were labelled with the fluorescent dyes Calcein (20 µM; ThermoFisher, Waltham, USA), Hoechst (32.5 µM; ThermoFisher) and/or PI (7 µM; ThermoFisher) in 200µl medium per well in a black CellCarrier Ultra-96 well plate with a clear bottom especially designed for high content imaging readers (#6055300, PerkinElmer) and incubated for 2 h at 37°C. Next, the wells were washed twice with PBS for 30 min. Alternatively, cells expressing dTomato were used for HCA.

Results

Comparing ArtiCYT-p and Matrigel for supporting 3D cancer cell growth We explored 3D tumour cell growth properties of a panel of breast, lung and ovarian cancer cell lines using the manufactured standard ArtiCYT-p (0.7%) and Matrigel, and compared spheroid growth and morphology by bright-field microscopy. All cell lines tested were able to form spheroids in ArtiCYT-p and Matrigel (Fig. 1). Notably, fewer spheroids grew in ArtiCYT-p when plating the same cell numbers as in Matrigel (Fig. 1). Therefore, the number of seeded cells that resulted in optimal spheroid growth for the different cell lines, as indicated in Table 1, are different for ArtiCYT-p and Matrigel. An inverse correlation between the number of seeded cells and spheroid size was observed by microscopy, as illustrated for MCF7 in Matrigel (Fig. 2A).

Table 1. Cell numbers plated for optimal spheroid growth

Cell line	ArtiCYT	Matrigel
BT474	5000-10000	1000-5000
MDA-MB-361	5000-10000	1000-5000
MCF-7	1000-5000	500-1000
A549	500-1000	100-1000
H460	100-1000	100-500
OVCAR3	1000-5000	500-1000
SKOV3	1000-5000	500-1000

Quantitative measurements of the spheroid size of Calcein stained A549 lung cancer and BT474 breast cancer spheroids with the Operetta confirmed that in both ArtiCYT-p and

Matrigel the spheroid size decreased with increasing seeded cell numbers (Fig. 2B). Spheroids cultured in ArtiCYT-p had a more irregular morphology when compared to those cultured in Matrigel, which was observed for all cell lines (Fig. 1). In general, spheroids cultured in Matrigel were larger than spheroids cultured in ArtiCYT-p for the same time period as shown by microscopic evaluation and high content analysis (HCA) (Fig. 1 and 2B). Thus, ArtiCYT-p supported 3D growth of these cancer cell lines similarly as Matrigel, although differences in morphology and spheroid number were observed.

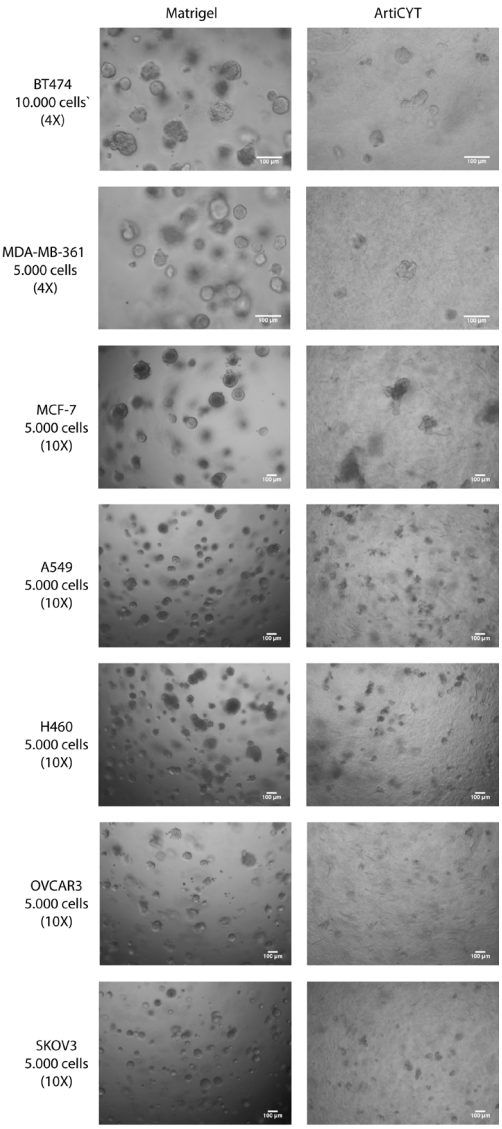


Figure 1. Spheroid formation of cancer cells in ArtiCYT-p and Matrigel *The indicated cancer cell lines were seeded in 96 well plates at the indicated cell numbers in Matrigel or ArtiCYT-p. After 15 days of growth spheroids were analysed by bright field microscopy for size and number. Photos were taken with 4x magnification. All cell lines tested were able to form spheroids in both matrices (N=3).*

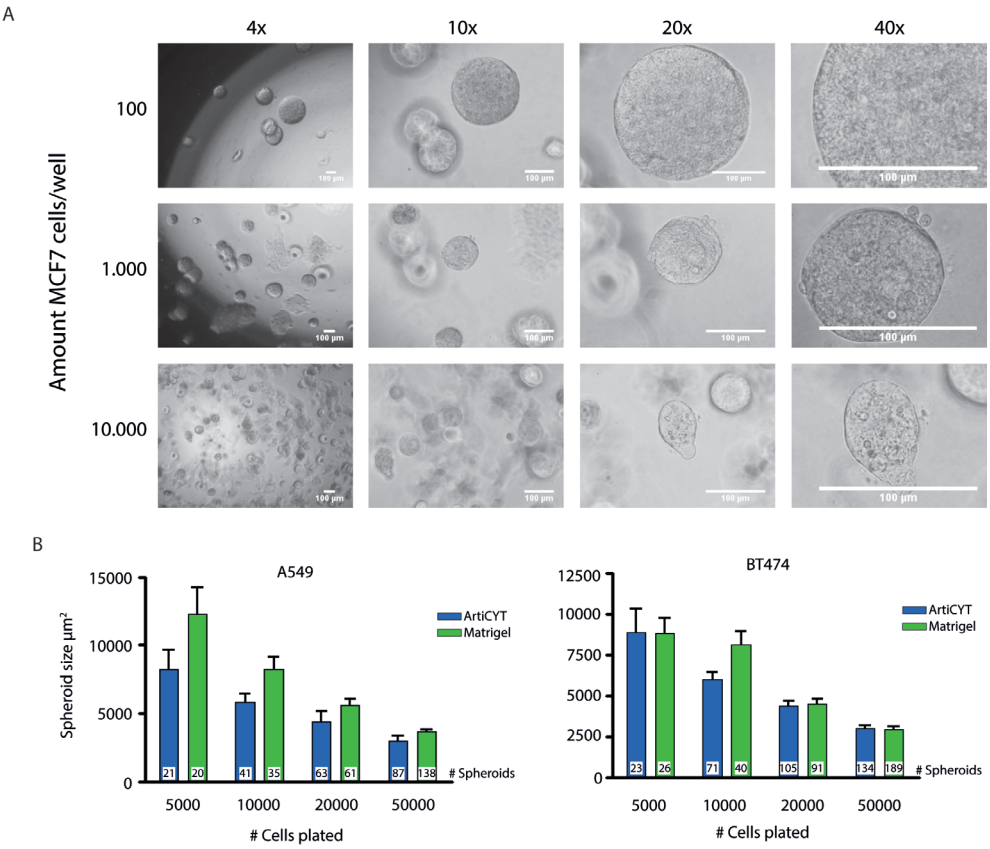


Figure 2. Spheroid size is affected by the number of cancer cells plated in ArtiCYT and Matrigel ***(A)*** *The indicated cell numbers of MCF7 cells were seeded and cultured in Matrigel for 7 days and spheroid growth was analysed with brightfield microscopy. Photos of the spheres were taken with 4x, 10x, 20x or 40x magnifications (N=1).* ***(B)*** *5.000, 10.000, 20.000 or 50.000 A549 or BT474 cells were plated in ArtiCYT-p or Matrigel. After 25 days of culturing the spheres were stained with Calcein and pictures were taken with the Operetta imaging system (N=1). Subsequently the size (µm²) and number of Calcein expressing spheroids was analysed with the Harmony software. The number of cells seeded affected the final size of the spheroids.*

Optimizing ArtiCYT-p conditions for high content analysis The standard ArtiCYT-p concentration (0.7%) resulted in the growth of irregular shaped spheroids which posed a problem for HCA with the Operetta as it hampered automated software-based detection. Therefore, several parameters were altered to optimize spheroid growth. The use of ArtiCYT without peptides (ArtiCYT-np) and the effect of different FCS concentrations on spheroid growth and morphology was assessed. Spheroid growth of A549 cells was reduced in ArtiCYT-np compared to ArtiCYT-p (Fig. 3A/B). ArtiCYT-p and Matrigel supported a round

and more dense spheroid morphology, whereas spheroids in ArtiCYT-np in general had a loose and grapelike structure (Fig. 3A/B). Reducing the FCS concentration resulted in decreased spheroid growth in all three matrices (Fig 3A). This effect was most evident in ArtiCYT-np, in which 5% FCS already resulted in impaired spheroid growth and a loose and more irregular spheroid morphology. In the absence of FCS, Matrigel still supported spheroid growth, although spheroid size was strongly reduced (Fig. 3A). Besides spheroid morphology, we found that matrices containing the standard ArtiCYT-p concentration (0.7%) were moderately turbid. To further optimize spheroid growth and to reduce gel turbidity, we tested different ArtiCYT-p concentrations ranging from 0.3 to 0.9%. The stiffness of the 0.3% gel was poor, such that it was impractical to handle for cell culturing. The turbidity increased with increasing concentrations ArtiCYT-p. We found that spheroid growth was optimal in 0.5% and 0.7% ArtiCYT-p, however, the clarity was most optimal in 0.5% ArtiCYT-p gels (Fig. 3C). Thus, an 0.5% ArtiCYT-p concentration and 10% FCS were optimal for supporting spheroid growth and use in brightfield microscopy and HCA.

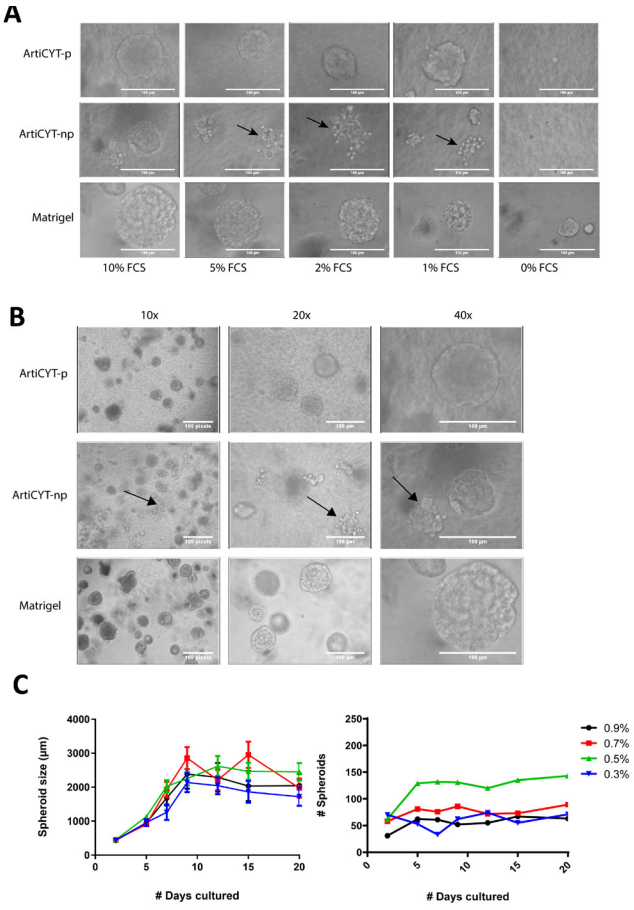


Figure 3. The effect of FCS, ArtiCYT composition and concentration on spheroid growth (A) *The effect of FCS on A549 spheroid growth was investigated in ArtiCYT-p, ArtiCYT-np and Matrigel. 2500 cells per well were plated and grown for 14 days in matrices with 10%, 5%, 2%, 1% or without FCS. Spheroids were analysed by brightfield microscopy and pictures taken with 40x magnification (N=1).* ***B)*** *The effect of ECM-binding peptides to ArtiCYT on spheroid growth was investigated by brightfield microscopy. 2500 A549 cells were cultured in Matrigel, ArtiCYT-p and ArtiCYT-np with 10% FCS for 14 days. Pictures were taken with a brightfield microscope with 10x, 20x or 40x magnification (N=1).* ***(C)*** *Different concentrations ArtiCYT-p, ranging from 0.3-0.9%, ArtiCYT-p were used for culturing A549 cells for the indicated number of days (N=1). The spheres were stained with Calcein and the average size and number was calculated by the Harmony software after stacked pictures were taken with the Operetta.*

High throughput screening and high content analysis assays using 3D ArtiCYT-p and Matrigel models Next, we investigated the applicability of fluorescently labelled A549-dTomato cells to analyse spheroid growth and morphology over time in our 3D ArtiCYT-p and Matrigel models with HTS and HCA assays. We observed, however, adherence of tumour cells to the bottom of the wells that resulted in background signals and interfered with automated analysis. To reduce cell adhesion, we tested ultra-low-attachment plates and different low-adherent coatings. Although cell adhesion was prevented as expected in ultra-low-attachment plates, ArtiCYT-p based gels did not sufficiently adhere to the wells, in contrast to Matrigel, hampering the use of these plates. PolyHEMA coated plates effectively prevented cell adherence, while allowing adherence of ArtiCYT-p but not Matrigel. Then, pre-coating the wells with a 1% agarose solution was tested, which provided sufficient adherence for both Matrigel and ArtiCYT-p, while preventing cell adherence to the bottom of the wells. Under these conditions, spheroids could be detected using A549-dTomato cells, which was comparable to spheroid detection with the nuclear Hoechst dye (Fig. 4A).

