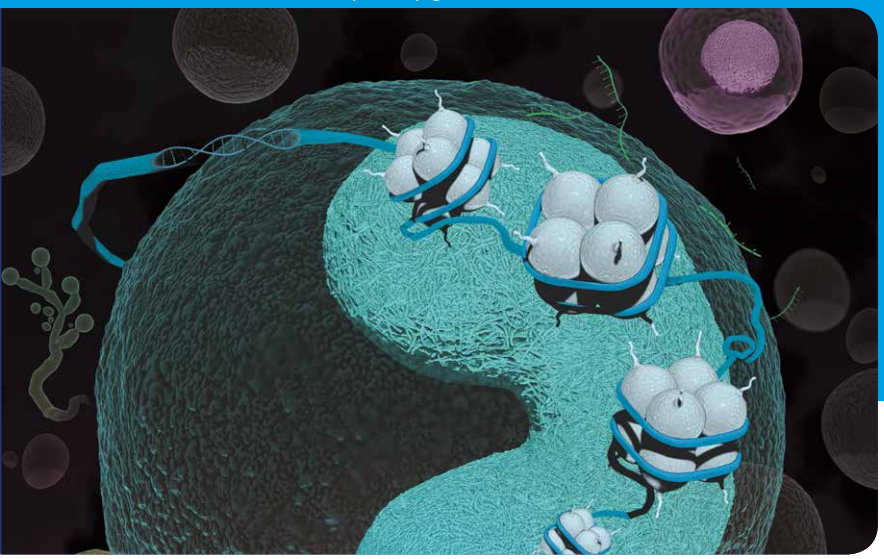

The watchmen of immunity need signals and sugar

Epigenetic processes determine whether innate immune cells actively protect a tissue, take up posts there, or sleep on the job

Cells called monocytes constantly circulate through our bloodstream, like night watchmen or paramedics, on the lookout for attacks, injuries or infections. Monocytes generally stay on patrol for three to five days. During this time they can become an upgraded and more specialized member of the task force: they can either notice a problem and take action or they take up specialized posts in a particular tissue. In both cases they differentiate into macrophages, which can carry out some basic repairs, digest intruding pathogens, or call in reinforcements. Like human guards, macrophages adopt different styles in carrying out their jobs. Some take on more responsibilities to protect the host, while others are ineffective and fail to be roused by a threat.

In two papers in the Sept. 26, 2014, edition of Science, BLUEPRINT scientists identified a number of changes that monocytes undergo as they differentiate into macrophages. Their studies capture a detailed picture of epigenetic processes that alter the activity of a huge number of genes. Several of the changes seem to involve signals that help macrophages react to threats; others are made so that the cell can metabolize an increased amount of sugar. The study shows how macrophages obtain their specialized instructions. These results have strong implications for the understanding of the fast immune response in our body and how we can potentially interfere with failures in the future.

"The first type of cell - monocytes - experiences a sort of 'trauma' when it encounters a severe infection or trauma," says Henk Stunnenberg of Radboud University in the Netherlands, head of BLUEPRINT and a key investigator in the study. "The next time something happens, it mounts a very poor response, which is what you see in cases of severe sepsis, where an infection triggers an inflammation across the entire body, or other conditions. These monocytes develop into macrophages that produce a very low number of inflammatory signals. They often fail to draw other types of cells to the tissue, which is necessary for a full-fledged immune response and a defense against secondary infections."

Other macrophages react very differently – faced with one threat, they expand their response to others. This represents a type of "training" that is stimulated by their first taste of trouble. Without such cells, tissues would experience the biological equivalent of looting during a blackout. Any infection would throw open the door to a variety of opportunistic, secondary infections. Trained macrophages keep them at bay, also playing a role in the way the body responds to live vaccines. Immunization against tuberculosis or measles, for example, stimulates macrophages to respond more powerfully to additional microbial infections.

"Training" is often used to refer to a quite different aspect of the immune system – the process by which our bodies produce antibodies against a pathogen, "remember"

it, and mount a defense if it ever returns. That process, called adaptive immunity, is targeted at a very specific pathogen and involves other types of cells. They behave like snipers that will only fire at a precise target.

