might be due to altered constitutive priming by diaminopimelic acid–type peptidoglycan[1]. Because the gut provides the greatest source of this bacterial molecule, they decided to turn their attention to a possible link between intestinal microflora and general function of neutrophils[1].

Clarke *et al.*[1] isolated bone marrow–derived neutrophils from antibiotic-treated and germ-free mice and found that these cells had reduced killing capacity *in vitro*. By comparing the results obtained in Nod1-knockout, Nod2-knockout and Toll-like receptor 4–knockout mice, the authors identified Nod1 as a crucial mediator of the systemic priming of neutrophils by components of the intestinal microflora[1].

Next, they found that soluble peptidoglycan, released by bacteria present in the intestinal lumen, could translocate into the circulation and serve as a molecular mediator responsible for the remote systemic priming of neutrophils in the bone marrow[1]. To show this, Clarke *et al.*[1] colonized the gut of mice with a strain of *Escherichia coli* with radiolabeled diaminopimelic acid and found radioactivity in cells of the bone marrow three days after inoculation.

In addition, the authors designed an original technique relying on *in vitro* luciferase reporter assays to monitor the presence of peptidoglycan in the serum. In agreement with their model, the researchers found reduced quantities of peptidoglycan in sera from germ-free or antibiotic-treated mice[1]. Finally, the authors demonstrated that administration of Nod1 ligand (given intraperitoneally) to antibiotic-treated mice was sufficient to restore systemic neutrophil killing capacities, whereas Nod2 ligand was unable to do so[1]. Peptidoglycan released into the circulation from gut bacteria, by enhancing the general killing capacity of neutrophils, may affect host responses to a wide range of pathogenic microorganisms—not only bacteria—that are targeted by these innate immune cells.

The observations reported by Clarke *et al.*[1] suggest a much more complex relationship between the intestinal microflora and innate immunity than previously anticipated. They also raise new questions for future investigations. It is unclear how Nod1 ligands diffuse from the gut to the circulation; is it a passive or active mechanism? It will also be important to determine whether other gut-derived microbial products—such as flagellin, lipoproteins or CpG DNA—can also circulate and modulate the innate immune system remotely. Are immune cells, such as antigen-presenting cells or lymphocytes, affected by a similar mechanism?

The findings also dovetail with observations that peptidoglycan products can modulate slow-wave sleep[8,9]. Indeed, early investigations had shown that a long-sought sleep-promoting factor found in the circulation was a peptidoglycan moiety; however, the source of this bacterial molecule was not identified. In light of the results from Clarke *et al.*[1], it will be interesting to characterize the role of the intestinal microbiota on sleep parameters.

Finally, for patients receiving broad-spectrum antibiotic therapies, it will be crucial to determine the duration of the prophylactic effect provided by Nod1 ligands on neutrophils to avoid a general blunting of innate immune capacities after treatment. Therefore, in addition to favoring the emergence of antibiotic-resistant pathogenic bacterial strains, imprudent use of antibiotics may have unexpected adverse effects on systemic innate immunity, a topic that will undoubtedly require further investigation in the near future. On the flip side, the results by Clarke *et al.*[1] illustrate that the rational targeting of the intestinal microflora by drugs or specific diets may hold great prophylactic and therapeutic potential.

COMPETING INTERESTS STATEMENT
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Inflammatory proteinase slips into tumor cells

Barbara Fingleton

Inflammatory cells can promote tumor cell proliferation, but the range of mechanisms has not been fully explored. A proteinase produced by neutrophils is now shown to enter tumor cells and promote their proliferation (pages 219–223).

A recent report from the US National Institutes of Health indicates that cancer rates are falling[1]. But despite this welcome trend, for many people with cancer, there are still far too few treatment options. Overall, cancers of the lung and bronchus have the highest incidence and mortality rates.

In this issue of *Nature Medicine*, Houghton *et al.*[2] provide a possible new therapeutic approach for lung cancer while outlining a previously unknown way that inflammation can promote tumorigenesis. The authors show that neutrophils, as purveyors of their own specific proteinase, neutrophil elastase, can profoundly influence lung cancer growth and development[2]. They further illustrate how targeting neutrophil elastase with already available inhibitors can affect a crucial intracellular signaling pathway, ultimately blocking tumor growth[2]. These results suggest a new treatment approach that could move rapidly to clinical testing and perhaps into the therapeutic arsenal for patients who need a new option.