Next, we determined cisplatin sensitivity of A549-dTomato spheroids using the optimized ArtiCYT-p 3D culture conditions in comparison to the Matrigel model (Fig. 4B-G). First, cisplatin sensitivity of A549-dTomato cells in 3D ArtiCYT-p and Matrigel culture was examined using MTS as an HTS read-out for cell survival. No differences in cell viability were found between ArtiCYT-p and Matrigel (Fig. 4B). IC50 in these 3D cultures was comparable to the IC50 found in 2D cultures (2D data not shown). HCA analysis showed that continuous cisplatin treatment for 4 days resulted in decreased spheroid numbers and spheroid size in both ArtiCYT-p and Matrigel, reflecting reduced colony formation and spheroid growth, respectively (Fig. 4C-D). However, large variations in spheroid size were found (Fig. 4D). Finally, cisplatin sensitivity of spheroids that were cultured in 3D media for 4 days before treatment (preformed), was determined and compared to cells that were directly exposed to cisplatin upon seeding in 3D media (Fig. 4E-G). HTS analyses showed that preformed spheroids were less sensitive to cisplatin than directly exposed seeded cells (Fig. 4E). HCA analyses demonstrated that number and size of the preformed spheroids were also less affected by cisplatin treatment when compared to directly exposed cells (Fig. 4F-G). Taken together, ArtiCYT-p is suitable to detect spheroid numbers and size by HTS and HCA approaches, giving similar results as Matrigel models.

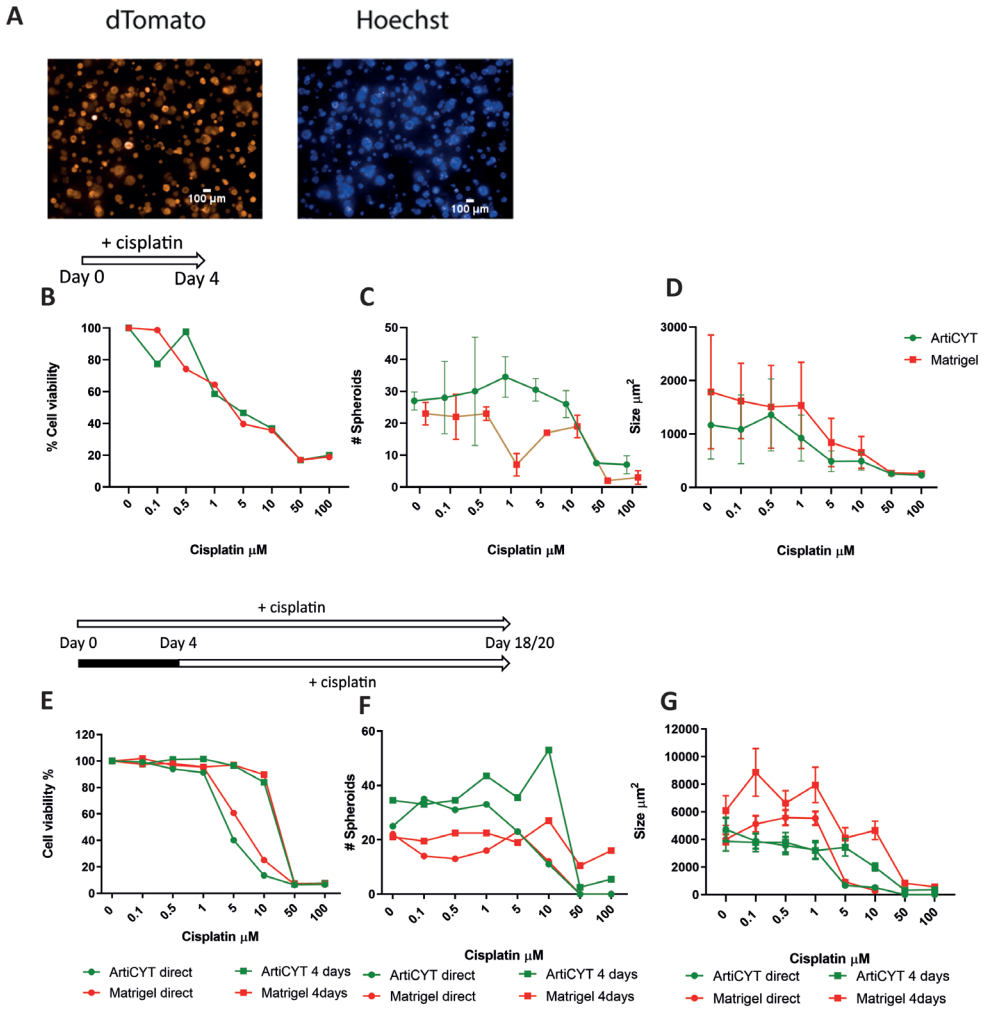


Figure 4. Use of ArtiCYT for drug responsiveness studies using high content analyses (HCA) and high throughput screening (HTS) approaches (A) *A549 cells were transfected with d-Tomato and 20.000 cells/well were plated and cultured for 7 days in Matrigel. Spheres were stained with Hoechst and pictures were taken with the Operetta.* ***(B)*** *1.000 A549-dTomato cells per well were cultured in ArtiCYT-p or Matrigel and treated with different cisplatin concentrations (0.1-100 µM) for 4 days added immediately after plating. Cell viability was determined with* ***(B)*** *an MTS assay for HTS (N=1) and* ***(C)*** *the number and (D) size of the spheres was determined by HCA (Operetta) and SD is indicated (N=1).* ***(E-G)*** *1.000 A549-dTomato cells per well were cultured in ArtiCYT-p or Matrigel. The cells were treated with 0.1 to 100 µM cisplatin directly for 18 days or after allowing first spheroid formation for 4 days and subsequent 14-day cisplatin exposure (N=1). (E) Cell viability after 20 days was determined with MTS assay and HCA analysis was performed on the number and size of the spheroids after 18 or 4 + 14 days (F-G). Thus, with ArtiCYT-p spheroid numbers and size can be detected by HTS and HCA approaches, similarly to Matrigel.*

rhTRAIL sensitivity in ArtiCYT-p and Matrigel models Next, we investigated the efficacy of apoptosis-inducing ligand rhTRAIL in the ArtiCYT-p and Matrigel 3D models using HCA to determine spheroid viability and cell death. For this the spheroids were co-stained with Hoechst, Calcein and PI. Stainings were tested first in parental TRAIL resistant A549 cells, since PI fluorescence overlaps with dTomato fluorescence. Hoechst showed strong nuclear staining in the spheroids with little variation between the spheres (Fig.4A/5A). Heterogenous Calcein staining patterns were detected between the spheroids, varying depending on spheroid size. In untreated cells, PI staining was mainly seen in spheroid cores, likely representing necrotic areas. Combined Calcein/PI staining appeared most suitable for use in the rhTRAIL sensitivity assay, allowing both detection of vital and dead cells, respectively (Fig. 5A). However, in ArtiCYT, but not Matrigel, we found increased scattering of fluorescent light as compared to Matrigel, which was possibly caused by the turbidity of ArtiCYT, hampering automated analysis of the pictures with the Harmony software (Supplementary fig. 1). Next, we administered different concentrations of rhTRAIL either directly to seeded single cells or to preformed spheroids from TRAIL resistant A549 and sensitive H460 NSCLC cells [29]. As expected, A549 cells showed no considerable change in viability after continuous rhTRAIL treatment in ArtiCYT-p or Matrigel and spheroid formation (number and size) was not affected (Fig. 5B). Interestingly, increased PI fluorescence was observed in A549 spheroids cultured in Matrigel with high concentrations of rhTRAIL, but not in ArtiCYT-p (Fig. 5B). Preformed A549 spheroids grown for 6 days and treated with rhTRAIL for 1 day, however, were not PI positive (Fig. 5C). In contrast, H460 spheroid formation and viability were strongly reduced after 7 days of continuous rhTRAIL treatment (Fig. 5D). High PI positivity in untreated H460 spheroids indicated high levels of spontaneous cell death in both ArtiCYT-p and Matrigel. Surprisingly, low PI positivity was observed at high rhTRAIL concentrations (Fig. 5D). TRAIL is known to rapidly induce high levels of apoptosis in H460 and thus, most seeded cells were killed by rhTRAIL before being able to form spheroids. Interestingly, a few TRAIL-resistant H460 spheroids were detected, suggesting the presence of a resistant subfraction of H460 cells. Treatment of preformed H460 spheroids with rhTRAIL for 1 day caused a decrease in cell viability and an increase in cell death in both ArtiCYT-p and Matrigel (Fig. 5E). Of note, cell death was most abundant in H460 spheroids cultured in Matrigel. These experiments were repeated but then A549 and H460 cells were treated for 8 days with rhTRAIL or 6 days without rhTRAIL and the lasty two days with rhTRAIL, with similar results as described in Figure 5 (results not shown).

Overall, Calcein/PI staining is suitable for detecting viable and dead cells by HCA using the Operetta and results were largely comparable between ArtiCYT-p and Matrigel 3D models.

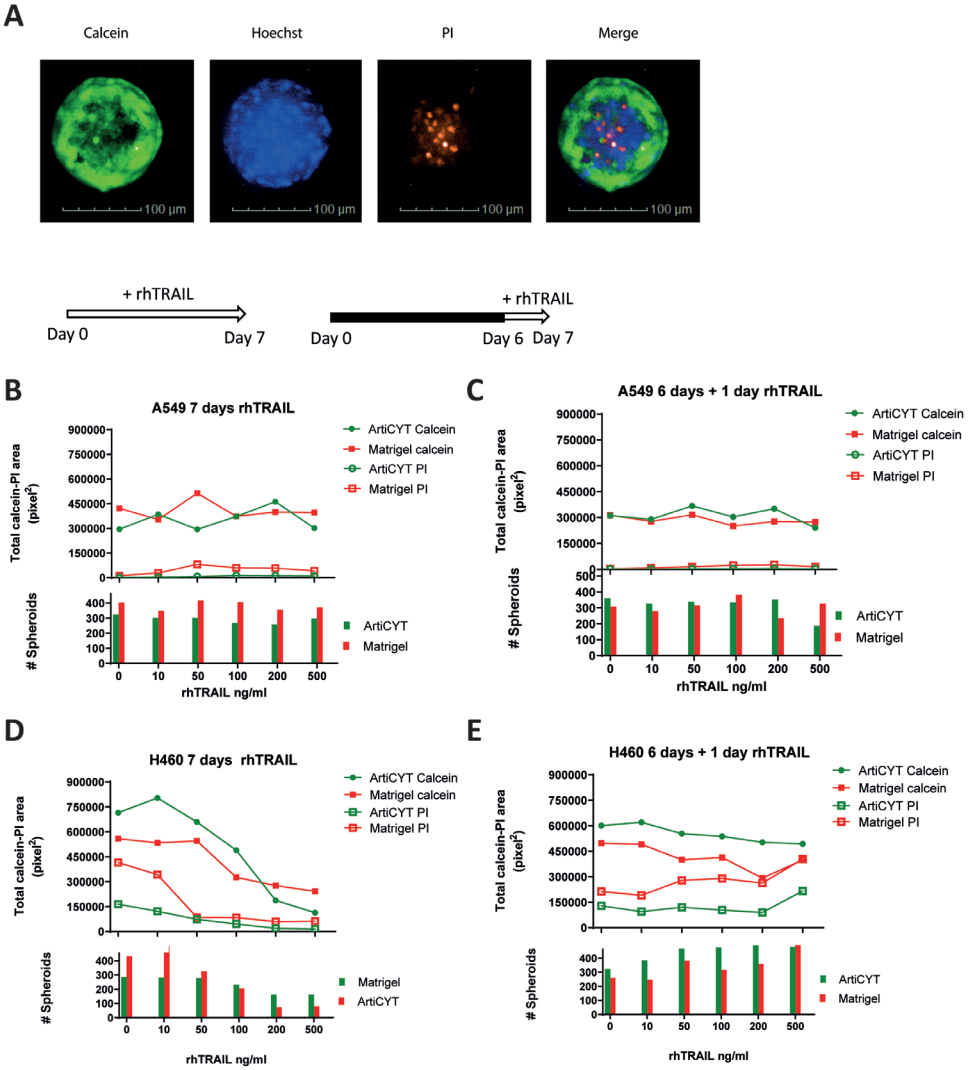


Figure 5. Determining rhTRAIL sensitivity of ArtiCYT and Matrigel cultured A549 spheroids (A) *10.000 A549 cells were plated per well in ArtiCYT-p and the spheroids were allowed to grow for 33 days. The spheroids were stained with Calcein, Hoechst and PI. The plates were scanned with the Operetta with a 10x magnification and the spheroids were analysed by the Harmony software.* ***(B-C)*** *2.500 A549 and* ***(D-E)*** *2.500 H460 cells were plated in ArtiCYT-p or Matrigel and were exposed to different concentrations of rhTRAIL for 7 days immediately after plating, or for 24 h after allowing spheroid formation for 6 days (N=1). After treatment the spheroids were stained with Calcein and PI and spheroid numbers and total area of both Calcein (cell viability) and PI (cell death) were determined by HCA using the Operetta. The pixel² surface of both Calcein and PI expression, as measurement for cell viability and cell death respectively, and the number of cells was calculated with ImageJ. Calcein and PI staining was applicable for detecting viable and dead cells by HCA using the Operetta.*