"Macrophages, on the other hand, are a part of the innate immune system, and their behavior is much more generic," Henk says. "They can usually distinguish between their own body and something foreign, but have only a moderate capacity to distinguish between various invading microbes. When they see a pathogen, their response is more like throwing hand grenades at it. So their 'training' is quite basic and has little specificity. It means the next time an invading microbe appears, they'll have more grenades on hand, and throw them more quickly."

Innate immunity is a blunt response, but a quick one. During later phases of a disease adaptive immunity steps in, bringing along an array of highly specialized cells to handle persistent problems and create a lasting memory of infections. There is some sort of hand-off between the two systems, probably involving trained macrophages, but this process is poorly understood.

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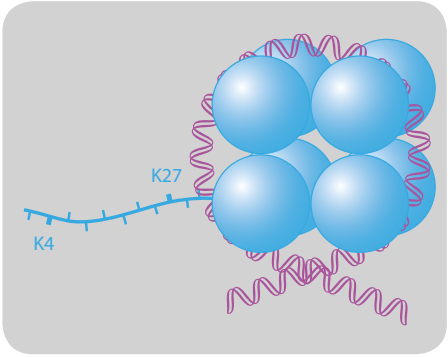
Decoding the epigenetic factors that influence the specialization of monocytes required years of work on the part of several labs across Europe, a number of cutting-edge technology platforms, and a tremendous computational effort to analyze the data. The first step was to collect blood from healthy human volunteers and purify it by removing other types of cells, leaving only monocytes. They were grown in cell cultures in a medium that included human serum, to replicate conditions in the bloodstream as closely as possible.

Now the monocytes needed to be prompted to develop in different ways. Scientists knew that exposing the cells to toxins such as lipopolysaccharides (LPS) from bacteria can trigger a state of immunotolerance – the cells produce fewer protective molecules. This echoed the situation in sepsis. Alternatively, treating monocytes with a molecule called beta-glucan (BG), which occurs in fungal infections, led to trained macrophages. A third group of cells was exposed to neither, causing them to develop into the type of naive macrophages that take up residence in tissues. Then the three types would be compared to monocytes in hopes of understanding why the cells specialized in different ways.

The cells were stimulated with LPS or BG, or allowed to differentiate without introducing foreign molecules, and were then observed over five to six days. The wait was necessary to observe immunotolerance or training effects; earlier studies had failed to track their behavior over more than a day or two. During the longer incubation period, macrophages acquired characteristics that allowed them to be classified into the three types. Immunotolerant cells produced molecules that provoked only weak inflammation, and were thus unable to recruit other types of cells needed to effectively fight infections. BG-stimulated cells increased their output of inflammatory molecules.

Several methods were used to study the epigenetic features of the cells. The first involved a survey of DNA, looking for chemical changes in the proteins that were attached to it. This could provide indications that particular genes were being switched on or off. Active genes produce RNA molecules that may be used in the synthesis of proteins. When a gene is inactivated, this process – called transcription – comes to a halt.