Although the contribution of inflammation to tumor progression is well accepted for several cancers, it is debated for others, including lung cancer. Perhaps the strongest evidence comes from studies of tumor-associated macrophages in breast cancer[3]. High levels of these cells are linked to aggressive, metastatic disease and, ultimately, shorter survival. The various growth, survival and angiogenic factors released by macrophages may explain the cells' potent tumor-promoting capabilities[3].

Neutrophils, innate inflammatory cells that carry granules containing various bactericidal proteins, have received far less attention—although they have recently been shown to polarize into populations that either promote or quell tumorigenesis[4]. Neutrophils may be neglected in part because they are considered representatives of an 'acute' inflammatory response, whereas macrophages indicate a chronic response. This

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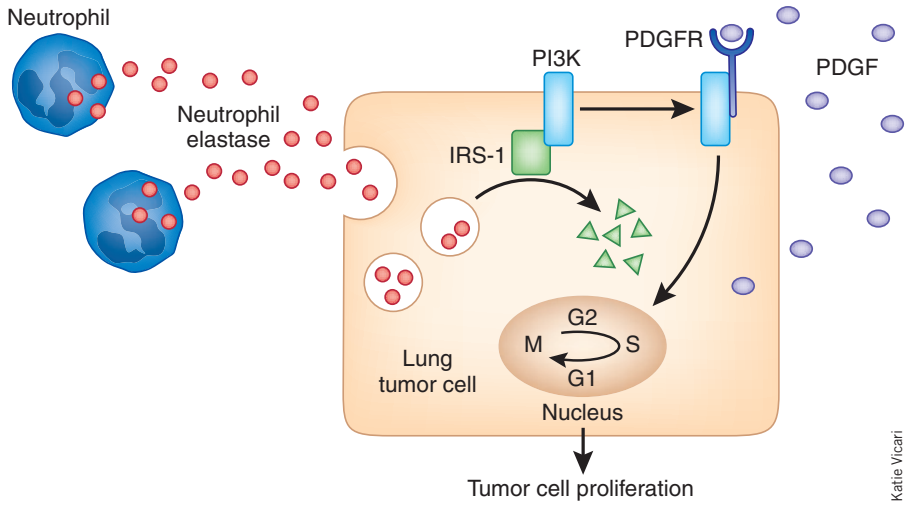

Figure 1 Neutrophil elastase controls tumor cell proliferation. Houghton *et al.*[2] show that activated neutrophils within the lung release neutrophil elastase, which is taken up by adjacent epithelial tumor cells. IRS-1, associated with PI3K, is degraded by the neutrophil elastase, thus freeing PI3K to interact with the cytoplasmic domain of ligand-bound PDGFR. This transmits a mitogenic signal and results in proliferation of the tumor cell.

overly simplistic delineation fails to take into account a dynamic tumor environment where different signals are constantly being released, thus altering the recruited cell populations.

The airways of the lungs are a ready entryway for environmental irritants, such as the components of cigarette smoke, as well as airborne microorganisms. As first-line responders to extraneous agents, neutrophils are numerous in the lungs. One protease released by activated neutrophils is neutrophil elastase, which was originally defined as an extracellular matrix–modifying enzyme responsible for the elastolytic damage in emphysema. Recent work, however, has greatly expanded the possible substrates of this protease to include cytokines and cytokine receptors[5], making neutrophil elastase a potential regulator of the inflammatory process.

To assess the dominant roles for neutrophil elastase in lung cancer, Houghton *et al.*[2] used neutrophil elastase–deficient mice in both transgenic and graft lung tumor models. They observed a marked survival advantage in mice lacking the enzyme[2].

Unexpectedly, none of the known extracellular substrates of neutrophil elastase seemed to contribute to this effect. Instead, by examining neutrophil elastase localization, the authors discovered a new way that this proteinase can function[2]. The enzyme, they found, is taken up into a specific endosomal compartment of epithelial tumor cells, where it degrades the adaptor molecule insulin receptor substrate-1 (IRS-1)[2]. By degrading IRS-1, neutrophil elastase frees a subunit of phosphoinositide 3-kinase, which normally interacts with IRS-1, to instead interact with another protein, the platelet-derived growth factor receptor (PDGFR). This receptor is present with its ligand in lung tumor cells but not normal lung epithelium. The interaction, in turn, amplifies PDGF signaling and accelerates cell proliferation and tumor growth (**Fig.1**). The findings suggest that it is IRS-1 abundance that controls tumor proliferation.