rhTRAIL sensitivity in 3D co-culture models The tumour microenvironment (TME) composed of non-cellular and cellular components, such as the ECM and various stromal cells, affects tumour cell behaviour and morphology [30]. As a consequence, therapeutic efficacy of rhTRAIL can be modulated by both direct and indirect interactions with the TME [30]. Therefore, we investigated the effect of fibroblasts on rhTRAIL sensitivity in our 3D NSCLC models. The spheroids were grown on a "feeder" layer of human fibroblasts (HS27a cells), which were seeded prior to adding ArtiCYT-p or Matrigel mixed with tumour cells. Interestingly, we found an increase in cell death in normally resistant A549 spheroids after rhTRAIL treatment (Fig. 6A). Co-culturing with HS27a cells reduced the growth of A549 and H460 spheroids as demonstrated by a decrease in Calcein staining and number of spheroids (Fig. 6). Additionally, the presence of HS27a feeder cells resulted in less rhTRAIL induced cell death of both A549 and H460 spheroids rhTRAIL in ArtiCYT-p as well as Matrigel, suggesting a protective effect secreted by feeder cells (Fig. 6A-D). However, we cannot exclude that reduced spheroid growth was caused by competition for nutrients in co-cultures that diminishes the sensitivity for rhTRAIL induced apoptosis. Thus, we demonstrated that the ArtiCYT-p model is suitable for use in co-culture models and the outcome of drug sensitivity was comparable to Matrigel.

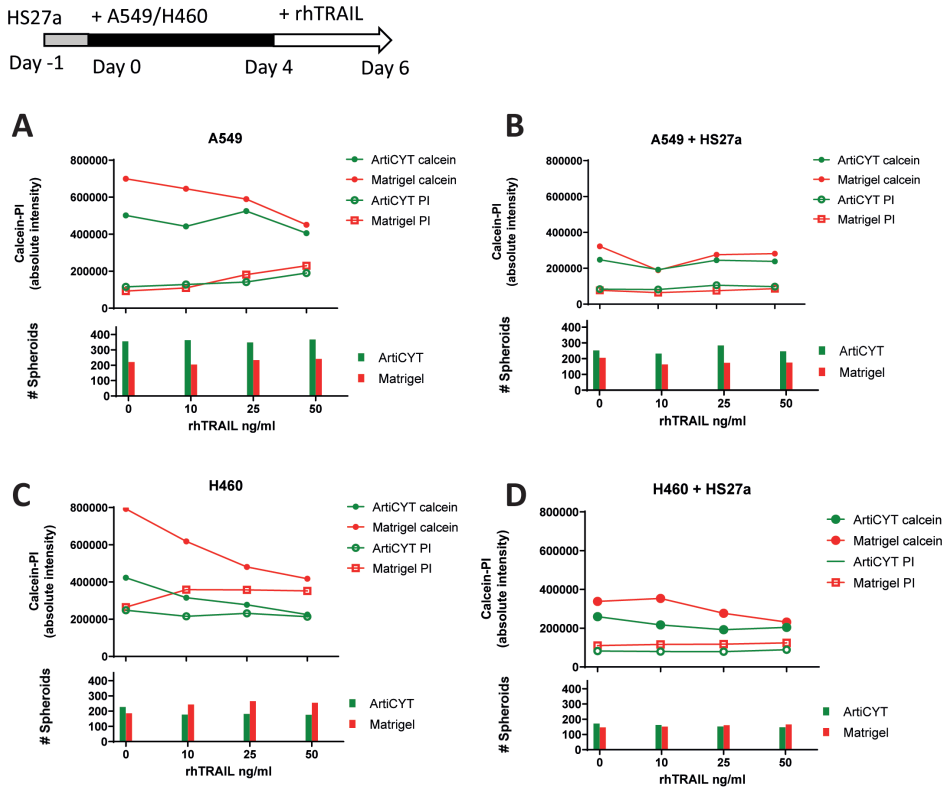


Figure 6. The effect fibroblast co-culturing on NSCLC spheroid formation and growth in ArtiCYT and Matrigel *1*10^4 HS27a cells were plated and grown overnight. Next, 2.500 A549* ***(A-B)*** *or H460* **(C-D)** *cells were seeded in ArtiCYT-p or Matrigel. Spheroids were allowed to grow for 4 days and were subsequently treated with the indicated concentrations of rhTRAIL for 48 h. The plates were scanned with the Operetta and the number of spheroids and the Calcein and PI intensity (mm^2) per spheroid were measured with the Harmony software (N=1). ArtiCYT-p as a model is suitable for use in co-culture models.*

Discussion

Synthetic matrices are being increasingly used for organoid and spheroid growth *in vitro* for studying biology and therapeutic responses and present promising prospects for cancer research (30,31). Here we investigated the applicability of the synthetic matrix ArtiCYT-p as a substitute for Matrigel to support 3D tumour spheroid growth. Similar to Matrigel, ArtiCYT-p provided a suitable matrix that supported 3D growth of a panel of cancer cell lines. We optimized various conditions to obtain the most optimal circumstances for 3D culturing of these cancer cell lines. The optimized ArtiCYT-p and Matrigel 3D models were both eligible for HTS and HCA analyses to determine drug sensitivity, and revealed largely comparable results. Overall, we show that ArtiCYT is applicable to investigate drug efficacy in spheroids with HTS and HCA imaging tools using multiple read-outs such as cell viability, cell death, morphology and growth.

We found that use of Matrigel as compared to ArtiCYT resulted in somewhat higher basal levels of both cell growth and treatment-induced cell death. However, synthetic matrices such as ArtiCYT compared to Matrigel have advantages such as a consistent and well-defined composition and little variability between different batches. Matrigel composition is variable and contains unknown concentrations of growth factors and cytokines that can affect proliferation and therapeutic efficacy. Moreover, the composition of ArtiCYT can be modified by adjusting the nanofiber concentration and the linkage of different ECM-related peptides. We showed that the highest ArtiCYT-p concentration resulted in smaller average size and less uniform morphology of the spheroids, which is likely due to a denser fibre network and a concomitant increase in matrix stiffness. The ability to adjust the concentration nanofibers of ArtiCYT-p makes it possible to modulate stiffness to mimic the process of tumour stiffening [31,32]. A stiff tumour matrix is known to promote EMT, metastasis and even chemoresistance, demonstrating the possibilities that the use of ArtiCYT-p offers [33–35].

Indirect and direct cell-cell and cell-ECM interactions are known to affect TRAIL sensitivity (29). In line with this, we found that drug sensitivity of preformed spheroids reflecting established tumours more closely, have reduced sensitivity to cytostatic and targeted drugs as compared to applying treatment during spheroid formation. Furthermore, fibroblast- 3D

lung cancer cell co-culturing appeared to protect the cells from rhTRAIL driven cell death, although we cannot exclude this also involves competition for nutrients. The protective effect was especially apparent in H460 spheroids cultured in Matrigel, implying that also unknown factors in Matrigel can affect therapeutic efficacy. These additional effects of Matrigel underscore the advantage of ArtiCYT, as this allows to study more precisely how stromal/feeder cells affect tumour growth. Additionally, also other cells can be included, such as immune cells, endothelial cells and/or mesenchymal cells that have been used in other 3D platforms (41–43). Further studies, in which variable stromal cell types and TME conditions are used in the ArtiCYT 3D model, could further elucidate the more precise role of these cellular and non-cellular components in regulating tumour growth, death and therapeutic efficacy.

Full application of HTS and HCA in 3D models remains a hurdle due to matrix variability with respect to light scattering as well as reproducibility and scalability [8,36]. We found that 3D cell culturing in ArtiCYT-p combined with the Operetta scanner imager provides a suitable HCA approach. However, the turbidity and the consequential scattering of fluorescent light with ArtiCYT needs to be taken into account when performing HCA. Others have investigated the applicability of HTS or HCA in 3D cultures with biological matrices, however, the suitability of matrixes for use in both HTS and HCA has not been examined [37]. In general, our drug responsiveness studies in the 3D cancer cultures analysed with HTS and HCA gave similar outcomes. We found particularly substantial variation of the spheroids size as determined by HCA for both ArtiCYT and Matrigel models. This variation may be related to differences in intrinsic properties of the colony forming cells. Alternatively, only small areas of the individual wells were analysed to generate high resolution scans and stacked pictures in a time-efficient manner, which may have introduced variation as well. An advantage of HCA is that more information can be obtained regarding growth, cell viability and cell death of individual spheroids.

In conclusion, ArtiCYT-p is a synthetic and convenient matrix that can substitute Matrigel for generating 3D cancer cell models and is suitable for drug sensitivity studies in a more controllable fashion using HTS and HCA.

Acknowledgements
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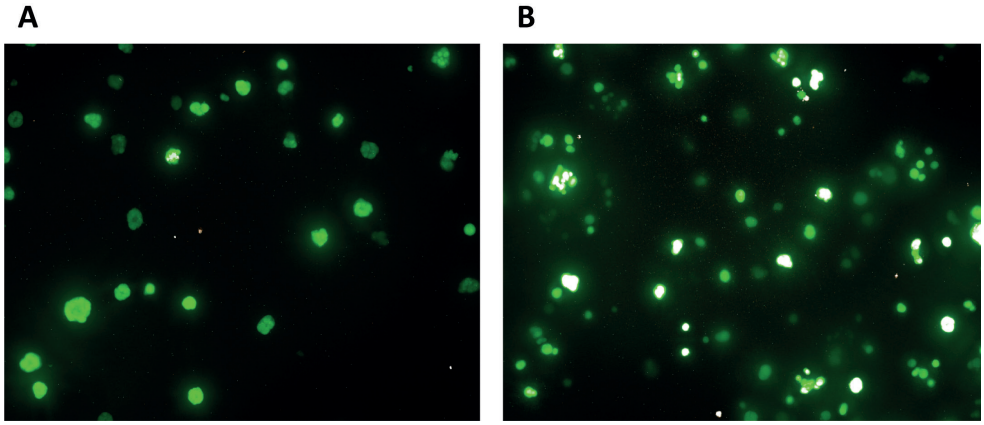
References