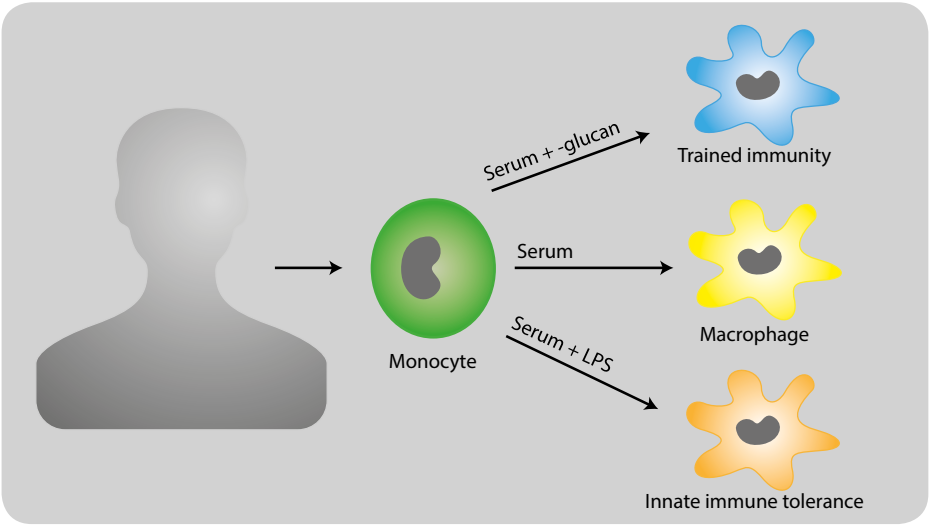
DNA in the cell nucleus is wrapped at regular intervals around spool-like clusters of eight proteins called histones. The molecules used for the DNA packing, particularly one histone called H3, receive chemical modification tags that loosen or tighten the connection of H3 to DNA sequences. Generally, loosening the connection to the histones makes DNA accessible to other molecules that read the information in a gene and transcribe it into RNA. Tightening things up silences the gene.
H3 has a long tail that hangs outside of the spool, where other molecules can bind to it and modify its chemistry through the attachment of tags. This process can either activate a gene or silence it, depending on the type of tag, its position on the tail, and the function of nearby DNA sequences. In the current study the scientists studied two types of very simple tags that exert a powerful influence on gene activity. Specialized enzymes attach methyl and acetyl groups to the H3 proteins at amino acids called lysines (abbreviated as "K"). The tags can be removed again to change the activity of a gene.



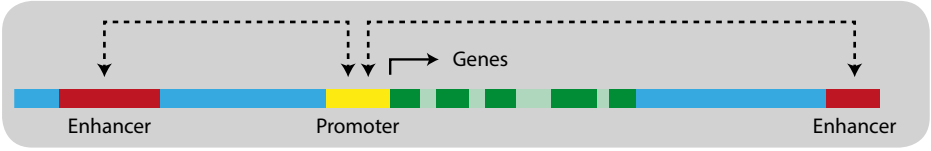
At regular intervals, DNA in the cell nucleus is wrapped around complexes of histone proteins (blue). The long tail of histone 3 can be marked with chemical tags at specific positions (such as lysine 27, or K27), which influences the activity of nearby genes.

The H3 tail contains several lysines that can be tagged by methyl or acetyl groups. Especially important in gene activation are a lysine near the end of the tail, called H3K4, and another closer to the point at which the tail is connected to the main body of the protein, called H3K27. (The numbers and letters represent the H3 protein, K represents lysine, and 4 tells its position: this lysine is the fourth amino acid from the tip of the tail. H3K27 tells you that the 27th amino acid from the tip is also a lysine.)

Both sites can either receive methyl or acetyl tags, but never both at the same time. They have different effects. Finding acetylation at H3K27 or methylation at H3K4 usually turns on the process of transcribing RNA from a



Three types of treatments were applied to monocytes to trigger their development along three routes, giving scientists a way to study processes of differentiation.



Enhancers and promoters are DNA sequences that work together to influence the activation and productivity of genes. Histones within these regions can receive the same chemical tag at the same position in their tails, but the effects are often different.

nearby gene; methylation at H3K27 turns it off.

For a gene to be transcribed, a start reaction must take place at a nearby sequence called a promoter. A second signal, located in a more distant sequence called an enhancer, amplifies this reaction. Proteins called transcription factors bind to these sites and orchestrate the assembly of the very complex machine needed to synthesize RNAs. Promoters are the docking site for this machine and it is here that transcription is initiated. Enhancers tune this activity up and down, so what happens at these sequences ultimately determines how many RNAs are produced from a gene.

The researchers measured the acetylation and methylation of histones along the entire genome, hoping to see different patterns of "packing" DNA that were clearly associated with monocytes or macrophages. The effort revealed that thousands of promoters and enhancers were receiving special chemical tagging at H3K27 and H3K4. In all, about 0.6% of the entire genome underwent such changes during the transformation of monocytes to macrophages. The number of tags that were applied was roughly equal to the amount stripped off, leading to the activation or silencing of hundreds of genes. Most importantly, the cells changed in different ways depending on whether they would become immunotolerant macrophages, with lower inflammatory activity, or trained macrophages that increased their response to pathogens.