The authors provide experimental relevance to people with cancer by showing in human lung tumor specimens an inverse relationship between neutrophil elastase and IRS-1 expression[2]. Finally, they return to mouse models to show antitumor efficacy of drugs that inhibit neutrophil elastase[2]. One such neutrophil elastase inhibitor, silvelestat (ONO-5046), is already approved in Japan for use in acute respiratory distress syndrome and is in clinical trials for other indications[6].

Perhaps the most exciting aspect of the study of Houghton *et al.*[2] is their finding of an intracellular function of neutrophil elastase. There are reports of several normally secreted proteinases, such as matrix metalloproteinase-2 (ref. 7), having apparent intracellular functions, but this activity is within the cells that produce the enzymes. What is remarkable about the current study is that neutrophil elastase is apparently released normally by neutrophils but then undergoes uptake by adjacent tumor cells. The internalizing mechanism, which remains unidentified, causes the proteinase to be localized to particular endosomal vesicles, where it acts on its substrate IRS-1. The localization of neutrophil elastase inside the cell could occur similarly to the way granzymes enter target cells in association with perforin[8] or, perhaps more likely, via receptor-mediated internalization, as has been proposed for pepsin[9].

Determining the mechanism of entry will presumably allow us to narrow down the possible cell types that can be entered by neutrophil elastase. Although neutrophil elastase is regarded as quite a promiscuous enzyme, its substrate profile was, before this study, at least limited to the proteins it could access within the local extracellular milieu of activated neutrophils. This newly identified internal localization enhances the range of possible substrates and may greatly complicate our understanding of how neutrophils influence their environment.

Although IRS-1 is the direct substrate of neutrophil elastase, it is the subsequent effect on phosphoinositide 3-kinase (PI3K) function that alters tumor behavior. PI3K is regarded as one of the key control nodes of intracellular signaling, as it can link to processes regulating cellular metabolism, migration, proliferation and survival. It is an enzyme containing both regulatory and catalytic subunits that catalyzes a seemingly straightforward reaction—the addition of a phosphate to the lipid second messenger phosphatidylinositol biphosphate. This deceptively simple function can unleash an enormous range of cellular responses explaining the important role PI3K has in development, normal cellular function and diverse diseases, including cancer, cardiovascular and autoimmune problems[10].

The indirect regulation of PI3K function by neutrophil elastase described by Houghton *et al.*[2] suggests that inhibitors directed against proteinases can have far-reaching effects on particular signaling pathways. Although direct pharmacological inhibitors of PI3K are in clinical development for a range of disorders, there is reason to approach their use with caution, given the myriad of processes in all cell types that could be affected. Using a more subtle approach such as a neutrophil elastase inhibitor to alter the downstream output of PI3K activity may be a much more attractive alternative.

A recent study suggests that chronic inflammatory diseases of the lungs, such as emphysema, are clearly associated with tumor development, independent of smoking[11]. Additionally, studies in mouse models have indicated roles for inflammatory mediators in lung tumor progression[12]. All of these studies identify various inflammatory populations or molecules as being crucial; however, few are done using the same model. One of the advantages of the study of Houghton *et al.*[2] is the use of several models, which may indicate that the neutrophil elastase—IRS-1 axis is a major pathway. Clearly, however, all of these studies together strongly argue for considering lung cancer as an inflammation-associated disease that may respond to anti-inflammatory drugs.

It remains to be seen whether IRS-1 is the only intracellular target for neutrophil elastase

or whether there are other diseases with inflammatory components in which neutrophil elastase–regulated signaling occurs. What is clear is that the report by Houghton *et al.*[2] suggests a new, readily testable therapeutic approach to lung cancer, which could be a welcome option for patients in need.

COMPETING INTERESTS STATEMENT
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Gene therapy activates EVI1, destabilizes chromosomes

Cynthia E Dunbar & Andre Larochelle

One hazard of gene therapy is that the vector will insert into an inappropriate location, causing aberrant expression of genes that can lead to disease. A new study reveals how such events occurred in a recent gene therapy trial using a vector that has now fallen out of favor (pages 198–204).