1. Mak IW, Evaniew N, Ghert M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res*. 2014;6(2):114-118. http://www.ncbi.nlm.nih.gov/pubmed/24489990.
2. Kimlin LC, Casagrande G, Virador VM. In vitro three-dimensional (3D) models in cancer research: An update. *Mol Carcinog*. 2013;52(3):167-182. doi:10.1002/mc.21844
3. Ravi M, Ramesh A, Pattabhi A. Contributions of 3D Cell Cultures for Cancer Research. *J Cell Physiol*. 2017;232(10):2679-2697. doi:10.1002/jcp.25664
4. Langhans SA. Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol*. 2018;9:6. doi:10.3389/fphar.2018.00006
5. Nyga A, Cheema U, Loizidou M. 3D tumour models: novel in vitro approaches to cancer studies. *J Cell Commun Signal*. 2011;5(3):239-248. doi:10.1007/s12079-011-0132-4
6. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. *J Cell Sci*. 2010;123(24):4195. doi:10.1242/JCS.023820
7. Kim J, Tanner K. Recapitulating the Tumor Ecosystem Along the Metastatic Cascade Using 3D Culture Models. *Front Oncol*. 2015;5(July):1-14. doi:10.3389/fonc.2015.00170
8. Ryan S-L, Baird A-M, Vaz G, et al. Drug Discovery Approaches Utilizing Three-Dimensional Cell Culture. *Assay Drug Dev Technol*. 2016;14(1):19-28. doi:10.1089/adt.2015.670
9. Benton G, Arnaoutova I, George J, Kleinman HK, Koblinski J. Matrigel: From discovery and ECM mimicry to assays and models for cancer research. *Adv Drug Deliv Rev*. 2014;79-80:3-18. doi:10.1016/j.addr.2014.06.005
10. Asghar W, El Assal R, Shafiee H, Pitteri S, Paulmurugan R, Demirci U. Engineering cancer microenvironments for in vitro 3-D tumor models. *Mater Today*. 2015;00(00):1-15. doi:10.1016/j.mattod.2015.05.002
11. Kibbey MC. Maintenance of the EHS sarcoma and Matrigel preparation. *J Tissue Cult Methods*. 1994;16(3-4):227-230. doi:10.1007/BF01540656
12. KANNAN S, BALARAM P, CHANDRAN GJ, et al. Alterations in expression of basement membrane proteins during tumour progression in oral mucosa. *Histopathology*. 2007;24(6):531-537. doi:10.1111/j.1365-2559.1994.tb00571.x
13. WILLEBRAND D, BOSMAN FT, GOEIJ AFPM. Patterns of basement membrane deposition in benign and malignant breast tumours. *Histopathology*. 1986;10(12):1231-1241. doi:10.1111/j.1365-2559.1986.tb02567.x
14. De Vries EGE, De Jong S. Exploiting the apoptotic route for cancer treatment: A single hit will rarely result in a home run. *J Clin Oncol*. 2008;26(32):5151-5153. doi:10.1200/JCO.2008.18.3160
15. Engbring JA, Kleinman HK. The basement membrane matrix in malignancy. *J Pathol*. 2003;200(4):465-470. doi:10.1002/path.1396
16. Sawai H, Okada Y, Funahashi H, et al. Basement Membrane Proteins Play an Important Role in the Invasive Processes of Human Pancreatic Cancer Cells. *J Surg Res*. 2008;144(1):117-123. doi:10.1016/j.jss.2007.03.023
17. Mouw JK, Ou G, Weaver VM. Extracellular matrix assembly: a multiscale deconstruction. *Nat Rev Mol Cell Biol*. 2014;15(12):771-785. doi:10.1038/nrm3902
18. Walker C, Mojares E, Del Río Hernández A. Role of Extracellular Matrix in Development and Cancer Progression. *Int J Mol Sci*. 2018;19(10). doi:10.3390/ijms19103028
19. Gelain F, Bottai D, Vescovi A, Zhang S. Designer Self-Assembling Peptide Nanofiber Scaffolds for Adult Mouse Neural Stem Cell 3-Dimensional Cultures. Herman C, ed. *PLoS One*. 2006;1(1):e119. doi:10.1371/journal.pone.0000119
20. Brizard A, Stuart M, van Bommel K, Friggeri A, de Jong M, van Esch J. Preparation of Nanostructures by Orthogonal Self-Assembly of Hydrogelators and Surfactants. *Angew Chemie Int Ed*. 2008;47(11):2063-2066. doi:10.1002/anie.200704609
21. Nibourg LM, Gelens E, de Jong MR, Kuijer R, van Kooten TG, Koopmans SA. Nanofiber-based hydrogels with extracellular matrix-based synthetic peptides for the prevention of capsular opacification. *Exp Eye Res*. 2016;143:60-67. doi:10.1016/j.exer.2015.10.001
22. Cui H, Webber MJ, Stupp SI. Self-assembly of peptide amphiphiles: From molecules to nanostructures to biomaterials. *Biopolymers*. 2010;94(1):1-18. doi:10.1002/bip.21328

23. Nibourg LM, Gelens E, Nibourg SAF, et al. Effects of peptide ratios in nanofibre-based hydrogels for the prevention of capsular opacification. *Acta Ophthalmol*. 2016;94(7):721-729. doi:10.1111/aos.13047
24. Ashkenazi A, Holland P, Eckhardt SG. Ligand-Based Targeting of Apoptosis in Cancer: The Potential of Recombinant Human Apoptosis Ligand 2/Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand (rhApo2L/TRAIL). *J Clin Oncol*. 2008;26(21):3621-3630. doi:10.1200/JCO.2007.15.7198
25. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of Apoptosis by Apo-2 Ligand, a New Member of the Tumor Necrosis Factor Cytokine Family. *J Biol Chem*. 1996;271(22):12687-12690. doi:10.1074/jbc.271.22.12687
26. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 1995;3(6):673-682. http://www.ncbi.nlm.nih.gov/pubmed/8777713.
27. Holland PM. Death receptor agonist therapies for cancer, which is the right TRAIL? *Cytokine Growth Factor Rev*. 2014;25(2):185-193. doi:10.1016/j.cytogfr.2013.12.009
28. Lawrenson K, Grun B, Gayther SA. Heterotypic three-dimensional in vitro modeling of stromal-epithelial interactions during ovarian cancer initiation and progression. *J Vis Exp*. 2012;(66):e4206. doi:10.3791/4206
29. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3 dependent invasion in resistant non-small cell lung cancer cells. *J Cell Sci*. 2012:4651-4661. doi:10.1242/jcs.109587
30. de Looff M, de Jong S, Kruyt FAE. Multiple Interactions Between Cancer Cells and the Tumor Microenvironment Modulate TRAIL Signaling: Implications for TRAIL Receptor Targeted Therapy. *Front Immunol*. 2019;10:1530. doi:10.3389/fimmu.2019.01530
31. Capeling MM, Czerwinski M, Huang S, et al. Nonadhesive Alginate Hydrogels Support Growth of Pluripotent Stem Cell-Derived Intestinal Organoids. *Stem Cell Reports*. 2019;12(2):381-394. doi:10.1016/J.STEMCR.2018.12.001
32. Cruz-Acuña R, Quirós M, Farkas AE, et al. Synthetic hydrogels for human intestinal organoid generation and colonic wound repair. *Nat Cell Biol*. 2017;19(11):1326-1335. doi:10.1038/ncb3632
33. Hui L, Zhang J, Ding X, Guo X, Jiang X. Matrix stiffness regulates the proliferation, stemness and chemoresistance of laryngeal squamous cancer cells. *Int J Oncol*. 2017;50(4):1439-1447. doi:10.3892/ijo.2017.3877
34. Rice AJ, Cortes E, Lachowski D, et al. Matrix stiffness induces epithelial–mesenchymal transition and promotes chemoresistance in pancreatic cancer cells. *Oncogenesis*. 2017;6(7):e352-e352. doi:10.1038/oncsis.2017.54
35. Reid SE, Kay EJ, Neilson LJ, et al. Tumor matrix stiffness promotes metastatic cancer cell interaction with the endothelium. *EMBO J*. 2017;36(16):2373-2389. doi:10.15252/embj.201694912
36. Rimann M, Graf-Hausner U. Synthetic 3D multicellular systems for drug development. *Curr Opin Biotechnol*. 2012;23(5):803-809. doi:10.1016/J.COPBIO.2012.01.011
37. Kota S, Hou S, Guerrant W, et al. A Novel 3-dimensional High Throughput Screening Approach Identifies Inducers of a Mutant KRAS Selective Lethal Phenotype. *Oncogene*. 2018;37(32):4372. doi:10.1038/S41388-018-0257-5

Supplementary figures



Supplementary figure 1. Scattering of fluorescent light in ArtiCYT *2.500 A549 were plated in* ***A)*** *Matrigel or* ***B)*** *ArtiCYT-p for 7 days. The spheroids were stained with Calcein and PI and spheroid and the spheroids were analysed HCA using the Operetta and Harmony software. Increased scattering is seen from spheroids cultured in ArtiCYT.*

Chapter 6

Summary, general discussion and future perspectives

Summary

Lung cancer is the second most common type of cancer and responsible for most cancer related deaths worldwide [1]. The majority of lung cancers (85%) belong to the non-small cell lung cancer (NSCLC) subtype and the minority (15%) is classified as small cell lung cancer (SCLC) [2]. Nowadays, NSCLC can be treated with surgery, radiotherapy, platinum-based chemotherapies, targeted therapies and immune therapies. Which therapy is preferred depends on the stage, histologic subtype and genetic profile of the disease. However, despite the successful development of targeted therapies for NSCLC in the last decades, such as receptor tyrosine kinase (RTK) and immune checkpoint inhibitors, the overall 5-year survival rate remains low and needs to be further improved [1,3,4].

Tumour-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors 1 (R1) and TRAIL-R2 are promising therapeutic targets as they can induce apoptosis selectively in tumour cells. The activation of TRAIL-R1/R2 by TRAIL-R agonists results in intracellular complex formation at the death domains of these receptors. Depending on the proteins recruited, the death inducing signalling complex (DISC) or the secondary complex is established. The DISC is assembled when FADD and caspase 8 are recruited to the receptors, resulting in cleavage and activation of caspase 8, and consequently the activation of the canonical TRAIL apoptotic pathway. However, in apoptosis resistant NSCLC cells TRAIL treatment can induce survival, proliferation and even migration/invasion [5]. This so called non-canonical TRAIL signalling is induced when the secondary complex is formed at the TRAIL receptors. In this case, TRAF2, TRADD, RIPK1, FADD and caspase 8 are recruited to the receptors and non-canonical TRAIL signalling is activated via, among others, the RIPK1-Src-STAT3 pathway that we identified in previous work [5]. Src, a non-receptor tyrosine kinase, is a known oncogenic driver and often overexpressed or hyperactivated in cancers, including NSCLC. It is involved in many cellular biological processes like survival, proliferation and migration [6,7]. Whether Src activation plays an important role in TRAIL signalling in NSCLC cells has not been well examined and needs to be further elucidated.

The outcome of TRAIL-R activation is not only determined by intrinsic cellular factors, but also extrinsic factors from the tumour microenvironment (TME) play an important role. The TME consists of cellular components, including immune cells, stromal cells and fibroblasts, and non-cellular components such as the extracellular matrix (ECM). Reciprocal (in)direct cell-cell or cell-ECM interactions can modulate TRAIL signalling and thereby the susceptibility towards TRAIL-R-driven cell death [8]. For instance, it has been demonstrated that TRAIL-resistant NSCLC cells can adjust the TME by secreting cytokines that attract pro-tumorigenic immune cells. This results in a pro-tumorigenic environment that can stimulate metastasis, as we have extensively reviewed in chapter 2 [8,9].

Monolayer cancer cell lines are widely used in oncology research, although the translation of findings from these *in vitro* 2D models to the complex 3D *in vivo* tumour situation in patients remains poor. The use of pre-clinical *in vivo* models as an intermediate step to clinical translation provides a more realistic tumour model, still these have species dependent discrepancies in important molecular, immunologic, genetic and cellular mechanisms, and moreover animal models are labour intensive and costly [10]. The use of 2D models partly explains why less than 8% of the *in vivo* pre-clinically tested drugs make it to clinical phase 1 trials [10]. Since 3D models represent better the *in vivo* situation these models have become more popular. To support 3D cell culturing, the biological matrix Matrigel, consisting of ECM isolated from Englebreth-Holm-Swarm mouse sarcoma cells, is most often used. However, the presence of unknown concentrations of growth factors, cytokines and other proteins secreted by the mouse sarcoma cells affect tumour cell behaviour and result in reduced reproducibility due to batch-to-batch variations. Synthetic matrices that support 3D culturing may overcome these limitations of biological matrices. To represent the *in vivo* situation better, both in 2D and 3D models, components of the TME can be included, thereby providing models that better represent the *in vivo* tumour setting. Such models allow examination of TME-tumour cell interactions and give more realistic and clinically relevant results in drug sensitivity studies. The use of inappropriate pre-clinical models that showed encouraging activity of TRAIL therapy has likely hampered effective clinical translation.

As mentioned above, the underlying mechanisms that determine activation of canonical or non-canonical TRAIL signalling in cancer cells are still incompletely understood. Also, the mechanisms that contribute to non-canonical TRAIL signalling have not been well investigated. A better understanding of these mechanisms is expected to provide possible prognostic markers and therapeutic targets that could be exploited for optimizing TRAIL-based cancer therapies. The main aim of this thesis was to explore in greater detail the role of Src in regulating apoptotic and non-canonical TRAIL signalling and to study how TRAIL-induced Src activation contributes to pro-tumorigenic effects. This included examination of possible involvement of Src in TRAIL-induced alterations in cytokine secretion and possible immune modulatory effects. To advance the development of 3D pre-clinical *in vitro* models, the synthetic matrix ArtiCyt was tested for supporting growth of 3D cancer cell cultures and to examine the efficacy of cisplatinum and TRAIL based therapies in NSCLC in comparison to the biological matrix Matrigel.

In **chapter 1** a general introduction on lung cancer, TRAIL (non-)canonical signalling, the TME and 3D models was provided. Firstly, lung cancer, its current therapeutic interventions and the requirement for new therapeutics were described. TRAIL and TRAIL-R1/R2 apoptotic and non-canonical signalling pathways were introduced in more detail. Src was introduced

as a potential important player in TRAIL signalling in NSCLC and the need to further unravel mechanisms underlying TRAIL signalling was indicated. Next, the effect of the TME on tumorigenesis and the possible involvement of TRAIL-Src signalling on TME dynamics and tumour development were explained. Subsequently, the necessity to utilise 3D models in cancer research and the need for synthetic matrices to support 3D culturing of NSCLC were explained. Lastly, the aims and outline of the thesis were described.