One important additional finding was that while the modifications of H3K27 and H3K4 form combined signatures at active genes the status of H3K27 seems to be more important. And the enhancers and promoters for single genes are usually chemically tagged in a similar way, which means they reinforce each other's activity as a decision is made to transcribe the gene or prevent that from happening.

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Modifications in the tails of histone 3 were a good sign that genes were being switched on or off. But the researchers wanted to know more. How are these epigenetic patterns translated into gene activity? What shifts do epigenetic patterns undergo as monocytes developed? Three new experiments – once again making use of high-throughput technologies and huge sets of data – were designed to provide some answers.

First, the scientists introduced an enzyme called DNase I into the cells. This molecule cuts DNA when it is accessible, in a region that permits easier access by gene-activating molecules. The scientists discovered that DNase I indeed cleaves DNA more easily in enhancers and promoters that have been tagged in a way that loosens the chromatin and allows gene transcription. When the sequences bear a signature of densely packaged DNA, it is unable to carry out the cleavage.

Another experiment took a direct look at which RNA molecules were being produced by the cells. This, too, confirmed the findings – the monocytes and macrophages were transcribing most of the genes with permissive tags in their enhancers and promoters.

The final test was to take a look at specific sequences in promoters. The genetic code in these regions contains patterns that permit particular transcription factors to bind. Here, too, the results were encouraging. The regions were full of motifs known to be docking sites for particular proteins that stimulate transcription.

All three methods confirmed that the epigenetic architecture of monocytes significantly changes upon stimulation and that these changes trigger the development of macrophages with different characteristics and functions.

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How, exactly, were these changes altering the behavior of the cells? The final aim of the two studies reported in Science was to search for patterns unique to the monocytes and the different types of macrophages they produced. Somewhere in these sets of genes should be key molecules that could explain the differences.

Here the researchers focused exclusively on tags that changed during the specialization of the monocytes – those that remained constant couldn't explain the way cells acquired new properties. A first finding was no surprise: immunotolerant macrophages, whose immune responses had been downtuned by applying lipopolysaccharides from bacteria, were suppressing the activity of a lot of molecules that are typically produced during inflammations. Monocytes stimulated by beta-glucan, or those that had been grown in serum alone, were turning out more. In other words, immunotolerant macrophages maintained epigenetic patterns that were more like those found in monocytes that hadn't yet been challenged. Another set of macrophages developed different patterns.

What caused the cells to be programmed in these different ways? Was it only that they were receiving different biochemical signals? One answer to this question came through observations of changes in metabolic enzymes, energy-producing molecules that are considered typical markers of most macrophages. "Resident" macrophages were increasing the production of these enzymes; BG-stimulated cells were churning out even more. These changes in metabolic programs were accompanied by an increase in the production of transcription factors that help stimulate responses to inflammations. This was something new and important – it meant that macrophages coordinate the two extremely complex biochemical systems those that regulate metabolism and immunity.

Scientists obtained a deeper view of that connection through a second study, also spearheaded by scientists from Radboud University Medical Center. Here the focus was on trained cells – monocytes that had been stimulated, acquired a first "memory", and were then restimulated. The researchers found that these cells were significantly increasing their use of glucose –sugars – to produce energy and go in action quickly. Glucose metabolism is also stimulated in cells that divide very quickly, including adaptive immune cells that begin multiplying like mad when they respond to an infection. Such increases are also found in a number of types of cancer.

Mihai Netea, whose lab took a leading role in the project, explains that several pieces of evidence pointed toward enhanced glycolysis as a driving force in the training process.

"We had already identified these genes as targets of chemical alterations during the epigenetic studies," Mihai says. "Now we

looked specifically at enzymatic processes in the cells and their output of RNAs."

The scientists examined macrophages from mice that had been exposed to beta-glucogen. The cells consumed more glucose; they also produced more lactate and other byproducts of glycolysis.