In the 20 years since the first subjects enrolled in gene therapy trials, the field has been on a roller coaster. The 1990s provided no clear evidence of clinical benefit, and a seminal 1995 report commissioned by the US National Institutes of Health director suggested that enthusiasm had outstripped knowledge[1]. Scientists went back to the laboratory to design better vectors and to hone their understanding of target cells and diseases.

The advances in the laboratory came to fruition in 2000–2002, when Fischer, Cavazzano-Calvo and their coworkers in France successfully treated subjects with a form of severe combined immunodeficiency (SCID) by expressing the corrective gene, *IL2RG* (encoding interleukin-2 receptor-γ (IL-2Rγ)), in their autologous hematopoietic stem and progenitor cells (HSPCs)[2]. Unfortunately, the elation after this success lasted only until 2003, when several subjects in the trial developed acute T cell leukemia. The gene therapy vector, Moloney murine leukemia virus (MLV) had inserted near the locus for *LMO2* and had bumped up the expression of this protooncogene through the vector's enhancer activity[3]. Researchers initially held out hope that these adverse events were unique to the SCID trial. They thought that in this form of SCID there could be a unique interaction between constitutive expression of the IL2-Rγ signaling receptor carried by the vector and overexpression of LMO2, and that therefore this complication would not be seen in gene therapy protocols targeting HSPCs in other diseases.

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A study by Stein *et al.*[4] in this issue of *Nature Medicine* suggests that adverse events linked to integrating vectors are more general. The researchers attempted to correct another type of immunodeficiency disorder, chronic granulomatous disease (CGD), using the same class of vector (MLV) and the same autologous transplantation approach used by the French team. They found that the vector activated expression of the *MDS–EVI1* protooncogene, resulting in eventual dominance of single vector-containing clones and myelodysplasia/acute myeloid leukemia (MDS/AML)[4]. The authors provide tantalizing clues as to the mechanism of leukemogenesis due to *MDS–EVI1* overexpression[4]. The findings shut the door on the use of MLV vectors, at least for HSPC gene therapy, and add momentum to the search for safer integrating vectors. The work also provides insights into the mechanisms of certain forms of human leukemia.

Nature Medicine published the first part of this story in 2006. Ott *et al.*[5] reported the initial results of their gene therapy trial for CGD, which is characterized by the inability to fight bacterial and fungal infections due to mutations in the NADPH oxidase complex, which is necessary for neutrophil function. The researchers corrected the gene in autologous HSPCs that were then transplanted back into the subjects[5]. The first two young men treated resolved life-threatening infections and were doing well, stably engrafted with 50–70% gene-corrected cells (**Fig. 1**).

Even in the initial study, however, the authors documented an unexpected increase in the proportion of gene-modified myeloid cells over time, completely accounted for by an overrepresentation of cells with vector insertions at the *MDS1–EVI1* genomic locus. A variety of gene products are encoded by this locus via alternative splicing, including the zinc finger transcription factors Evi1 and Mds1-Evi1. Their function is poorly understood, but, given the involvement of the *MDS1–EVI1* locus in spontaneous chromosomal translocations in human leukemias and in viral-induced leukemias in animal models, they are classified as protooncogenes and have been shown to affect the cell cycle and apoptosis.

The CGD findings dovetail with work in experimental systems published after the CGD clinical trial began. A high frequency of MLV vector insertions activating expression from the *MDS1–EVI1* locus was reported in a rhesus macaque model[6] and in mouse bone marrow cells immortalized *in vitro* after transduction with MLV vectors[7].

In the current study, Stein *et al.*[4] report progressive decline in blood counts—including platelets, red blood cells and neutrophils—in the two CGD subjects at 15 and 28 months, respectively, after gene therapy. Bone marrow examination showed myelodysplasia, a preleukemic clonal marrow failure syndrome characterized by ineffective and disordered myeloid maturation. Despite the persistent high frequency of vector-corrected neutrophils, expression of NADPH oxidase dropped precipitously in both subjects over time. The silencing of NADPH oxidase, they found, occurred through progressive CpG methylation of the promoter contained in the long terminal repeat (LTR) of the vector, a region crucial for transgene expression[4]. Cytopenias and loss of oxidase function in one of the men resulted in a series of infections that led to his death. The second subject was referred for unrelated donor stem cell transplantation while still infection free.