An extensive overview of literature (**chapter 2**) was provided discussing the complex bidirectional interactions between tumour cells and the TME which modulate the efficacy of both endogenous and exogenously administered TRAIL receptor agonists. We thoroughly discussed how the different components of the TME, including immune effector cells, neutrophils, macrophages, and non-hematopoietic stromal cells, affect TRAIL induced signalling. Also considered were the non-cellular biochemical and biophysical properties of the TME, including mechanical stress, acidity, hypoxia and glucose deprivation. Tumour-TME interactions resulted in both tumour stimulating and inhibiting effects, depending on the tumour-model and -type. We therefore concluded that for effective TRAIL treatment critical mechanisms and factors responsible for TRAIL resistance need to be identified that include a better understanding of the contribution of different TME components on TRAIL signalling.

In **chapter 3** we explored the contribution of the non-receptor tyrosine kinase Src and its underlying mechanisms in response to TRAIL induced (non-)canonical signalling in NSCLC cell lines. Different time-dependent rhTRAIL-induced Src phosphorylation patterns were found between sensitive H460 and resistant A549 NSCLC, with an early Src-Tyr418 phosphorylation, and thus activation, in A549 cells. Sensitivity towards rhTRAIL driven cell death was not altered by decreased Src expression, or by pharmacological Src inhibition. Silencing of TRADD, that is part of the secondary complex, impaired TRAIL dependent Src activation in A549 cells. Co-IP-LC-mass spectrometric analysis was performed to identify mediators of Src-dependent TRAIL signalling in H460 and A549 cells upon rhTRAIL exposure. In A549 cells the number of Src-interacting proteins increased after rhTRAIL treatment, whereas the opposite was seen for H460 cells. Known tumorigenic proteins were found in complex with Src in rhTRAIL-treated A549 cells, including components of the RAF/MEK/ERK, Wnt and SMAD3 signalling pathways. Functional analyses showed that Src mediated the phosphorylation of MEK1/2 and ERK, prevented phosphorylation of SMAD3 and was required for (peri)nuclear translocation of ERK and β-catenin in A549 cells. Clonogenic growth of both Src proficient and deficient A549 cells was not affected by rhTRAIL exposure, although Src depletion and/or MEK1/2 inhibition reduced colony size and number of clones significantly. We concluded that TRAIL dependent Src activation subsequently modulates MEK-ERK, SMAD3 and β-catenin signalling via (in)direct interactions. However, the exact mechanisms need to be further elucidated, as well as the potential therapeutic value of

targeting these pathways together with TRAIL-R agonists.

In **Chapter 4** the possible involvement of Src in determining the composition of the rhTRAIL-induced secretome of resistant A549 cells was examined. Next, the subsequent impact of the secretome on monocyte differentiation, macrophages polarization and $CD8^+$ T cell activation was analysed. Conditioned media (CM) was obtained from Src proficient (S+) and deficient (S-) A549 cells either treated with rhTRAIL (T+) shortly (1-2 h, CM-S), long (24 h, CM-L) or untreated (T-). The effect of the CM on differentiation and polarization of monocytes and differentiated M0 macrophages was determined by flow cytometry analyses of M0 (CD11b, CD68), M1 (CD80, CD86) and M2 (CD163, CD206) marker expression. All different CM polarized macrophages, independent from A549 Src status and rhTRAIL treatment. T cell activation decreased moderately when cultured in CM-S(S+/T+), but no effect was seen with the other CM-S. Cytokine array analysis for CM-S showed higher cytokine levels in CM-S from Src proficient cells, especially for GRO-α/β/γ and GRO-α (CXCL1). The levels of these cytokines decreased in CM-S (S-/T-) and were further reduced in CM-S (S-/T+). More in depth analyses of the CM-S and CM-L with mass spectrometry demonstrated the presence of cytokines that were annotated to immune related GO biological processes in CM-S(S+/T-), CM-S(S+/T+) and CM-L(S+/T-). In conclusion, our preliminary results suggest that both CM-S and CM-L modulated macrophage differentiation and polarization independent of Src status and rhTRAIL treatment. Src in A549 cells appeared to regulate the secretion of immune related cytokines and might be involved in reducing CD8+ T cell activation. However, further studies are needed to confirm these findings.

In **chapter 5** the utility of the synthetic peptide-linked matrix, ArtiCYT-p, for 3D cancer cell culturing was investigated in comparison to the biological matrix Matrigel. Firstly, ArtiCYT composition was optimised for spheroid growth of 7 different human breast, lung and ovarian cancer cell lines. Next, the applicability of ArtiCYT-p for both high throughput screening (HTS) and high content analysis (HCA) was explored by determining sensitivity to the chemotherapeutic drug cisplatin and the targeted drug rhTRAIL. ArtiCYT-p was suitable for use in both HTS and HCA studies and no substantial differences in drug sensitivity were observed between Matrigel and ArtiCYT-p based 3D models. Additionally, the presence of HS27a feeder cells resulted in less rhTRAIL induced cell death of both A549 and H460 spheroids rhTRAIL in ArtiCYT-p as well as Matrigel, suggesting a protective effect secreted by feeder cells. Thus, we found that ArtiCYT-p was a suitable synthetic substitute for Matrigel in generating 3D cancer cell (co-culture) models and performing drug sensitivity studies.

General discussion and future perspectives

Src as a potential therapeutic target for intervention in TRAIL non-canonical signalling In a wide range of cancer types, Src overexpression and hyperactivation are associated with oncogenic properties like migration, invasion and proliferation [7,11–14]. Previously, we and others reported that Src plays an important role in non-canonical TRAIL signalling in resistant NSCLC cells by downstream activation of proliferative, survival and migration pathways such as RAS/RAF/MEK/ERK, NFκβ, JNK, RIPK1 and AKT [5,15,16]. Here, we identified MEK1/2, SMAD3 and β-catenin in the Src interactome of rhTRAIL exposed A549 cells and found that Src regulated MEK1/2 and subsequent ERK phosphorylation, SMAD3 phosphorylation and β-catenin translocation. Others have reported that simultaneous treatment with Src and MEK inhibitors in NSCLC and ovarian cancer has synergistic effects on anti-tumour responses [17,18]. Further, TRAIL activation of both pro- or anti-apoptotic signalling in HT-29 colon cancer cells was found to be regulated by TRAIL dependent MEK-ERK activation leading to upregulation of anti-apoptotic MCL1[19,20]. MEK/ERK inhibition sensitized for TRAIL-induced mitochondrial apoptosis and also increased caspase-8 cleavage suggesting that the balance between the MEK/ERK-TRAIL/apoptosis pathways determines the outcome. In our experiments, neither MEK1/2 nor SMAD3 could be directly linked with rhTRAIL-induced Src-dependent pro-tumorigenic activity as no effects on colony formation were seen. However, further research using *in vivo* NSCLC models is required to examine the contribution of Src and downstream MEK1/2, SMAD3 and β-catenin in mediating pro-tumorigenic effects of rhTRAIL. In these models the possible therapeutic benefit of combining rhTRAIL with pharmacological Src, MEK1/2, SMAD3 and Wnt inhibitors should be tested.

Src has also been identified as a direct regulator of TRAIL-induced apoptosis. In gastric, hepatoma and breast cancer cells inhibition of Src was associated with increased sensitivity towards TRAIL driven cell death accompanied by enhanced levels of caspase 8 and FADD [15,21–23]. Src can phosphorylate caspase 8 at Tyr380, impeding its apoptotic activity and preventing FAS and TRAIL induced apoptosis [21,24]. Inactivated pTyr380 caspase 8 can bind to Src at the SH2 domain, thereby promoting Src Y416 autophosphorylation and activation [25]. Furthermore, Src can inhibit intrinsic apoptotic signalling by stimulating degradation of pro-apoptotic BIK via RAS/RAF/MEK/ERK-dependent BIK ubiquitination and subsequent proteasomal degradation [26]. However, in our experiments we found no indications of Src being involved in regulating apoptotic TRAIL signalling, neither in apoptosis resistant nor sensitive NSCLC cell lines, indicative of cell dependent differences in Src function.

In resistant A549 cells the presence of TRADD, a component of the secondary complex, was required for rapid Src activation upon TRAIL treatment. In non-canonical TRAIL signalling, TRADD is known to mediate RIPK1 recruitment to the secondary complex and

the subsequent activation of Src, MAPK and ERK [23,27]. Interestingly, TRADD deficient mouse embryonic fibroblasts showed increased sensitivity for TRAIL induced apoptosis due to enhanced FADD recruitment to the receptor complex [23]. Although we did not specifically examine apoptosis in A549 cells after depletion of TRADD, increased cleavage of caspase 8 after rhTRAIL treatment was observed, suggesting that decreased expression of TRADD resulted in activation of TRAIL apoptotic signalling. This implies that TRADD could act as a switch between canonical and non-canonical TRAIL signalling. In fact, targeting of TRADD could be an interesting approach to sensitize apoptosis resistant NSCLC cells as well as to suppress non-canonical TRAIL signalling. Pharmacological inhibitors of TRADD have been developed and could provide possible therapeutic agents to be used in combination with TRAIL agonists [28]. However, further examination is required to determine the role of TRADD in TRAIL induced apoptosis and as a potential therapeutic target.

TRAIL, Src and the secretome of NSCLC cells

TRAIL treated resistant NSCLC cells were reported to produce a pro-tumorigenic secretome resulting in attracting monocytes and pushing macrophages towards a pro-tumorigenic subtype [9]. The production of this pro-tumorigenic secretome after TRAIL treatment was FADD dependent and the chemokine CCL2 was found to play a key role in immune modulation [9]. However, as described in **Chapter 4,** we did not observe significant rhTRAIL dependent effects of the secretome on macrophage polarization. Although speculative, the use of different TRAIL receptor agonists in our study and previously reported work, in which isoleucine zipper TRAIL was applied [9], might have affected the composition of the secretome differently. Isoleucine zipper TRAIL has a higher cytotoxic activity than rhTRAIL due to inducing multimerization of TRAIL/TRAIL-R trimers resulting in higher biological activity [29]. In another study, TRAIL sensitive cells were shown to produce pro-tumorigenic inflammatory cytokines after TRAIL treatment and inhibition of caspase 8 was found to decrease apoptosis and enhance cytokine production [30]. In future studies, it would be important to investigate the effects of various TRAIL receptor agonists on the composition of TRAIL-induced secretome as this likely will determine the outcome of TRAIL therapy.

Src is known to be involved in the production of cytokines in tumour cells via NFκβ, ERK, JNK and STAT3 [31,32]. In macrophages, Src can be activated by various inflammatory cytokines, among others monocyte chemoattractant protein-1, macrophage inflammatory protein-1/2 and stromal cell-derived factor. Src activation in macrophages was shown to stimulate the production of inflammatory cytokines TNF-α, IL-1β and IL-6, which can activate Src in surrounding tumour cells and subsequently lead to increased invasion of inflammatory pro-tumorigenic immune cells. In this way a continuous loop of pro-tumorigenic activation occurs [33]. This illustrates that Src is a potential immune modulatory therapeutic target, however, in our studies we found no clear role of Src in modulating the activity of the secretome of

TRAIL resistant A549 cells. Previously, it was demonstrated that TRAIL via a FADD-RIPK1-caspase-8 complex produces a pro-tumorigenic secretome [9,16]. Although a possible role for Src was not examined, it appears that different TRAIL-dependent mechanisms can affect the production of a pro-tumorigenic secretome in TRAIL resistant NSCLC.

Novel TRAIL agonists Besides targeting intracellular factors to improve the therapeutic efficacy of TRAIL agonists, TRAIL potency could be augmented at the receptor level by developing TRAIL-R1 or -R2 specific TRAIL agonist or agonists that increase receptor clustering and thus apoptotic signalling. Depending on the cancer cell type, either TRAIL-R1 or -R2 or both can mediate ligand induced apoptosis activation. Notably, in NSCLC cells, particularly TRAIL-R2 activation has been associated with non-canonical signalling [34,35]. TRAIL receptor specific agonists have been developed which, depending on the cancer type, induce canonical/ apoptotic TRAIL signalling in pre-clinical models [36–38]. However, in clinical studies with TRAIL receptor specific agonists thus far no clearly improved anti-tumour effects were observed [39]. Aside from TRAIL-R specific targeting, increased TRAIL receptor clustering results in increased apoptosis activation. Soluble rhTRAIL mostly induces trimerization of TRAIL receptors, whereas cell membrane bound TRAIL propagates receptor oligomerisation [40]. The increased efficacy of membrane bound TRAIL can be mimicked by modified TRAIL agonists that also stimulate receptor oligomerization, such as earlier mentioned Isoleucine zipper TRAIL. Increased oligomerisation of TRAIL receptors is known to facilitate DISC assembly resulting in effective caspase 8 and 3 activation, not requiring co-activation of the intrinsic apoptotic pathway to fully engage apoptosis activation [41]. Currently, several potent TRAIL receptor agonists have been developed to improve TRAIL-R oligomerisation [36,37,42]. For example, BI 905711, a bispecific antibody targeting TRAIL-R2 and cadherin 17 is currently in phase 1 trial for patients with advanced gastrointestinal cancers [37,43]. Eftozanermin Alfa (ABBV-621), a TRAIL-R1/2 agonist which enhances TRAIL-R clustering, is currently under investigation in a phase 1 clinical trial for previously treated solid cancers and hematologic malignancies and in phase 2 clinical trial for relapsed or refractory multiple myeloma (NCT03082209; NCT04570631; [34]). Interestingly, combining a TRAIL-R2 specific antibody with soluble TRAIL could greatly augment the efficacy of soluble TRAIL by promoting bridging between TRAIL-R trimers leading to enhanced receptor oligomerisation and apoptosis [45,46]. Other encouraging approaches to advance TRAIL therapeutic efficacy are under development, including TRAIL gene monotherapy and TRAIL cell based/immune therapies. Adeno-associated viruses mediated delivery of the TRAIL gene into tumour cells results in local production of high levels of TRAIL at the tumour and apoptosis, that can be further increased by combined application of standard chemotherapy [47]. Cell based/ immune therapies include the use of mesenchymal stem cells expressing TRAIL to deliver the death ligand at the tumour site resulting in efficient tumour cell death [48,49]. In addition, TRAIL fusion proteins that bind both to tumour and immune cells can potentiate tumour

cell killing by triggering both TRAIL and immune effector cell dependent apoptosis [47,50–52]. For example, the tumoricidal toxicity of T cells can be improved with TRAIL-anti CD3/CD7 fusion proteins that in addition to directly triggering tumour cell death also stimulate T cell dependent cell death [53]. Currently, it remains to be determined whether, and which of these TRAIL therapeutic strategies, will be most effective in patients.