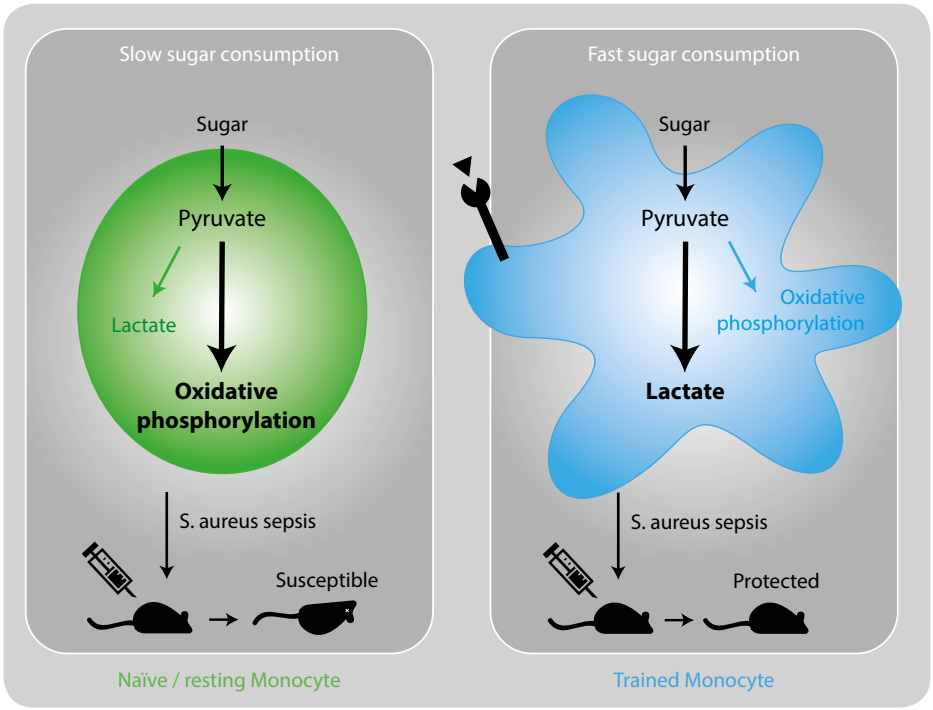
Researchers had already discovered biochemical signaling pathways that enhance glucose metabolism in cells, usually in response to external signals. Were monocytes using the "usual suspects" to do so as they differentiated into macrophages, and needed more energy? Mihai and his colleagues discovered that as they make this transition, monocytes raise their production of two molecules called mTOR and HIF-1 alpha.

That was interesting because these two molecules were already known to play important roles in immune reactions. Drugs have been developed that suppress their activity in cases where weaker immune responses are desirable – for example in patients who have undergone organ transplantations. Normally a patient's body would reject almost any new organ, but this reaction can be dampened by blocking mTOR activity. But in healthy people the system needs to be intact – defects in mTOR signaling are found in diabetes, depression, many types of tumors, and other diseases.

Higher amounts of mTOR probably meant a more active signaling network, which might be driving the training of macrophages. This needed to be tested in an animal model as well as cell cultures to confirm that the signal had the same function. The scientists treated mice with a substance that blocks mTOR signals and discovered that the animals lost their ability to defend the body against Staphylococcus bacteria –tissues infected by the pathogen failed to mount a proper inflammatory response and developed sepsis instead.

"This means that glucose metabolism is fundamental to that process – it provides the extra boost of energy needed for an increased activation of immune cells," Mihai says.

Henk Stunnenberg sees broader implications for the findings of the two studies. "What we observed was that epigenetic changes represented a global shift in the metabolic programs of these cells," Henk says. "This has potential therapeutic implications. When a patient experiences an excessive drop in inflammatory responses, in cases of sepsis and other types of conditions, what you would like is to reactivate pathways such as glycolysis that have been tuned down. Epigenetic studies can point you toward genes that are essential in that process."



„Unchallenged" monocytes and those that have been trained through encounters with bacteria activate different processes involved in the metabolism of glucose. These changes are governed by epigenetic processes and contribute to the cells' development and their roles in future immune responses.

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