Synthetic matrices for 3D cell culture models Patient tumours contain ECM, endothelial, immune and other stromal cells that are part of the TME. In addition, also chemical and biophysical properties are part of the TME such as oxygen levels, pH and ECM stiffness. The composition of the TME is not identical in tumours and depends on the tissue origin and stage of the tumour. For example, lung tissue greatly differs from breast tissue and the TME of the primary tumour differs from a metastatic TME. Reciprocal interactions between the tumour and TME are known to modulate TRAIL signalling and affect the therapeutic outcome, which we extensively reviewed in chapter 2 [8]. However, 2D cell culture models are most often used in cancer research though they lack the 3D structure and TME of a patient tumour which hinders adequate clinical translation of *in vitro* findings. To improve clinical translation, 3D cancer cell models are increasingly used, mostly in combination with Matrigel. Matrigel is a basement membrane extract obtained from mouse sarcoma cells. Despite its frequent use in *in vitro* and *in vivo* pre-clinical xenograft mouse studies, Matrigel has several disadvantages. For example, it lacks high quantities of collagen-1 and hyaluronic acid that are major ECM components in human tumours and contains variable concentrations of unknown growth factors, cytokines and other proteins that will affect cell behaviour. Therefore, Matrigel has limitations and poses a hurdle for experimental reproducibility. This has stimulated the development of well-defined synthetic matrixes that also allow the study of functional consequences of specific ECM macromolecular-cell interactions by incorporating cell adhesive peptide binding sites in these matrices [54]. In **chapter 5,** we tested a newly developed synthetic 3D matrix, ArtiCYT, for supporting 3D cancer cell growth. ArtiCYT is a nanofiber hydrogel that can be linked with ECM derived peptides. We found that ArtiCYT can replace Matrigel for generating *in vitro* 3D cancer cell spheroids and provides a controllable and adjustable system for drug sensitivity studies. ArtiCYT and other synthetic hydrogels allow adjustments in both the type, ratio and concentration of cell adhesion related peptides, to better mimic specific TME components [55,56]. For example, in **chapter 5**, we showed that ArtiCYT linked with ECM peptides from laminin, fibronectin and collagen (ArtiCYT-p) resulted in increased tumour spheroid growth with compact and regular morphology when compared to regular ArtiCYT. The ECM can interact with cells via cell membrane bound receptors resulting in intracellular signalling. For example, in a recent study an optimal synthetic matrix formulation that binds to specific integrins was developed for maintaining growth of endometrial organoids [57].

Presently, different matrices/hydrogels are widely used to study the impact of stiffness, or elasticity, and subsequent mechano-transduction on tumour cell behaviour and aggressiveness [58]. The ECM is a dynamic structure requiring continuous cellular adaptation to a changing microenvironment. In order to capture the dynamics of the ECM *in vivo,* hydrogels have been developed which properties can be changed by time-dependent hydrolytic or enzymatic degradation of the matrices [58]. ArtiCYT may also be useful to mimic altered mechanical TME properties in cancer, however, this remains to be investigated.

We demonstrated in **chapter 5** that rhTRAIL had comparable cytotoxic effects in both Matrigel and ArtiCYT-based 3D H460 and A549 spheroids. TRAIL sensitivity in our 3D models was decreased in H460 cells when they were allowed to grow into spheroids before treatment. It would be interesting to examine pro-tumorigenic TRAIL signalling and to evaluate efficacy of different TRAIL agonists in such 3D models. Furthermore, some properties of ArtiCYT need to be improved to optimize use for HTS and HCA analyses. ArtiCYT-p was more turbid compared to Matrigel, impeding analysis with brightfield and fluorescent microscopy due to light scattering. This complicated the automatic analysis of tumour spheroids, which could be partly solved by adjusting the settings of the HTS and HCA software. Overall, the development and application of synthetic matrices is important for generating better and more realistic 3D cell culture models.

In conclusion in this thesis we attempted to unravel in greater detail the different mechanisms and factors involved in (non-)canonical TRAIL signalling modulated by Src. We particularly focused on the role of TRAIL activated Src in intracellular non-canonical signalling and modulation of the secretome in resistant NSCLC cells. We found that Src is involved in non-canonical, but not apoptotic TRAIL signalling in NSCLC. Several clues for Src involvement in non-canonical signalling and Src-dependent composition of the secretome were identified. However, their involvement in pro-tumorigenicity TRAIL signalling could not be clearly demonstrated and requires further study. This thesis demonstrates that TRAIL and Src mediated non-canonical signalling in NSCLC is complex and involves various downstream mechanisms that affect both cellular and extracellular/TME properties. Additional research is needed to further delineate the mechanisms determining canonical or non-canonical TRAIL signalling. The use of more realistic 3D cancer models in artificial matrices could help to further unravel the relevance of reciprocal tumour cells and TME interactions in TRAIL signalling and facilitate clinical translation.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. 2020;70(1):7-30. doi:10.3322/caac.21590
2. Travis WD, Brambilla E, Nicholson AG, et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances since the 2004 Classification. *J Thorac Oncol*. 2015;10(9):1243-1260. doi:10.1097/JTO.0000000000000630
3. Oberndorfer F, Müllauer L. Molecular pathology of lung cancer: current status and perspectives. *Curr Opin Oncol*. 2018;30(2):69-76. doi:10.1097/CCO.0000000000000429
4. Lemjabbar-Alaoui H, Hassan OUI, Yang YW, Buchanan P. Lung cancer: Biology and treatment options. *Biochim Biophys Acta - Rev Cancer*. 2015;1856(2):189-210. doi:10.1016/j.bbcan.2015.08.002
5. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. *Kinome Profiling of Non-Canonical TRAIL Signaling Reveals RIP1-Src-STAT3 Dependent Invasion in Resistant Non-Small Cell Lung Cancer Cells*.; *J Cell Sci*. 2012;125(Pt 19):4651-4661. doi:10.1242/jcs.109587
6. Irby RB, Yeatman TJ. Role of Src expression and activation in human cancer. *Oncogene*. 2000;19(49):5636-5642. doi:10.1038/sj.onc.1203912
7. Simatou A, Simatos G, Goulielmaki M, Spandidos DA, Baliou S, Zoumpourlis V. Historical retrospective of the SRC oncogene and new perspectives. *Mol Clin Oncol*. 2020;13(4):1-10. doi:10.3892/mco.2020.2091
8. de Looff M, de Jong S, Kruyt FAE. Multiple Interactions Between Cancer Cells and the Tumor Microenvironment Modulate TRAIL Signaling: Implications for TRAIL Receptor Targeted Therapy. *Front Immunol*. 2019;10:1530. doi:10.3389/fimmu.2019.01530
9. Hartwig T, Montinaro A, von Karstedt S, et al. The TRAIL-Induced Cancer Secretome Promotes a Tumor-Supportive Immune Microenvironment via CCR2. *Mol Cell*. 2017;65(4):730-742.e5. doi:10.1016/j.molcel.2017.01.021
10. Mak IW, Evaniew N, Ghert M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res*. 2014;6(2):114-118. http://www.ncbi.nlm.nih.gov/pubmed/24489990. Accessed August 21, 2019.
11. Rothschild S, Gautschi O, Haura E, Johnson F. Src inhibitors in lung cancer: Current status and future directions. *Clin Lung Cancer*. 2010;11(4):238-242. doi:10.3816/CLC.2010.n.030
12. Guarino M. Src signaling in cancer invasion. *J Cell Physiol*. 2010;223(November 2009):14-26. doi:10.1002/jcp.22011
13. Voortman J, Resende TP, Abou El Hassan M a I, Giaccone G, Kruyt F a E. TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. *Mol Cancer Ther*. 2007;6:2103-2112. doi:10.1158/1535-7163.MCT-07-0167
14. Giaccone G, Zucali PA. Src as a potential therapeutic target in non-small-cell lung cancer. *Ann Oncol*. 2008;19(7):1219-1223. doi:10.1093/annonc/mdn048
15. Xu L, Qu X, Li H, et al. Src/caveolin-1-regulated EGFR activation antagonizes TRAIL-induced apoptosis in gastric cancer cells. *Oncol Rep*. 2014;32(1):318-324. doi:10.3892/or.2014.3183
16. Azijli K, Weyhenmeyer B, Peters GJ, de Jong S, Kruyt FAE. Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ*. 2013;20(7):858-868. doi:10.1038/cdd.2013.28
17. Simpkins F, Jang K, Yoon H, et al. Dual Src and MEK inhibition decreases ovarian cancer growth and targets tumor initiating stem-like cells. *Clin Cancer Res*. 2018;24(19):4874-4886. doi:10.1158/1078-0432.CCR-17-3697
18. Yuan M, Xu L, Zhang J, et al. SRC and MEK Co-inhibition Synergistically Enhances the Anti-tumor Effect in Both Non-small-cell Lung Cancer (NSCLC) and Erlotinib-Resistant NSCLC. *Front Oncol*. 2019;9(JUN):586. doi:10.3389/fonc.2019.00586
19. Vaculová A, Hofmanová J, Souček K, Kozubík A. Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells. *FEBS Lett*. 2006;580(28-29):6565-6569. doi:10.1016/j.febslet.2006.11.004
20. Holoch PA, Griffith TS. TNF-related apoptosis-inducing ligand (TRAIL): A new path to anti-cancer therapies. *Eur J Pharmacol*. 2009;625(1-3):63-72. doi:10.1016/j.ejphar.2009.06.066
21. Xu J, Xu Z, Zhou J, Zhuang Z, Wang E. Regulation of the Src-PP2A Interaction in Tumor

Necrosis Factor (TNF) -related Apoptosis-inducing Ligand (TRAIL) -induced Apoptosis *. 2013;288(46):33263-33271. doi:10.1074/jbc.M113.508093
22. De Toni EN, Kuntzen C, Gerbes AL, et al. P60-c-src suppresses apoptosis through inhibition of caspase 8 activation in hepatoma cells, but not in primary hepatocytes. *J Hepatol*. 2007;46(4):682-691. doi:10.1016/j.jhep.2006.10.010
23. Cao X, Pobezinskaya YL, Morgan MJ, Liu Z. The role of TRADD in TRAIL-induced apoptosis and signaling. *FASEB J*. 2011;25(4):1353-1358. doi:10.1096/fj.10-170480
24. Cursi S, Rufini A, Stagni V, et al. Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *EMBO J*. 2006;25(9):1895-1905. doi:10.1038/sj.emboj.7601085
25. Tsang JLY, Jia SH, Parodo J, et al. Tyrosine phosphorylation of caspase-8 abrogates its apoptotic activity and promotes activation of c-Src. *PLoS One*. 2016;11(4). doi:10.1371/journal.pone.0153946
26. Lopez J, Hesling C, Prudent J, et al. Src tyrosine kinase inhibits apoptosis through the Erk1/2-dependent degradation of the death accelerator Bik. *Cell Death Differ*. 2012;19(9):1459-1469. doi:10.1038/cdd.2012.21
27. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3-dependent invasion in resistant non-small cell lung cancer cells. *J Cell Sci*. 2012;125(19):4651-4661. doi:10.1242/jcs.109587
28. Xu D, Zhao H, Jin M, et al. Modulating TRADD to restore cellular homeostasis and inhibit apoptosis. *Nature*. 2020;587(7832):133-138. doi:10.1038/S41586-020-2757-Z
29. Han JH, Moon AR, Chang JH, et al. Erratum: Potentiation of TRAIL killing activity by multimerization through isoleucine zipper hexamerization motif [BMB Rep., 49, 5, (2016) (282-287)]. *BMB Rep*. 2017;50(12):647. doi:10.5483/BMBRep.2016.49.5.245
30. Henry CM, Martin SJ. Caspase-8 Acts in a Non-enzymatic Role as a Scaffold for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation. *Mol Cell*. 2017;65(4):715-729.e5. doi:10.1016/j.molcel.2017.01.022
31. Ramnath RD, Sun J, Bhatia M. Involvement of Src family kinases in substance P-induced chemokine production in mouse pancreatic acinar cells and its significance in acute pancreatitis. *J Pharmacol Exp Ther*. 2009;329(2):418-428. doi:10.1124/jpet.108.148684
32. Ungefroren H, Sebens S, Groth S, Gieseler F, Fändrich F. Differential roles of Src in transforming growth factor-β regulation of growth arrest, epithelial-to-mesenchymal transition and cell migration in pancreatic ductal adenocarcinoma cells. *Int J Oncol*. 2011;38(3):797-805. doi:10.3892/ijo.2011.897
33. Liu ST, Pham H, Pandol SJ, Ptasznik A. Src as the link between inflammation and cancer. *Front Physiol*. Published 2014 Jan 16. doi:10.3389/fphys.2013.00416 doi:10.3389/fphys.2013.00416
34. van Roosmalen IAM, Quax WJ, Kruyt FAE. Two death-inducing human TRAIL receptors to target in cancer: Similar or distinct regulation and function? *Biochem Pharmacol*. 2014;91(4):447-456. doi:10.1016/J.BCP.2014.08.010
35. Azijli K, Weyhenmeyer B, Peters GJ, de Jong S, Kruyt F a E. Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ*. 2013;20(7):858-868. doi:10.1038/cdd.2013.28
36. Lemke J, von Karstedt S, Zinngrebe J, Walczak H. Getting TRAIL back on track for cancer therapy. *Cell Death Differ*. 2014;21(9):1350-1364. doi:10.1038/cdd.2014.81
37. de Miguel D, Lemke J, Anel A, Walczak H, Martinez-Lostao L. Onto better TRAILs for cancer treatment. *Cell Death Differ*. 2016;23(5):733-747. doi:10.1038/cdd.2015.174
38. Kaplan-Lefko PJ, Graves JD, Zoog SJ, et al. Conatumumab, a fully human agonist antibody to death receptor 5, induces apoptosis via caspase activation in multiple tumor types. *Cancer Biol Ther*. 2010;9(8):618-631. doi:10.4161/CBT.9.8.11264
39. Amarante-Mendes GP, Griffith TS. Therapeutic applications of TRAIL receptor agonists in cancer and beyond. *Pharmacol Ther*. 2015; 155:117-131. doi:10.1016/j.pharmthera.2015.09.001
40. Wajant H. Principles of antibody-mediated TNF receptor activation. *Cell Death Differ 2015 2211*. 2015;22(11):1727-1741. doi:10.1038/cdd.2015.109
41. Naval J, Miguel D De, Lleyda AG, Anel A, Lostao LM. Importance of TRAIL Molecular Anatomy in Receptor Oligomerization and Signaling. Implications for Cancer Therapy. *Cancers (Basel)*.

2019;11(4):444. doi:10.3390/cancers11040444
42. Kretz A-L, Trauzold A, Hillenbrand A, et al. TRAILblazing Strategies for Cancer Treatment. *Cancers (Basel)*. 2019;11(4):456. doi:10.3390/cancers11040456
43. García-Martínez JM, Wang S, Weishaeupl C, et al. Selective tumor cell apoptosis and tumor regression in CDH17-positive colorectal cancer models using BI 905711, a novel liver-sparing TRAILR2 agonist. *Mol Cancer Ther*. 2021;20(1):96-108. doi:10.1158/1535-7163.MCT-20-0253
44. Tahir SK, Smith ML, Solomon LR, et al. Abbv-621 Is a Novel and Potent TRAIL Receptor Agonist Fusion Protein That Induces Apoptosis Alone and in Combination with Navitoclax and Venetoclax in Hematological Tumors. *Blood*. 2017;130(Supplement 1):2812-2812. doi:10.1182/BLOOD.V130.SUPPL_1.2812.2812
45. Graves JD, Kordich JJ, Huang TH, et al. Apo2L/TRAIL and the death receptor 5 agonist antibody AMG 655 cooperate to promote receptor clustering and antitumor activity. *Cancer Cell*. 2014;26(2):177-189. doi:10.1016/J.CCR.2014.04.028
46. Tuthill MH, Montinaro a, Zinngrebe J, et al. TRAIL-R2-specific antibodies and recombinant TRAIL can synergise to kill cancer cells. *Oncogene*. 2014;34(April):1-7. doi:10.1038/onc.2014.156
47. Zhong H hai, Wang H yuan, Li J, Huang Y zhuo. TRAIL-based gene delivery and therapeutic strategies. *Acta Pharmacol Sin*. 2019;40(11):1373-1385. doi:10.1038/s41401-019-0287-8
48. Guiho R, Biteau K, Grisendi G, et al. TRAIL delivered by mesenchymal stromal/stem cells counteracts tumor development in orthotopic Ewing sarcoma models. *Int J Cancer*. 2016;139(12):2802-2811. doi:10.1002/ijc.30402
49. Grisendi G, Bussolari R, Cafarelli L, et al. Adipose-derived mesenchymal stem cells as stable source of tumor necrosis factor-related apoptosis-inducing ligand delivery for cancer therapy. *Cancer Res*. 2010;70(9):3718-3729. doi:10.1158/0008-5472.CAN-09-1865
50. Wiersma VR, De Bruyn M, Shi C, et al. C-type lectin-like molecule-1 (CLL1)-targeted TRAIL augments the tumoricidal activity of granulocytes and potentiates therapeutic antibody-dependent cell-mediated cytotoxicity. *MAbs*. 2015;7(2):321-330. doi:10.1080/19420862.2015.1007811
51. El-Mesery M, Trebing J, Schäfer V, Weisenberger D, Siegmund D, Wajant H. CD40-directed scFv-TRAIL fusion proteins induce CD40-restricted tumor cell death and activate dendritic cells. *Cell Death Dis*. 2013;4(11):e916-e916. doi:10.1038/cddis.2013.402
52. De Bruyn M, Wei Y, Wiersma VR, et al. Cell surface delivery of TRAIL strongly augments the tumoricidal activity of T cells. *Clin Cancer Res*. 2011;17(17):5626-5637. doi:10.1158/1078-0432.CCR-11-0303
53. de Bruyn M, Wei Y, Wiersma VR, et al. Cell Surface Delivery of TRAIL Strongly Augments the Tumoricidal Activity of T Cells. *Clin Cancer Res*. 2011;17(17):5626-5637. doi:10.1158/1078-0432.CCR-11-0303
54. Kusters I, Mukherjee N, Jong MR de, Tans S, Koçer A, Driessen AJM. Taming Membranes: Functional Immobilization of Biological Membranes in Hydrogels. *PLoS One*. 2011;6(5):20435. doi:10.1371/JOURNAL.PONE.0020435
55. Nibourg LM, Gelens E, Nibourg SAF, et al. Effects of peptide ratios in nanofibre-based hydrogels for the prevention of capsular opacification. *Acta Ophthalmol*. 2016;94(7):721-729. doi:10.1111/aos.13047
56. Nibourg LM, Gelens E, de Jong MR, Kuijer R, van Kooten TG, Koopmans SA. Nanofiber-based hydrogels with extracellular matrix-based synthetic peptides for the prevention of capsular opacification. *Exp Eye Res*. 2016;143:60-67. doi:10.1016/j.exer.2015.10.001
57. Hernandez-Gordillo V, Kassis T, Lampejo A, et al. Fully synthetic matrices for in vitro culture of primary human intestinal enteroids and endometrial organoids. *Biomaterials*. 2020;254. doi:10.1016/J.BIOMATERIALS.2020.120125
58. Kyburz KA, Anseth KS. Synthetic Mimics of the Extracellular Matrix: How Simple is Complex Enough? *Ann Biomed Eng*. 2015;43(3):489. doi:10.1007/S10439-015-1297-4

Chapter 7

Nederlandse samenvatting
(Dutch summary)

Samenvatting

Longkanker is de meest voorkomende vorm van kanker en is verantwoordelijk voor de meeste kanker gerelateerde sterfgevallen wereldwijd [1]. De meerderheid van de longkanker types (85%) behoort tot het niet-kleincellige longcarcinoom (non-small cell lung cancer; NSCLC) subtype en de minderheid (15%) wordt geclassificeerd als kleincellige longkanker (small cell lung cancer; SCLC) [2]. Tegenwoordig kan NSCLC worden behandeld met chirurgie, radiotherapie, chemotherapieën, doelgerichte therapieën en/of immuuntherapieën. Welke therapie de voorkeur heeft, hangt af van het stadium, het histologische subtype en het genetische profiel van de ziekte. Ondanks de succesvolle ontwikkeling van doelgerichte therapieën voor NSCLC in de afgelopen decennia, waaronder receptor tyrosine kinases (RTK) en immuun checkpoint remmers, is de 5-jaarsoverleving relatief laag [1,3,4].

Tumornecrosefactor (TNF) gerelateerde apoptose-inducerende ligand (TRAIL) is een cytokine dat apoptose kan activeren in cellen door binding aan TRAIL receptoren, waaronder TRAIL-R1 en TRAIL-R2. TRAIL receptor agonisten zijn veelbelovende antikankermiddelen, omdat ze selectief apoptose in kankercellen kunnen induceren. De activering van TRAIL-R1/R2 door agonisten resulteert in receptor trimerisatie en intracellulaire complexvorming aan de zogenoemde doodsdomeinen van deze receptoren. Afhankelijk van de eiwitten die worden gerekruteerd wordt het dood-inducerende signaleringscomplex (DISC) of het secundaire complex geassembleerd. Bij de assemblage van de DISC worden de Fas-Associated protein with Death Domain (FADD) en caspase 8 gerekruteerd, hetgeen resulteert in caspase 8 activatie gevolgd door irreversibele activatie van apoptose, ook wel de canonieke TRAIL-apoptotische signaleringsroute genoemd. TRAIL receptor activatie in apoptose resistente cellen leidt tot vorming van het secundaire complex dat onder andere bestaat uit receptor-interacting serine/threonine protein kinase 1 (RIPK1), TNF receptor associated factor 2 (TRAF2), de TNF receptor type 1 associated death domain (TRADD), FADD en caspase 8. Dit complex activeert cel proliferatie en overlevingsmechanismen en stimuleert ook cel migratie en invasie, hetgeen tumorprogressie bevordert, en ook wel niet-canonieke TRAIL-signalering genoemd wordt [5]. Bij deze niet-canonieke TRAIL-signalering wordt onder meer de RIPK1-Src-STAT3-route geactiveerd die wij in een eerdere studie reeds hebben geïdentificeerd [5]. Src is een niet-receptor tyrosine kinase en een bekend oncogen en tumor promotor die vaak tot over-expressie komt of hyperactief is bij kanker, waaronder NSCLC. Src is betrokken bij vele cellulaire processen zoals cel overleving, proliferatie en migratie [6,7]. Of Src-activering een belangrijke rol speelt bij niet-canonieke TRAIL-signalering in NSCLC-cellen is nog niet goed onderzocht.

Het resultaat van TRAIL-R activatie, apoptose of tegenovergestelde effecten wordt niet alleen bepaald door intrinsieke cellulaire factoren, maar ook door signalen afkomstig van

buiten de cellen, de zogenaamde extrinsieke factoren uit de tumor micro-omgeving (TME). De TME bestaat uit verschillende celtypen, waaronder immuun cellen, stromale cellen en fibroblasten, maar ook uit niet-cellulaire componenten zoals de extracellulaire matrix (ECM). Wederzijdse (in)directe cel-cel of cel-ECM interacties kunnen de intracellulaire TRAIL-signalering moduleren en bepalen hiermee de gevoeligheid van cellen voor TRAIL-R geïnduceerde celdood [8]. Zo is aangetoond dat TRAIL-resistente NSCLC cellen de TME kunnen beïnvloeden door de secretie van cytokinen die immuun cellen aantrekken met een tumorstimulerende werking. Dit resulteert in een kankergroei bevorderende TME die ook metastase kan stimuleren, zoals uitvoerig en gedetailleerd is uiteengezet in **hoofdstuk 2** [8,9].

In oncologisch onderzoek wordt veel gebruik gemaakt van *in vitro* kankermodellen, zoals kankercellijnen gekweekt als een monolaag in kweekflessen. Echter, de vertaling van resultaten verkregen in deze 2-dimensionale (D) *in vitro* modellen naar de complexe 3D *in vivo* structuur van tumoren bij patiënten is slechts beperkt. Het gebruik van preklinische diermodellen als tussenstap naar klinische vertaling levert een realistischer tumormodel op, maar deze modellen vertonen soortafhankelijke verschillen in belangrijke moleculaire, immunologische, genetische en cellulaire mechanismen. Bovendien is het gebruik van diermodellen arbeidsintensief en kostbaar [10]. Het gebruik van 2D-modellen en de beperkingen van de *in vivo* modellen verklaart gedeeltelijk waarom minder dan 8% van de preklinisch geteste geneesmiddelen ook werkzaam blijkt in klinische studies[10]. *In vitro* 3D-modellen, ten opzichte van 2D modellen, zijn een betere weergave van de *in vivo* situatie en worden daarom steeds vaker gebruikt. Om het kweken van sferoïde tumorcellen in een 3D-model te ondersteunen wordt vaak de biologische matrix Matrigel gebruikt. Matrigel bestaat uit ECM geïsoleerd uit Englebreth-Holm-Swarm-muissarcoomcellen. Echter, Matrigel beïnvloedt het gedrag van tumorcellen door de aanwezigheid van onbekende concentraties groeifactoren, cytokinen en andere eiwitten afkomstig van de muissarcoomcellen. Daarnaast komen zogenoemde ‘batch to batch’ variaties vaak voor bij Matrigel wat leidt tot verminderde reproduceerbaarheid van experimenten. Het gebruik van synthetische matrices voor 3D tumor celkweken kan de beperkingen van biologische matrices voorkomen. Om de *in vivo* tumor situatie beter te kunnen nabootsen, zowel in 2D- als in 3D- *in vitro* modellen, kunnen ook componenten van de TME worden toegevoegd aan de synthetische matrix. Op deze manier is het mogelijk om interacties tussen TME en tumorcellen te onderzoeken en geven deze modellen tevens meer realistische en klinisch relevante resultaten om bijvoorbeeld de werking van mogelijke geneesmiddelen bij patiënten te voorspellen. Het gebruik van minder geschikte preklinische modellen waarin anti-kanker activiteit van TRAIL-therapie gezien werd, hebben waarschijnlijk de klinische vertaling belemmerd.

Zoals hierboven vermeld, zijn de onderliggende mechanismen die de activering van canonieke of niet-canonieke TRAIL-signalering in kankercellen bepalen nog niet volledig begrepen. Daarnaast zijn de mechanismen die bijdragen aan niet-canonieke TRAIL-signalering niet goed onderzocht. Een beter inzicht in deze mechanismen zal naar verwachting prognostische markers en therapeutische doelen kunnen opleveren die bijdragen aan het optimaliseren van anti-kanker TRAIL therapieën. Het doel van dit proefschrift was om de rol van Src bij het reguleren van apoptotische en niet-canonieke TRAIL-signalering in meer detail te onderzoeken en om te bestuderen hoe TRAIL-geïnduceerde Src-activering bijdraagt aan tumor stimulerende effecten. Hierbij werd ook de mogelijke betrokkenheid van Src bij TRAIL geïnduceerde veranderingen in cytokinesecretie onderzocht en de mogelijke gevolgen voor immuun cel activatie. Verder werd de nieuwe synthetische matrix ArtiCYT getest als mogelijke vervanger van Matrigel bij 3D tumormodellen en werd de werkzaamheid van cisplatinum en TRAIL bij NSCLC onderzocht.

In **hoofdstuk 1** werd een algemene inleiding gegeven over longkanker, TRAIL signalering, TME en 3D-modellen. Longkanker en huidige therapeutische behandelingen werden kort beschreven en de behoefte aan nieuwe therapieën benoemd. TRAIL-R1/R2 apoptotische en niet-canonieke signaalroutes werden in detail geïntroduceerd. De rol van Src als een mogelijke belangrijke schakel bij TRAIL-signalering in NSCLC werd uitgelegd en de noodzaak om onderliggende mechanismen van TRAIL-signalering verder te ontrafelen. Het effect van de TME op tumorvorming en de mogelijke betrokkenheid van TRAIL-Src-signalering hierbij werd toegelicht. Daarna werd de urgentie voor het gebruik van 3D-modellen in kankeronderzoek uitgelegd alsmede de noodzaak van het toepassen van synthetische matrices voor 3D- tumorcelkweken. Ten slotte werden de doelstellingen en de opzet van het proefschrift uiteengezet.

In **hoofdstuk 2** werd een uitgebreid literatuuroverzicht gegeven van de complexe en bi-directionele interacties tussen tumorcellen en de TME en hoe deze de werkzaamheid van zowel endogeen als exogeen toegediende TRAIL-receptoragonisten kunnen beïnvloeden. Hiertoe behoren verschillende componenten van de TME, waaronder immuun-effectorcellen, neutrofielen, macrofagen en niet-hematopoëtische stromale cellen. Ook werden de niet-cellulaire biochemische en biofysische eigenschappen van de TME besproken die ook een effect hebben op TRAIL signalering, zoals mechanische stress, zuurgraad, hypoxie en glucosedeprivatie. Tumor-TME interacties kunnen zowel tumor stimulerende als remmende effecten hebben, mede afhankelijk van het tumortype en model. We concludeerden dat voor het ontwikkelen van een effectieve TRAIL-behandeling de mechanismen en factoren die verantwoordelijk zijn voor TRAIL-resistentie moeten worden opgehelderd, waarbij ook de bijdrage van verschillende TME-componenten aan

TRAIL-signalering onderzocht moet worden.

In **hoofdstuk 3** hebben we de bijdrage van Src en de onderliggende mechanismen in TRAIL geïnduceerde (niet-)canonieke signalering in NSCLC-cellijnen onderzocht. Tijdsafhankelijke verschillen in rhTRAIL-geïnduceerde Src-fosforylatie patronen werden gevonden tussen de TRAIL gevoelige H460 en resistente A549 NSCLC. TRAIL leidde tot snelle Src-Tyr418-fosforylering en activering in A549-cellen, maar niet in H460. De gevoeligheid voor rhTRAIL geïnduceerde celdood was niet afhankelijk van Src aangezien het verlagen van Src-expressie of farmacologische Src-remming geen effect had op apoptose in A549. Het verlagen van TRADD expressie, een eiwit dat deel uitmaakt van het secundaire complex, met behulp van siRNA leidde tot verminderde TRAIL-afhankelijke Src-activering in A549-cellen. Co-IP-LC-massaspectrometrische analyse werd uitgevoerd om eiwitten betrokken bij Src-afhankelijke TRAIL-signalering in H460- en A549-cellen te identificeren. In A549-cellen nam het aantal eiwitten toe dat interacteerde met Src na behandeling met rhTRAIL, terwijl het tegenovergestelde werd waargenomen bij H460-cellen. Bekende tumor-geassocieerde eiwitten die aan Src binden werden gevonden in rhTRAIL behandelde A549-cellen, waaronder componenten van de RAF/MEK/ERK-, Wnt- en SMAD3-signaleringsroutes. Functionele experimenten toonden aan dat Src de fosforylering van MEK1/2 en ERK medieerde en de fosforylering van SMAD3 voorkwam. Tevens bleek dat Src nodig was voor (peri)nucleaire translocatie van ERK en β-catenine in A549-cellen. Klonale groei van zowel Src tot expressie brengende als Src-deficiënte A549-cellen werd niet beïnvloed door blootstelling aan rhTRAIL, hoewel Src-depletie en/of MEK1/2-remming de koloniegrootte en het aantal kolonies significant verminderde. We concludeerden dat TRAIL-afhankelijke Src-activering vervolgens MEK-ERK-, SMAD3- en β-catenine-signalering moduleert via (in)directe interacties. De exacte mechanismen moeten echter verder worden opgehelderd, evenals de potentiële therapeutische toepassingen van deze eiwitten en signaleringsroutes in combinatie TRAIL-R-agonisten.

In **hoofdstuk 4** werd de mogelijke betrokkenheid van Src bij de samenstelling van het door rhTRAIL-geïnduceerde secretoom van resistente A549 NSCLC onderzocht en de daaropvolgende impact van het secretoom op monocyt differentiatie, macrofagen polarisatie en CD8+ T-cel activering. Geconditioneerd medium (CM) werd verkregen van Src-proficiënte (S+) en deficiënte (S-) A549-cellen die ofwel kort (1-2 uur, CM-S) of lang (24 uur, CM-L) met rhTRAIL (T+) waren behandeld of juist niet (T-). Het effect van CM op differentiatie en polarisatie van monocyten en gedifferentieerde M0-macrofagen werd bepaald door flowcytometrische-analyses van M0 (CD11b, CD68), M1 (CD80, CD86) en M2 (CD163, CD206) markerexpressie. Blootstelling aan elke van de CM leidde tot polarisatie van macrofagen, onafhankelijk van Src-status en rhTRAIL-behandeling. T-celactivatie nam iets af na blootstelling aan CM-S(S+/T+), alhoewel niet significant, maar bij de andere

CM-S werden geen effecten gezien. Een cytokine-array analyse van de CM-S liet hogere cytokineniveaus zien in CM-S van Src-proficiënte cellen; deze toename was vooral bij GRO-α/β/γ en GRO-α (CXCL1). De levels van deze cytokines namen af in CM-S (S-/T-) en waren verder verlaagd in CM-S(S-/T+). Meer diepgaande analyses van de CM-S en CM-L met massaspectrometrie toonden de aanwezigheid aan van cytokines die geannoteerd zijn aan immuun-gerelateerde biologische GO-processen in CM-S(S+/T-), CM-S(S+/T+) en CM-L(S+/T-). Concluderend, onze voorlopige resultaten suggereren dat zowel CM-S als CM-L macrofaag differentiatie en polarisatie bewerkstelligen, onafhankelijk van Src-status en rhTRAIL-behandeling. Src in A549-cellen bleek de secretie van immuun gerelateerde cytokinen te reguleren en zou betrokken kunnen zijn bij het verminderen van CD8+ T-celactivering. Verdere studies zijn echter nodig om deze preliminaire bevindingen te bevestigen.

In **hoofdstuk 5** werd de bruikbaarheid van de synthetische peptide-gekoppelde matrix, ArtiCYT-p, voor het kweken van 3D kanker sferoïden onderzocht en vergeleken met de biologische matrix Matrigel. Allereerst werd de ArtiCYT-samenstelling geoptimaliseerd voor de 3D sferoïde groei van 7 verschillende menselijke borst-, long- en eierstokkankercellijnen. Vervolgens werd de toepasbaarheid van ArtiCYT-p voor zowel *high throughput screening* (HTS) als *high content analysis* (HCA) onderzocht door de gevoeligheid van NSCLC cellen te bepalen voor cisplatine chemotherapie en de doelgerichte therapie met recombinante humane (rh)TRAIL. ArtiCYT-p bleek geschikt voor gebruik in zowel HTS als HCA studies en er werden geen substantiële verschillen in geneesmiddelgevoeligheid waargenomen tussen Matrigel en ArtiCYT-p 3D-modellen. Daarnaast resulteerde de aanwezigheid van HS27a feeder cellen in minder rhTRAIL-geïnduceerde celdood van H460-sferoïden in ArtiCYT-p en Matrigel, wat een beschermend effect tegen rhTRAIL door uitgescheiden factoren van de feeder cellen suggereert. We hebben in deze studie gevonden dat ArtiCYT-p mogelijk een geschikte synthetische vervanger is voor Matrigel voor 3D-kanker modellen en voor het uitvoeren van HCA en HTS geneesmiddelen onderzoek.

Referenties

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. 2020;70(1):7-30. doi:10.3322/caac.21590
2. Travis WD, Brambilla E, Nicholson AG, et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances since the 2004 Classification. *J Thorac Oncol*. 2015;10(9):1243-1260. doi:10.1097/JTO.0000000000000630
3. Oberndorfer F, Müllauer L. Molecular pathology of lung cancer: current status and perspectives. *Curr Opin Oncol*. 2018;30(2):69-76. doi:10.1097/CCO.0000000000000429
4. Lemjabbar-Alaoui H, Hassan OUI, Yang YW, Buchanan P. Lung cancer: Biology and treatment options. *Biochim Biophys Acta - Rev Cancer*. 2015;1856(2):189-210. doi:10.1016/j.bbcan.2015.08.002

5. Azijli K, Yuvaraj S, Peppelenbosch MP, et al. Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3-dependent invasion in resistant non-small cell lung cancer cells. *J Cell Sci*. 2012;125(19):4651-4661. doi:10.1242/jcs.109587
6. Irby RB, Yeatman TJ. Role of Src expression and activation in human cancer. *Oncogene*. 2000;19(49):5636-5642. doi:10.1038/sj.onc.1203912
7. Simatou A, Simatos G, Goulielmaki M, Spandidos DA, Baliou S, Zoumpourlis V. Historical retrospective of the SRC oncogene and new perspectives. *Mol Clin Oncol*. 2020;13(4):1-10. doi:10.3892/mco.2020.2091
8. de Looff M, de Jong S, Kruyt FAE. Multiple Interactions Between Cancer Cells and the Tumor Microenvironment Modulate TRAIL Signaling: Implications for TRAIL Receptor Targeted Therapy. *Front Immunol*. 2019;10:1530. doi:10.3389/fimmu.2019.01530
9. Hartwig T, Montinaro A, von Karstedt S, et al. The TRAIL-Induced Cancer Secretome Promotes a Tumor-Supportive Immune Microenvironment via CCR2. *Mol Cell*. 2017;65(4):730-742.e5. doi:10.1016/j.molcel.2017.01.021
10. Mak IW, Evaniew N, Ghert M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res*. 2014;6(2):114-118. http://www.ncbi.nlm.nih.gov/pubmed/24489990. Accessed August 21, 2019.

Appendix

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